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THE CONTROL OF RENIN RELEASE FROM ISOLATED RENAL CORTICAL CELLS AND THE PRESENCE OF RENIN IN SUBCELLULAR FRACTIONS *IN VITRO*

By

Margaret Eleanor Dew

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Approved:	Date:
Andrew M. Michelakis	Aug. 29, 1975
Dorman Davenport	Aug 29, 1975
B. V. Rama Sastry	Aug 29, 1975
Allan D Bass	Aug 29 1975
Erwin J. Landon	aug 29 1975

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS ii

LIST OF TABLES vii

LIST OF ILLUSTRATIONS ix

LIST OF ABBREVIATIONS xiii

Chapter

I. STATEMENT OF THE PROBLEM 1

II. INTRODUCTION 5

A. Physiological Role of Renin and Its Source in Kidney 5
B. Regulation of Renin Release 12
1. Baroreceptor Theory 12
2. Macula Densa Theory 13
3. Contribution of the Sympathetic Nerves 14
4. Hormonal Influences 17
5. Influence of Ions and Osmolality . . 20
6. Influence of Autocoids ("Local Hormones") 24

III. METHODS 27

A. Analytical Methods 27
1. Acid Phosphatase 27
2. Succinate Dehydrogenase and Cytochrome-C Oxidase 27
3. Renin 29
4. Other Enzyme Assays 39
5. Protein 40
6. CAMP Assay 41
7. PGE Extraction and Assay 50
B. Selection of Species for Preparation of Isolated Cells 52
1. Yield of Isolated Cells 52

Chapter | Page

2. Contamination with Tubule Fragments 53
3. Cell Viability 53
4. Size and Anatomy of Kidneys 53
5. Availability and Uniformity of the Animal 54

C. Methods of Cell Isolation 54
1. Preparation of Kidney Cortical Cell Suspensions by Enzymatic Dissociation 54
2. Preparation of Rabbit Kidney Cortical Cell Suspensions by Combined Enzymatic and Mechanical Methods 57
3. Preparation of Rabbit Kidney Cortical Cells by Sodium Citrate and Mechanical Methods 59

D. Viability of Isolated Cells 61
1. Oxygen Consumption 61
2. Trypan Blue Dye Exclusion 63

E. Separation of Kidney Cortical Cells: Enrichment into Cell Types 63
1. Buoyant Density Separation 64
2. Separation of Kidney Cortical Cells by Preparative Free-Flow Electrophoresis 66
3. Fluorochrome Staining of Renin-Containing Cells 70

F. Subcellular Fractionation 71
1. Preparation and Assay of Rat Kidney Subcellular Fractions . . . 71
2. Preparation of Renin Granules . . . 71

G. _In Vitro_ Incubation of Kidney Cortical Cells with Ions, Hormones or Pharmacological Agents 74
1. Incubation of RKC Cells with Different Ionic and Osmolar Earles Buffer 75
2. Incubation of RKC Cells with Easily Degraded Biomolecules . . . 76
3. Incubation of RKC Cells with Inhibitors of Biomolecules 76
4. Incubation of RKC Cells with Agents which are not Degraded by Kidney Cortex 77

H. Preparation of Labile Chemicals 77

Chapter Page

1. Prostaglandin and Unsaturated Fatty Acid Solutions 77
2. Catecholamines 78
3. Acetylcholine Bromide 79
4. Vasopressin 79
5. Other Chemicals 79

I. Statistical Analysis 80

IV. RESULTS 81

A. Renin Radioimmunoassay 81
1. Reproducability of the Assay 81
2. Correlation of Bioassay with Radioimmunoassay 81
3. Effect of Pharmacological Agents on the Reaction of Renin with Its Substrate 81

B. Isolation of Kidney Cortical Cells . . . 82
1. Yield of Cells and Loss of Renin and Protein 82
2. Selection of Conditions for Isolation of Cells with Adequate Renin Levels 84

C. Viability of Isolated Cells 85
1. Oxygen Consumption 85
2. Ability of Isolated Cells to Secrete Renin with Physiological Stimulants 88

D. Integrity of the Isolated Cell Plasma Membrane 88

E. Separation of Kidney Cortical Cells into Cell Types 90
1. Buoyant Density Centrifugation . . . 90
2. Free-flow Electrophoresis 93
3. Fluorochrome Staining of Juxtaglomerular Cells 95

F. Subcellular Fractionation 97

G. *In Vitro* Incubation of Kidney Cortical Cells with Ions, Hormones or Pharmacological Agents 99
1. Incubation of RKC Cells with Different Ionic and Osmolar Composition of Earles Buffer 99
2. Incubation of RKC Cells with Easily Degraded Biomolecules 101
3. Renin Release with Neurohormones 103

Chapter Page

4. Renin Release with Hormones 108
5. Renin Release with Autocoids . . . 114

V. DISCUSSION 128

A. Comparison of Cell Preparation Methods 128
B. Control of Renin Release 135
1. Ionic and Osmolar Regulation . . . 135
2. Autonomic Nervous System Regulation 140
3. Hormonal Regulation 142
4. Regulation by Autocoids 145
C. Intracellular Renin and Possible Parameters Affecting Release from the Cell 150

VI. SUMMARY 153

APPENDIXES

A. TABLES 158

B. ILLUSTRATIONS 198

REFERENCES 283

LIST OF TABLES

Table Page

1. Loss of Renin Activity with Time 159
2. Effect of Pharmacological Agents on the Reaction of Renin with Renin Substrate 160
3. Loss of Renin Activity and Protein in the Preparation of Renal Cells by Enzymatic Dissociation 162
4. Loss of Renin Activity and Protein in the Preparation of Renal Cells by Combined Enzymatic and Mechanical Dissociation 163
5. Loss of Renin Activity and Protein in the Preparation of Renal Cells by Citrate and Mechanical Methods 164
6. Isolation of Renal Cortical Cells. Loss of Renin at 4° C and 24° C 165
7. Isolation of Renal Cortical Cells. Effect of Calcium on Loss of Renin 166
8. Effect of 3% BSA on Oxygen Consumption of Kidney Cortical Cells 167
9. Effect of NEAA on Oxygen Consumption of Kidney Cortical Cells 168
10. Release of Intracellular Enzymes and Protein as a Measure of Membrane Integrity 169
11. Centrifugation of High Renin Activity Fractions after Free-flow Electrophoresis . . 170
12. Rat Kidney Subcellular Fractions. Purity and Renin Activity 171
13. Purity of Rabbit Kidney Renin Granules 173
14. Release of Renin from RKC Cells in Response to Magnesium with and without Calcium 174

Table | Page

15. Release of Renin from Furosemide Pre-treated RKC Cells in Response to Sodium Chloride Made Iso-osmotic with Choline Chloride 176

16. Response of RKC Cells to Catecholamines . . . 178

17. Effect of Exogenous CAMP, CAMP Analogues and SC 2964 on Renin Release from RKC Cells . . . 180

18. Inhibition of Renin Release from RKC Cells by Aldosterone 182

19. Effect of Argenine Vasopressin on Renin Release from RKC Cells 183

20. Effect of Parathyroid Hormone with EGTA or Calcium on Release of Renin from RKC Cells . . 184

21. Effect of Insulin on Renin Release from RKC Cells with and without Glucose 186

22. Release of Renin from RKC Cells with Glucagon 187

23. Effect of Serotonin on Renin Release from RKC Cells 188

24. Time Course of PGE_2 Action on Renin Release from RKC Cells 189

25. Influence of Calcium or EGTA on the Decrease of Renin Release from RKC Cells with $PGF_{2\alpha}$ 191

26. Effect of Arachidonic Acid on Renin Release from RKC Cells and the action of Prostaglandin Synthesis Inhibitors on this Effect 193

27. Cell Preparations and Responsiveness to Calcium 195

28. Stimulation and Inhibition of Renin Release 197

LIST OF ILLUSTRATIONS

Figure	Page
1. Time course of the reaction of cell renin with substrate	200
2. Time course of the reaction of supernatant (released from cells) renin with substrate	202
3. Scatchard plot of renin radioimmunoassay standard curve	204
4. Determination of ^{125}I-A I by isotope dillution	206
5. Purification of commercial ^{3}H-CAMP	208
6. Calibration of Dowex 50W AG x 8 H^{+} 0.7 x 20 cm column	210
7. Purification of rabbit muscle protein kinase by protamine sepharose	212
8. Continuous fractionation scheme for obtaining subcellular components from rat kidney	214
9. Correlation between human plasma renin (PRA) values obtained with bioassay and with the radioimmunoassay	216
10. Oxygen consumption of RKC cells and combined wash supernatants from collagenase-prepared cells	218
11. Effect of 3% BSA and NEAA on oxygen consumption of isolated dog cortical cells	220
12. Effect of 3% BSA and NEAA on oxygen consumption of isolated RKC cells	222

Figure		Page

13. Effect of 10 mM D-glucose on the oxygen consumption of dog kidney cortical cells . . . 224

14. Effect of 8 mM α-ketoglutarate on oxygen consumption of dog kidney cortical cells . . . 226

15. Effect of 17 mM α-ketoglutarate on oxygen consumption of dog kidney cortical cells . . . 228

16. Trypan blue uptake of cells dissociated from kidney cortex by the collagenase-mechanical method 230

17. Trypan blue uptake of cells dissociated from tissue by the sodium citrate-mechanical method 232

18. Bands of renal cortical cells separated by Ficoll density gradient centrifugation 234

19. Separation of normal dog kidney cortical cells by Ficoll density gradient centrifugation 236

20. Separation of kidney cortical cells from dogs pretreated with furosemide, 80 mg/day i.m for 2 days 238

21. Comparison of renin-containing cells obtained from five normal dogs and five dogs pretreated with furosemide, 40 mg/day i.m for 2 days 240

22. Separation of normal RKC cells by discontinuous Ficoll density gradient centrifugation 242

23. Renin-containing cells from normal and sodium-deficient rabbits 244

24. Responsiveness to epinephrine of renin-containing cells separated by Ficoll density gradient centrifugation 246

Figure | Page

25. Increase in renin activity and CAMP levels caused by isoproterenol in renin-containing cells separated by Ficoll density gradient centrifugation 248

26. Renin-rich fraction of RKC cells isolated by Ficoll density gradient centrifugation . . . 250

27. Separation of normal RKC cells and renin granules by free-flow electrophoresis . . . 252

28. Separation of ^{3}H-aldosterone pretreated RKC cells by free flow electrophoresis 254

29. Electrophoretic separation of a heavy mitochondrial fraction from rabbit kidney cortex in a free-flow electrophoresis apparatus . . 256

30. Electron micrograph of anodic fractions 13-15 obtained after electrophoresis and centrifugation at 4,500 x g in 5% BSA and OsO_4 1:1 (v:v) 258

31. Effect of changes in calcium concentration on renin release by isolated RKC cells . . . 260

32. Effect of osmolarity change on renin release by RKC cells 262

33. Effect of variation in ratios of sodium to potassium on renin release from RKC cells 264

34. Increase in renin release from RKC cells with epinephrine, norepinephrine, or isoproterenol, and specific blockade of this release by or blockers 266

35. Effect of acetylcholine and histamine on renin release from RKC cells and blockade of the response by atropine and diphenhydramine 268

36. Renin release from RKC cells in response to angiotensin I and angiotensin II 270

Figure | Page

37. Formation of PGE_2 and $PGF_{2}\alpha$ from arachidonic acid, and structures of important prostaglandin compounds 272

38. Release of renin from RKC cells in response to various levels of prostaglandins or oleic acid 274

39. Release of renin from RKC cells in response to the E- and F-type prostaglandins 276

40. The effect of PGE_2 on renin release from RKC cells with and without calcium 278

41. Effect of arachidonic acid and indomethacin on the release of renin and formation of PGE_2 in RKC cells 280

42. Interaction of catecholamines and prostaglandin on the release of renin from RKC cells . 282

LIST OF ABBREVIATIONS

α-KG	Alpha-Ketogluturate
A I , A II	Angiotensin I, angiotensin II
BSA	Bovine serum albumen
BAL	British Antilewisite
BES	N, N-bis (2 hydroxyethyl)-2-aminoethane sulfonic acid
Cpm, dpm	Counts per minute, disintegrations per minute
CAMP	Cyclic AMP
DOCA	Desoxycorticosterone acetate
EGTA	Ethyleneglycol-bis (β-amino-ethyl ether) N, N'-tetraacetic acid
EDTA	Ethylenediamine tetraacetate
JG	Juxtaglomerular
JGA	Juxtaglomerular apparatus
M (g, Ci)	Mole (gram, Curie)
mM (mg, mCi)	Millimole (milligram, millicurie)
µM (µg, µCi)	Micromole (microgram, microcurie)
nM (ng)	Nanomole (nanogram)
pM (pg)	Picomole (picogram)
µS	Microsiemens $(\frac{1}{mhos})$
NAD	Nicotinamide adenine nucleotide
NADH	Reduced nicotinamide adenine nucleotide

NADH	Reduced nicotinamide adenine nucleotide
OsO_4	Osmium tetroxide
PIPES	Piperazine-N-N' -bis (2-ethane-sulfonic acid)
pNP	Paranitrophenyl phosphate
KP_i	Potassium phosphate
PGA_1	Prostaglandin A_1
PGA_2	Prostaglandin A_2
PGE	Prostaglandin E
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
RKC	Rabbit kidney cortical
S.E.M.	Standard error of the mean
U, µU	Units, microunits
Tris	Tris-(hydroxymethyl)-aminomethane
V:v	Volume by volume
W:v	Weight by volume

CHAPTER I

STATEMENT OF THE PROBLEM

The regulation of local hormone, or autocoid, activity is an important aspect in the control of greater physiological functions, such as blood pressure, nerve activity, and metabolic activity of organs and glands. This regulation of local hormones could be termed "fine control" of body physiology and is possibly responsible for the delicate balance of complex functions that are necessary for physiological and biochemical normalcy. It is valuable to study control of autocoids in order to understand their role in homeostasis.

Renin, an enzyme that is produced by the juxtaglomerular apparatus in kidney cortex, acts on a 13-amino acid portion of an alpha-2 globulin in plasma to produce a decapeptide, angiotensin I. This circulating decapeptide is then further cleaved to an octapeptide, angiotensin II, by "converting enzyme," which is located in the lung. Angiotensin is generally classified as a local hormone; it seems to maintain body homeostasis in several ways. The most immediate action of angiotensin II is to constrict arterioles by stimulating smooth muscle of the vessel walls, thereby increasing both blood pressure and

cardiac output. A more prolonged effect of angiotensin II is its action on the adrenal gland, causing release of aldosterone, which in turn acts on the renal distal tubule to cause sodium conservation. The increase in body sodium then increases plasma osmolality, blood volume, and blood pressure, all of which inhibit further release of renin. Other actions of angiotensin II which have been less completely defined are: effects on kidney, causing diuresis, natiuresis, local changes in blood perfusion, and regulation of prostaglandin release; effects on the nervous system, causing release of epinephrine from adrenal medulla, norepinephrine from sympathetic nerve endings, and vasopressin from the neurophypophysis; and effects on smooth muscle, influencing contractions in gut, uterus and bronchioles.

Since angiotensin II is the final product of renin release, the regulation of renin release ultimately controls angiotensin II levels, and has a part in some aspects of homeostatic modulation. The release of renin and the control of this release could thus serve as a model for regulation of local hormones. However, the regulation of renin release is difficult to study in the whole animal because many feedback control loops exist and it is difficult to ascertain the relative importance of each

influence. Tissue slices simplify the problem, but often slices are relatively insensitive to chemical agents because few of the cells are in direct contact with the medium. This system must therefore rely on diffusion, since blood pressure is not present to aid penetration of substances into the tissue. Cell free homogenates are not useful in release studies which require cellular integrity. The best method would be to study the effects of individual agents on isolated kidney cortical cells. Such cells retain their cellular intactness, are easily exposed to variations in incubation medium, and are free from changes in blood pressure, osmotic pressure, nerve activity, and hormone levels that are present in the whole animal. Hence, each influence on renin release can be separately studied.

The purpose of this dissertation is to elucidate the regulation of renin release by elements both external and internal to the juxtaglomerular cell. To study the effect of agents external to the cell, it was necessary to develop a method for dissociating kidney cortex tissue into isolated cells; these cells had to be viable, morphologically intact, and sensitive to changes in their surrounding environment. The isolated cells were then exposed to ionic, chemical, and hormonal changes in order to "dissect" the relative importance of these agents,

which other investigators had shown to control renin release in whole animal and tissue. To elucidate influences on renin release internal to the cell, methods were developed to isolate an enriched population of renin-containing cells. These cells were then studied to observe changes in their renin content, and changes in cyclic AMP levels with various stimuli. The possible events in renin granule formation inside the cell, which would ultimately affect renin release, were delineated by isolation of the renin granule and by measuring renin content of kidney subcellular fractions.

CHAPTER II

INTRODUCTION

A. Physiological Role of Renin and Its Source in Kidney

Renin, which was first described as a hypertensive agent in 1898 by Tigerstedt and Bergman, was shown by Skeggs *et al.* in 1957 to act on a tetradecapeptide portion of an α-2 globulin to produce angiotensin I (A I); Skeggs *et al.* (1956) also demonstrated that this product was changed to the biologically active form angiotensin II (A II) by converting enzyme. A II causes constriction of the aorta (Furchgott and Bhadrakom, 1953), arterial resistance vessels (Bohr and Uchida, 1967) and veins (Somlyo and Somlyo, 1966), producing an increase in blood pressure lasting from 3 to 5 minutes and changes in regional blood flow in many organs (Peart, 1965). It also causes contraction of uterine and intestinal smooth muscles (Paiva and Paiva, 1960; Bisset and Lewis, 1962). A II is a primary factor in the regulation of aldosterone secretion (Ganong *et al.* (1962). In this role, it starts a negative feedback mechanism which results in inhibition of renin release (Davis, 1971). Aldosterone may directly

decrease renin release (Greco and Murphy, 1972), but probably a greater effect is achieved by aldosterone-stimulated renal tubular sodium reabsorption, which increases osmolality and thereby decreases renin release (Weinberger and Rosner, 1972).

A II has important local effects on kidney hemodynamics, tubular urine flow and reabsorption of sodium, and on secretion of renin and prostaglandin. Aukland (1968) demonstrated a decrease in both cortical and medullary blood flow in response to A II, and Porusch et al., (1967) observed a decrease in osmolal and free water clearances with A II, and suggested that its site of action might be the ascending loop of Henle and the proximal tubule. However, part of this change in tubular osmolality could be caused by the release of vasopressin by A II (Bonjour and Malvin, 1970). A negative feedback effect of A II has been amply demonstrated in conscious sheep (Blair-West el al., 1971), isolated rat kidney (Vandogen et al., 1974), nonfiltering dog kidney (Shade et al., 1973), and in a dog renal cell suspension (Michelakis, 1971). Another effect of A II which could lead to complexities, is the stimulation of prostaglandin E (PGE) release from medulla into renal venous blood (McGiff et al., 1970) and urine (Frölich et al., 1975).

PGE is a determinant of intrarenal distribution of blood flow (Itskovitz *et al.*, 1974), is as influential in the maintenance of resting blood flow (Itskovitz and McGiff, 1974), and is a stimulant of sodium and fluid excretion, especially when intake of sodium is low (Fülgraff *et al.*, 1973).

A II also has effects on the nervous system. It can stimulate ganglia, release acetylcholine from nerve endings, modulate the effects of norepinephrine on its end organs and stimulate epinephrine release from adrenal medulla (Khairallah, 1971).

The exact mechanism of action of A II has not been elucidated. However, it has been shown by Schreier-Muccillo *et al.*, (1974) that A II causes a conformational change in phospholipid spin-labeled membranes from guinea-pig ilial smooth muscle. Robertson and Khairallah (1971) also showed that intracardiac injection of A II caused an increase in the number of pinocytotic vesicles in heart muscle and that labeled A II was found primarily in the nuclei of smooth and cardiac muscle cells 30 to 40 seconds after injection. A II can cause release of calcium from smooth muscle cell microsomes (Baudouin *et al.*, 1972). Thus it seems that A II may be incorporated into the cell by pinocytosis, which could explain the membrane conformational changes, and that A II may mobilize calcium

once it gets into the cytoplasm. Long-term effects might occur by stimulation of messenger RNA from the nucleus. Munday et al. (1971) reported that A II increased extrusion of sodium in rat kidney cortex slices, an effect which was not blocked by ouabain or a potassium-free medium. They attributed these effects to stimulation of a potassium-independent ouabain-insensitive sodium pump. This hypothesis provides a mechanism of action for the reabsorption of sodium in kidney in response to A II.

The spectrum of normal responses caused by A II shows that it is an important factor in the homeostatic mechanisms of many systems. Certain kinds of hypertension are correlated with abnormal A II levels. An understanding of the source of renin and the factors regulating its release would give a key to how some normal body functions are maintained and what happens in pathological states when some of the maintenance factors are disturbed.

Juxtaglomerular (JG) cells, located on the afferent arteriole of the glomerulus, were first described by Ruyter (1925). In 1939 and 1940, Goormaghtigh noticed that other structures near to the JG cells seemed to connect to these cells, and suggested the term "juxtaglomerular apparatus"

(JGA) to include the JG cells, Polkisson (lacis) cells located in the apex of the angle formed by the afferent and efferent glomerular arterioles, and cells of the macula densa located in a part of the distal tubule next to the afferent arteriole. Goormaghtigh noticed that the granulation of the JG cells increased in chronic renal ischemia and suggested that the JGA might have an endocrine function. However, demonstration that the degree of JGA granulation was correlated with changes in renin activity, and that renin was located in the JGA was more difficult. Taquini et al. (1950) assayed the renin content of successive layers through the kidney cortex of dog, cat, pig and ox, and found the most renin in the superficial layers of all species. In dog, these areas were aglomerular, so they concluded that renin could not be associated with the glomerulus. However, more careful examination by Cook et al. (1956) showed that high renin was present in areas where glomeruli first appeared in outer cortex but not in the thin subcapsular cortex region, and that although glomeruli were present near the medulla, that area had very low renin activity. Brown et al. (1966) showed that if rabbits were made hypertensive by renal artery constriction, glomeruli from the deep cortex as well as the superficial cortex had much renin, whereas

in the normal animal renin was high only in glomeruli from superficial cortex. Localization of renin activity in structures near to the glomerulus but not in the glomerulus was demonstrated by Bing and Kazimierczak (1959), who selectively destroyed glomeruli by heat in frozen cortex sections and found no decrease in renin activity, but when areas in the immediate vicinity of the gomeruli were destroyed the activity was lost. This observation was also confirmed by Cook and Pickering (1959) in iron oxide-filled glomeruli isolated by magnet from a glomerular suspension. The glomeruli which included the JGA had higher activity than those without these associated structures. Localization of renin to the area of the JGA was demonstrated in magnetically isolated glomeruli that had been bisected so that one-half always included the JGA region (Cook and Pickering, 1962). Faarup (1967, 1968), using microdissection of freeze-dried cat kidney sections, found 90% of the renin activity in the afferent arteriole close to the glomerulus, about 5% in macula densa cells and 3% in lacis cells. Hartroft et al. (1964), using a fluorescent antibody against partially purified renin also demonstrated bright fluorescence only in JG cells, and no fluorescence in macula densa or lacis cells. It is presently thought

that most renin is located in the JGA, primarily in the JG cells of the afferent arteriole.

Electron microscopic studies of the JGA have shown electron-dense membrane bound granules from 0.7 to 1.5 μ in diameter, which were often associated with the Golgi apparatus in animals with renal artery constriction (Barajas, 1966). This evidence supported the concept that the Golgi apparatus of the JG cell might concentrate renin and form the membrane surrounding the granule; an example of this process was elegantly shown in pancreas cells by Caro and Palade (1964). Granules high in renin activity and with similar electron microscopic morphology to the granules described by Barajas (1966) have also been isolated from guinea-pig (Schmidt _et al._, 1971) and rabbit (Gross and Barajas, 1975); (Dew and Heidrich, 1975). Thus, it appears that renin in the JG cell is contained in a membrane-bound subcellular particle, or granule, which seems to have been formed by the Golgi apparatus of the cell and remains stored in the cytoplasm until release. The release process of the granule has not been clarified. Whether the entire granule is extruded by the cell by exocytosis, or is broken at the cell membrane and its contents released into the blood has not been determined.

B. Regulation of Renin Release

1. Baroreceptor Theory

In 1959, Tobian *et al.* hypothesized that the JG cells might act as stretch receptors since they were located in the walls of the afferent arteriole and would be subject to pressures within the arteriole. With decreased pressure, the JG cell would become less distended and renin release would rise, and with increased pressure the JG cell would be stretched, which would inhibit renin release. The actual pressures on the JG cell would thus be intravascular, transmural and intracellular (the product of arteriolar diameter and transmural pressures). Factors which could influence these pressures would be: the renal interstitial pressure, which is relatively constant unless ureteral or renal venous occlusion, nephritis, or osmotic diuresis is present; the arterial blood pressure; and afferent arteriolar vascular tone, which could be regulated by sympathetic nerves. Evidence for the effect of a change of arterial blood pressure on renin release was obtained by hemorrhage (Huidobro and Braun-Menedez, 1942; McKenzie *et al.*, 1966). Hypovolemic shock released renin in all experiments. However, the renal nerves or catecholamine output from adrenal medulla could have also caused this. Blaine *et al.* (1971) showed that, in dogs with nonfiltering kidneys (which

eliminated any action of osmolality changes in the macula densa) and no renal nerves or adrenals, hemorrhage caused a rise in renin release; this was the best evidence for the existence of the JG cell as a renal vascular stretch receptor. Skinner *et al*. (1963) also found that increased renin release occurred with only 10 mm reduction in renal artery pressure.

2. Macula Densa Theory

Since the JG cell is intimately connected with the cells of the afferent arteriold (Barajas and Latta, 1963), it was thought that the release of renin was controlled by changes in sodium load, or osmolality changes, to the macula densa. Although osmolality and sodium concentration of the macula densa has never been measured, Gottschalk (1964) showed that distal tubular fluid osmolality and sodium concentration was always lower than that of plasma. Also hypertonic mannitol caused increased distal tubular osmolality (Ullrich *et al*., 1963). To separate the baroreceptor effect of decreased arteriolar pressure caused by decreased glomerular filtration rate (GFR) from the macula densa effect, Vander and Miller (1964) reduced arterial pressure as osmotic diuretics were administered in order to enhance the sodium load to the macula densa, and decreased

renin release by this process, in spite of a large decrease in renal plasma flow. These investigators also found that renin release increased with increasing ureteral pressure. This manipulation caused increased intratubular pressure, and decreased GFR, resulting in decreased sodium excretion.

Barajas (1972) reported new anatomical evidence that, although some areas of the distal tubule impinged on granular (JG) cells, there were also granular cells far removed from the area. He also saw granular cells in the efferent arteriole, and occasionally saw contact between the granular cells and the proximal tubule. He proposed that any change in volume of either proximal or distal tubule cells could change the degree of contact with the granular arteriolar cells, hence modifying any chemical exchange that occurred. Thus, the entire renal tubule, not just the macula densa region could regulate renin release.

3. Contribution of the Sympathetic Nerves

Sympathetic nerves end very near JG cells (Wågermark _et al._, 1968) and many nonmyelinated nerves impinge on the evidence has stimulated much research into the role of these

primarily the adrenergic fibers, in renin release. Release of renin could occur either by direct stimulation of the JG cell by released norepinephrine, by contraction of the vascular smooth muscles, reducing contact between the arterioles and the tubules, or by stimulation of other JGA elements such as the macula densa. Vander (1965) reported that both catecholamines and renal nerve stimulation increased renin release in anesthetized dogs; however this study did not clarify whether the action was directly on the JG cell or via other JGA components. Johnson et al. (1971) studied the effect of epinephrine, norepinephrine and renal nerve stimulation in dogs with nonfiltering kidneys, in an experimental design that excluded any effects of the macula densa or renal tubules, on renin release. They also tested the above procedures in the presence of papaverine, a drug which relaxed smooth muscles and would therefore reduce any effects caused by stimulation of arteriolar smooth muscle. They found that although epinephrine enhanced renin release, this response was blocked by the simultaneous infusion of papaverine and epinephrine. Norepinephrine and renal nerve stimulation also increased renin release, but this increase was not blocked by concomitant papaverine infusion. They concluded that norepinephrine and renal nerve stimulation acted

directly on the JG cell, but that epinephrine was primarily acting indirectly via renal arterioles. Michelakis et al. (1969) demonstrated a direct effect of both epinephrine and norephinephrine on renin release from a renal cortical cell suspension. Since cyclic AMP (CAMP) also stimulated renin release in this preparation, it was possible that the catecholamines were activating adenylcyclase in the cell. According to the hypothesis of Robinson and Sutherland (1970), the release of renin by the adrenergic nervous system would be through β-receptor stimulation. The renin response to epinephrine was slightly increased by the α-adrenergic blocker phenoxybenzamine and abolished by the β-blocker propranolol (Assaykeen et al., 1970), which suggested that the stimulatory effect of ephinephrine was related to a β-receptor mechanism. Infusion of a potent β-agonist, isoproterenol has been shown to markedly stimulate renin release (Allison et al., 1970; Vandogen et al., 1973). This release was blocked by dl-propranolol but not d-propranolol (which had only local anesthetic effects but no β-blocking effects) or phenoxybenzamine (Vandogen et al., 1973). CAMP and dibutyryl CAMP also stimulated renin release when infused into the renal vein (Allison et al., 1972), as did theophylline,

an effect that was not blocked by phenoxybenzamine or propranolol (Reid *et al.*, 1972). Although there has been no direct report of an increase in CAMP levels and increased renin secretion in response to β-agonists, the pharmacological evidence indicates that the increase in renin release by sympathetic stimulation is via the β-receptor.

4. Hormonal Influences

It is possible that circulating hormones have direct influences on renin release as well as indirect ones. Since A II acts on endocrine glands to release aldosterone, ephinephrine and vasopressin, it would be reasonable to expect that negative feedback controls on renin release exist with these hormones. As was discussed above, this was not the case for ephinephrine, for its predominant action on renin release was stimulatory, although an inhibitory component could be illustrated by the use of the α-blocker phenoxybenzamine (Assaykeen *et al.*, 1970). Vasopressin, however, has been shown by many authors to inhibit renin release (Bunag *et al.*, 1967; Vander, 1968; Tagawa *et al.*, 1971). In the experiments of Tagawa *et al.* (1971), the infused vasopressin changed endogenous vasopressin only within normal limits and did not change plasma

osmolality, sodium or mean arterial pressure. It probably did not change renal hemodynamics or sodium excretion, although these parameters were not measured. Gutman and Benzakein (1971) reported interesting results with normal rats and rats with diabetes insipidus. In normal rats, administration of vasopressin caused a decrease in renin activity, but in rats with diabetes insipidus the same dose of vasopressin caused increased renin activity. Although the effects seen may have been caused by differences in hydration of the animals, the results might imply that elevated vasopressin levels decreased renin release whereas low vasopressin levels stimulated renin release. Thus, vasopressin seemed to have true negative feedback control on release of renin.

In the case of aldosterone, the results were not so clear. Chronic adrenal insufficiency increased renin release and JGA granulation (Sokabe _et al_., 1963), whereas chronic administration of aldosterone, DOCA and salt, or hyperaldosteronism decreased renin release (Gross _et al_., 1957; Tobian, 1960; Luetscher and Beckerhoff, 1972). However, most data indicate that the changes in sodium balance affected renin release more than a direct negative feedback. Greco and Murphy (1972), using perfused isolated dog kidney showed some evidence

for negative feedback of renin release with exogenous aldosterone and sodium loading, but not without sodium loading.

The influence of other hormones on renin release have not been sufficiently studied. Meyer *et al*. (1972) noticed that patients with hyperparathyroidism had increased renin activity, high urinary calcium, hyperaldosteronism and hypokalemia; the majority of these patients also were hypertensive. All of these patients had normal vascular volume, serum albumen and sodium concentrating ability. It is possible that either the high levels of parathyroid hormones or the resultant high tubular calcium concentration caused eventual stimulation of renin secretion, but probably the symptoms seen were caused by more complicated interactions on the renin system.

The effects of insulin-induced hypoglycemia on renin release was studied by Otsuka *et al*. (1970). The experiments, which were done in anesthetized dogs, showed that insulin hypoglycemia produced a significant increase in renin release. However, infusion of glucose, potassium or removal of the adrenal medulla abolished this effect. It therefore seems clear that in the intact animal, regulation of renin release by insulin is at best doubtful. Insulin effects have not been extensively studied *in vitro*.

It was found that renin release with glucose was higher than without glucose in kidney slices from decapitated rats, or from rats that had been bled to increase sympathetic nervous system activity. There was no renin release in slices from normal animals (Bozović and Efendić, 1969). These results suggest that insulin as well as epinephrine and norepinephrine may be involved in the release of renin by glucose, but the circulating levels of these hormones were not measured.

Vandogen _et al_. (1973) reported a stimulation of renin release by infusion of glucagon (0.6 μg/min/g) into isolated rat kidneys, an effect which was not blocked by propranolol. This finding implies a direct effect of glucagon on renin cells, not one mediated by sympathetic nerves. Nolly _et al_. (1974) also found a direct stimulatory effect of glucagon (10^{-3}M) on rat kidney slices. These results suggested that glucagon had a partially regulated renin secretion.

5. Influence of Ions and Osmolality

Calcium and magnesium are necessary for the release of many hormones and the transmitter substances (Rubin, 1970; Milner and Hales, 1967; Leclerq-Meyer _et al_., 1973). The effect of calcium on renin release has been studied

in vitro but not *in vivo*. Morimoto *et al.* (1970) found that renin release from slices of dog kidney cortex was minimal in calcium-free medium, and even less in calcium- and magnesium-free medium. Low release occurred with 1.25 mM calcium and even greater release was caused by 2.50 mM calcium. However, in the presence of calcium, 0.59 mM magnesium and 1.18 mM magnesium did not significantly affect renin release. Michelakis (1971b) also found maximum release with 2.5 mM calcium and less release with 1.25 mM and 3.75 mM calcium. Omission of calcium from the medium resulted in the least release. Aoi *et al.* (1974), using a higher osmolality buffer than Morimoto or Michelakis, found no correlation between increasing calcium concentration and renin release, although they did not test the effects of calcium-free medium. These reports suggest that, although there appears to be a necessity for calcium in renin release, the ratio between sodium concentration, or osmolality, and calcium concentration may be more important. Renin release will occur in the presence of magnesium if calcium is absent; if calcium is present, magnesium acts synergistically with it.

Sodium depletion, either by dietary deprivation or with natiuretic agents such as the diuretic furosemide,

always produced an increase in plasma renin activity (Winer, 1962; Brown *et al.*, 1964; Bunag *et al.*, 1966) as did water deprivation for 12 hours (Gross *et al.*, 1965). Increased sodium in diet (Brown *et al.*, 1962; Gross *et al.*, 1965) or retention of sodium caused by administration of DOCA and salt or mineralocorticoids (Gross *et al.*, 1957; Tobian, 1960) decreased renin activity. However, Young and Rostorfer (1973) showed *in vivo* that changes in renal artery osmolality with NaCl, dextrose or urea all caused equivalent changes in renin activity, which increased with increasing osmolality. Weinberger and Rosner (1972) also showed that osmolality, not just NaCl, was important for renin release. They observed no change in renin release when NaCl osmolality was constant, but when the osmolality was not constant, they observed high release at low osmolality and low release at high osmolality.

Vander (1970) found that infusion of KCl into the renal artery inhibited renin release and natiuresis, but did not affect renal hemodynamics and blood pressure. Likewise, high potassium intake diminished renin secretion and potassium depletion increased renin release (Brunner *et al.*, 1970; Abbrecht and Vander, 1970). Hypokalemia in various diseases often resulted in high renin activity (France *et al.*, 1973; Leutscher and Beckerhoff, 1972).

The mechanism of altered renin release by potassium is still unclear. Schneider et al. (1972) demonstrated that infusion of 8 to 16 μEq/min/kg of KCl into the dog renal artery did not significantly affect proximal tubular reabsorption of sodium or glomerular filtration rate, but caused a significant drop in renin secretion. The decreased renin release could have been caused by a direct effect on the JG cells or potassium-induced inhibition of sodium reabsorption in the loop of Henle and distal tubule, thereby inhibiting the JG cells via increased sodium load in the macula densa. This hypothesis was supported by the observation of Shade et al. (1972), who found that potassium infusion into a nonfiltering kidney did not change renin secretion. The change in renin release in response to potassium concentration thus appears to be dependent on an intact tubule system. However, the ratio of sodium to potassium presented to the JG cell may also be important in the renin-angiotensin system. Dluhy et al. (1974) found that in normal subjects given low sodium-low potassium diets followed by low sodium-high potassium diets, no significant difference in plasma renin activity was detected. A week later the same subjects were given high sodium-low potassium diets followed by high sodium-high potassium diets. Although

all renin values were significantly lower with the high sodium diet than with the low sodium diet, there was again no difference between the potassium diets. Because low sodium-high potassium diet increased aldosterone output, it is hard to draw definitive conclusions from this experiment. _In vitro_ experiments are needed to clarify the relationship of sodium-potassium ratios on renin release.

6. Influence of Autocoids ("Local Hormones")

Of all the local hormones tested on the renin-angiotensin system, only A II and PGE had regulatory effects. Bunag _et al_. (1966) found no release of renin in anesthetized dogs when serotonin was infused into their kidneys. Likewise, infusion of histamine, dopamine, bradykinin or khallidin had no effect on renin release from dog kidneys (Vander and Luciano, 1967).

The A II is active in a short-loop negative feedback on renin release, as was shown by many investigators _in vivo_ and _in vitro_ (Genest _et al_., 1965; Vander and Geelhoed, 1965; Shade _et al_., 1973; Michelakis, 1971a). Vandogen _et al_. (1974) demonstrated that the decrease in renin secretion caused by A II _in vivo_ could not be attributed to renal vasoconstriction and subsequent rise

in renal perfusion pressure. The effect of A II still occurred in a nonfiltering kidney (Shade *et al.*, 1973), which eliminated the possibility of sodium reabsorption changes by angiotensin and effects via the macula densa. It was interesting that infusion of A II after vasopressin greatly potentiated the inhibitory effect on renin release. The inhibition of renin release by A II was shown to be a direct one on the renin-producing cells by Michelakis (1971a), using dog renal cortical cell suspensions. This direct action was also observed by Russet and Veyrat (1971) using human kidney cortex slices. They also observed that A I had no effect on renin release.

Werning *et al.* (1971) reported a significant increase in renin activity, along with increases in natiuresis, kaliuresis and diuresis 30 minutes after infusion of very large amounts (625 ng/kg/min) of prostaglandin E_1 (PGE_1). Since the effect was independent of changes in blood pressure, heart rate, serum calcium, magnesium, sodium potassium, or hematocrit, the authors concluded that the mechanism of action might be via electrolyte and water losses, which would implicate the macula densa in the stimulation of the JG cell. This hypothesis was supported by Corsini *et al.* (1974) *in vitro*, using rat kidney cortex slices incubated with high concentrations (1 mg/ml) of

PGE_1. There was no effect on renin release in this system, indicating that intact tubular transport must be present for stimulation of release with PGE_1 to occur. Other prostaglandins, especially those made in the kidney medulla (PGE_2 and $PGF_{2\alpha}$) were not tested, although it is apparent that A II could affect their release (McGiff et al., 1970; Frölich et al., 1975).

It is evident that the regulation of renin release is very complicated. The most effective control is probably intrarenal. These mechanisms include the amount of stretch on the arteriole which causes lengthening or rounding of the JG cell, the amount of sodium, potassium or the osmolar concentration in the macula densa area, the activity of the sympathetic nerves impinging on the JGA and the amount of A II formed in the kidney. In addition other extrarenal control mechanisms exist, exerting direct and indirect influences on the JG cell. These involve changes in amounts of circulating hormones such as aldosterone, vasopressin, epinephrine, glucagon, and possibly insulin and parathyroid hormone, as well as gross circulatory and neural changes. No one theory at this time can explain the regulation of renin release; it must be viewed as a fluid pattern of forces, and many of the forces are still poorly understood. Also, which described effects are physiological and which are experimental artifacts remain to be determined.

CHAPTER III

METHODS

A. Analytical Methods

1. Acid Phosphatase

Acid phosphatase, a marker enzyme for lysosomes, was measured spectrophotometrically according to the method of Bessey *et al.* (1946). The method utilized paranitrophenyl phosphate (pNP) (Fisher Scientific Co.) as a substrate and measured phosphatase activity by extinction at 400 mμ in a 1 cm cell. The assay total volume was 0.3 ml and contained 0.2 ml of 0.0055 M pNP in 0.05 M sodium citrate buffer and 0.1 ml of enzyme preparation at suitable dilution (approximately 0.3 units/ml) in water. This mixture was incubated for 1 hour at 37° C, the reaction was stopped by addition of 0.8 ml 0.1 N NaOH and the extinction of 400 mμ was read in a Gilford spectrophotometer. The enzyme activity was calculated using the formula:

$$\text{Units } (\mu\text{M pNP}) = \frac{\text{E } 400 \text{ nM - blank}}{18.8/\text{ml reaction mixture}} \times \frac{1}{60 \text{ min}}$$

2. Succinate Dehydrogenase and Cytochrome-*c* Oxidase

Succinate dehydrogenase or cytochrome-*c* oxidase was used as a marker enzyme for mitochondrial activity.

Succinate dehydrogenase was assayed by the method of Slater and Bonner (1952). The rate of succinate dehydrogenase reduction of 0.001 M $K_3Fe(CN)_6$ was measured spectrophotometrically in the presence of 0.01 M KCN, which inhibited cytochrome oxidase, by following the rate of optical density decrease at 400 mμ. The assay total volume was 1.0 ml and contained 0.1 M $K_3Fe(CN)_6$ (Fisher Scientific Co.), 0.1 ml 0.1 M KCN (Fisher Scientific Co.), 0.1 ml of 0.15 M sodium succinate (Fisher Scientific Co.), 0.3 ml of 0.5 M phosphate buffer, pH 7.24, enzyme preparation (about 0.1 mg protein) and water to equal a final volume of 1.0 ml. Each experimental sample was read at 400 mμ in a Gilford 240 recording spectrophotometer against a control cuvette which contained 0.3 ml of phosphate buffer, enzyme and water to a final volume of 1.0 ml. The average rate between 1 and 5 minutes was used to calculate the activity according to these equations:

$$\text{Rate } (\Delta A)/\text{ml} = \frac{\Delta \text{O.D./min} - \Delta \text{blank/min}}{\text{aliquot}}$$

$$\text{Rate } (\Delta A)/\text{mg protein} = \frac{\Delta \text{O.D./min} - \Delta \text{blank/min}}{\text{aliquot}} \times \frac{1}{\text{mg protein}}$$

Cytochrome-*c* oxidase was tested according to Heidrich *et al.*, (1972).

3. Renin

There are two general methods for measurement of kidney renin in tissue and biological fluids: bioassay and radioimmunoassay. Of these two methods, only radioimmunoassay was suitable for kidney tissue for these reasons: many pressor and depressor substances are made in the kidney and these could affect the animal used for bioassay; many samples can be analyzed at once by radioimmunoassay, whereas only a few can be measured by bioassay; and the radioimmunoassay is much more sensitive and reproducible than the bioassay.

In all studies, kidney renin was measured by an indirect method which was specific for the first product of renin reacting with its substrate, i.e., specific for the formation of A I. This product was analyzed by a radioimmunoassay developed by Haber _et al_. (1969), but substantially modified to increase sensitivity for experiments with cells and small amounts of renal tissue.

a. _Reagents_. Inhibitors of "converting enzyme" and "angiotensinases": EDTA (J. T. Baker Chemical Co., Phillipsburg, N. J.) 10%, pH 6.5-15 µl for every ml of blood or tissue solution; BAL (Hynson, Wescott and Dunning, Inc., Baltimore, Maryland) 100 mg/ml in 680 mg peanut oil and 210 mg benzyl benzoate - 2 µl for every ml plasma or tissue solution; 8-quinolinol sulfate (Eastman Kodak Co.,

Rochester, N. Y.) 0.34 M-10 µl for every ml plasma or tissue solution; and incubation diluent buffer - 0.1 M tris buffer adjusted to pH 7.4 with glacial acetic acid.

Radioimmunoassay reagents were: radioimmunoassay (RIA) buffer--0.1 M tris buffer containing 0.25% BSA, adjusted to pH 9.0 with glacial acetic acid (E. R. Squibb and Sons, Inc., Princeton, N. J., catalog number 09509) stored frozen until use to prevent excessive bubbling in the Micromedic automatic pipette; antiserum to A I (E. R. Squibb and Sons, Catalog number 09507)--a large amount of one lot of antiserum that gave a bound/free (B/F) of above 1.0 and showed a steep standard curve was bought, diluted 1:2 or 1:3 and 50 µl was added per assay tube; ^{125}I-A I--The concentrated solution provided in the commercial radioimmunoassay kit was diluted so that a constant amount, 4-6 picograms per 50 µl (3,000 cpm) was added to the assay and 50 µl of this solution was added to each assay tube; standard A I--7-100 picograms of the 100 pg/10 µl solution provided in the commercial kit was added to the assay standard curve; charcoal solution--3.75 grams of the neutral charcoal provided in the commercial kit was added to barbital buffer, consisting of 7.65 grams NaCl, 1.471 grams sodium barbital, 0.9714 grams sodium acetate $\cdot 3H_2O$ in 1 liter of distilled water adjusted to pH 9.0 with 1 N HCl.

b. Preparation of renin substrate. Bilaterally nephrectomized male rabbits were kept for three days without food or water, and were then exsanguinated by cardiac puncture. The blood was added to chilled tubes containing 10% EDTA, 15 µl/ml blood, and centrifuged at 2,500 x g for 20 minutes. The pH of the separated plasma was adjusted to 5.5 was stored frozen at -20° C. To assess the maximum endogenous renin activity of the plasma, an aliquot of each sample was incubated for 3 hours at 37° C according to the procedure outlined in III.A.3.c. (without homogenate), and assayed for renin activity by radioimmunoassay. Plasma with 2.0 ng A I/ml/3 hours or less was pooled and was as substrate for the formation of A I.

c. Incubation of cell homogenate with plasma substrate.

(1) α-2 globulin-leu-leu-tetradecapeptide $\xrightarrow{\text{Renin}}$ decapeptide (A I)

(2) A I $\xrightarrow{\text{"Converting enzyme"}}$ octapeptide (A II)

(3) A II, A I $\xrightarrow{\text{Angiotensinases}}$ small inactive peptides

Renin is an endopeptidase which cleaves the leucine-leucine bond in a tetradecapeptide portion of a plasma alpha - 2 globulin to form the decapeptide A I [equation (1)]. A I is further cleaved by "converting enzyme," located primarily in lung, to an octapeptide A II [equation (2)]. This

peptide is a potent pressor agent, and is inactivated by "angiotensinases" located in most tissues and in plasma [equation (3)].

Kidney tissue renin is very quickly degraded by lysosomal proteolytic enzymes if the tissue has been disrupted. To protect the renin from these proteolytic enzymes, all disrupted tissue samples were immediately resuspended in 0.01 M BES pH 7.15 in 0.9% saline containing two chelating agents, 0.0034 M 8-quinolinol sulfate and 0.15% EDTA, and a sulfhydryl inhibitor, 0.2 mg BAL/ml tissue, and stored at -70° C. The tissue, or supernatant from cells, was always incubated with substrate within a few days of the experiment, since it was found that there was a marked decrease in renin activity with time, even if samples were stored at -70° C. The decrease in activity was less if the renin was protected as described, e.g., cells (table 1).

Before combining the enzyme renin with its substrate, inhibitors were added to the substrate and to the enzyme, if it was tissue renin, to prevent conversion to A II and destruction of the peptide product. BAL, 8-quinolinol sulfate and EDTA (see a. Reagents) were most effective in this regard and did not inhibit the renin-substrate reaction (Haber et al., 1969). The reaction, in 0.5 ml total volume, was started by incubating the

enzyme-substrate mixture at 37° C, pH 5.5. To determine the amount of enzyme protein needed to give reproducible renin-substrate incubations, the linearity of the enzyme-substrate reaction with time and increase in enzyme (protein) concentration was determined. Rabbit kidney cortical homogenate was diluted in 0.01 M BES-0.9% saline, pH 7.15, to a concentration of 0.42 mg/ml and inhibitors were added as described above. 10 µl, 25 µl and 50 µl of this mixture was added to plasma from nephrectomized rabbits in a final volume of 0.5 ml. Likewise, 50 µl, 100 µl and 200 µl of supernatant from a rabbit renal cortical cell suspension, which had been incubated for 20 minutes at 37° C for 5, 10, 15, 20, 25, 30, 45, 60, 90 minutes, 2 hours and 3 hours. It was found that the reaction was linear up to 30 minutes when cell protein was used (figure 1) and was linear only with low amounts of protein in supernatants of cell suspensions (figure 2). Therefore, all incubations were stopped at 30 minutes. The reaction was stopped by chilling the mixture and adding an equal volume of cold 0.1 M tris acetate buffer, pH 7.4. Larger proteins in the mixture were removed by boiling for 10 minutes, centrifuging at 2,500 x g 15 minutes and decanting the supernatant. This supernatant was diluted 1:1 with RIA buffer and 50 µl was added to the radioimmunoassay for A I. If necessary, the supernatant was further diluted with RIA

buffer. If the samples were not assayed immediately, they were stored at -20° C. A "blank" of plasma without added enzyme was included in all incubations and was subtracted from unknowns incubated with enzyme.

The recovery of A I using this method of incubation was measured by adding a known amount of standard A I (Squibb) to plasma substrate without enzyme and incubating as described. Recovery was calculated according to the equation:

$$\frac{\text{Actual recovery}}{\text{Expected recovery}} \times 100$$

The recovery of angiotensin I was found to be 85-95%.

d. Radioimmunoassay of A I. Since very small amounts of renal tissue were used in all studies, the basic radioimmunoassay of Haber *et al*. (1969) was modified to detect from 1 to 100 picograms of A I. The commercial radioimmunoassay kit from E. R. Squibb and Sons was found to give identical values for all samples and provided antiserum of sufficient binding capacity to give a reproducibly steep standard curve.

The assay total volume was 250 µl. For the standard curve, 0.7, 1.4, 2.8, 5 and 10 µl of standard A I was added to 10 x 75 mm Falcon polystyrene (catalog number 2038) tubes with Unimetrics 1, 5 and 25 µl syringes. Radioimmunoassay buffer was added with a Micromedic auto-

matic pipette to a final volume of 250 µl. For the unknown samples, 50 µl of the sample and 50 µl of a 1:1 dilution of the sample were used as duplicates in the assay and buffer was added to a final volume of 250 µl. Both sample and buffer were added with the Micromedic. Fifty µl of ^{125}I-angiotensin I and 50 µl of antibody were added with Hamilton repeating dispensors. The tubes were agitated well, wrapped in aluminum foil and incubated at 4°C for 24 hours. The bound moiety was separated from the free moiety by quickly adding 250 µl of charcoal solution to each tube with an Eppendorf 250 µl pipette, centrifuging at 2,500 x g for 15 minutes in the cold and separating a 350 µl aliquot of the supernatant from the charcoal pellet, using the Micromedic automatic pipette. Both bound (supernatant) and free (charcoal) moieties were counted for 4 minutes in a Nuclear Chicago gamma well-scintillation counter with teletype-punch tape attached. The picograms A I in each unknown was calculated on a Sigma VII computer using a BASIC program for Scatchard regression analysis. A typical standard curve is shown in figure 3.

The total and specific activities of unknowns were calculated with a Wang 720C computer program using the basic formulae:

(A x B - C)D = total activity, ng/total volume

$$\frac{(A \times B - C)D}{E} = \text{specific activity, ng/mg protein}$$

where:

A = ng angiotensin I

B = correction factor for aliquot of incubation

C = plasma blank (no enzyme)

D = correction factor for aliquot of enzyme put into the incubation mixture from the total volume of the "unknown" sample

E = total protein, mg protein/sample.

e. Determination of ^{125}I-A I specific activity. The concentration of labelled A I used becomes important in an assay detecting small amounts of A I, and in order to obtain linear Scatchard regressions it is necessary to calculate the specific activity of each new lot of ^{125}I-A I. The isotope dilution method of Smigel and Fleischer (1974) was used to determine this value. Briefly, the amount of antibody was the same as in the radioimmunassay, and the total radioactivity was increased. The assay was incubated, separated and counted as described in III.A.3.d. Binding of radiolabelled angiotensin with antibody varied according to the formula:

$$\frac{(1 + S)}{ES} \frac{1}{\mu l_i} = \frac{S_T}{E_T} + \frac{K_S}{E_T} \frac{(1 + ES)}{S} \frac{1}{\mu l_i}$$

where:

S_T = amount of ^{125}I-A I in 1.0 ul of solution

μl_i = amount of radioactive angiotensin added to the assay

K_s = dissociation constant of the antibody

E_T = saturation binding capacity of the antibody

S = unbound angiotensin

E = unbound angiotensin receptor

S/ES = ratio of free/bound angiotensin

K_s/E_T = slope

S_T/E_T = intercept

A typical plot of experimental data is shown in Figure 4.

f. Correlation of bioassay with radioimmunoassay plasma renin values. Although bioassay of renin was not used in any experiments, the values obtained by both methods were checked for correlation. Nine samples of human plasma were analyzed for renin activity by bioassay and by radioimmunoassay. The bioassay method of Boucher *et al.* (1964), which measured A II, was used. Plasma was adjusted to pH 5.5, and contained 15 µl of 10% EDTA pH 6.5/ml plasma. It was incubated 3 hours at 37° C in siliconized glass 50 ml Erhlenmeyer flasks with 0.7 ml Dowex 50 W-X_2/ml plasma added to protect the angiotensin II from angiotensinases. The incubated mixture was transferred into a non-siliconized glass chromatographic column filled with 1.5 ml washed Dowex 50 W-X_2 over glass wool. The flask containing the mixture was rinsed with 15 ml of each

of the following reagents and sequentially transferred to the column: 0.2 M ammonium acetate, pH 6.0, 10% acetic acid, and distilled water. The column was eluted with 15 ml each of 0.1 N diethylamine and 0.2 N ammonium hydroxide and collected into a 100 ml non-siliconized boiling flask containing 0.6 ml glacial acetic acid. The eluate was evaporated to dryness in a flash evaporator at 48° C under less than 1 mm mercury vacuum. The ammonium acetate was removed by dissolving the dry residue in 4 ml 80% ethanol and evaporating to dryness as before. This procedure was repeated until no more cystals formed in the flask, i.e., about 7 times. The dry residue was dissolved in 2 ml of 0.9% saline which contained 0.1% polyvinylpyrrolidine (PVP) and 0.2% neomycin. The samples were then stored in 10 x 75 mm non-siliconized glass tubes at -20° C until bioassay.

The standard angiotensin used in the bioassay was prepared from 2.5 mg pure dry A II (Hypertensin, Ciba). A 1 mg/ml solution was prepared in 0.9% saline containing 0.1% PVP (to prevent angiotensin from sticking to the glass) and 0.2% neomycin (a preservative) and was diluted 1:100,000 to give a solution 0.1 ng/µl.

16 - 24 hours before the bioassay, 140 to 240 gram male Holtzman rats were nephrectomized by flank incision. At the time of the bioassay, the animal was anesthetized with pentobarbital and urethane; supplemental anesthesia

was given as required during the assay. The trachea was cannulated with 2 cm of PE 205 tubing; one jugular vein was cannulated with two 15 cm lengths of PE 10 tubing filled with heparinized saline (100 units/ml), and on the opposite side of the neck a carotid artery was cannulated with a 20 cm length of PE 50 tubing filled with heparanized saline. The animal was given 1% pentolinium, i.m. (0.125 ml/100 gram) to prevent sympathetic compensatory reflexes during the assay. After 30 minutes the bioassay began. Blood pressure was measured from the arterial cannula by a Statham transducer attached to a Grass polygraph. A response of 5 mm or more (from baseline to the bottom of the peak tracing) to 0.4 ng of standard A II was required for an adequately sensitive assay. When this was achieved, 0.2 ng of standard was injected i.v. and enough unknown was injected to match the pressor response of the standard within 1.5 mm. This was repeated for 0.6 ng and, if possible, for 0.8 ng of standard A II. The exact amount of A II in the unknown was calculated by interpolation.

4. Other Enzyme Assays

In studies with subcellular fractions from rat kidney, marker enzymes were used to estimate the contamination of each purified fraction with other subcellular fractions. The methods for marker enzyme determinations have been well described in the following references:

Ouabain-sensitive ATPase (Fleischer and Zambrano, 1974) was used as a marker enzyme for plasma membrane; succinate-cytochrome c reductase (Fleischer and Fleischer, 1967) was used as a marker enzyme for kidney mitochondria, glucose-6-phosphatase (VanGolde et al., 1971) was used as a marker enzyme for endoplasmic reticulum; galactosyltransferase was used as a marker enzyme (Fleischer et al., 1969) for the Golgi apparatus.

5. Protein

Protein was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumen (BSA) (Sigma, Fraction V) as a standard. The blue color was read at 750 nM in a Gilford spectrophotometer. For routine determination of cell protein, each sample was either sonicated 5 seconds with a Heat Systems Model W140 sonifier cell disrupter or was solubilized by addition of 0.1% sodium dodecyl sulfate (lauryl sulfate) (SDS) (Fisher) to the Folin A reagent. 8-quinolinol sulfate interfered with the protein assay and was not added until protein had been determined. For samples obtained from the free-flow electrophoresis, all trace of the triethanolamine buffer was removed from the fractions, since it interfered with the protein assay, and the cells were resuspended in 0.01 M BES (Sigma) in 0.9% saline, pH 7.15 before protein determination.

6. CAMP Assay

There are several methods for measurement of CAMP which are quite sensitive in the range of 1-2 picomoles CAMP. The method of Steiner *et al.* (1969) was not used, although it was an easy and specific radioassay which required 2-10 mg wet weight tissue. However, the antibody was difficult to make and by the time this assay was commercially available, assays had already been done for CAMP studies using the method of Gilman (1970). The Gilman method was a competitive protein binding assay that was sensitive in the range of 1-2 picomoles CAMP and required only a few miligrams of tissue. This method was rather tedious but very reproducible once mastered, and since the binding protein (protein kinase) had already been prepared, further studies utilized this method.

a. "*Unknowns.*" Cells in fractions obtained from cell separation were incubated at 37°C, as previously described, III.A.3.c. At the end of incubation, further formation and degradation of CAMP was stopped by immediate addition of a proper volume of ice-cold concentrated (12 N) perchloric acid, making a final concentration of 0.3 N. Samples were agitated well and immediately frozen in liquid nitrogen. Cells were then broken by rapid freeze-thaw three times to release the CAMP. Each sample was then analyzed for CAMP

by Gilman's method (1970). Before assay, CAMP had to be separated from other nucleotides by column fractionation.

b. Column procedure. One pound of Bio-Rad analytical grade exchange resin, AG 50 W - x 8 (100-200 mesh, hydrogen form) was stirred into 1 liter of distilled water, allowed to settle, and decanted. This was repeated until wash water was no longer pink. A slurry of the resin was made in distilled water and added to an 8 cm diameter column with scintered glass filter attachment and spring fasteners. After the water ran off, 3 liters of 0.5 N NaOH was added followed by 3 liters of water (color disappeared), followed by 4 liters of 2 N HCl, a wash of 4 liters of distilled water, and 3 liters of 0.05 N HCl (the last liter was made up in glass distilled water, as were all solutions throughout the procedure). The prepared Dowex-50 was stored refrigerated in 0.05 N HCl and could be kept indefinitely this way.

Commercial ^{3}H-CAMP (New England Nuclear adenosine-^{3}H-3',5'-cyclic phosphate, ammonium salt, Lot #642-178; specific activity 22.1 curies per mM; 0.25 mCi in 0.5 ml 1:1 ethanol:water, stored frozen at -40°C) was packaged in a 1:1 (v:v) ethanol: water solution. The ethanol volume had to be reduced so that it could not interfere with the assay. The ^{3}H-CAMP was purified by a column procedure to remove contaminating $^{3}H_2O$, ^{3}H-3'-AMP, ^{3}H-5'-AMP

and ^{3}H-adenosine from the CAMP. The ethanol was measured and an equal volume of 0.2 N HCl was added, making a final concentration of 0.1 N HCl. This solution was then diluted to 2 ml with 0.1 N HCl. The solution was applied to a 0.7 x 20 cm Dowex-50 column. When the sample was down into the resin, 2 ml of 0.1 N HCl was added as a wash and collected in a "rinse vial." When the wash was through the resin, the column resevoir was filled with excess 0.1 N HCl and 2 ml fractions were collected (80 ml of effluent). Fifty µl of each fraction was counted in 10 ml of Bray's scintillation liquid; counts were plotted to locate the $^{3}H_2O$ peak and the ^{3}H-CAMP peak (figure 5). The CAMP fractions were pooled, neutralized to pH 7.0 with 1 N NaOH, divided into 2 ml aliquots and quickly frozen in an acetone-dry ice bath. Stock ^{3}H-CAMP was stored frozen at -40°C and diluted as needed.

Calibration of columns for CAMP. A standard calibration solution was made by diluting 10 mM AMP, ADP and ATP 1:10 with glass distilled water (1 mM) and adding 10,000 cpm of ^{3}H-CAMP. Volume of the mixture was the same as sample volumes were expected to be (2 ml). This standard calibration mixture was applied to the size column to be used for ordinary samples--0.7 x 20 cm of prepared Dowex-50W AG x 8 H^{+}-resin. It was washed through with an equal volume of 0.1 N HCl, which was discarded, and was

then eluted with 36 ml of 0.1 N HCl and collected in 2 ml fractions. Fifty μl of each fraction was counted in a Packard Tri Carb beta scintillation counter in 10 ml of Bray's scintillation cocktail. Each fraction was also read at 259 mμ in a Beckman DU spectrophotometer to determine the location of other nucleotides. The O.D., cpm and fraction number were plotted (figure 6) and from this graph the amount of eluate to discard and the amount of eluate to save was determined.

CAMP column procedure for renal cortical cell fractions. To check recovery of CAMP from the columns, 10 μl containing 10,000 cpm ^{3}H-CAMP was added to each 2 ml sample, making a final tracer concentration of 1,000 cpm/50 μl (50 μl was taken from each sample after the column was run and counted in 10 ml Bray's cocktail). Samples were then added to a 0.7 x 10 cm Dowex-50 AG x 8 column and rinsed through with an equal volume of 0.1 N HCl to remove the perchloric acid. They were then eluted with 10 ml 0.1 N HCl, which was discarded, and with 18 ml 0.1 N HCl, which was collected into siliconized pear-shaped flasks (Kontes). The eluate was shell frozen and lyophilized overnight; the residue was taken up in two washes 0.25 ml each (total 0.5 ml) of freshly made 0.05 M acetate buffer pH 4.0. These samples were then transferred to polypropylene 12 x 75 mm tubes with a pasteur pipette and stored frozen until assay.

c. <u>The binding assay</u>. Components of the binding assay were: ^{3}H-CAMP; cold standard CAMP in a series of dilutions for the standard curve, or "unknown" sample CAMP; binding protein and buffer. Preparation of these components are described below. Glass distilled water was used in all preparations to avoid contamination with bacterial CAMP.

Preparation of ^{3}H-CAMP was described in the column procedure, III.A.6.b.

Preparation of unlabelled CAMP standards. A stock solution of 1 x 10^{-3}M was prepared using CAMP (Sigma 0.03292 g/100 ml). This was diluted 1:20, to 5 x 10^{-5}M (50% of the molar extinction coefficient of CAMP), measured 259 mμ at pH 7.0 in a Beckman DI Spectrophotometer and the correct molarity was calculated. This solution was diluted separately (i.e., not serial dilutions) with 0.1 M acetate buffer, pH 4.0, to obtain appropriate standard concentrations for the assay--1 x 10^{-6}M, 1, 2, 3, 5 and 7 x 10^{-7}M.

Preparation of "unknown" CAMP samples-- see III.6.a.

d. <u>Preparation of rabbit muscle protein kinase using protamine sepharose</u>. Back and leg skeletal muscles were excised from two large rabbits, were placed on ice and passed through a meat grinder and weighed. The tissue was then homogenized in a Waring blender for 1 minute at its highest speed with a solution (1:2, w:v) of 40 mM potassium

phosphate (KP_i) and 4 mM EDTA, pH 6.5. The homogenate was centrifuged at 10,000 rpm for 30 minutes in a Sorvall RC-3 refrigerated centrifuge (SS 34 rotor). The supernatant was added to a 4.5 x 80 cm protamine-sepahrose column, equilibrated, and washed with 20 mM KP_i, 2 mM EDTA, pH 6.5. This was allowed to stand overnight and was then washed with several liters of KP_i-EDTA (above molarity and pH) buffer until protein began to appear (checked by monitering O.D. at 280 mμ). The column was eluted with a linear gradient of: 2 liters of 20 mM KP_i, 2 mM EDTA pH 6.5 and 2 liters of 20 mM KP_i, 2 mM EDTA pH 6.5, and 2 M NaCl. Ninety 20 ml fractions were collected with a peristaltic pump attached to the column, and each fraction was checked for protein and CAMP (50 mM acetate buffer pH 4.0, 5 picomoles ^{3}H-CAMP and 20 μl from each fraction).

The protein kinase binding of ^{3}H-CAMP occurred in two peaks as seen in figure 7. The fractions with the highest binding were dialyzed overnight in a continuous flow dialysis vessel using 5 mM KP_i buffer pH 7.5 with 2 mM EDTA. Since the dialysate was cloudy, it was centrifuged at maximum rpm in a Sorvall RC-2B, SS 34 rotor for 60 minutes. The supernatant was then divided into 25 x 100 mm tubes and frozen at -70° C. No dilution was necessary for assay.

e. <u>Assay procedure</u>. 10 x 75 mm Kimax glass culture tubes in racks were placed in ice and numbered so that duplicates were the first and the last tubes in the rack. This was a measure of the timing error caused by addition of assay components. Each assay component was added in this order: CAMP as either standard (10 µl of 5×10^{-8}M, 1, 2, 3, 5 and 7×10^{-7}M and 1×10^{-6}M), or sample (50 µl); acetate buffer, 0.1 M pH 4.0 to make the reaction mixture to a final volume of 110 µl; 10 µl ^{3}H-CAMP, which contained 1 picomole CAMP, i.e., about 11,000 cpm; 50 µl of a mixture consisting of albumen (1 mg/ml BSA in water), concentrated protein kinase and glass distilled water in a ratio of 20:20:10 µl/tube. The first two tubes of the assay represented a "blank" for background counts: 10 µl glass distilled water, 50 µl acetate buffer, and 50 µl of mixture. The second set of duplicates represented a "0% competition": 10 µl of ^{3}H-CAMP, 50 µl acetate buffer and 50 µl mixture. The next 7 sets of duplicates represented the standard curve: 10 µl of various standard solutions, 10 µl ^{3}H-CAMP, 50 µl acetate buffer and 50 µl of mixture. The remaining duplicates were samples containing an unknown amount of CAMP: 50 µl sample (no acetate buffer), 10 µl ^{3}H-CAMP, and 50 µl of mixture. Tubes were well agitated after addition of cold and hot CAMP

and after addition of the kinase mixture. They were then incubated on ice in the cold room for 1-2 hours and the contents of each tube were emptied onto a Millipore filter (HAWP 02500, 0.45 μ, 25 mm) which had been moistened with a few drops of cold 20 mM phosphate buffer pH 6.0, under vacuum. The tube was rinsed with 9 ml ice cold 20 mM phosphate buffer. The vacuum pump was turned off as soon as the wash ran through, and the filter was placed in 10 ml Bray's solution, allowed to dissolve, mixed well, and counted 10 minutes in a Packard Tri-Carb beta counter.

A "standard recovery" was prepared using 10 μl of ^{3}H-CAMP (11,000 cpm) in 2 ml water and was well mixed. Fifty μl of this was added to 10 ml Bray's cocktail and counted 10 minutes in the beta counter. ^{3}H-CAMP recovery of each sample after the column procedure was determined by adding 50 μl from each sample to 10 ml Bray's cocktail and counting 10 minutes in the beta counter; the ^{3}H-CAMP sample counts were compared to the "standard recovery" counts.

The amount of CAMP in picomoles in each sample was calculated after correcting for column recovery. The samples were calculated by using a BASIC computer program which compared unknown values to standard curve values obtained by linear regression.

7. PGE Extraction and Assay

Extraction and assay for the E prostaglandins in tissue and biological fluids has been described by Frölich *et al*. (1974). Their method was utilized to assess the endogenous levels of PGE_2 in rabbit kidney cortex and to estimate the ability of the cortex to form PGE_2 from arachidonic acid, its precursor.

a. Preparation of arachidonic acid salt. Twenty-five mg of arachidonic acid, 99% pure (lot Y-70A-YY from Nu-Check Prep, Inc.) was dissolved under nitrogen, in 5 ml ice cold 100% ethanol (U.S. Industrial Chemical Co., N.Y.). 1.0 ml of this solution was added to 0.3 ml ice cold distilled water and 2 drops of 1% phenolphthalein in 100% ethanol under nitrogen. The solution was then titrated on ice, under nitrogen, with 3 drops of 0.1 N NaOH and about 14 drops of 0.01 N NaOH, until the solution was slightly pink. This was evaporated to dryness under nitrogen at 32° C and stored overnight at -20° C. The next morning it was reconstituted in 5 ml 80% ethanol and used in rabbit kidney cortical cell suspensions, at a final concentration of 0.1 µg/ml, as a precursor to PGE_2. This salt was prepared because it was soluble in aqueous buffers, but the original compound was not.

b. Extraction of PGE_2 from renal cortical cells. (All solvent mixtures expressed as v:v). 3,000 dpm ^3H-PGE_1 (New England Nuclear) was added as tracer to the samples, which contained about 2.5 mg protein in about 30 ml, and the tissue was sonicated 5 seconds with a Heat Systems Model W140 sonifier. An equal volume of 100% ethanol was added to destroy enzymes which form and degrade PGE_2, i.e., to precipitate the protein, and an equal volume of distilled water was also added. The samples were centrifuged at 1,500 x g in a Sorvall RC-3 centrifuge and the supernatants were put into 250 ml separating funnels. These supernatants were washed twice with petroleum ether (Allied Chemicals, Reagent grade) to remove the neutral lipids, and the petroleum ether was discarded. The pH of the samples was then adjusted to 3.5 with concentrated formic acid (Matheson, reagent grade), and were quickly shaken about 5 minutes with 100 ml chloroform (Matheson, spectrophotometric quality) to extract the prostaglandin into the chloroform. The chloroform was removed by flash evaporation at 32° C and the samples were reconstituted in 100:100:30:2 chloroform:heptane:ethanol:acetic acid, added to Sephandex LH-20 (Pharmacia) 1 x 13 cm columns and eluted with 20 ml 100:100:30:2, chloroform:heptane:ethanol:acetic acid mix. The first 10 ml was discarded and the second 10 ml was

collected. The solvent was removed by flash evaporation at 32° C, and the samples were taken up in 1:9 ethyl acetate:toulene (both from Fisher Scientific). They were then added to 1 gram silicic acid (Fisher) columns to remove PGA_2, a metabolite of PGF_2, and sequentially eluted with 35 ml each of the following ratios of ethyl acetate: toluene, 1:9, 3:7 (PGA_2 came off here), 4:6, and 6:4. The last elution was collected and the solvent was removed by flash evaporation. The samples were rinsed with 100% ethanol and flash evaporated five times to insure that they were free of any residual solvent. They were taken up in 100 µl of 100% ethanol; for recovery, 10 µl was added to 7 ml of Triton-toluene scintillation cocktail (2 liters toluene, 666 grams Triton x-100, 10 grams PPO and 1 gram POPOP) and counted 10 minutes in a Nuclear Chicago Mark II liquid scintillation counter. The recovery from the column procedure was 76-95%. The samples were stored under nitrogen in teflon-lined screw cap vials at -20° C until assay.

c. Assay of PGE_2. To two 2 ml cellulose nitrate centrifuge tubes (5/16 x 1 15/16 inches, Beckman 303369) was added 25 µl 0.3 M NaCl-0.02 M PIPES buffer, pH 6.25, 15 µl water, 1 µl ^3H-PGE_2 (New England Nuclear, approx. 50 Ci/mM) in ethanol containing from 0-100 pg nonradioactive PGE_2, and, last, 15 µl of rat liver plasma membrane

preparation (Smigel and Fleischer, 1974) of about 10 mg protein/ml. The contents of the tubes were agitated for 5 minutes and incubated at 37° C for 60 minutes. They were then placed on ice, and centrifuged 10,000 rpm for 5 minutes in a Spinco Ti-50 rotor using plastic adaptors. Forty µl of the supernatant was added to 5 ml Triton-toluene scintillation fluid. Total radioactivity was determined using control tubes with 15 µl of water substituted for the membrane suspension. The amount of PGE_2 in the samples was estimated by calculation of the amount of binding of ^3H-PGE_2 to plasma membrane in standards and samples by Scatchard regression analysis.

B. Selection of Species for Preparation of Isolated Cells

In several experiments, cell preparations from rabbit, cat, and dog kidneys were compared with respect to: yield of isolated cells; contamination with tubule fragments and glomeruli; cell viability; size and anatomy of the kidney; availability of the animal; and the condition of its kidneys.

1. Yield of Isolated Cells

The wet weight yield of isolated cells was approximately the same in all three species for the collagenase

dissociation method, i.e., about 6% of the cortical wet weight. Only rabbits were used for the other two methods.

2. Contamination with Tubule Fragments

Cell preparations from rabbit kidneys contained more tubules than those from dogs or cats. However, sequential filtering through 40 μ and 20 μ nylon monofilament meshes (Nitex, Griffin Supply Co., Nashville, Tennessee) completely removed the tubules from the cells.

3. Cell Viability

Viability of cell preparations from all three species was determined by 0.1% trypan blue exclusion and oxygen consumption (see below).

4. Size and Anatomy of Kidneys

The weight of one kidney from a rabbit was 12-25 grams, from a cat, 11-17 grams and from dog about 34 grams. Cat and dog kidneys had slightly more cortex than rabbits, but rabbit cortex contained more renin than renal cortex from dogs or cats (Schaffenburg *et al*., 1960). They were therefore the animal of choice for experiments using cell suspensions. The renal capsule was quite thick in dogs and thinner in cats and rabbits. Thus pressure perfusion was more effective in dogs than in the other two species.

5. Availability and Uniformity of the Animal

Dogs and rabbits were easily obtained from commercial suppliers, but cats were often unavailable. As might be expected from mongrel animals, dogs and cats were extremely variable and often had diseased kidneys, a problem which was circumvented by using young animals. Rabbits were the most available and uniform animal, but only animals that were under 4 kg were used because older animals often had damaged kidneys (see III.C.2.a.). Young mongrel dogs were used for some experiments, but in most experiments 2-3 kg New Zealand white rabbits were used. Male Holtzman albino rats, 200-250 grams, were used for batch preparation of subcellular organelles.

In all studies, male animals were used because they had less extrarenal renin-like substances in their tissues (Smeby and Bumpus, 1968). Animals were allowed access to standard laboratory chow (Purina Food Company) and water *ad libitum* except in those experiments where indicated otherwise.

C. Methods of Cell Isolation

1. Preparation of Kidney Cortical Cell by Enzymatic Dissociation

a. *Materials*. Young male dogs, cats or rabbits were used in all experiments.

Solutions were kept oxygenated with 95% O_2-5% CO_2 at 4° C. Three perfusing solutions were used: 0.9% sterile saline (Cutter); Spinner-modified (calcium-free) Eagles medium without glutamine (Baltimore Biological Laboratory, Baltimore, Maryland) with 2.6 mM sterile l-glutamine (Grand Island Biological Company, New York) pH 7.2; and 0.15-0.3% (depending on the lot) crude collagenase (originally from Nutritional Biochemicals Corp., Cleveland, Ohio, and later from Worthington Biochemical Corp., Freehold, N. J.) made up in Eagles medium.

b. Procedure. The procedure used was a modification of that used by Burg and Orloff (1962). Dogs, cats, or rabbits were anesthetized with pentobarbital and their kidneys were removed through a midline abdominal incision, placed in ice-cold 0.9% saline and the renal artery was cannulated with the appropriate size polyethylene tubing. The kidneys were perfused with 0.9% saline until the solution was clear. They were then perfused with Eagles medium and the renal veins were clamped. The kidneys were then perfused under pressure with 0.15-0.3% crude collagenase made up in Eagles medium. The capsule was peeled back, the cortex was sliced off with a Stadie-Riggs knife and minced in a cold petri dish containing collagenase solution until the pieces were 1-3 mm^3. The tissue was gently rinsed three times with collagenase solution and

placed in 30-50 ml of the same solution for digestion. This mixture was slowly stirred with a magnetic stirrer for one hour at room temperature under 95% O_2-5% CO_2. It was then filtered through a double layer of 20 x 12 mesh sterile surgical gauze and the filtrate was centrifuged in the cold in round bottom 50 ml plastic entrifuge tubes at 50 x g in an IEC PR-6 centrifuge for 3 minutes. The supernatant was discarded and the pellet was saved and washed (resuspended and centrifuged) with Eagles medium four times to remove the collagenase. The cell pellets were either resuspended in fresh Eagles medium and used for oxygen consumption experiments or were resuspended before density gradient centrifugation.

The entire procedure took about 2 hours but the tissue was without nutrient medium or oxygen only about 15 minutes of that time. Metabolic processes in the tissue were probably slowed because of the cold environment, so that not much nutrient or oxygen was needed.

c. Appearance of the cells and their properties. Cells isolated by this method appeared normal but were only 50-70% viable as judged by 0.1% trypan blue dye exclusion. The procedure produced more tubule fragments than isolated cells, and the cells had a pronounced tendency to clump. These effects were most apparent in cells

from rabbit kidneys and were least troublesome in cells from cat kidneys.

2. Preparation of Rabbit Kidney Cortical Cell Suspensions by Combined Enzymatic and Mechanical Methods

a. Materials. Male New Zealand white rabbits weighing 3-4 kg were used in all experiments. Small rabbits were used because their kidneys were healthier and gave a better yield of isolated cells than larger rabbits.

Calcium and magnesium-free Earles buffer was used in the isolation of cells because it was found that both divalent ions released renin at physiological levels (figure 31 and table 14), and it was thought that more renin would remain in the cells if these ions were left out. The Earles buffer consisted of: 116 mM NaCl, 5.4 mM KCl, 0.09 mM $NaH_2PO_4 \cdot H_2O$, 26 mM $NaHCO_3$, 10 mM dextrose, 2.6 mM l-glutamine and 0.001% BSA (Fraction V, Sigma) in deionized water.

Perfusing solutions were kept at 24° C and were well oxygenated with 95% O_2-5% CO_2. Three solutions were used: 0.9% saline; Earles physiological buffer, pH 7.4, without calcium or magnesium and fortified with 2.6 mM sterile l-glutamine; and 0.2% crude collagenase (Worthington, Freehold, New Jersey, protease-free type III, or trypsin-free, type IV) made up in the Earles buffer. Four hundred,

40 and 20 μ Nitex nylon monofilament meshes were obtained from Griffin Supply Co., Nashville, Tennessee.

b. Procedure. The male New Zealand white rabbits were pre-anesthetized with 8 mg/kg pentobarbital (i.m.) and further anesthetized with ether. A lateral incision was made, each kidney was removed after ligation of ureters, renal arteries and renal veins, and was immediately placed in saline. Within 2-5 minutes, the renal artery was cannulated with 20 mm of beveled PE 50 tubing connected to a disposable 22-gauge needle. The kidneys were then perfused with 10 ml 0.9% saline and 30 ml Earles buffer. The renal veins were clamped and each kidney was slowly perfused with 5 ml warm 0.15-0.3% collagenase solution. The capsule was peeled back and the cortex cut in thin (1-2 mm) slices with a Stadie-Riggs knife, placed in a tared dish containing Earles buffer and weighed. The slices were very gently rubbed through 400 micron nylon monofilament mesh with a Parafilm-covered weighing spoon, while buffer was constantly dripped onto the tissue to remove the collagenase (about 100 ml total volume). This suspension was then sequentially filtered through nylon meshes (40 micron once and 20 micron twice) to remove tubule fragments. The filtrate was centrifuged in 50 ml round bottom plastic centrifuge tubes at 50 x g for 5 minutes in a PR-6 IEC centrifuge.

c. Appearance of cells and their properties. The supernatant, which contained subcellular particles, renin granules and free renin was either discarded or used for preparation of renin granules; the pellet, which contained single cells, some nuclei, and a few small tubule fragments, was resuspended in 2 ml of calcium and magnesium-free Earles buffer or electrophoresis buffer. Clumps of tissue were removed by passing the suspension through glass wool or surgical gauze which had been washed with buffer. The resulting cell suspension was essentially free of subcellular particles and tubules. The cell membranes were intact, and cells 60-80% viable as determined by 0.1% Trypan blue dye exclusion.

The entire isolation procedure took 20 minutes, but the tissue was without nutrient medium and oxygen for only 2 minutes, before cannulation of the renal artery.

3. Preparation of Rabbit Kidney Cortical Cells by Sodium Citrate and Mechanical Methods

a. Materials. Materials were the same as those in III.C.2.a. Sodium citrate, reagent grade, was obtained from Fisher Scientific Company.

b. Procedure. Crude collagenase preparations, even those in which some proteolytic enzyme activity had been removed, contained a mixture of proteolytic enzymes. Trypsin in particular was shown to damage cell membranes (Poste,

1971) and receptors for some hormones located on the membranes (Kono, 1969). Because of this possibility, the method of Jacob and Bhargava (1962), which used no enzymes but instead employed a chelator to dissociate cells from tissue, was modified in the following manner: kidneys were prepared as described in the collagenase preparations but were perfused for about 5 minutes, without clamping the renal vein, with well-oxygenated 0.027 M sodium citrate solution pH 7.4, 25° C, made up in calcium and magnesium-free Earles buffer. The cortex was sliced off with a Stadie-Riggs microtome blade and the tissue was quickly minced with a sharp razor blade until the pieces were about 3 mm^3. The minced tissue was placed in a modified homogenizer made according to the specifications of Jacob and Bhargava (1962) and homogenized carefully by hand with two strokes. The tissue was then quickly diluted with freshly oxygenated calcium and magnesium-free Earles buffer to dilute the citrate and gently rubbed through 400 micron nylon mesh with a Parafilm-covered spoon while about 100 ml of the Earles buffer was continually dripped onto the mesh. The cell suspension was filtered once through 40 μ nylon monofilament mesh, twice through 20 μ nylon monofilament mesh and centrifuged in round bottom plastic 50 ml centrifuge tubes at 50 x g in an IEC PR-6 centrifuge. The supernatant was decanted and the cell pellet was resuspended in Earles buffer without calcium or magnesium, checked for

viability with 0.1% trypan blue and used in various experimental incubations.

The procedure took 20 minutes but the tissue was without nutrients or oxygen only for about 3 minutes.

The cortex could also be sliced thinly and gently rubbed through the 400 μ mesh as in the collagenase preparation, but the two stroke homogenization gave a greater yield of undamaged cells and fewer tubules.

c. Appearance and properties of cells obtained by this method. This preparation gave a very high yield of isolated cells which were 80-90% viable as determined by 0.1% trypan blue exclusion. The cells were not contaminated by nuclei and did not clump at all, as did cells isolated with either collagenase method.

D. Viability of Isolated Cells

1. Oxygen Consumption

One way to estimate cellular viability is to measure the ability of the cells to respire normally. If oxygen consumption is low, metabolic functions of the cells will be lowered. It is likewise important to determine the substrates, or other components, necessary for normal cell respiration so that these substrates can be added to buffers in high enough concentration that they will not be depleted during the experiment.

The oxygen consumption of dog, rabbit and cat renal cortical cells isolated by collagenase digestion was determined using a Warburg respirometer and standard manometric techniques described by Umbreit (1964). In a typical experiment, 0.5 ml of rabbit, cat, or dog kidney cell suspension, containing 100 to 300 mg wet weight of cells, was added to 1.5 ml of oxygenated suspending medium in a 20 ml Warburg Flask (Bronwill Scientific Co.) which had 0.2 ml 20% KOH on Whatman No. 1 filter paper in the center well to absorb all CO_2. The cells were equilibrated under 95% O_2-5% CO_2 atmosphere for 10 minutes while they were shaken at a constant rate at 37.5° C. All stopcocks were closed, manometers adjusted to 0 and oxygen uptake was recorded at 4 minute or 15 minute intervals for 30 minutes or one hour. The data was calculated for interval uptake of oxygen according to the formula:

$$x = h \frac{Vg(273/T) + Vf\alpha}{Po}$$

where:

x = µl of gas

Vg = volume of gas phase in flask and connecting tubes to the reference point on the manometer

Vf = volume of fluid in the vessel

Po = standard pressure, i.e., 760 mm Hg

T = temperature of bath in absolute degrees (273 + °C)

α = solubility of oxygen in the reaction liquid.

The data was expressed as µl O_2/gram wet weight or µl O_2/gram wet weight/minute.

2. Trypan Blue Dye Exclusion

0.3% trypan blue solution was prepared in Earles buffer and one drop of this solution was added to freshly prepared cells, giving a final concentration of about 0.15%. This method indicated whether a cell was dead or alive by staining the nucleus of the dead cell. However, it was not a very critical test of viability.

E. Separation of Kidney Cortical Cells: Enrichment into Cell Types

There are several methods available to separate cells into specific types. These methods utilize physical properties that differentiate one cell from another. These properties may be inherent to the cell; examples are an increased density because of a large nucleus or the presence of cytoplasmic granules, low density caused by cytoplasmic lipid droplets or by a small nucleus and much cytoplasm, or a difference in membrane charge. The physical properties may also be given to the cell by its surroundings. Some examples of these are cell shrinkage or swelling with exposure to a hyper- or hypotonic medium, or changes in

charge by absorption of ions to cell plasma membranes.

Two methods for cell separation were used to enrich renin-containing cells. These methods were buoyant density centrifugation with discontinuous gradients, and free-flow electrophoresis.

1. Buoyant Density Separation

Cells from dog or rabbit kidneys were dissociated using the collagenase digestion method. Since the procedure was very long, the cells were prepared at room temperature, gradually adapted to 4° C and kept at this temperature so that cell metabolism would be decreased enough to preserve viability to the end of the procedure. To insure constant osmolarity, discontinuous gradient solutions were made with Ficoll (Pharmacia), a high molecular weight compound which has no capacity to bind ions and well-oxygenated Eagles medium. The Ficoll gradients were made by slowly pumping each layer onto the next with a Harvard infusion pump. The cells, in freshly oxygenated Eagles medium, were added last and the gradients were centrifuged for 10,173 x g for 60 minutes in a Beckman ultracentrifuge (SW 25.1 rotor). Fractions were collected by pumping the gradients out through a conical Teflon cap that fit tightly onto the centrifuge tube. Wet weight of t[illegible] cells was determined by weighing

the cell pellet after centrifugation at 250 x g (IEC PR-6 centrifuge).

a. Methods for increasing the renin content of reinn-containing cells. The renin in cells to be separated by Ficoll density gradients was augmented by treatment of the animals with a potent diuretic and naturetic agent and by low sodium diet. Five normal male mongrel dogs weighing about 20 kg were pretreated with intramuscular injections of furosemide (Hoechst Pharmaceuticals, Somerville, N. J.) for two days, 80 mg/day in two doses. Their kidneys were removed and the cortical cells were isolated and separated by Ficoll gradient centrifugation. The amount of renin present in the renin-containing cell fraction was compared to the amount of renin in the same fraction from five normal dogs. The second method used to increase the cell content of renin was to feed normal 2-3 kg male New Zealand rabbits low sodium diet (Nutritional Biochemical Corp., Cleveland, Ohio) for a week and treat their kidneys as outlined above. The amount of renin present in the renin-rich fraction from rabbits on low sodium diet was compared to the amount of renin in the same fraction from rabbits on normal diet.

b. Responsiveness of rabbit enriched renin-containing cells to epinephrine and isoproterenol. Cell fractions

obtained from Ficoll density gradient centrifugation were centrifuged at 250 x g for 5 minutes in an IEC PR-6 centrifuge and the cell pellets were resuspended in 3 ml of freshly oxygenated Eagles medium. 0.5 ml of this cell suspension was added to 1.5 ml of Eagles medium in plastic vials and these vials were incubated at 37° C in a Dubnoff metabolic shaker under 95% O_2-5% CO_2 atmosphere. Either Eagles medium, as a control, or 2.5 µg/ml epinephrine was added every 5 minutes for 30 minutes. The total renin activity in the treated samples was compared to control samples and to a starting total kept at 4° C throughout the experiment. In a similar experiment, both renin and CAMP levels in cell fractions were tested with and without addition of 2.5 µg/ml isoproterenol every 5 minutes for 30 minutes. The values in treated cell fractions were compared to controls incubated at 37° C and 4° C.

2. Separation of Kidney Cortical Cells by Preparative Free-Flow Electrophoresis

a. Materials. RKC cells were prepared by combined enzymatic and mechanical methods, as described in III.C.2. For electrophoretic cell fractionation, cells were resuspended in 310 mosm/liter buffer containing 2.1 grams triethanolamine, 2 grams glucose, 0.399 grams potassium acetate, 18 grams glycine, 10.26 grams sucrose per liter, and acetic acid to pH 7.4.

Fractionated cell pellets were resuspended in 0.01 M BES in 0.9% saline pH 7.15.

^{3}H-Aldosterone (New England Nuclear) was purified with paper chromatography according to the method of Flood <u>et al</u>. (1967) and 3×10^{-8}M (200 μCi) in 10 ml of collagenase solution. Triton-toluene scintillation cocktail used for counting ^{3}H-aldosterone was described in III.A.7.b.

b. <u>Procedures</u>. Separation of RKC cells. The cell suspensions were kept at 4° C in electrophoresis buffer and quickly fractionated in the preparative free-flow electrophoresis apparatus developed by Hannig (1971) with 1090-1180 V and 190-210 mA. The entire process took approximately 15-30 minutes. The cells were then sonicated 5 seconds at 35 watts with a Heat Systems-Ultrasonics Inc. Model W 140 Sonifier and assayed for renin activity. In some experiments, the cells were centrifuged at 250 x g for 15 minutes, the pellet was resuspended, and both pellet and supernatant were assayed for renin activity. The amount of cell tissue recovered was too small for protein assay except in the experiment using labelled aldosterone as a marker for distal tubule cells.

Separation of RKC cells treated with ^{3}H-aldosterone. Aldosterone is a steroid which specifically binds to cytoplasmic receptors in distal tubular cells of the kidney

and regulates sodium transport in its target tissue by inducing RNA and protein synthesis (Edelman *et al.*, 1963; Williamson, 1963; Edelman and Fimognari, 1963). It has been shown by Marver *et al.* (1972) that kidney tissue will bind 2.6×10^{-6}M of aldosterone per mg protein and that the binding of the steroid with its ctyoplasmic receptor is complete within 15 minutes at 37° C. Since the amount of time needed to isolate kidney cortical cells was about 20 minutes, well within the amount of time needed for aldosterone to bind with its receptor, it was feasible to try to label the distal tubule cells with ^{3}H-aldosterone by perfusing rabbit kidneys with a high concentration of ^{3}H-aldosterone. This large amount of labelled steroid competed with the endogenous bound aldosterone in the cells and also provided enough cpm to detect after electrophoretic cell separation.

For a typical rabbit weighing about 3 kg the amount of protein in the kidney cortices was roughly 10% of their wet weight (5 grams), i.e., 500 mg protein. If 2.6×10^{-16}M of aldosterone were bound per mg protein, then approximately 2×10^{-12}M of endogenous aldosterone should be bound in the whole cortex. Less than half of this amount, 3×10^{-8}M of ^{3}H-aldosterone was used for the experiment outlined below.

This competition method eliminated the need to use adrenalectomized rabbits. The tight connection of one adrenal gland with the vena cava in the rabbit made adrenalectomy extremely difficult.

^{3}H-Aldosterone in collagenase solution was perfused slowly into two isolated rabbit kidneys according to the protocol for collagenase-mechanical isolation of kidney cortical cells and the cells were separated by free-flow electrophoresis using disposable laboratory apparatus for all steps. Electrophoresis conditions were similar to previous cell fractionations, except that the buffer was continually oxygenated while the electrophoresis occurred. No clumping of cells was observed.

Every electrophoresis fraction was centrifuged at 250 x g for 15 minutes. Supernatants were saved and cell pellets were resuspended in 2 ml of 0.01 M BES-saline. 0.5 ml of supernatant and 0.5 ml of pellet were added to 5 ml Triton-toluene and counted for 20 minutes in a Packard Tri-Carb liquid scintillation counter. One hundred µl of the pellet and 250 µl of the supernatant was incubated in a total volume of 0.5 ml plasma substrate, pH 5.5, 37° C for 30 minutes, and the extracted angiotensin I was assayed for radioimmunoassay. The cells in each fraction were counted with a hemocytometer under a Zeiss phase-contrast microscope. These cells appeared completely

normal and there were no mitochondria or tubules present. Red blood cells were observed towards the cathode in some experiments, and were noted as marker cells.

3. Fluorochrome Staining of Renin-Containing Cells

Very few fresh cells (about 3,000) from each Ficoll gradient centrifugation fraction were diluted with 3 ml of freshly oxygenated Eagles medium. The cells were collected onto a clean glass slide covered with a cytocentrifuge filet filter paper (Shandon Scientific Company) by centrifugation at 1900 rpm 5 minutes in a Shandon Elliot Cytocentrifuge. The slides were immediately placed in 95% ethanol for at least 30 minutes for fixation. They were then taken up to water by 30 second immersion in each solution of 95%, 80%, 60% and 30% ethanol and tap water. The hydrated cells were stained according to the method of Janigen (1965): they were first stained with Harris' hematoxylin for 20 seconds to quench nuclear fluorescence, and washed 2 minutes in running tap water; subsequently, they were stained for 6-7 minutes in freshly made 1% thioflavin T (Matheson Scientific) solution which had been filtered through Whatman No. 1 filter paper (Fisher Scientific Co.); immersed in 1% (v:v) acetic acid 5 minutes; and washed with two changes of distilled water about 1

minute each. Slides were mounted in glycerol, which did not interfere with fluorescence, and viewed at 250 x or 630 x under a Zeiss fluorescent microscope using a dark field condensor, BG 12/4 excitation filter, which excluded the red spectrum, and barrier filters 53 and 47 which absorbed wavelengths below 470 mμ and above 530 mμ.

F. Subcellular Fractionation

1. Preparation and Assay of Rat Kidney Subcellular Fractions

Subcellular fractions from rat kidney were prepared by Drs. Becca Fleischer and Fernando Zambrano by methods previously described (Fleischer and Zambrano, 1974). The general scheme for continuous subcellular fractionation is shown in figure 8. The purity of each fraction was determined by assay of enzymes known to mark mitochondria, Golgi apparatus, rough endoplasmic reticulum smooth endoplasmic reticulum and plasma membrane, and also by electron microscopy. Renin activity in each fraction was determined by radioimmunoassay to elucidate the possible sequence of packaging, storage and secretion of the renin granule, and to determine the location of renin-like enzymes in these fractions.

2. Preparation of Renin Granules

a. Sucrose density gradient centrifugation. Granules from normal rabbit kidney cortex were prepared

according to the method of Morimoto *et al.* (1972). Twenty grams of rabbit cortex was minced in 0.45 M sucrose and homogenized in two 40 ml batches at 800 rpm for 40 seconds with a Teflon pestle cut to a clearance of 0.974 inches. The homogenate was centrifuged at 500 x g 10 minutes in a JA-20 Beckman centrifuge. The supernatant was decanted and layered onto two SW 25.1 gradients containing 3.5 ml of each, 1.2 M (35.9% sucrose), 1.3 M (38.3%), 1.4 M (41.0%), 1.5 M (43.5%), 1.6 M (45.5%), and 1.7 M (48.1%) and the gradients were centrifuged at 60,000 x g for two hours in a Beckman L5-65 ultracentrifuge. The bands were removed by careful aspiration with a glass syringe and curved needle attached. These bands were assayed for protein, acid phosphatase, succinate dehydrogenase, and renin activity.

In a modification of this method, the supernatant obtained from a cell preparation (collagenase and mechanical methods) was diluted 1:1 (v:v) with 0.9 M sucrose and 8 ml was layered onto 6 discontinuous gradients containing 5 ml each of 1.3 M (38.3%), 1.35 M (39.7%), 1.4 M (41.0%), 1.6 M (45.5%) sucrose, with a 50% sucrose cushion. The gradients were centrifuged 60,000 x g for 2 hours in a Beckman L5-65 ultracentrifuge SW-27 rotor. The fractions were removed by aspiration and were assayed for marker enzymes and renin.

b. Preparative free-flow electrophoresis. The kidneys of male New Zealand (black and white) rabbits were perfused with an isolation medium containing 10 mM triethanolamine, 10 mM acetic acid, 0.25 mM EDTA and 337 mM sucrose, pH 7.4 (2 N NaOH), 400 mOsm, 5.2 x 10^2 µS. The cortices were homogenized gently with a loose Dounce homogenizer in the same isolation medium and the homogenate centrifuged and resuspended in fresh isolation medium three times at 650 x g for 5 minutes. The supernatant was centrifuged at 4500 x g for 5 minutes, the resulting pellet carefully resuspended in 3-10 ml of the isolation medium and then used for further separation. The free-flow electrophoresis separated renin granules from this heavy mitochondrial fraction. The electrophoresis was done with isolation medium (120 mA, 140 V/Cm, flow rate 2.1 ml per fraction per hour, and t = 4° C). Ten to 15 mg of protein were separated per hour (Dew and Heidrich, 1975).

Fractions 8-14 and 28-34 were centrifuged at 650 x g for 10 minutes in a Sorvall SS-34 rotor. Renin activity was tested in the pooled fractions before centrifugation, in the supernatants after centrifugation, and in the pellets after they had been resuspended in a volume equal to the total volume before centrifugation.

For electron microscopy, three fractions each from fractions 10-15 were pooled, 5% BSA and OsO_4 1:1 (v:v)

were added and the pooled material was centrifuged at 4,500 x g for 10 minutes in a SW-40 rotor IEC superspeed centrifuge. There was not enough material in fractions 10-12 to make a pellet, but fractions 13-15 were processed for electron microscope study.

G. *In Vitro* Incubation of Kidney Cortical Cells with Ions, Hormones or Pharmacological Agents

A series of experiments was designed to elucidate the mechanism and control of renin release *in vitro*, using cells dissociated from rabbit kidney cortex. In all studies, 0.5 ml (0.3-2.0 mg protein or approximately 3,000-20,000 cells) of cells from two pooled rabbit kidney cortices was quickly added to marked 25 ml plastic scintillation vials containing 4.5 ml oxygenated Earles buffer which had been made with appropriate ionic variations or with physiological ionic concentration. The osmolality of these solutions varied from 283 to 289 milliosmoles. Total cell renin activity was estimated by including a similar nonincubated vial in every experiment. The cells were incubated for 20 to 35 minute intervals, depending on the agent used, and were gassed under an oxygenating hood with 95% O_2-5% CO_2 and continuous shaking so that the cells would not settle in the vials. At the end of the incubation, each sample was quickly transferred to similarly

marked 17 x 100 mm polypropylene tubes (Falcon Plastics) and centrifuged at room temperature 250 x g for 5 minutes in a Sorvall RC-3 centrifuge. An aliquot of the supernatant was taken and the rest of the supernatant poured off. The cell pellet was resuspended in 5 ml 0.01 M BES in 0.9% saline and saved for protein determination; since negligible amounts of protein were in the supernatants, they were not analyzed. Renin activity was determined in supernatants and total samples.

1. Incubation of RKC Cells with Different Ionic and Osmolar Earles Buffer

The effect of physiological osmolality and ionic concentration was compared to the effect of high or low osmolality or ionic concentration on renin release from isolated cells (table 15). In some experiments, choline chloride was added to osmotically balance low osmotic strength NaCl solutions. In other experiments, choline chloride was used at high osmolality as a comparison for the renin release caused by high osmolality NaCl solutions. Cells were added to these solutions, incubated and separated as described above.

2. Incubation of RKC Cells with Easily Degraded Biomolecules

Many of the compounds that affected renin release were quickly metabolized by kidney cortical cells, and might not have acted with full potency if administered only at the beginning of the incubation. To circumvent this problem, such agents were added to the cell suspension at the beginning of the incubation before the cells were added, every 10 minutes during the incubation, and at the end of the experiment before the cells were centrifuged. Compounds administered in this manner were: catecholamines and serotonin, which were metabolized by monamine oxidase in mitochondria of all cells; prostaglandins E_1, E_2, $F_{2\alpha}$, A_1, A_2, which were degraded by prostaglandin dehydrogenase located in kidney cortex; oleic acid, which was oxidized by fatty acid dehydrogenases after being converted to the trans configuration by cis-trans isomerases; A I and A II and bradykinin, which were made inactive by peptidases; acetylcholine, which was destroyed by cholinesterases; and histamine, which was metabolized by diamine oxidases of kidney.

3. Incubation of RKC Cells with Inhibitors of Biomolecules

In some experiments, specific blockade of renin release caused by an agonist was investigated. To insure

that the inhibitor bound completely to the cell membrane receptors, the cells were preincubated with the antagonist 15 minutes before the agonist was added. The agonist was then added every 10 minutes during the incubation and at the end of the incubation preceding centrifugation. Antagonists incubated in this manner were: atropine; phentolamine; propranolol; diphenhydramine; and indomethacin.

4. Incubation of RKC Cells with Agents which Are Not Degraded by Kidney Cortex

Agents which were not appreciably metabolized by the kidney cells were added to the buffer before addition of cells and cells were then incubated as described, normally for 20 minutes. The compounds administered in this manner were: EGTA; glucose; parathyroid hormone; insulin; glucagon; vasopressin; aldosterone; all CAMP derivatives; theophylline; SC 2964; 16,16-dimethyl PGE_2; and 15-S-15-methyl PGE_2.

H. Preparation of Labile Chemicals

1. Prostaglandin and Unsaturated Fatty Acid Solutions

Prostaglandins E_1, E_2, $F_{2\alpha}$, A_1, and A_2 were the kind gifts of Dr. John E. Pike of the Upjohn Company. Upon arrival, the vials were wrapped well in parafilm and immediately stored at -20° C. The 16,16-dimethyl PGE_2 and

15-S-15-methyl PGE_2 were generously provided by Dr. Sidney Fleischer's laboratory.

Concentrated solutions of all the prostaglandins were made up in 100% ethanol except the $F_{2\alpha}$ tromethamine salt, which was water soluble, and was made up in Earles physiological buffer. These stock solutions were stored under nitrogen at -20° C in glass vials that were well sealed with Teflon lined caps and a tight wrapping of parafilm. Concentrations of stock solutions were:

PGE_1 and PGE_2 -- 0.0005 M, 0.18 mg/ml in 80% ethanol

$PGF_{2\alpha}$ -- 0.005 M, 0.24 mg/ml in Earles buffer

PGA_1 and PGA_2 -- 0.025 M, 8.75 mg/ml in absolute ethanol

16,16-dimethyl PGE_2 and 15-S-15 methyl-PGE_2 -- 0.00014 M, 50 µg/ml

Oleic acid (Nu Check Prep Inc.) was made up in 100% ethanol and stored under nitrogen at -20° C.

Dilutions were made into 80% ethanol or Earles buffer at 4° C, used once and discarded. Fresh dilutions were made for each experiment on the day of the experiment.

2. Catecholamines

The catecholamines epinephrine, norepinephrine and isoproterenol were obtained from Sigma Chemical Company, St. Louis, Missouri. The night before each experiment the

catecholamines were weighed, placed in aluminum foil wrapped test tubes to protect them from light and were sealed with parafilm. The proper amount of Earles buffer was added immediately before the experiment.

3. Acetylcholine Bromide

This chemical was obtained from Sigma Chemical Company, St. Louis, Missouri. It was weighed out the night before the experiment, sealed well with parafilm and stored at -20° C in a dessicator. The proper amount of Earles buffer was added immediately before the experiment.

4. Vasopressin

Argenine vasopressin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and was made up in 0.2 N acetic acid at a concentration of 10 U/ml and stored frozen at -70° C. On the day of the experiment, it was diluted to the desired concentration.

5. Other Chemicals

The following chemicals were made up in concentrated solution in Earles buffer the night before each experiment, stored at -20° C and diluted to the appropriate concentration on the day of the experiment: CAMP, N^6-monobutyryl CAMP N^6,O'^2-dibutyryl CAMP, 5'-AMP, serotonin, glucagon and parathyroid hormone from Sigma Chemical Company, St. Louis,

Missouri; A I from Beckman, Palo Alto, California; A II from Ciba Pharmaceutical Company, Summit, N. J.; histamine dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio; insulin from Schwarz-Mann, Orangeburg, N. Y.; diphenhydramine from Applied Science Laboratories, Inc., State College, Pa. Aldosterone (Sigma Chemical Company, St. Louis, Missouri), indomethacin (Merck, Sharp and Dhome, West Point, Pa.) and aspirin (E. R. Squibb and Sons, Princeton, N. J.) were made up in 100% ethanol before use. Propranolol (Ayerst Laboratories, Inc., New York, N. Y.), phentalamine (Ciba Pharmaceutical Company, Summit, N. J.), theophylline (Nutritional Biochemicals Corp., Cleveland, Ohio), SC 2964 (kindly supplied by Dr. Sidney Fleischer's laboratory), and atropine (Eli Lilly and Company, Indianapolis, Indiana) were made up fresh in Earles buffer the day of the experiment. Furosemide (Hoechst Pharmaceuticals, Somerville, N. J.) was injected intramuscularly into animal from the vial.

I. Statistical Analysis

The statistical significance of differences between mean values was analyzed with a two-tailed student's t-test. A p - value of < 0.05 was selected as the criterion of significance.

CHAPTER IV

RESULTS

A. Renin Radioimmunoassay

1. Reproducibility of the Assay

Plasma pooled from twelve nephrectomized male rabbits was incubated at 37° C for 30 minutes and renin activity was measured in ten different radioimmunoassays. The renin activity (mean ± S.E.M.) was 0.121 ± 0.008 ng A I in 0.5 ml of plasma. Addition of 11 μg protein and 22 μg protein of rabbit kidney cortex homogenate to this plasma gave renin activities of 2.6 ± 0.05 and 4.8 ± 0.10 ng A I in 0.5 ml plasma.

2. Correlation of Bioassay with Radioimmunoassay

Plasma values obtained by bioassay and by radioimmunoassay were highly correlated (figure 9) with a correlation coefficient of 0.995.

3. Effect of Pharmacological Agents on the Reaction of Renin with Its Substrate

Since the renin radioimmunoassay does not directly measure renin, it seemed possible that some pharmacological agents that appeared to inhibit or increase renin release actually might have inhibited or enhanced the reaction of

renin with its substrate. To test this possibility, pharmacological agents were added to plasma substrate at levels that were 40 to 400 times those that would be added to the substrate from cell suspension experiments. This substrate was then incubated with 0, 1.0 μg or 4.0 μg protein of kidney cortical cell suspension in a total volume of 0.5 ml for 30 minutes at 37° C. Table 2 shows the results of this experiment, with blank values subtracted. Only indomethacin at 0.1 μg/ml inhibited the reaction at both enzyme concentrations, but aspirin at 10 μg/ml and 1 mg/ml, and indomethacin at 10 μg/ml stimulated the reaction. These results did not explain the increase in renin activity in cell suspensions treated with indomethacin, for the final concentration of indomethacin in cells reacted with plasma substrate was 0.025 μg/ml. They also did not explain the decrease in renin activity with 10 μg/ml aspirin in cell suspensions (0.25 μg/ml final concentration in cells reacted with plasma substrate). Therefore, the stimulation or inhibition of renin by pharmacological agents were effects on the renin cells and not on the reaction of renin with plasma substrate.

B. Isolation of Kidney Cortical Cells

1. Yield of Cells and Loss of Renin and Protein

a. Enzymatic (collagenase) dissociation. Table 3 shows a summary of the steps used in this procedure and the total amount of renin and protein left after each step. The

large loss in protein and renin occurred by removal of tubules during the gauze filtration. With each wash of the cell pellets more renin was lost from the cells.

The yield of cells was assessed by determining the wet weight of the cortex before cell dissociation and the wet weight of cells after dissociation. Percent yield of cells was calculated according to the formula:

$$\frac{\text{wet weight of cells}}{\text{wet weight of cortex}} \times 100 = \%\text{ yield of cells}$$

The average yield of cells by this method (20 experiments) was 5.5% (range = 3.2-10.1%) of the cortical wet weight. The yield of renin in the cells was 3% of the total cortex renin with one centrifugation and 1% if the cells were washed once.

b. Combined enzymatic and mechanical dissociation. Table 4 shows a summary of the steps in this procedure and the amount of renin and protein left after each step. As in preparation A., much renin activity and protein were lost by sequential filtering to remove tubules. The yield of cells was calculated as in A. The average yield of 30 experiments was 9.9% (range = 5.6-21%). The yield of renin in the cells was 3.3% of the cortex renin with one centrifugation and 0.77% if the cells were washed once.

c. Citrate and mechanical dissociation. Table 5 is a summary of the steps in this procedure and the amount of renin and protein left after each step. The yield of cells in 30 experiments was 17.4% (range = 12-28%) of the cortex wet weight. The yield of renin in the cells was 4.9% of the total cortex renin with one centrifugation and 1.5% with one wash.

2. Selection of Conditions for Isolation of Cells with Adequate Renin Levels

In several RKC cell preparations dissociated from tissue by collagenase digestion, conditions were varied to obtain cells with minimum loss of renin. In many experiments that were time consuming, it seemed advantageous to slow the metabolism of the tissue by cooling to 4° C so that it would live longer. However, it also seemed possible that prolonged exposure of tissue to this abnormally low temperature might cause damage and death of cells. To test this possibility, cells were isolated at 4° C and at 25° C and samples from each step were collected and tested for renin activity. Table 6 shows the results of this experiment. Release of renin from cells prepared in the cold was greater than from cells prepared at room temperature. Therefore, all subsequent preparations were done at room temperature.

It is well established that calcium is an essential element for stimulus-secretion coupling in many systems (Douglas, 1968). It was therefore suspected that if calcium

were present in the medium used for cell isolation, more renin would be released during the procedure. Using room temperature conditions (25° C), the renin release was monitored throughout the preparation. Table 7 summarizes the results of this control experiment. More renin was released when calcium was included in the medium than when it was not. Later it was found that magnesium also caused release of renin (Table 14). For these reasons, calcium and magnesium were left out of the buffers used in the preparation of cells from kidney cortex.

Before the cell isolation procedure was extensively modified, it was thought that several wash steps were needed to remove collagenase from the cells. However, the cell suspensions had very low renin levels after many washes, and it was suspected that renin was lost with each wash. Tables 2-4 show the effect of wash procedures on the amount of renin left in the cells. If cells were not washed, 4% of the total renin remained, but only 1% of the total cortex renin was left in cells after one wash.

C. Viability of Isolated Cells

1. Oxygen Consumption

The respiration of 4 pooled wash supernatants from the cell suspension preparation was compared to the respiration of the cells to see whether subcellular particles released into the supernatants utilized as much oxygen as

cells. As is seen in figure 10, the supernatants had very low oxygen consumption, but cells had a higher oxygen consumption of 4.7 μl O_2/gram wet weight/minute.

Addition of 3% BSA (Fraction V, Sigma) to Eagles medium (Spinner modified, without glutamine) fortified with 2.6 mM l-glutamine caused a marked increase in oxygen consumption of cells from all three species (Table 8). This increased oxygen consumption with BSA was possibly the result of enhanced oxygen distribution to cells, which did not precipitate as fast because the medium with BSA was more viscous.

Addition of physiological concentrations of the NEAA argenine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine (MEM Eagle, Baltimore Biological Laboratory, diluted 1:100) to rabbit and dog kidney cells in Eagles medium with or without BSA caused a very slight increase in cell oxygen consumption that was not statistically significant (table 9). Figures 11 and 12 illustrate that the addition of 3% BSA caused more cellular oxygen consumption than addition of NEAA.

Glucose is a primary energy-yielding substrate in most tissues, and would be expected to enhance oxygen consumption. Dog kidney cortical cells were incubated with or without a physiological amount (10 mM) d-glucose in 10 mM potassium phosphate buffer pH 7.5 with 3% BSA added. Eagles

medium was not used because it contained 10 mM d-glucose. Instead, the Warburg flasks contained 1.2 ml phosphate buffer, 0.5 ml cells (about 150 mg wet weight), and 0.3 ml 10 mM dextrose in the sidearms. The cells were equilibrated as described; the dextrose was tipped into the main compartment and oxygen consumption was measured for one hour at 15 minute intervals. The results are shown in figure 13. The addition of glucose to the cells caused a slight increase in oxygen consumption, which was not statistically significant, from (mean $\pm$ S.E.M.) 5.7 $\pm$ 0.3 µl O_2/g wet weight/minute to 7.7 $\pm$ 0.9 µl O_2/gram wet weight/minute. The results showed that glucose was used as a substrate in the dog kidney cortex and caused an increase in oxygen consumption.

α-KG has been shown to be avidly utilized by the kidney (Selleck and Cohen, 1965) and it is the only substrate except glucose which produces high energy phosphate as it is anaerobically oxidized (Racker, 1961). The oxidative decarboxylation of α-KG can occur in kidney cortex by three coupled reactions which produce succinate, coenzyme A, GTP and NADH. The production of high energy phosphate would be expected to enhance cellular metabolism and increase oxygen consumption. To test this possibility, two concentrations of α-KG (Mann Research Labs, N.Y.), 8 or 17 mM in 0.2 ml Eagles medium, were placed in the sidearms of Warburg flasks which contained 1.3 ml Eagles medium with 3% BSA, 0.67 mM ADP (grade 1, Sigma) and 0.5 mM NAD (type III, Sigma) as

cofactors and 0.5 ml cells (50-80 mg wet weight) in the main compartment. Control flasks contained 0.2 ml Eagles -BSA-cofactors in the sidearms and the same components in the main compartment. After a 10 minute incubation under 95% O_2-5% CO_2, at 37° C, α-KG was tipped into the main compartment and oxygen consumption was measured for one hour at 15 minute intervals. The results are shown in figures 14 and 15. The addition of 8 mM α-KG to the cells caused a slight but statistically insignificant increase in oxygen consumption from (mean ± S.E.M.) 12.1 µl ± 0.51 O_2/g wet weight/min. to 14.3 ± 2.1 µl O_2/g wet weight/min., and addition of 17 mM α-KG increased oxygen consumption of the cells from 16.1 ± 2.2 µl O_2/g wet weight/min. to 21.2 ± 1.9 µl O_2/g wet weight/min.

2. Ability of Isolated Cells to Secrete Renin with Physiological Stimulants

A very important estimation of the isolated kidney cell viability for later experiments was the release of renin with physiological concentrations of stimulants. It will be shown in IV. G. that this goal was achieved for both collagenase and citrate cell preparations.

D. Integrity of the Isolated Cell Plasma Membrane

The uptake of certain dyes by cells is an indication that the cell membranes are "leaky." This is a common estimation of cellular viability, but it is more a measure of

membrane damage. Cell preparations were always checked for membrane integrity by exposure to 0.1% trypan blue for 4 minutes. The number of stained cells and total amount of cells in a field were counted with a hemocytometer and % viable cells was calculated by the formula:

$$\frac{\text{stained cells}}{\text{total cells}} \times 100 = \%\ \text{viable}$$

It was found that cells obtained by the collagenase incubation method were 50-70% viable, by the collagenase-mechanical method, 60-80% viable and by citrate-mechanical method 80-90% viable. Figures 16 and 17 illustrate the appearance of cells isolated by collagenase-mechanical and by citrate-mechanical methods when treated with trypan blue.

Cells should not release intracellular organelles, or marker enzymes for these organelles, if their membranes are intact. To test whether the membranes of cells prepared by collagenase or citrate dissociation were intact, the prepared cells (0.2-0.4 mg protein) were incubated for 20 minutes in 5 ml of Earles buffer at 37° C under an atmosphere of 95% O_2-5% CO_2. They were then centrifuged 5 minutes at 200 x g in a Sorvall RC-3 centrifuge and the supernatants were separated from cell pellets. Both pellets and supernatants were analyzed for protein, renin activity, acid phosphatase (a marker enzyme for lysosomes) and succinate dehydrogenase (a marker enzyme for mitochondria)

activity. The results were expressed as amount of activity in supernatant, cell pellet, and total per 5 ml sample, and as release into the supernatant, % of total, calculated by the formula:

$$\text{Release \% of total} = \frac{\text{supernatant activity}}{\text{total activity}} \times 100$$

The results in table 10 show that the membranes of cells prepared by the collagenase methods are more damaged than those of cells prepared by the citrate dissociation. Protein and renin released by the collagenase-prepared cells were about twice that of the citrate-prepared cells and succinate dehydrogenase, a marker enzyme for mitochondria, was released by collagenase-prepared cells about seven times that of the citrate-prepared cells. These data indicate that there was more cell disruption in collagenase-prepared cells than in cells dissociated from tissue with citrate.

E. Separation of Kidney Cortical Cells into Cell Types

1. Buoyant Density Centrifugation

It was found that cells which had been dissociated from tissue by collagenase would form bands if centrifuged on Ficoll-Eagles medium density gradients (figure 18), but would not band on similar gradients of BSA, sucrose or high molecular weight dextrans. Therefore, Ficoll gradients were used to attempt purification of renin-containing cells from dog and rabbit kidney cortex.

Ten-20% (w:v) Ficoll density gradients were used to enrich dog renin-containing cells. For 5 normal dogs, it was found that the peak renin activity and maximum number of thioflavin T-stained cells was located between 14% and 16% Ficoll (figure 19). The renin activity of this peak was (mean ± S.E.M.) 28 ± 2.0 ng A I/mg wet weight/3 hours. To increase the renin content of these cells, 5 dogs were pretreated with 80 mg/day furosemide i.m. for 2 days, and their kidney cells were centrifuged on 10-20% Ficoll gradients. The peak renin activity of these cells was also located at the interface between 14% and 16% Ficoll (figure 20), but the renin activity of this peak fraction was about 7 times higher than the peak fraction from normal dogs, at (mean ± S.E.M.) 208 ± 10.0 ng A I/mg wet weight/3 hours (figure 21) (Dew and Michelakis, 1972). The approximate specific gravity, as extrapolated from equivalent % sucrose, of these enriched dog renin-containing cells was 1.055-1.061.

Since rabbit kidneys contain more renin granules than dog kidneys (Schaffenburg *et al.*, 1960), their renin cells should be heavier than dog renin cells. These cells were therefore centrifuged on 14-22% Ficoll gradients with 50% cushion at 10,173 x g for one hour. The peak renin activity and thioflavin T fluorescence was located between 20% and 22% Ficoll (figure 22). The peak fraction average of three fractionations was (mean ± S.E.M.) 62 ± 3.0 ng A I/mg wet weight/3 hours, and the approximate specific gravity

of this fraction was 1.073. To increase the cell renin content, two rabbits were kept on a sodium-deficient diet for 7 days and their kidney cortices were separated on 20-32% (and 50% cushion) Ficoll gradients. The peak renin activity was located in 32% Ficoll and was about twice as high as the peak from normal rabbits (mean $\pm$ S.E.M.), 134 $\pm$ 34.0 ng A I/mg wet weight/3 hours (figure 23). The approximate specific gravity of this fraction was 1.110.

Since the procedure for purifying renin cells was very long, it was conceivable that the cells would have lost most of their renin, lack responsiveness, or be dead after long exposure to cold and the centrifugation process. As was shown in the previous paragraph, cells from rabbits kept a week on low sodium diet had the most renin activity. The effects of epinephrine and isoproterenol on renin release from these cells were determined. Thioflavin T staining indicated that the cell fraction was homogeneous; the levels of CAMP were determined in control and isoproterenol-stimulated cells. Figure 24 illustrates that these cells were responsive to epinephrine, which increased the renin activity from (mean $\pm$ S.E.M.) 102.3 $\pm$ 2.5 to 178.3 $\pm$ 2.3 ng A I/mg wet weight/3 hours for samples incubated at 37° C (56.9 $\pm$ 35.1 for 4° C control). That the renin-containing cells were also responsive to isoproterenol is shown in figure 25. The renin activity/mg wet weight increased from 176.0 (control) to 312.0 (isoproterenol-treated) in samples

which were incubated at 37° C (160.0 for 4° C control). The same cells in this experiment increased CAMP in response to isoproterenol at 37° C. The control levels were 4.3 pM/mg wet weight (2.2 for 4° C control) and the isoproterenol-stimulated levels were 20.7 pM/mg wet weight. These data provided evidence that increased CAMP was responsible for β-agonist-stimulated renin release. The CAMP values probably reflected the true levels of this compound in renin-containing cells, since the cell population was homogeneous.

The renin cells enriched by Ficoll gradient centrifugation appeared intact under the microscope (figure 26) and were viable, as determined by their responsiveness to catecholamines. However, the method was too time-consuming and had too low a yield of cells to be practical for detailed studies on the mechanism of renin release. For such experiments a "crude" kidney cortical cell suspension was adequate.

2. Free-flow Electrophoresis

Because renin cells are located in a specialized region of the kidney cortex and have been postulated to be modified smooth muscle cells (Page and McCubbin, 1968), it seemed possible that the net charge on their plasma membranes differed from the net charge of other kidney cortex cell plasma membranes. The availability of the free-flow electrophoresis developed by Hannig (1971) made it possible to test this hypothesis. As is shown in figure 27, three main peaks

of renin activity were found after electrophoretic separation of RKC cells at 1150-1160 V, 200 mA, 4° C. Two of these peaks, at fractions 19 and 22, were in nucleated cell regions. The total renin activity of fraction 19, which contained 1.06×10^3 cells, or 5.6% of the total cells, was 0.172 ng A I, and the renin activity of fraction 22, which had 1.76×10^3 cells, or 9.3% of the total cells, was 0.53 ng A I. The renin peak nearest to the anode, fraction 10, was thought to be granule-bound renin. For this reason, renin granules were prepared as described in III.F.2.b. and separated by free-flow electrophoresis on the same day using the same conditions as those in the cell separation. Granule-bound renin activity was distinguished from free renin activity by centrifugation (table 11). The renin activity of the peak granule-bound fractions towards the anode had 75% of the total activity in the pellet; the peak renin activity towards the cathode had 62% of the total activity in the supernatant. The peak granule-bound renin in this separation was located in the same fractions as the "unknown" peak near the anode in the cell separation; thus, this fraction was probably renin granules. There was no free renin in the cell preparation; either the cells were not damaged by the electrophoresis or they released renin only in the form of granules.

The renin activity was present in discrete peaks in the region of nucleated cells, but it was possible that

other cell types were contaminating the fractions. Proximal or distal tubule cells, which constitute a large part of the kidney cortex, would be the most obvious contaminants. To distinguish distal tubule cells from renin-containing cells, ^{3}H-aldosterone was used as a "marker" for the distal tubule cells, since it was taken up and bound to cytoplasmic and nuclear receptors (Edelman et al., 1963). Another advantage to its use as an intracellular marker was that it remained intact in the cell for a long time and would not be dislodged during electrophoresis. Figure 28 shows the result of labeling kidney cells with ^{3}H-aldosterone and separating them by free-flow electrophoresis. The ^{3}H-aldosterone peak was in fraction 29, which was 531 cpm/fraction compared to a baseline of about 270 cpm/fraction. This fraction was in the main cell peak; hence, distal tubule cells were enriched in this peak. There was no ^{3}H-aldosterone label in the renin peak towards the anode. However, when the larger renin peak was calculated per 10^{4} cells, it became smaller than the anodic renin peak (Fraction 23 had 78.4 mg A I/10^{4} cells and fraction 28 had 25.6 ng angiotensin I/10^{4} cells, compared to a baseline activity of about 30 ng A I/10^{4} cells). The ^{3}H-aldosterone-labeled cell peak was clearly separated from the renin cell peak when this calculation was made.

3. Fluorochrome Staining of JG Cells

To see whether thioflavin T actually stained only

the renin-containing cells, kidneys from a normal dog were treated in two different ways. The first kidney was perfused with saline and Eagles medium and a cross section was fixed in 10% neutral buffered formalin, embedded, and stained with hematoxylin and eosin (H and E), and with H and E and thioflavin T. The second kidney was perfused with 0.3% collagenase, fixed, embedded and stained the same way as the first kidney. Microscopic examination of the H and E sections showed normal kidney structure with well-defined glomeruli and juxtaglomerular apparatus. Collagenase treatment appeared to destroy the glomerular area and surrounding arterioles first. Microscopic examination of thioflavin T stained sections from saline-Eagles perfused kidneys showed that the stain fluoresced golden only in the area of the afferent arteriole; granules appeared only in that region. The dog renin granules were small and sparsely distributed. Sections stained with thioflavin T from collagenase-treated kidneys fluoresced golden in areas that were below the juxtaglomerular region. As was seen in the H and E sections, the collagenase preferentially disrupted the glomerulus and juxtaglomerular regions.

The renin-containing cells isolated by Ficoll density gradient centrifugation appeared bright golden, in contrast to the background of pale blue-green autofluorescence of other kidney cells. If the microscope was switched to phase-contrast, the structure of the brightly-stained cells

could be discerned. The cytoplasm was densely packed with granular material and the nucleus was small and centrally located. An example of the appearance of fluorescent cells seen under fluorescent and phase-contrast microscope is shown in figure 27.

Renin values obtained by radioimmunoassay were always highest in the same fractions that the bright golden fluorescent cells appeared.

F. Subcellular Fractionation

Rat kidney subcellular fractions which were relatively free of contaminating organelles (table 12) were tested for renin activity because it was possible that some of the organelles would contain renin granules. If this were so, the sequence of synthesis, packaging and eventual secretion of the renin granule might become clearer. Table 12 shows that the renin activity of the subcellular fractions was highest in mitochondria and golgi fractions. Mitochondria had an activity of 92.4 ng A I/mg protein, pure golgi had 22.4 ng A I/mg protein, and less pure golgi had 10.7 ng A I/mg protein. The high renin activity of mitochondria was not mitochondria *per se*, but was renin granule contamination of this fraction, as was shown by the low renin activity of mitochondria prepared by lysis of plasma membrane sacs. These sacs, which had trapped mitochondria during homogenization and centrifugation of the tissue, were located in a

region of lower sucrose density than the free mitochondria. The mitochondria released from these plasma membranes had no renin activity at all, nor did the plasma membrane.

Since it was suspected that the high renin activity of the mitochondrial fraction was caused by renin granule contamination, two methods were used to separate kidney mitochondria from renin granules.

Discontinuous sucrose gradients were designed to favor purification of the heavier renin granule. As is seen in table 3, the peak renin activity, 4.29 µg A I/30 minutes/mg protein, was located in 1.40 M sucrose. The highest mitochondrial activity, 0.067 µM/min/mg protein, was located in 1.30 M sucrose. However, some mitochondrial activity remained in the peak renin fractions, indicating that the two particles had similar densities. There was also some lysosomal contamination of the renin granule fractions but the main acid phosphatase activity was located in the supernatant and in 1.6 M sucrose. It is possible, however, that the acid phosphatase activity occurred in the renin granule fraction because the renin granule itself had acid phosphatase activity.

Free-flow electrophoresis was used more successfully to separate the renin granule from mitochondria and lysosomes, and gave an 18-fold increase in renin activity over the starting homogenate. Figure 29 shows that the renin granule migrated farther toward the anode than the

mitochondria and lysosomes. This showed that it was a unique particle with a different membrane charge than the lysosome or mitochondria (Dew and Hendrich, 1975). The activity towards the anode was "bound" in granule form and the activity towards the cathode was free, as was shown by centrifugation of the pooled peaks. Table 11 contains results which show that the anode peak had high activity in the pellet (747 ng A I/30 minutes/ml (75% of the total activity) but the cathode peak had high activity in the supernatant (119 ng A I/30 minutes/ml, or 62% of the total activity). Figure 30 is an electron micrograph of anodic fractions 13-15. The renin granules in these fractions were slightly contaminated by lysosomes, mitochondria and plasma membranes. We were unable to obtain enough material for electron microscope study from fractions further towards the anode. The electron micrograph shows membrane-bound granules that were mostly undamaged by electrophoresis and centrifugation. These granules were similar in appearance to those reported by Barajas (1966); the maximal diameter of these granules was 1.4 μ.

G. In Vitro Incubation of Kidney Cortical Cells with Ions, Hormones or Pharmacological Agents

1. Renin Release with the Divalent Cations, Magnesium and Calcium

The divalent cations calcium and magnesium are necessary for secretion of glucagon (Leclercq-Meyer *et al.*, 1973),

insulin (Milner and Hales, 1967) and many other hormones and transmitter substances (Rubin, 1970); calcium is also necessary for the release of renin from dog kidney cortex cells (Michelakis, 1971).

The effect of different calcium and magnesium concentrations on renin release was studied using RKC cells. Figure 31 shows the effect of calcium on renin release. Maximum increase in release occurred with 1.0 mM and 1.5 mM calcium; calcium-free medium inhibited release, as did high calcium. Renin release from the control samples, 2.5 mM calcium, was (mean ± S.E.M.) 69.9 ± 1.3 ng A I/mg cell protein. Release increased to 87.8 ± 4.3 ng A I/mg cell protein ($p < 0.02$) with 1.0 mM calcium and to 87.4 ± 5.1 ng A I/mg cell protein ($p < 0.02$) with 1.5 mM calcium. Calcium-free medium, however, caused a decrease in renin release to 57.7 ± 4.9 ng A I/mg cell protein ($p < 0.01$) and 4 mM calcium likewise inhibited renin release to 61.9 ± 4.3 ng A I/mg cell protein (not significant).

Table 14 shows the effect of magnesium on renin release with and without calcium. In the presence of calcium, only 0.5 mM magnesium increased renin release. At other concentrations, renin release was inhibited. Calcium was necessary for the stimulation of renin release with 0.5 mM magnesium, for when it was removed, no release occurred. Renin release from control cells, incubated with 2.5 mM calcium and 1 mM magnesium, was (mean ± S.E.M.) 69.9 ± 1.20 ng

A I/mg cell protein. With 2.5 mM calcium and 0.5 mM magnesium, renin release increased to 76.7 ± 3.5 ng.

A I/mg cell protein ($p < 0.001$), but with no calcium and 0.5 mM magnesium, release was prevented, 68.2 ± 3.0 ng A I/mg cell protein (not significant). The maximum decrease in renin release was obtained with 2.5 mM calcium and 1.5 mM magnesium, 31.2 ± 2.7 ng A I/mg cell protein ($p < 0.001$).

2. Renin Release with Monovalent Cations

Many investigators have shown that low sodium increased renin release and that high sodium inhibited renin release (Bunag *et al.*, 1968).

However, it was probably osmotic change and not sodium alone that caused the changes in renin release (Young and Rostorfer, 1973; Weinberger and Rosner, 1972). To test this hypothesis, RKC cells were incubated in Earles buffer with different concentrations of NaCl or choline chloride which were not iso-osmotic. Figure 32 shows that isolated RKC cells released more renin in response to low concentrations of NaCl or choline chloride. Renin release increased maximally from a control level of (mean ± S.E.M.) 153.5 ± 5.3 ng A I/mg cell protein with 150 mM NaCl or 145.3 ± 5.4 ng A I/mg cell protein with 150 mM choline chloride to 207.2 ± 7.1 ng A I/mg cell protein with 10 mM or 191.0 ± 3.1 ng A I/mg cell protein with 10 mM choline chloride ($p < 0.001$, but there was no significant difference between values for

NaCl and choline chloride). At 300 mM NaCl or choline chloride, renin release decreased to 91.7 ± 2.6 ng A I/mg cell protein (NaCl) or 94.8 ± 2.7 ng A I/mg cell protein (choline chloride) ($p < 0.001$). There was no significant difference between NaCl and choline chloride.

It was possible that the renin cells released renin in response to low sodium only if they were loaded with renin or had been exposed to low sodium levels for a longer period of time. To test this possibility, male New Zealand white rabbits were pre-treated with 0.65 mg/kg furosemide i.m. twice a day for two days before their kidneys were removed. Kidney cortical cells were then prepared by the collagenase-mechanical dissociation method and exposed to low concentrations of NaCl made iso-osmotic by appropriate concentrations of choline chloride. Table 15 shows that when NaCl was iso-osmotic, there was no significant effect of sodium on renin release from sodium-depleted RKC cells. These data indicate that osmotic change, not sodium chloride levels *per se*, was responsible for high or low renin release.

The ratio of sodium to potassium outside the cell is very important for release of some hormones (Douglas and Poisner, 1964; Grodsky and Bennett, 1966; Hales and Milner, 1968b). To test whether the ratios of these ions were of significance to the release of renin from RKC cells, the cells were incubated 30 minutes in Earles buffer which had various ratios of sodium to potassium. Figure 33

illustrates that, in the presence of low potassium, renin release from the cells was not increased or decreased by low sodium chloride concentrations. With higher potassium levels, renin release increased in response to low concentrations of sodium chloride. 2.5 mM KCl and 50 mM NaCl caused increased renin release; the control was (mean ± S.E.M.) 5.19 ± 4.6 ng A I/mg protein, and treated was 64.8 ± 4.2 ng A I/mg protein ($p < 0.05$). 5.0 mM KCl and 50 mM NaCl caused increased renin release to 66.4 ± 2.9 ng A I/mg cell protein, and 10 mM KCl and 50 mM NaCl caused a maximum increase in renin release to 78.9 ± 1.9 ng A I/mg cell protein ($p < 0.001$). A decrease in renin release with 200 mM NaCl was seen at all concentrations of KCl but was significantly decreased from control levels only with 10 mM KCl, which produced a renin activity of 40.2 ± 3.9 ng A I/mg cell protein ($p < 0.05$). From these data it is evident that the ratio of sodium to potassium is an important factor regulating the release of renin.

3. Renin Release with Neurohormones

Many investigators have shown that renin release is stimulated both *in vivo* and *in vitro* by catecholamines, especially adrenergic β-stimulants (Wathen *et al.*, 1965; Michelakis *et al.*, 1969; Aoi *et al.*, 1974).

Since sympathetic nerve endings surround the JG cells (Wågermark et al., 1968), it is believed that the

sympathetic nervous system regulates renin release to some degree. The influences of the sympathetic nerves on renin secretion, however, may be overestimated because of concurrent effects on renal blood flow and ion-water fluxes. Michelakis et al. (1969) showed that epinephrine and norepinephrine directly increased renin release from dog renal cortical cell suspensions. The RKC cell suspension was used to extend these observations. Catecholamine concentration-dependent release of renin was prevented by the presence of α or β blockers. Table 16 gives the dose-related response of RKC cells to epinephrine, norepinephrine and isoproterenol. Cells were incubated 20 minutes and catecholamines were added to Earles normal buffer as described in III.G.2. Low concentrations, 3×10^{-7} M, of l-epinephrine slightly stimulated renin release from (mean $\pm$ S.E.M.) 51.8 $\pm$ 2.5 mg A I/mg cell protein to 55.3 $\pm$ 1.4 mg A I/mg protein (107% of control, N.S.). High amounts, 1.5×10^{-5} M, of epinephrine decreased renin release to 39.1 $\pm$ 1.6 ng A I/mg protein (75% of control, $p < 0.001$). The same effect was seen with l-norepinephrine; 3×10^{-8} M l-norepinephrine increased renin release from 48.6 $\pm$ 2.0 ng A I/mg protein to 53.8 $\pm$ 2.3 ng A I/mg protein (111% of control, N.S.), but high levels of l-norepinephrine, 1.5×10^{-5} M decreased renin release to 39.7 $\pm$ 1.9 mg A I/mg protein (82% of control, $p < 0.01$). Isoproterenol, however, decreased renin release at 3×10^{-7} M from 54.1 $\pm$ 1.4 ng A

I/mg protein to 48.9 ± 1.8 ng A I/mg protein (90% of control, $p < 0.05$) and high concentrations, 1.5×10^{-5} M, stimulated release to 69.7 ± 3.3 ng A I/mg protein (129% of control, $p < 0.001$). In a similar experiment, RKC cells were pre-incubated 15 minutes with or without 5×10^{-7} M of the α-blocker, or 1×10^{-4} M of the β-blocker propranolol, and then norepinephrine (total concentration 3×10^{-8} M), epinephrine (total concentration 3×10^{-8} M) or isoproterenol (total concentration 5×10^{-7} M) was added as described above. Figure 34 shows that norepinephrine increased renin release from a control of (mean ± S.E.M.) 31.4 ± 1.8 ng A I/mg protein to 50.1 ± 2.9 ng A I/mg protein ($p < 0.001$). This increase was blocked slightly by phentolamine, which did not affect renin release itself, and was prevented by propranolol, which by itself slightly stimulated renin release. Renin activity (ng A I/mg protein) with phentolamine was 36.7 ± 1.6 (N.S.), with phentolamine and norepinephrine was 42.4 ± 6.6 (N.S.) and with propranolol and norepinephrine was 36.3 ± 3.3 (N.S.). Epinephrine also increased renin release from 28.3 ± 1.9 ng A I/mg protein to 43.8 ± 5.2 ng A I/mg protein ($p < 0.05$). Neither phentolamine nor propranolol affected renin release in this experiment, but phentolamine allowed the β-agonist capacity of epinephrine to appear by blockade of the α-receptor, and propranolol blocked the increase in renin activity caused by epinephrine. The renin activity (ng A I/mg protein) with

phentolamine was 29.3 $\pm$ 2.0 (N.S.), with propranolol was 29.5 $\pm$ 1.7 (N.S.), with phentolamine and epinephrine was 53.7 $\pm$ 3.1 ($p < 0.001$) and with propranolol and epinephrine was 32.5 $\pm$ 2.1 (N.S.). Isoproterenol stimulated renin release from 55.1 $\pm$ 3.0 ng A I/mg protein to 64.4 $\pm$ 3.9 ng A I/mg protein ($p < 0.05$). Propranolol, which had no effect on renin release (47.9 $\pm$ 10.4 ng A I/mg protein, N.S.), blocked this increase caused by isoproterenol (49.6 $\pm$ 2.0 ng A I/mg protein, N.S.). The release of renin was thus directly affected by the sympathetic nervous system, and β-activity appeared to be dominant. However, α-receptors were present on the renin cells, as evidenced by the increase in renin activity caused by epinephrine after α-receptor blockade. The α-receptor action of epinephrine depressed renin release, causing a net lowering of the β-receptor action of epinephrine, which increased renin release.

Since the effects of β-adrenergic stimulation are thought to be mediated by CAMP (Robison and Sutherland, 1970), and increased renin activity *in vivo* and *in vitro* is accomplished by dibutyryl CAMP (Yamamoto *et al.*, 1973), a series of experiments was done to determine the effect of high CAMP levels on renin release. Table 17 shows the effect of exogenous CAMP, monobutyryl CAMP, dibutyryl CAMP, 5'AMP, and the phosphodiesterase inhibitor SC-2964 on release of renin from RKC cells. These cells, which were incubated in Earles normal buffer with each agent

20 minutes, released more renin with high levels of exogenous CAMP and dibutyryl CAMP, but released less renin with low levels of CAMP, monobutyryl CAMP and with 5'AMP. These diverse results can be explained by the fact that phosphodiesterase was not inhibited in these experiments and was able to produce 5'AMP from low levels of CAMP, which then depressed renin release. Higher levels saturated the enzyme, thus allowing CAMP to enhance renin release. The results with monobutyryl CAMP and dibutyryl CAMP are more difficult to explain. Possibly the monobutyryl derivative did not penetrate the cell as well as the dibutyryl derivative. Active esterases in kidney cells then might have split off the butyric acid moiety, which might have decreased renin release. The small amount of CAMP formed was quickly metabolized to 5'AMP by phosphodiesterase; this 5'AMP could also decrease renin release. When 0.25 mM SC 2964, an xanthine which only inhibits phosphodiesterase activity and not adenyl cyclase, was incubated with RKC cells, renin activity increased from 50.5 $\pm$ 2.8 ng A I/mg cell protein to 57.1 $\pm$ 4.5 ng A I/mg protein (113% of control, but not statistically significant). Because the renal cell suspension used was a mixed population of cells, it was not pertinent to directly measure CAMP levels. However, results from renin-containing cells enriched by Ficoll gradients showed that isoproterenol, a β-stimulator which increased renin release, also increased CAMP levels (figure 26). These data, together with the

exogenous CAMP and the phosphodiesterase inhibitor results implied that an increase in CAMP caused renin release, and that this was the probable mechanism of renin stimulation by the β-adrenergic agonists.

The JG area of the kidney is richly surrounded by nerves, many of which are sympathetic (Barajas, 1972). It is possible, however, that some of these nerves are cholinergic. To test this possibility, RKC cells in Earles normal buffer were pre-incubated 15 minutes with or without 2×10^{-5} M atropine, and then acetylcholine was added every 10 minutes for 20 more minutes, making a total concentration of 1.5×10^{-5} M. In figure 35, it is seen that acetylcholine increased renin release from a control level of (mean $\pm$ S.E.M.) 25.2 ± 1.5 ng A I/mg cell protein to 32.1 ± 5.7 (atropine, N.S.) and 31.9 ± 2.8 ng A I/mg protein (acetylcholine, $p < 0.05$). Acetylcholine added after atropine did not affect renin release (28.3 ± 2.0 ng A I/mg protein, N.S.). This experiment showed that cholinergic nerve discharge could affect renin release as much as adrenergic nerve activity.

4. Renin Release with Hormones

Renin release results in production of A II, which increases aldosterone secretion from the adrenal cortex. The aldosterone then triggers a "negative feedback" loop, primarily by increasing kidney distal tubule reabsorption of

sodium, which increases tissue osmolality and decreases renin release. There may be, however, a minor direct effect of aldosterone to inhibit renin release from the JGA. Evidence for this inhibition by aldosterone has been found by Leutscher and Beckerhoff (1972) in patients with hyperaldosteronism or low renin hypertension. In both situations aldosterone levels did not change with sodium intake, and renin levels remained low. The authors attributed these low renin levels to expansion of extra cellular fluid volume and to non-suppressible aldosterone production. Additional evidence for a slight direct effect of aldosterone on renin release from isolated dog kidneys was found by Greco and Murphy (1972). These investigators reported a decrease in renin release with high salt infusion only in the presence of aldosterone. No change in renin release occurred with low salt infusions but a rise in plasma renin substrate occurred, indicating a slight decrease in renin release. These effects were abolished by perfusion of spironolactone and aldosterone with high or low salt. However, in this preparation decreased renal blood flow, glomerular filtration rate, increase in the ratio of sodium to potassium or production of A II could have contributed to the decrease in renin release. To elucidate this problem, RKC cells in normal Earles buffer were incubated 20 minutes with aldosterone levels that were within the range normally found in peripheral plasma (20-200 pg/ml plasma). Table 18 gives the

results of these experiments in RKC cells from two different rabbits. In the first rabbit, very low concentrations of aldosterone inhibited renin release (ng A I/mg cell protein) from (mean $\pm$ S.E.M.) 39.7 $\pm$ 3.7 to 23.3 $\pm$ 1.6 ($p < 0.01$) with 0.002 pg/ml aldosterone and 19.4 $\pm$ 0.8 ($p < 0.001$) with 0.2 pg/ml aldosterone. The second rabbit had a higher control release, 135.4 $\pm$ 4.7, but this was also significantly inhibited by 0.2 pg/ml aldosterone to 115.5 $\pm$ 5.4 ($p < 0.05$), by 2.0 pg/ml aldosterone to 102.2 $\pm$ 2.9 ($p < 0.001$) and by 20 pg/ml aldosterone to 94.7 $\pm$ 6.6 ($p < 0.01$). These results showed that, at normal ionic levels, and in the absence of any other chemical inhibitor such as A II, aldosterone could directly inhibit renin release.

Several studies have shown that A II caused excessive drinking (Severs _et al._, 1967; Epstein _et al._, 1969) and release of vasopressin (Bonjour and Malvin, 1970). Also vasopressin decreased renin secretion in high doses but not low doses of vasopressin (Bunag _et al._, 1967). Renin activity also was shown by Gutman and Benzakin (1971) to be higher in rats with hereditary diabetes insipidus than in control rats. These authors confirmed that in normal rats there was a decrease in renin activity with high amounts of vasopressin. The data suggest there is a negative feedback system for renin and vasopressin as well as for renin and aldosterone. This would make a very effective mechanism for volume and osmolarity regulation. It is possible, however,

that osmolality changes that occurred with vasopressin administration were influencing renin release. Therefore, RKC cells in normal Earles buffer were incubated for 20 minutes with low (1 μU/ml and 10 μU/ml) and high (50 μU/ml and 100 μU/ml) concentrations of argenine vasopressin. In table 19 it is shown that low levels of vasopressin stimulated renin release from a control (ng A I/mg cell protein, mean ± S.E.M.) of 47.7 ± 3.8 to 68.0 ± 2.4 ($p < 0.001$) with 1 μU/ml and 68.7 ± 2.5 ($p < 0.001$) with 10 μU/ml, but high levels of vasopressin did not change renin release. These results support the negative feedback hypotheses that low levels of vasopressin should stimulate renin release (and the resulting A II increase vasopressin release), and high levels of vasopressin should either depress renin release or not alter the basal release.

Parathyroid hormone mobilizes calcium from bone, aids uptake of calcium in some tissues and promotes calcium excretion. Since it has been shown in figure 32 that increasing calcium concentrations decreased renin release, it was of interest to see if parathyroid hormone affected renin release when calcium was or was not available. RKC cells were incubated 20 minutes in normal Earles buffer containing 2.5 mM EGTA, a specific calcium chelator, or 2.5 mM $CaCl_2$ with or without 1 ng/ml and 10 ng/ml parathyroid hormone. Table 20 gives the results of this experiment. 2.5 mM EGTA caused an increase in renin release from (ng A I/mg

cell protein, mean ± S.E.M.) 26.0 ± 2.3 to 63.7 ± 1.5 ($p < 0.001$). When 2.5 mM EGTA was present, renin release changed from 63.7 ± 1.5 to 58.1 ± 8.2 (N.S.) with 1 ng/ml parathyroid hormone and to 47.7 ± 2.2 ($p < 0.001$) with 10 ng/ml parathyroid hormone. When 2.5 mM calcium was present, renin release decreased, only with 10 ng/ml parathyroid hormone, from 26.0 ± 2.3 to 19.3 ± 1.4 ($p < 0.05$). Since parathyroid hormone inhibited renin release with and without external calcium available, it could either act directly on the renin cell to decrease renin release, or cause intracellular calcium mobilization to internally inhibit release of renin from the cell.

Božović and Efendić (1969) found that renin release from rat kidney cortex slices was glucose-dependent and concluded that the process was energy-dependent. Increased glucose could also activate glycolytic pathways in the cell and generally increase metabolism, including _de novo_ synthesis of renin. It is well known that insulin facilitates glucose transport in many cells and also increases anabolism, such as lipid, protein and glycogen synthesis. Insulin could therefore be important for the utilization of glucose by the JG cell. To test this hypothesis, RKC cells in normal Earles buffer were incubated for 20 minutes with or without 10 mM glucose in the presence or absence of 2.5 μU/ml insulin. Table 21 gives the results of this experiment. As was observed by Bozović and Efendić (1969), 10 mM

glucose increased renin activity from (mean ± S.E.M.) 17.6 ± 1.2 ng A I/mg cell protein to 21.2 ± 0.9 ng A I/mg cell protein ($p < 0.05$). However, the presence of insulin did not further increase renin release when incubated with 10 mM glucose, but did increase release in the absence of glucose. When RKC cells were incubated without glucose, renin release was increased from 17.6 ± 1.2 ng A I/mg protein to 30.0 ± 1.2 ng A I/mg protein with 2.5 uU/ml insulin. The data suggest that insulin acted on intracellular anabolic processes, which increased net synthesis and release of renin. It is interesting that the action of insulin in this regard was only apparent when glucose was absent, indicating that insulin acted on the JG cell only in "emergency" situations when glucose levels dropped. This result was supported by Otsuka _et al_. (1970), who observed that insulin-induced hypoglycemia increased plasma renin activity in intact dogs. Denervation of the kidneys did not affect the response, but unilateral adrenalectomy and denervation of the other adrenal blunted the response. In this system, however, the catecholamines were dominant in control of renin release.

Some of the known actions of glucagon, such as gluconeogenesis, enhanced amino acid uptake and metabolism might give this hormone a role in regulation of renin release. RKC cells in normal Earles buffer were incubated with and without glucagon. Table 22 shows that renin release was increased from control levels (ng A I/mg cell

protein, mean $\pm$ S.E.M.) of 39.9 $\pm$ 2.8 to 48.0 $\pm$ 2.6 with 0.5 pg/ml glucagon ($p < 0.001$). The concentrations of glucagon used were within normal physiological levels. This showed that glucagon also had a role in renin secretion regulation.

5. Renin Release with Autocoids

Local "hormones" or autocoids are a broad range of compounds that are naturally produced by tissues and also have actions on tissue near their site of origin. Such actions require small amounts of the autocoid, and often involve fine regulation of local physiological responses such as blood flow, ionic and water fluxes or modulation of other hormone or nervous effects. These compounds have definite roles in kidney autoregulation and some of them regulate renin release. For these reasons, the effects of histamine, serotonin, A I and A II and the prostaglandins were used in the RKC cell suspension system to study their role in renin release regulation.

Histamine is readily made by the action of histidine decarboxylase on the amino acid histidine. The enzyme for formation of histamine is present in most tissues, including kidney, but the most histamine is found in the ubiquitous mast cell. Histaminases, which inactivate histamine, are likewise found in cell tissues. The effect of histamine on smooth muscle is primarily stimulatory; since the JG cells

are supposed to be modified smooth muscle cells, histamine would be expected to stimulate them. RKC cells in normal Earles buffer were pre-incubated 15 minutes with or without 6 ng/ml of the antihistaminic agent diphenhydramine. Histamine, 6 ng/ml, was then added to all samples but control (Earles buffer was given in a volume equal to that of the histamine) every 10 minutes for 20 more minutes. In figure 35 it can be seen that histamine increased renin release from (ng A I/mg cell protein, mean $\pm$ S.E.M.) 24.5 $\pm$ 2.7 to 31.3 $\pm$ 1.5 ($p < 0.005$). Diphenhydramine, which by itself did not significantly affect renin release (21.0 $\pm$ 3.2), when incubated with histamine blocked the increase in renin release caused by histamine (22.0 $\pm$ 2.0, N.S.). Therefore, the local hormone histamine, which can be released from tissue by basic drugs, allergy or shock, could regulate renin release to some degree in these conditions, and its site of action at the JG cell could be specifically blocked by antihistamines.

Serotonin (5-hydroxytryptamine) is made from the amino acid tryptophan by tryptophan-5-hydroxylase, to make 5-hydroxytryptophan, and by 5-hydroxytryptophan decarboxylase, which removes the carboxyl group from the intermediate compound to make serotonin. Serotonin is stored in intestine, brain and blood platelets. It can be released from platelets by shock, injury or hypersensitivity; in these situations serotonin could enter the kidney and affect renin

release. However, its effects would be very transient, since serotonin is inactivated by monamine oxidase present in mitochondria. To assess the amount of influence serotonin had on renin release, RKC cells in normal Earles buffer were incubated for 20 minutes with different concentrations of serotonin or with the same volume of Earles buffer (control), added every 10 minutes to circumvent breakdown of serotonin by monamine oxidases. Table 23 shows that serotonin inhibited renin release at 8.0 and 80 ng/ml. 8.0 ng/ml serotonin decreased renin release from (ng A I/mg cell protein, mean $\pm$ S.E.M.) 68.1 $\pm$ 4.0 to 53.5 $\pm$ 5.3 ($p < 0.02$) and 80 ng/ml decreased renin release to 50.6 $\pm$ 5.9 ($p < 0.05$). However, increased capillary permeability and antidiuretic actions of serotonin (Little *et al.*, 1961) would counteract this direct decrease in renin release. Hence, *in vivo*, there is probably no renin release regulation by serotonin.

Angiotensin I and II are the products of renin cleaving a 13-amino acid portion of α-2 globulin and tissue "converting enzyme" which is present in kidney, heart, liver and aorta. Several investigators have shown that there is an inhibition of renin release with A II which is independent of aldosterone secretion or renal artery pressure changes (Vander and Geelhoed, 1965; Vandogen *et al.*, 1974), and Michelakis (1971) showed that the effect was directly upon the renin-producing cells. Vandogen *et al.* (1974) also

showed that A I inhibited renin release, but this effect could have been secondary to local changes in vascular resistance or renal tubular sodium load. The RKC cell system was used to investigate the relative effects of A I and A II on renin release. The effects seen in this system were direct ones on the renin-containing cells. RKC cells in Earles normal buffer were incubated 20 minutes and Earles buffer as control, A I or A II was added every 10 minutes to circumvent breakdown of the peptides by kidney angiotensinases. Because the radioimmunoassay for renin measured A I, the samples containing A I were corrected for background A I by subtracting the amount of angiotensin measured from the total amount of angiotensin that had been exogenously added. Figure 36 shows that angiotensin I was a more potent inhibitor of renin release than A II. A I decreased renin activity from a control of (ng A I/mg cell protein, mean $\pm$ S.E.M.) 24.4 $\pm$ 1.2 to 10.9 $\pm$ 1.5 with 0.6 ng/ml ($p < 0.001$) 6.0 $\pm$ 2.4 with 6.0 ng/ml ($p < 0.001$) 1.3 $\pm$ 1.1 with 60 ng/ml and 0 with 600 ng/ml ($p < 0.001$). In contrast to this marked effect with low levels of A I, A II significantly decreased renin activity only at 600 ng/ml from a control of 27.4 $\pm$ 2.9 to 17.3 $\pm$ 3.0 ($p < 0.05$). Although it is possible that kidney converting enzymes changed some of the A I to A II, the fact that A II was much less effective in inhibiting renin release in the same experiment argues against this as a primary reason for the marked negative feedback effects with A I.

Prostaglandins are 20 carbon fatty acids with a cyclopentane ring which are synthesized from arachidonic acid (PGE_2 and $PGF_2\alpha$), or dihomo-γ-linolenic acid (PGE_1 and $PGF_2\alpha$) by prostaglandin synthetase. In kidney, only PGE_2 and $PGF_2\alpha$ are made by interstitial cells in the medulla. They are not stored, but are possibly transported to the cortex by tubular fluids, since they have been found in urine (Frölich *et al.*, 1975), or are endogenous to the cortex (Larsson and Änggård, 1973). However the prostaglandins get to the cortex, it is possible that they affect renin release either directly, or indirectly by changes in renal blood flow or ion-water fluxes (McGiff and Nasjletti, 1973). If this were so, yet another negative feedback system would exist for the renin-angiotensin system, since it has been shown that A II infusion increases PGE_2 and $PGF_2\alpha$ excretion (Frölich *et al.*, 1975) from kidney and releases a prostaglandin-like substance into renal venous blood (McGiff *et al.*, 1970). However, Änggård *et al.* (1971) have reported high levels of 15-hydroxyprostaglandin dehydrogenase in renal cortex, so that if the prostaglandins did reach the juxtaglomerular apparatus they would probably be acting in very low concentrations. A series of experiments were designed to elucidate the actions of various prostaglandins on renin release in RKC cells. In all experiments except those with prostaglandin dehydrogenase-resistant prostaglandins, RKC cells in Earles normal buffer

were incubated 20 minutes and the prostaglandins, or an equal volume of ethanol as control, were added every 10 minutes. Structures of the prostaglandins used, and of arachidonic acid, are shown in figure 37. It was found that the E prostaglandins increased renin activity at low concentrations but at higher concentrations this effect was less. PGA_1 and $PGF_{2}\alpha$ decreased activity more with increasing levels, and PGA_2 increased activity, but less at high concentrations than at low concentrations. Oleic acid, which was used as a control for nonspecific long chain (18 carbon) fatty acid effects, did not affect renin release at any concentration used for the prostaglandins. Figure 38 is a summary of the results. All results below are in ng A I/mg cell protein and mean $\pm$ S.E.M., and total concentrations are given. PGE_1 increased renin activity slightly but not significantly only at 3×10^{-8} M, from a control of 61.3 ± 4.0 to 65.8 ± 2.4 (110% of control). At 3×10^{-12} M, PGE_1 decreased release to 50.1 ± 3.0 ($p < 0.02$), 82% of control. PGE_2 increased release at low concentrations, 3×10^{-12} M, from a control level of 49.7 ± 2.6 to 71.6 ± 3.7 ($p < 0.001$, 144% of control). At 3×10^{-10} M, release was still high, 63.6 ± 1.7 ($p < 0.001$, 128% of control) as was release at 3×10^{-8} M, 54.7 ± 3.4 (N.S., 110% of control) but these higher levels of PGE_2 caused less release than 3×10^{-12} M PGE_2. PGA_2 at a 3×10^{-12} M, stimulated release from 40.1 ± 2.8 to 65.5 ± 3.0 ($p < 0.001$, 163% of control) whereas 3×10^{-10} M

increased release to 53.6 $\pm$ 4.0 ($p < 0.02$, 134% of control) and 3 x 10^{-8} M increased release to 53.1 $\pm$ 4.1 ($p < 0.02$, 132% of control). $PGF_2\alpha$ decreased renin at all levels tested, from a control of 55.5 $\pm$ 1.2 to 49.2 $\pm$ 1.4 ($p < 0.005$, 89% of control with 3 x 10^{-12} M, 43.1 $\pm$ 2.5 ($p < 0.005$, 78% of control) with 3 x 10^{-10} M and 40.7 $\pm$ 5.5 ($p < 0.05$, 73% of control). PGA_1 also decreased renin release at all concentrations, from 60.4 $\pm$ 3.3 to 56.3 $\pm$ 3.9 (N.S., 93% of control) with 3 x 10^{-12} M, 43.2 $\pm$ 4.5 ($p < 0.005$, 72% of control) with 3 x 10^{-10} M, and 35.1 $\pm$ 2.5 ($p < 0.001$, 58% of control) with 3 x 10^{-8} M. Oleic acid did not significantly affect renin release. Control levels were 110.1 $\pm$ 6.9; at 3 x 10^{-12} M, 113.6 $\pm$ 7.0 (N.S., 103% of control); at 3 x 10^{-10} M, 103.3 $\pm$ 6.6 (N.S., 94% of control); at 3 x 10^{-8} M 106.0 $\pm$ 10.3 (N.S., 96% of control).

Although all PGs were given every 10 minutes, to overcome degradation by dehydrogenases, some of the activity seen with the PGEs could have been caused by breakdown products. To test this possibility, RKC cells in normal Earles buffer were incubated with one dose of either 15-S-15-methyl PGE_2 or 16,16-dimethyl PGE_2. Figure 39 shows that higher concentrations of these dehydrogenase-resistant compounds were necessary to stimulate release than the endogenous PGE_2, but at high concentrations the stimulation was less than at lower concentrations, as was the case with PGE_2. 15-S-15-methyl PGE_2 stimulated renin release from a

control of 108.6 $\pm$ 4.1 to 115.6 $\pm$ 6.0 (N.S., 106% of control) with 5 pg/ml, to 134.1 $\pm$ 12.1 ($p < 0.05$, 124% of control) with 50 pg/ml, and to 138.3 $\pm$ 8.8 with 100 pg/ml ($p < 0.005$, 127% of control). At 500 pg/ml, release was 102.3 $\pm$ 7.9 (N.S., 94% of control). Likewise, 16,16-dimethyl PGE_2 increased renin release from a control of 35.8 $\pm$ 1.9 to 37.7 $\pm$ 3.8 (N.S., 105% of control) with 5 pg/ml, to 45.1 $\pm$ 6.5 (N.S., 126% of control) with 50 pg/ml to 44.2 $\pm$ 4.0 ($p < 0.05$, 123% of control) with 250 pg/ml and 30.8 $\pm$ 1.0 ($p < 0.05$, 86% of control) with 500 pg/ml.

The time course of PGE_2 action was investigated. 3×10^{-12} M PGE_2, or the same volume of ethanol as control, was added to normal Earles buffer just before RKC cells and the cells were incubated for 0, 5, 15 and 30 minutes. Table 24 shows that there was no action of PGE_2 on renin release until 15 minutes. Control renin activity at this time was (mean $\pm$ S.E.M.) 93.5 $\pm$ 5.4 ng A I/mg protein and PGE_2 increased the release to 113.4 $\pm$ 3.0 ng A I/mg protein ($p < 0.02$). At 30 minutes, release was also increased from 93.6 $\pm$ 5.5 control to 121.6 $\pm$ 3.5 ($p < 0.01$). These results implied that the action of prostaglandin on renin release involved some intracellular mechanisms that were activated by prostaglandins. Prostaglandins stimulated calcium uptake in several tissues, an effect which could be mediated by CAMP (Ramwell and Shaw, 1970), and PGE_1 has been postulated to be a "calcium ionophore" (Kirtland and Baum, 1972).

RKC cells incubated for 20 minutes with normal Earles buffer containing 2.5 mM EGTA, 1.5 mM or 2.5 mM calcium were used to determine whether or not calcium uptake by the cells was necessary for the action of PGE_2 and $PGF_{2}\alpha$. Figure 40 shows that if calcium exchange was prevented by the calcium chelator EGTA, the action of PGE_2 on renin release was prevented, and was slightly reversed. Control release with 2.5 mM EGTA was (ng A I/mg cell protein, mean $\pm$ S.E.M.) 79.4 $\pm$ 3.1 and addition of a total concentration of 3 x 10^{-12} M PGE_2 decreased release to 68.1 $\pm$ 4.3 (N.S., 86% of control). The stimulation of renin release was present, however, when calcium was present; this increase in activity was highest with 1.5 mM $CaCl_2$. 3 x 10^{-12} M PGE_2 increased release from a control of 56.7 $\pm$ 1.8 to 72.7 $\pm$ 3.3 ($p < 0.05$, 150% of control). With 2.5 mM $CaCl_2$, 3 x 10^{-12} M PGE_2 increased release from a control of 48.6 $\pm$ 3.4 to 63.5 $\pm$ 4.0 ($p < 0.02$, 131% of control). A similar effect was seen with $PGF_{2}\alpha$ in table 25. The inhibition of renin release with $PGF_{2}\alpha$ was prevented and slightly reversed when calcium flux was inhibited by chelation. Release with 2.5 mM EGTA was 35.7 $\pm$ 2.8 and addition of 3 x 10^{-12} M $PGF_{2}\alpha$ (total concentration) increased release to 41.9 $\pm$ 5.2 (N.S., 117% of control). The inhibition of renin release by $PGF_{2}\alpha$ returned when calcium was present; maximum decrease in release occurred with 1.5 mM $CaCl_2$. Control release was 42.3 $\pm$ 2.5 and 3 x 10^{-12} M $PGF_{2}\alpha$ decreased release to 35.8 $\pm$ 1.9 (p <

0.05, 85% of control). Control renin activity with 2.5 mM $CaCl_2$, 28.3 ± 5.2, was decreased to 23.0 ± 3.6 (N.S., 81% of control) with the addition of 3 x 10^{-12} M PGF_2 . These data prove that calcium is very necessary for the action of PGE_2 and $PGF_2\alpha$ on renin release and that the amount of renin release with these prostaglandins is dependent on calcium concentration.

The site of formation of PGE_2 and $PGF_2\alpha$ in rabbit kidney is primarily located in medulla (Crowshaw, 1971). However, although trace quantities of these prostaglandins have been found in rabbit kidney cortex (Crowshaw and Szlyk, 1970), the presence of active metabolizing enzymes, prostaglandin dehydrogenases, made it difficult to determine the real level of prostaglandin synthesis in this area. Recently, however, Larsson and Änggård (1973), using a microsomal fraction, reported that prostaglandin synthesis in rabbit kidney cortex was 10% of the medulla prostaglandin synthesis.

It has been shown above that very low levels of PGE_2 and $PGF_2\alpha$ have an effect on renin release. The stimulatory effect of PGE_2 may be "hit and run" if prostaglandin dehydrogenases are extremely active. Evidence for this kind of effect was provided by the time course of PGE_2 on renin release (table 24). Thus, even though very small amounts of these prostaglandins are made in the cortex, there would be enough to regulate renin. Also, there is a possibility that

the cyclic endoperoxides, which are intermediates of prostaglandin synthesis (Hamberg and Samuelsson, 1973), might have more potent effects on renin release than the prostaglandins, as has been found in other tissues (Hamberg _et_ _al_., 1974; Hamberg and Samuelsson, 1974). The effects of prostaglandin intermediates should be blocked by the prostaglandin synthetase inhibitors aspirin or indomethacin; these compounds have been shown to inhibit prostaglandin synthesis in kidneys (Somova, 1973). To see whether endogenous formation of prostaglandins occurred, affected renin release and could be blocked, RKC cells in normal Earles buffer were incubated 20 minutes in the presence of equal volumes of ethanol, 2.5 µg/ml arachidonic acid, 0.1 µg/ml indomethacin, these concentrations of arachidonic acid and indomethacin together, 10 µg/ml aspirin, or these concentrations of arachidonic acid and aspirin together. Table 26 summarizes the results of this experiment. Renin release was increased almost two-fold by the presence of arachidonic acid, from a control of (ng A I/mg cell protein, mean $\pm$ S.E.M.) 137.4 $\pm$ 6.9 to 242.9 $\pm$ 3.0 ($p < 0.001$, 177% of control). Indomethacin, which did not significantly affect renin release (134.7 $\pm$ 78, 98% of control), at the concentration used was not able to completely block this high release (211.3 $\pm$ 6.4, $p < 0.001$, 154% of control). However, the concentration of aspirin used, which significantly decreased renin release to 85.8 $\pm$ 4.3 ($p < 0.001$, 62% of control) also decreased renin release with

arachidonic acid to 137.0 ± 8.0 (N.S., 100% of control). The renin release with arachidonic acid and aspirin was significant, however, if aspirin was used as control ($p < 0.001$).

The amount of endogenous PGE formed was related to the amount of renin released in an experiment similar to the one just described. RKC cells in normal Earles buffer were incubated 20 minutes in either ethanol, 0.1 μg/ml arachidonic acid salt, 1.0 μg/ml indomethacin or these concentrations of arachidonic acid salt and indomethacin together. The amount of renin was determined in the supernatant, and immediately after incubation the amount of protein was determined from aliquots of the pellets, the cell pellets from each group were pooled, inhibitors (15 μl/ml 10% EDTA, 2 μg/ml BAL, 10 μl/ml 0.34 M 8-quinolinol sulfate) were added to inhibit prostaglandin dehydrogenases, and the E prostaglandins were extracted and assayed as described in III.7. Figure 41 shows the results of this experiment. Arachidonic acid increased renin release from (ng A I/mg cell protein, mean ± S.E.M.) 58.5 ± 2.6 to 140.6 ± 7.1 ($p < 0.001$, 240% of control) and also increased PGE formation from 2.78 ng/mg protein to 11.30 ng/mg protein. Indomethacin increased renin release to 98.1 ± 4.5 ($p < 0.001$, 168% of control) but PGE formation remained the same, 2.57 ng/mg protein. Arachidonic acid and indomethacin slightly decreased renin release to 118.5 ± 4.6 ($p < 0.001$, 202% of control) and reduced PGE

formation by half, to 5.94 ng/mg protein. These data proved that endogenously formed prostaglandins could increase renin release, and that both the formation of PGE and release of renin could be partially blocked by indomethacin; aspirin appeared to be more effective in blocking the arachidonic acid-stimulated release of renin, but the concentration used was 10-100 times that of indomethacin. The stimulation of renin release with arachidonic acid salt was far greater than with PGE_2 alone (240% of control for arachidonic acid salt and maximum of 150% of control with PGE_2). This could be explained by a detergent effect of arachidonic acid, or by a greater activity of the endoperoxide precurser to PGE_2 than the PGE_2 itself.

There have been many examples of modulation of catecholamine effects on their effector organ by prostaglandins (Weeks, 1972). It was therefore of interest to see whether prostaglandins changed the stimulatory effect of epinephrine and norepinephrine on renin release. For this experiment, a concentration of dehydrogenase-resistent PGE_2 which had not stimulated renin release was chosen, and release-stimulating levels of epinephrine or norepinephrine were used. RKC cells in normal Earles buffer were incubated for 20 minutes in the presence of ethanol as control or an equal volume of 5 pg/ml 16,16-dimethyl PGE_2. Three doses of epinephrine or norepinephrine were added to the cells with or without 16,16-dimethyl PGE_2 every 10 minutes, making a final concentration

of 3 x 10^{-8} M. Figure 42 gives the results of this experiment. Epinephrine stimulated renin release from (ng A I/mg cell protein, mean $\pm$ S.E.M.) 76.5 $\pm$ 2.2 to 99.0 $\pm$ 4.0 ($p <$ 0.001, 129% of control), as did 16,16-dimethyl PGE_2, to 94.1 $\pm$ 9.2 (N.S., 123% of control). However, when 16,16-dimethyl PGE_2 and epinephrine were incubated together, the stimulation of renin release did not occur, 70.7 $\pm$ 5.0 (N.S., 92% of control). A similar effect was seen when the cells were treated with 16,16-dimethyl PGE_2 and norepinephrine. Norepinephrine increased renin release from 87.9 $\pm$ 7.0 to 106.7 $\pm$ 5.5 ($p < 0.05$, 121% of control) and 16,16-dimethyl PGE_2 increased release to 99.8 $\pm$ 7.0 (N.S., 114% of control). When 16,16-dimethyl PGE_2 and norepinephrine were incubated together, the stimulation was not present, 72.1 $\pm$ 3.9 (N.S., 87% of control). The data suggest that there was an antagonism of some kind between the catecholamines and PGE_2. Such antagonism could be the result of receptor blockade, alterations in membrane architecture, changes in calcium availability or a change in some intracellular component, such as cyclic AMP or decreased metabolism, which would decrease release.

CHAPTER V

DISCUSSION

An important point should be emphasized at the beginning of this discussion. In any preparation of isolated cells, the normal modulating physiological controls on the cell in tissue and within the body are, of course, eliminated. The baseline metabolic activities and secretion in the isolated cell may therefore be very different from those of the same cell in the tissue state. In order to study effects which may be of physiological significance, the isolated cell must be kept in a milieu similar to that found in the body. The effects which are caused by each regulator of renin release should be assessed in view of similar studies by other investigators which have used tissue slice systems, animals with certain known control areas removed, or intact animals.

A. Comparison of Cell Preparation Methods

It should also be emphasized that the method of cell isolation directly influences the ability of the cells to react to chemical stimuli that are within physiological concentrations. Reproducible reactions from preparation to preparation are possible only if the cell isolation

technique reproducibly yields isolated cells with similar characteristics from each cell preparation. Since experimental procedures should be done with multiplicate cell incubations per treatment to reduce variation, a good yield of intact cells is also necessary.

Of the three tissue dissociation methods used, the best cell preparations were obtained with the citrate-mechanical method. This method was superior to the collagenase methods for several reasons. There were no tubules, glomeruli or cell clumps present in citrate preparations, but the collagenase-treated cell suspensions often included tubule fragments and cell clumps which had to be removed by several passes through 20 μ nylon mesh and glass wool. The yield of cells was 17% of the cortical wet weight with citrate isolation but only 10% of the total cortical wet weight with collagenase. The yield of renin was almost the same for both preparations; 5% of the total cortical renin for citrate preparations and 3% of the total cortex renin for collagenase preparations. However, since the renin assay was sensitive enough to detect renin released in both preparations, this low yield of renin was not important. With collagenase preparations, it was necessary to wash the cells several times to remove proteolytic enzymes contained in the crude collagenase, e. g., pronase, (Poste, 1971). Citrate was easily metabolized by the cell and was less of a problem. Since even one wash removed much renin activity from the

kidney cells (tables 3-5), it was a great advantage to have a material that did not require cell washing. The viability of the citrate-dissociated cells (90%) was much better than the collagenase-isolated cells (50-80%). Likewise, the membranes of the citrate-isolated cells were less leaky, as was shown by assay of intracellular enzymes released into the supernatant when cells were incubated at 37°C for 20 minutes (table 10). The citrate-prepared cells were also more reactive to changes in ionic environment than were the collagenase-prepared cells (table 27), and were exquisitely sensitive to treatment with hormones and drugs. The citrate-prepared cell membranes appeared more intact (figure 17) than did the collagenase-treated membranes (figure 16). These results were in complete agreement with Thimmappayya *et al*. (1970), who found that the yield of kidney cells prepared by citrate was very good and the cells were not contaminated with tubules or cell debris, did not take up trypan blue, and were not able to utilize oxygen in the presence of calcium. The yield of enzymatically dissociated (collagenase or collagenase-hyaluronidase) cells, on the other hand, was low, and the preparation included tubule and cell debris, the cells took up dye and the preparation could utilize oxygen in the presence of calcium. Another group (Yamada and Ambrose, 1966) reported a decrease in the electrophoretic mobility of cells with high levels of collagenase, a sign that some surface membrane components were destroyed by the

enzyme treatment. Segard et al. (1964) have also described superior cell preparations using similar concentrations of citrate. The morphological intactness of these cells, as seen by electron microscopy, was much better than that of cells that had been enzymically prepared.

The enhanced sensitivity of the citrate-isolated cells to calcium was also observed by Thimmappayya et al. (1970); they thought that when tissue was dispersed with enzyme some intracellular material adhered to the cell surface. This intracellular material might bind calcium, making it more difficult for calcium to enter the cell.

In this dissertation it was found that the gentlest conditions for kidney cell isolation were citrate dissociation at room temperature with no calcium or magnesium present in the buffers (to prevent renin release), and without cell washes. Scaife and Brohee (1967) have observed that cells dissociated at 0°C exhibited increased dye incorporation and ^{51}Cr-binding.

That normal inhibition of renin release was lacking in the isolated cell system was evidenced by a high release level of about 30-50% of the total cell renin in the case of collagenase-isolated cells and 10-20% release with citrate-isolated cells. This high release has been noticed by many other authors working with in vitro systems. DeVito et al. (1970) and Braverman et al. (1971) suggested that synthesis was taking place in vitro, since renin release calculated

in vitro was approximately ten times the release in *vivo*. It seemed to be related to metabolic rate and not to renin content of the kidneys, for renin release was decreased in a nitrogen atmosphere or by arsenate and arsenite treatment. The renal cell suspensions used in the experiments for this dissertation were always incubated in medium containing enough subtrate for continued metabolism and protein synthesis. The high rate of renin release in this system therefore probably reflected synthesis as well as release from pre-existing renin stores. Possibly the reason for the decreased basal renin release in the citrate-isolated cells was an overall decrease in metabolic activity caused by the well-known citrate-induced inhibition of glycolysis via phosphofructokinase. Renin release from collagenase-isolated cells increased with time but remained constant from citrate-isolated cells for periods up to 30 minutes (for an example of renin release with time from the citrate-isolated cells, see controls in table 24). Braverman *et al*. (1971) suggested that, *in vitro*, renin secretion might be occurring at a maximal rate; this view was shared by Corsini *et al*. (1974) who found maximal secretion with time, up to 60 minutes, at 37°C under an oxygen atmosphere and less under a nitrogen atmosphere. Since it was observed that in the cell suspension, control release increased after 30 minutes but was constant up to that time, few incubations were done beyond that amount of time in the present studies.

Because release of many intracellular enzymes, including renin, was lower in the citrate-isolated cell system, this dissociation procedure was used for all drug, hormone and autocoid work. Leakage of intracellular enzymes was observed in tissue slices by Morimoto et al. (1970) and DeVito et al. (1970), who remarked that the percentage release of enzymes other than renin was much lower than the percentage release of renin. This was true of the citrate-prepared cells, but not of the collagenase-treated cells (table 10).

Although several authors have found less control release at temperatures lower than at body temperature (37°C) (Yamamoto et al., 1967; Weinberger et al., 1972), all experimental incubations in these studies were done at 37° C because the inhibitory or excitatory stimuli on the system would be similar to physiological conditions. The membrane fluidity and metabolic activity of the cell at 37°C might be quite different than at lower temperatures. At 37°C and with the described incubation conditions, it was possible to observe both stimulation and inhibition of renin release; a fairly high control release was actually an advantage when factors inhibitory to the system were studied.

When the kidney cortical cells were separated into cell types, however, they were isolated at room remperature

and gradually adapted to lower temperature so that metabolism would be minimal. Of the two separation procedures tried, the most promising was free-flow electrophoresis. The main advantage of this procedure was that only 30 minutes was necessary for cell separation, whereas the density gradient centrifugation method took several hours before the cells were fractionated and washed free of Ficoll. The electrophoretically separated cells did not require washing, since they were collected into a physiological buffer. The physical system of free-flow electrophoresis also had less variation than did the density gradient centrifugation method. With step gradients, cells could aggregate in higher concentrations of macromolecules (e.g. Ficoll), pack at each interface, and also could stick to the centrifuge tube walls (Shortman, 1972). These artifacts were avoided by use of the correct buffer in the electrophoresis system. An added advantage of this system was that if dead or leaky cells were present, they were deflected towards the anode. A disadvantage of the electrophoresis system was that some cells might not differ as much in surface charge as in density or in a metabolic capacity; such cells would not be separated by this method.

The cell separation work in this dissertation was done primarily to ascertain whether or not there were biochemical differences between the renin-containing cells and

other kidney cortex cells. It was found that the renin-containing cells were indeed unique in their CAMP content and reactivity to CAMP and in their renin release to the - adrenergic stimulant isoproterenol (figure 25). In this experiment, all cell fractions were tested for release of renin or increase in CAMP in response to isoproterenol; only the renin-rich cell fraction reacted to this stimulus. The renin-rich cells stained with thioflavin T but other cortical cells did not, and the renin-rich cells did not incorporate aldosterone to any great extent, but other cortical cells did (figure 28). The separated renin-containing cells were used only for these studies because these measurements were very sensitive. The extremely low yield of enriched renin cells by both methods precluded their use in experiments studying control of renin release, since such studies required a large number of cells. For all detailed release studies, "crude" renal cortex cell suspensions were used.

B. Control of Renin Release

1. Ionic and Osmolar Regulation

The observed parabolic renin release response to increasing concentrations of calcium (figure 31) was also seen by Michelakis (1971b) in dog renal cortical cells, but not by Morimoto *et al.* (1970) in dog cortical slices.

although stimulation occurred, or Aoi et al. (1974) in rat kidney slices. This discrepancy between the isolated cell system and tissue slices was probably caused by a difference in permeability. It is difficult to achieve a uniform concentration in tissue slices, whereas cells are completely exposed to the medium. The increase in renin activity with increased calcium concentration seen by Morimoto et al. (1970) was probably a mixed response of JGA exposed to the medium and JGA deep inside the tissue which was exposed to less concentrated calcium. Aoi et al. (1974) used a high osmolality medium in their experiments, which may have blunted the response to calcium. The response of RKC cells to calcium confirmed the observations of Michelakis (1971b), who used dog renal cortical cells. However, there appears to be a species difference between dog and rabbit renal cortical cells, since renin release from RKC cells was highest at 1.5 mM calcium but in dog the maximum release was at 2.5 mM calcium. Renin release from RKC cells was not completely abolished but was inhibited by removal of calcium from the medium and this decreased release also occurred with 4 mM calcium. This effect was similar to the inhibition of glucose-induced insulin release by low or by high calcium (Hales and Milner, 1968a). The necessity for the presence of calcium is a common feature of many secretory systems (Douglas, 1968; Rubin, 1970).

Renin release from RKC cells was maximally stimulated by 0.5 mM magnesium in the presence of calcium. Morimoto et al. (1970) found that maximum release of renin from kidney slices occurred at 1.18 mM magnesium. This discrepancy could have been caused by poor penetration of buffer into the tissue chunks. Renin release with magnesium was different from insulin secretion (Hales and Milner, 1968a) or vasopressin release (Mikiten and Douglas, 1966) because magnesium did not inhibit renin release in the presence of calcium. The two ions were synergistic for renin release; this was also well demonstrated by Morimoto et al. (1970). The renin system seemed similar to glucagon secretion from pancreas, which was synergistically stimulated by low levels of calcium and magnesium (Leclercq-Meyer et al., 1973).

Stimulation of renin release by low sodium or low osmolality and inhibition of release by high sodium or high osmolality has been observed in vivo and in vitro by many investigators (Bunag et al., 1968; Weinberger and Rosner, 1972; Michelakis, 1971b), and is a direct effect on the renin-containing cell; high osmolality shrinks the cell, preventing release, and low osmolality causes the cell to swell and stretch, which enhances release. In the present studies it was found that if osmolality was kept constant by addition of choline chloride to equal the osmolality of 150 mM NaCl there was no significant change in renin release,

even if the kidneys had been "loaded" with renin by furosemide diuresis, which stimulates renin production (Meyer *et al.*, 1968). This observation was similar to that of Weinberger and Rosner (1972) using rat kidney slices, and to Young and Rostorfer (1973) who found no difference in the ability of hypertonic NaCl, dextrose or urea to change renin release *in vivo*. The increase in renin release with low osmolarity or low concentrations of NaCl could only occur when normal or high potassium was present (figure 33). With low potassium concentrations the effect of sodium concentration changes was blunted. Vander (1970), infusing KCl into dog renal arteries, reported a decrease in renin release and natiuresis, results which are consistent with the above observations; when extracellular potassium and sodium were high, renin release from RKC cells decreased. Likewise, when Shade *et al.* (1972) infused KCl into non-filtering kidneys where no sodium was present in the tubules to interact with JG cells, there was no change in renin secretion. This could have been caused by absence of control by the macula densa or by a lack of sodium to exchange with potassium. A stimulation of renin release with low sodium diet has been observed in rats (Sealy *et al.*, 1970) and man (Dluhy *et al.*, 1974) but was not observed in the present studies. The *in vivo* stimulation of renin release by decreased potassium was more related to the stimulation of aldosterone and concomitant increase in sodium

reabsorption than to a direct effect on the JGA. Dluhy et al. (1974) found that changes in potassium diet with either low or high sodium diet did not correlate with renin release, but changes in the sodium diet did. Increased secretion with high potassium has been reported in several hormone systems, always when the investigators lowered sodium concentrations to maintain isotonicity. The high extracellular potassium was thought to stimulate secretion by membrane depolarization, i.e., by mimicing nerve stimulation. Kraicer et al. (1969) found an increase in ACTH release in vitro when adenohypophysis were incubated in high KCl and low NaCl buffer. Samli and Geschwind (1968) obtained similar results with luteinizing hormone in vitro using high potassium-low sodium medium, and Hales and Milner (1968b) also saw stimulation of insulin secretion in vitro with high potassium. They observed that if the pancreas was incubated in completely sodium-free medium with high potassium, this stimulation did not occur. Malaisse-Lagae and Malaisse (1971) found that glucose-induced calcium uptake was increased by high potassium-low sodium buffer. It appears that secretion is stimulated by membrane depolarization, which initiates an inward flux of sodium and outward flux of potassium from the cell, which then stimulates uptake of extra cellular calcium. This increase of calcium in the secreting cell is thought to stimulate the processes necessary for secretion, such as activation of microtubule

systems that move the granule towards the plasma membrane, or perhaps the exocytosis of granules. The ions required for secretion from anterior pituitary and pancreas are also required for release of renin. The general mechanism for stimulus-secretion-coupling thus appears to be valid for the release of renin.

2. Autonomic Nervous System Regulation

The role of the sympathetic nervous system in regulation of renin release was studied by mimicing nerve stimulation with the β-adrenergic agonists norepinephrine and isoproterenol. Both of these catecholamines stimulated renin release but norepinephrine acted at lower concentrations than did isoproterenol (table 16). Epinephrine also stimulated renin release; the renin cell also had α-adrenergic receptors which, when activated, resulted in inhibition of renin release. This was shown by the increase in release with epinephrine when phentolamine was present. β-receptor blockade abolished the stimulation caused by all three catecholamines. The hypothesis that β-receptor stimulation is mediated by an increase in the "second messenger," CAMP (Robison and Sutherland, 1970) was supported by the observation that isoproterenol increased renin release and CAMP in renin-containing cells enriched by Ficoll density gradient centrifugation (figure 25). Exogenous CAMP also activated renin release at high concentrations, as did dibutyryl CAMP

and the specific phosphodiesterase inhibitor SC 2964. Monobutyryl CAMP decreased renin release, but this could have been caused by metabolites 5' AMP or butyrate. 5' AMP was shown to decrease renin release (table 17), but butyrate was not tested. The evidence that β-adrenergic stimulants act directly on the renin cells was also supported by Johnson _et al_. (1971) who found direct effects of norepinephrine and renal nerve stimulation on renin release in the nonfiltering kidney and by Michelakis, _et al_. (1969) using dog kidney cortical cell suspensions. Michelakis _et al_. (1969) likewise found a direct stimulation of the renin cells by epinephrine, as was shown in the present studies. Assaykeen _et al_. (1970) also reported enhancement of stimulation of renin release by epinephrine when phenoxybenzamine was present, as the present studies also showed (figure 34). Vandogen _et al_. (1973) obtained specific blockade of β-induced (isoproterenol) renin release by infusion of propranolol; these results were confirmed by the studies in this dissertation (figure 34). The role of CAMP in β-stimulation of renin release was indirectly shown with the increase in renin release caused by exogenous CAMP, its mono- and dibutyryl derivatives and by SC 2964. These results confirmed the earlier observations by Michelakis _et al_. (1969) that exogenous CAMP enhances renin release from dog renal cortical cells, Yamamoto _et al_. (1973) showed that CAMP and dibutyryl CAMP increased renin

release *in vivo* in dogs and *in vitro* from kidney cortex slices. They also reported that dibutyryl CAMP first stimulated and later depressed renin release. The depression could have been caused by metabolites of the dibutyryl CAMP, an effect which was observed in our studies using monobutyryl CAMP. Reid *et al.* (1972) demonstrated that theophylline stimulated renin release, and that this stimulation was not blocked by α- or β-blockers. The effects that they saw were much larger than those in this dissertation with SC 2964 on RKC cells *in vitro*; possibly theophylline had an intrarenal hypotensive effect which augmented the direct cellular effect at the JGA.

The cholinergic nervous system regulates renin release to some degree. A direct stimulation of renin release from the RKC cell was caused by acetylcholine and this response was specifically blocked by atropine, as would be expected of a smooth-muscle-derived cell. If some of the JGA nerve network were cholinergic, discharge from these nerves would add with the sympathetic nerves to cause release of renin. There were no *in vivo* or *in vitro* reports of renin release with acetylcholine. However, general renal nerve stimulation greatly increased renin release (Vander, 1965).

3. Hormonal Regulation

Negative feedback controls exist between the renin-angiotensin system and aldosterone and vasopressin release.

The direct inhibition of renin release by aldosterone (table 18) could be further accented _in vivo_ by sodium retention caused by aldosterone, which would increase tissue osmolarity and decrease renin release. The finding of direct inihibition of renin secretion from RKC cells was in agreement with Greco and Murphy (1972), but Rosset and Veyrat (1971) found no effect with 1 µg/ml aldosterone on human kidney slices. This could be a species difference, since DiVito _et al._ (1970) found direct inhibition of renin release in rat kidney slices with 0.05 to 0.5 µg/ml aldosterone. Generally, inhibition of renin release was found in patients with hyperaldosteronism (Luetscher and Beckerhoff, 1972) and in human subjects given mineralocorticoids chronically (Gross _et al._, 1957). It was thought that the latter effect was primarily caused by increased sodium retention as a result of the high mineralocorticoid levels.

A negative feedback was also found with low concentrations of vasopressin, which increased renin release; high levels, i.e., the amounts that would normally be found in the kidney if vasopressin was released in response to dehydration, did not change renin release.

Many authors have reported inhibition of renin release _in vivo_ with infusions or injections of vasopressin (Bunag _et al._, 1967; Vander, 1968), and Gutman and Benzakein (1971) demonstrated increased renin levels in rats with diabetes insipidus. These observations support

the data presented in this dissertation that renin release from RKC cells was stimulated by low amounts of vasopressin.

Glucagon was also found to increase renin release from RKC cells. This fact supports the *in vivo* results of Vandogen *et al.* (1973, 1974) and *in vitro* tissue slice data of Nolly *et al.* (1974). The effects of glucagon were possibly related to adenylcyclase activity, but through a different receptor than the adrenergic receptor (Robison *et al.*, 1971). Since the concentrations used, 0.5 pg/ml, were within normal plasma levels, it seemed that glucagon could normally regulate renin release to some degree.

Parathyroid hormone was inhibitory to renin release in the presence or absence of calcium. This finding was not consistent with one report (Meyer *et al.*, 1972) of several patients that had hyperparathyroidism and high renin activity. However, kidney damage caused by their hypercalcuria could have caused this. Possibly parathyroid hormone normally lowers renin release.

Since insulin increased renin release only when glucose was not present, it probably acted only in pathological states and normally had no role in regulation of renin secretion.

4. Regulation by Autocoids

In vivo infusion of histamine (Vander and Luciano, 1967) or serotonin (Bunag *et al.*, 1966b) had no effect on renin release. However, both autocoids were very quickly metabolized and may have never reached the JGA in great enough concentration to change renin release. Also, both compounds had smooth muscle effects and may have changed local blood flow enough to negate any direct effects on the renin cell. The *in vitro* RKC cell system released renin in response to histamine, and this release was blocked by an antihistamine. Serotonin inhibited renin release. However, probably these autocoids did not normally accumulate in great enough concentration to effect renin regulation.

The inhibition of renin release by A II reported *in vivo* (Vander and Geelhoed, 1965; Shade *et al.*, 1973) and *in vitro* with dog kidney cortex cells (Michelakis, 1971a) was also found with the RKC cells (figure 36). However, A I was the most powerful inhibitor, causing complete arrest of release at 600 ng/ml. This result was not in agreement with the work of Russet and Vegrat (1971) who used human kidney slices and found inhibition of renin release with A II but not A I. This lack of agreement could only be explained by species difference, since the inhibition was very marked with rabbit cells. The mechanism of action of renin release inhibition by angiotensin was not investi-

gated. However, Munday et al. (1971) have reported that angiotensin stimulated Na^{+}-K^{+} pump activity in kidney cortex slices, Baudouin and Meyer (1972) have reported calcium release when angiotensin interacted with smooth muscle microsomes, and Schreier-Muccillo et al. (1974) reported conformational changes in membrane caused by A II. It seems that angiotensin interacts with the cell membrane, causing changes in membrane architecture, and also enters the cell to cause release of stored calcium. It may also enhance calcium uptake from extracellular space. Increase in exogenous calcium inhibited renin release (figure 31) and possibly angiotensin promoted this calcium effect.

In RKC cells, it was found that the E prostaglandins and PGA_2 directly stimulated renin-containing cells to release renin, but only at low concentrations. $PGF_{2\alpha}$ and PGA, inhibited renin release. Of the prostaglandins tested, the only ones that could possibly reach the JGA intact were PGE_2 and $PGF_{2\alpha}$, which are made in kidney medulla, and PGA_1, which is not inactivated by the lung. Werning et al. (1971) also saw an increase in renin activity with PGE_1 infused into dog kidneys, and Corsini et al. (1974) saw no stimulation of renin release from rat kidney slices in vitro with high amounts of PGE_1. Varkarkis et al. (1975), reported a direct increase in renin release in response to PGA_1 from both infused and noninfused kidneys, a report that was contrary to the RKC cell results. However, infusion of

PGA_1 also caused sodium, potassium and water diuresis and increased PAH clearance, which might account for the increase in renin release. The results with RKC cells proved that PGE could be made in the cortex, as Larsson and Anggard (1973) postulated, and suggested that the endoperoxide intermediate was more active in releasing renin than the PGE_2 or $PGF_2\alpha$ (figure 40). The endoperoxide was found to be a more potent stimulant in other tissues (Hamburg et al., 1974). Prostaglandin synthesis was blocked by indomethacin (table 26, figure 40) as was reported by Somova (1973). Renin release was also blocked by indomethacin, however, aspirin was a better inhibitor of the renin release caused by arachidonic acid (table 26).

The fact that PGE_2 acted on renin release only after 15 minutes suggested that some slow interacellular mechanism was involved. Since prostaglandins stimulate calcium exchange in some tissues (Ramwell and Shaw, 1970; Kirtland and Baum, 1972), RKC cells were incubated with PGE_2 or $PGF_2\alpha$ in the presence of EGTA or different levels of calcium. No effect was seen with either prostaglandin unless calcium was present, and the magnitude of the effects changed proportionally with the calcium concentration in the buffer. This evidence verified the hypothesis that calcium was needed for prostaglandin action on renin release; it is possible that the prostaglandins act as calcium ionophores.

It is apparent, from the prostaglandin structures in figure 37, that hydroxyl and keto-groups on the cyclopentane ring were necessary for renin stimulation. If the hydroxyl group on the 20 carbon chain was blocked to prevent degradation by prostaglendin dehydrogenases, (16, 16-dimethyl PGE_2, 15-S-15-methyl PGE_1) or if the double bond was not present between carbons 5 and 6 (PGE_1) the compound was a less potent stimulant of renin release. Absence of the hydroxyl on the cyclopentane ring or of the 5-6 carbon double bond resulted in a compound that inhibited renin release (PGA_2, $PGF_{2\alpha}$ in figures 38 and 39).

Prostaglandins have been implicated in many tissues as modulators of catecholamines (Weeks, 1972). Experiments with RKC cells confirmed this observation (figure 42); the stimulatory effects of epinephrine and norepinephrine on renin release were prevented by the presence of 16, 16-dimethyl PGE_2. The stimulatory prostaglandins, however, were also self-regulatory, for they stimulated only at low concentrations; at higher concentrations, the effect became less, which perhaps reflected a saturation of prostaglandin receptors.

Table 28 summarizes the stimulatory and inhibitory regulators of renin release. The most potent regulators of release, according to the data presented, were those that could occur intrarenally. The most effective stimulant of renin release was the endogenous formation of PGE from

arachidonic acid. The next most active intrarenal stimuli were acetylcholine and β-adrenergic stimulation, which could be synergistic. However, the presence of prostaglandin E_2 effectively regulated stimulation by catecholamines, and also was "self-regulatory." The most potent inhibitor of renin release was A I. Probably it is formed very near to the JG cell, since secreted renin would quickly make it from α-2 globulin in blood flowing by the JGA (Granger et al., 1972). Although A I would probably not accumulate, only 600 pg/ml was needed for 45% inhibition of renin release; full inhibition was accomplished with 600 ng/ml. Another inhibitor that could occur near to the JGA was $PGF_{2\alpha}$. At 3×10^{-10} M this compound inhibited renin release 73%. It is easy to believe that this amount could be formed in the cortex or be carried to the cortex from the medulla by renal tubules. Other hormones or autocoids found to influence renin release probably do so only in conditions of physiological stress: fasting (insulin); temporary dehydration (vasopressin); infection (histamine, serotinin), or with gross abnormalities found in disease. The circulating hormones epinephrine, glucagon and aldosterone, which both directly and indirectly regulate renin release, probably play an important regulatory role normally. The prostaglandins modulated the effects of epinephrine, just as they did norepinephrine.

The most important intrarenal ionic parameters for control of renin release seem to be the ratio of sodium to potassium in the renal tubule versus the arteriole. These ions would vary considerably with diet, hydration and aldosterone output. If drugs, especially the natiuretic or the potassium-sparing diuretics were present, this also would greatly influence the ratio of sodium to potassium. Under normal conditions, calcium concentration in the body remains at a steady state of about 2.5 mM. At this concentration, renin release is low (figure 31); calcium concentration would probably never get low enough to stimulate renin release except in extreme cases of hyperparathyroidism or calcium wastage. Likewise, parathyroid hormone would normally not affect renin release. Magnesium levels also do not fluctuate enough to cause changes in renin secretion.

C. Intracellular Renin and Parameters Affecting Release from the Cell

Table 12 shows that, excluding mitochondria, pure Golgi had the highest renin activity of all subcellular fractions. The high renin activity of the mitochondria was not mitochondrial *per se* but renin granule contamination. This was shown by the low activity of "plasma membrane" mitochondria which did not sediment with renin granules. Partial separation of the renin granule from the mitochondria was achieved, as was shown in table 13, by density gradient

centrifugation; this showed that the renin sedimented into higher density sucrose than the mitochrondria. The renin granule was a distinct particle surrounded by a membrane that was differently charged than the lysosome or mitochondria (figure 29). The appearance of this high-renin activity particle (figure 30) was similar to granules seen *in vivo* in rats with renal arterial construction or in developing rats (Barajas, 1966). Since renin activity was high in Golgi subcellular fractions, and since granules have been seen near Golgi apparatus in animals with high plasma renin activity (Barajas, 1966), it seems possible that the Golgi apparatus is responsible for packaging the renin in the membrane and that the granule is then stored in the cytoplasm and released by exocytosis or by fusion of the granule membrane with the plasma membrane. Cleavage of both membranes released active renin into the blood stream.

The release of renin required energy (DiVito *et al.*, 1970; Bozovic and Efendic, 1969) as well as calcium (figure 31). Also, the fact that the ratio of sodium to potassium was important for renin release (figure 33) indicated that release of renin might be similar to release of neurotransmitter or excitation-secretion coupling in many endocrine glands. This process appeared to be aided by nerve stimulation, excess extracellular potassium, prostaglandins, catecholamines, glucagon and a rise in

intracellular CAMP, all of which promoted increased uptake of calcium from outside the cell and perhaps involved mobilization of calcium inside the cell. The net increase in intracellular calcium caused by these stimuli correlates with increased secretion of renin or renin granules from the cell.

CHAPTER VI

SUMMARY

The properties of enriched renin-containing cells were studied after these cells had been isolated by Ficoll gradient centrifugation or by free-flow electrophoresis. It was found that the renin-containing cells had more CAMP than other renal cortex cells, and that the intracellular levels of CAMP increased, as did renin release, in response to the β-adrenergic stimulant isoproterenol. These cells had a unique plasma membrane, and therefore could be separated from other renal cells by free-flow electrophoresis. They did not incorporate aldosterone as much as the other renal cells, and they fluoresced with thioflavin T, while cells in other fractions did not.

The external factors directly acting on the renin-containing cells were investigated using "crude" kidney cortex cells prepared by three methods. These methods were compared on the basis of cellular integrity, viability, yield and sensitivity. The citrate-mechanical method was chosen as the most adequate for extensive studies.

Both calcium and magnesium stimulated renin release

at low concentrations, but at physiological concentrations both divalent cations inhibited release. Osmolality change and not sodium per se was found to be important in renin release from cells of normal and sodium-depleted (renin-loaded) animals; low osmolality stimulated renin release and high osmolality inhibited renin release. The response to high sodium did not occur at low potassium concentrations, but at physiological and supraphysiological potassium concentrations, changes in sodium concentration changed renin release.

Catecholamines and acetylcholine both stimulated renin release. This stimulation was specifically blocked by atropine (for acetylcholine), or propranolol (for norepinephrine and for isoproterenol). In addition, phentolamine "unmasked" the β-stimulatory effect of epinephrine; its presence with epinephrine increased renin release more than did epinephrine itself. Exogenous CAMP and dibutyryl CAMP stimulated renin release, as did the phosphodiesterase inhibitor SC 2964. Monobutyryl CAMP, however, decreased renin release, possibly because of its rapid breakdown to butyrate and to 5'-AMP, both of which could depress renin release.

It was found that aldosterone directly inhibited renin release. Parathyroid hormone was also found to

directly inhibit renin release, both with and without calcium present. At physiological levels, insulin stimulated release only without glucose; glucagon and vasopressin also stimulated release.

Histamine stimulated renin release, an effect that was abolished by diphenhydramine. PGE_1, PGE_2 and PGA_2, as well as the dehydrogenase-resistant 16, 16-dimethyl PGE_2 and 15-S-15-methyl PGE_2 stimulated renin release at low concentrations but at higher concentrations this stimulation diminished. A I greatly inhibited renin release at very low concentrations and completely inhibited release at higher concentrations; A II was less effective in this regard. Serotonin, $PGF_{2\alpha}$ and PGA, all inhibited renin release. The stimulation of renin release caused by norepinephrine and epinephrine was abolished by the presence of 16,16-dimethyl PGE_2, indicating that prostaglandins modulate sympathetic nervous system activity in the kidney.

It was also found that PGE_2 could be formed in the cortex and it, or its precursor endoperoxide, greatly stimulated renin release. It was also found that both PGE_2 and $PGF_{2\alpha}$ required calcium for their actions on the renin-containing cell. Since it took 15 minutes for PGE_2

to act on renin release, it was probably acting on some intracellular mechanism, such as mobilization of intracellular calcium.

The intracellular formation of the renin granule was studied in kidney subcellular fractions. It was found that the highest renin activity was in the mitochondria, but that this activity was caused by renin granule contamination of the mitochondria. The second highest renin activity was found in the Golgi apparatus. It is suggested that the Golgi apparatus may be involved in the packaging of renin in a membrane to form the renin granule. Renin granules were isolated and studied electrophoretically and electron microscopically. The renin granule was found to be a unique particle with a membrane charge that was different from the mitochondrial or lysosomal membrane. The appearance of the granule was similar to those seen in the JGA in tissue sections. It had a double membrane, an electron-dense granular matrix and a maximal diameter of 1.4 μ. Classical stimulus-secretion coupling caused release of this granule from the cytoplasm, but the exact release process remains to be investigated.

The parameters directly affecting renin release are

very complex, but from the present work, it seems that the most important controls are local ones--presence of prostaglandins, sympathetic and parasympathetic nerve activity and the ionic balance of the tissue surrounding the JGA. Alterations in circulating hormones caused both direct and indirect effects. There was negative feedback control between the renin-aldosterone axis, the renin-vasopressin axis, the renin-prostaglandin axis and also within the renin-angiotensin system. These negative feedback loops could cause extremely fine regulation of fluid and osmotic balance in the body. The kidney cortical cell suspension was useful to this investigation because the physiological regulators active *in vivo* were removed. For this reason, each regulatory parameter could be assessed separately to obtain a model of the relative importance of each direct influence. Subcellular fractionation was useful for the study of intracellular renin granule formation, storage and release.

APPENDIX A

TABLES

TABLE 1

LOSS OF RENIN ACTIVITY WITH TIME

Incubation of Renin with Substrate ng A I/30 minutes/mg protein (mean ± S.E.M.)			
Day of experiment		1 month after experiment	
Cells[a]	Supernatant	Cells[a]	Supernatant
74.0 ± 11.8	38.3 ± 1.3	56.4 ± 5.2[b]	8.0 ± 2.1[c]

[a]Renin in cells was protected against degradation by addition of 0.0034 M 8-quinolinol sulfate/ml, 0.15% EDTA, 0.2 mg BAL/ml tissue and pH 7.15, whereas supernatants had no inhibitors added.

[b]N.S.

[c]$p < 0.001$.

TABLE 2

EFFECT OF PHARMACOLOGICAL AGENTS ON THE REACTION OF RENIN WITH RENIN SUBSTRATE[a]

	ng A I Formed/0.5ml Substrate					
Treatment of Substrate	1μg cell protein added	% of control	Effect	4 μg cell protein added	% of control	Effect
Control	0.49			1.65		
Theophylline, 1 mM	0.48	97	0	1.63	99	0
SC 2964, 2.5 mM	0.47	95	0	1.25	76	↓↓
Propranolol, 1 x 10^{-4}M	0.55	112	↑	1.53	93	0
Propranolol, 1 x 10^{-2}M	0.52	106	0	1.30	79	↓↓
Indomethacin, 0.1 μg/ml	0.42	86	↓	1.17	71	↓↓
Indomethacin, 10 μg/ml	0.84	171	↑↑↑	2.05	124	↑↑
Aspirin, 10 μg/ml	0.60	122	↑↑	1.78	108	↑
Aspirin, 1 mg/ml	0.56	114	↑	1.71	104	0
Ethanol, 0.2%	0.46	94	0	1.52	92	0

TABLE 2 - Continued

	ng A I Formed/0.5ml Substrate					
Treatment of Substrate	1μg cell protein added	% of control	Effect	4 μg cell protein added	% of control	Effect
Collagenase, 2 mg/ml	0.48	97	0	1.64	99	0

[a]pH 5.5, 37°C, 30 minutes incubation.

TABLE 3

LOSS OF RENIN ACTIVITY AND PROTEIN IN THE PREPARATION OF RENAL CELLS BY ENZYMATIC DISSOCIATION

	Total Renin Activity, μg A I	Total Protein, mg
Total cortex[a]	12.623	700
Saline perfusion	0.539	85
Eagles perfusion	0.451	26
Collagenase perfusion	0.144	17
Supernatant 1[b]	2.844	93
Cell pellet 1	0.388	5.8
Supernatant 2[c]	0.175	0.8
Cell pellet 2[c]	0.135	0.5

[a]Values from a cortex sample before collagenase digestion.

[b]Tubules removed by gauze filtration before this step.

[c]Supernatant and cell pellet of 50 x g centrifugation after cells had been washed once.

TABLE 4

LOSS OF RENIN ACTIVITY AND PROTEIN IN THE PREPARATION OF RENAL CELLS BY COMBINED ENZYMATIC AND MECHANICAL DISSOCIATION

	Total Renin Activity, µg A I	Total Protein mg
Total cortex[a]	11.759	1373
Saline perfusion	0.496	175
Earles perfusion	0.162	19
Collagenase	0.115	15
Supernatant 1[b]	6.975	53
Cell pellet 1	0.391	7.7
Supernatant 2[c]	0.193	1.2
Cell pellet 2[c]	0.090	2.2

[a]Values from a sample of cortex before rubbing through nylon mesh.

[b]Tubules were removed before this step.

[c]Supernatant and cell pellet of 50 x g centrifugation after cells had been washed once.

TABLE 5

LOSS OF RENIN ACTIVITY AND PROTEIN IN THE PREPARATION OF RENAL CELLS BY CITRATE AND MECHANICAL METHODS

	Total Renin Activity, μg A I	Total Protein, mg
Total cortex[a]	8.910	880
Saline perfusion	0.492	135
Citrate perfusion	0.518	57
Supernatant 1[b]	3.834	68
Cell pellet 1	0.436	15
Supernatant 2[c]	0.312	1.9
Cell pellet 2[c]	0.131	8.0

[a]Values from a cortex sample after tissue was minced.

[b]Tubules were removed before this step.

[c]Supernatant and cell pellet of 50 x g centrifugation after cells had been washed once.

TABLE 6

ISOLATION OF RENAL CORTICAL CELLS. LOSS OF RENIN AT 4°C AND 25°C

	Total Renin Activity			
	4°C		25°C	
	μg A I	% of Total	μg A I	% of Total
Total	373.64		453.82	
Saline Wash	19.10	5.1	9.88	2.2
Eagles Wash	3.52	0.9	5.82	1.3
Collagenase	0.39	0.0	1.67	0.0
Slice Solution[a]	203.13	54.4	187.54	41.3
Supernatant	151.00	40.4	107.81	23.8
Pellet	72.71	19.5	110.98	24.5

[a]Solution containing kidney slices before chopping.

TABLE 7

ISOLATION OF RENAL CORTICAL CELLS.
EFFECT OF CALCIUM ON LOSS
OF RENIN

	Total Renin Activity			
	Without Calcium		With Calcium	
	μg A I	% of Total	μg A I	% of Total
Total	38.10		35.89	
Saline Wash	0.48	1.3	0.92	2.6
Eagles Wash	0.36	0.9	0.38	1.1
Collagenase	0.14	0.0	0.14	0.0
Slice Solution[a]	3.81	10.0	6.06	16.9
Supernatant	23.38	61.4	20.57	57.3
Pellet	6.64	17.4	7.79	21.6

[a]Solution containing kidney slices before chopping.

TABLE 8

EFFECT OF 3% BSA ON OXYGEN CONSUMPTION OF KIDNEY CORTICAL CELLS

	$\mu l\ O_2/g$ wet weight/minute[a]								
	Control Eagles Medium				3% BSA in Eagles medium				
	Mean		S.E.M.		Mean		S.E.M.		p[b]
Dog	5.8	±	0.3	(4)	10.8	±	0.9	(4)	>0.001
Rabbit	5.1	±	1.2	(3)	13.7	±	0.4	(4)	<0.001
Cat	23.1	±	3.1	(3)	30.1	±	3.3	(4)	N.S.

[a]Number of Warburg flasks is indicated in parentheses.

[b]Control Eagles medium compared to 3% BSA in Eagles medium.

TABLE 9

EFFECT OF NEAA ON OXYGEN CONSUMPTION OF KIDNEY CORTICAL CELLS

	μl O_2/g wet weight/minute[a]			
	Eagles	Eagles + NEAA	3% BSA in Eagles	3% BSA in Eagles + NEAA
Dog	5.8±0.3 (4)	6.2±0.5 (4)[b]	10.8±0.9 (4)	13.6±3.1 (4)[b]
Rabbit	5.1±1.2 (3)	5.2±1.0 (4)[b]	13.7±0.4 (4)	16.4±2.0 (4)[b]

[a]Data presented as mean ± S.E.M. Number of Warburg flasks is indicated in parentheses.

[b]Not significant.

TABLE 10

RELEASE OF INTRACELLULAR ENZYMES AND PROTEIN AS A MEASURE OF MEMBRANE INTEGRITY

Cell Preparation		Protein mg/5ml Mean ± S.E.M.	Acid Phosphatase μMpNP/min/5ml Mean ± S.E.M.	Succinate Dehydrogenase ΔA 400/min/5ml Mean ± S.E.M.	Renin ng A I/30min/5ml Mean ± S.E.M.
Citrate	Supernatant	0.03±0.01	0.01 ±0.00	22.9± 20.5	22.5±1.6
	Pellet	0.41±0.02	1.07 ±0.00	390.0±108.5	74.7±3.1
	Total	0.44±0.02	1.13 ±0.00	412.9±117.1	97.2±2.9
	Release, % of total	7	1.2	5.5	23
	n[a]	(6)	(6)	(5)	(6)
Collagenase	Supernatant	0.03±0.00	0.03 ±0.00	37.9± 9.8	80.1±1.7
	Pellet	0.20±0.01	2.75 ±0.02	68.8± 7.7	92.7±4.9
	Total	0.24±0.01	2.78 ±0.02	103.7± 14.8	172.8±5.3
	Release, % of total	12.5	1.1	36	46
	n[a]	(6)	(6)	(6)	(6)

[a]Number of assay determinations is in parentheses.

TABLE 11

CENTRIFUGATION OF HIGH RENIN ACTIVITY FRACTIONS AFTER FREE-FLOW ELECTROPHORESIS

	Renin Activity, ng A I/30min/ml				
Pooled Fractions	Total	Pellet	% of Total	Supernatant	% of Total
Anode Fractions 8-14	996	747	75	263	26
Cathode Fractions 28-34	573	119	21	336	62

TABLE 12

RAT KIDNEY SUBCELLULAR FRACTIONS. PURITY AND RENIN ACTIVITY

	Golgi	Endo-plasmic Reticulum	Plasma Membrane			Mitochondria	
			ATP-ase[b]				
	Galactose trans-ferase[a]	Glucose-6-phosphatase[b]	Ouabain −	Ouabain +	+ oligo-mycin	Succinate-cytochrome c reductase[b]	Renin activity[c]
Homogenate	17.2	0.056	0.045	0.147	0.084	0.256	338.0
Pure golgi	552	0.104	0.518	0.589	0.378	0.043	22.4
Less pure golgi	212	0.176	1.22	0.964	0.787	0.097	10.7
Mitochondria	0.5	0.008	0.725	0.707	0	0.816	92.4
Plasma membrane	18	0.122	1.342	0.818	0.502	0.330	0
Plasma Membrane Mitochondria	-	-	-	-	-	0.465	0

TABLE 12 - Continued

[a]Activity expressed as nM/hour/mg protein.

[b]Activity expressed as μM/minutes/mg protein.

[c]Activity expressed as ng A I/30 minutes/mg protein.

TABLE 13

PURITY OF RABBIT KIDNEY RENIN GRANULES

Fraction	Sucrose (M)	Mitochondria Succinate Oxidase[a]	Lysosomes Acid Phosphatase[a]	Renin[b]	Protein mg/ml
1	(Supernatant)	0.054	38.0	1.818	1.95
2	1.30	0.067	25.0	1.572	1.75
3	1.35	0.059	16.0	3.570	3.05
4	1.40	0.050	17.0	4.290	2.45
5	1.60	0.056	31.0	1.290	4.00

[a]Activity - μM/min/mg protein.

[b]Activity - μg A I/30min/mg protein.

TABLE 14

RELEASE OF RENIN FROM RKC CELLS IN RESPONSE TO MAGNESIUM WITH AND WITHOUT CALCIUM

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
2.5 mM Calcium 0.25 mM Magnesium	5	60.7 ± 3.5	1.12 ± 0.04	54.0 ± 2.3	<0.001
2.5 mM Calcium 0.5 mM Magnesium	3	75.2 ± 3.2	0.98 ± 0.03	76.7 ± 3.5	<0.001
0 mM Calcium 0.5 mM Magnesium	3	63.7 ± 2.1	0.94 ± 0.03	68.2 ± 3.0	N.S.
2.5 mM Calcium 1.0 mM Magnesium (control)	6	49.6 ± 1.5	0.71 ± 0.02	69.9 ± 1.2	-
0 mM Calcium 1 mM Magnesium	3	62.7 ± 2.1	0.93 ± 0.01	67.2 ± 2.4	N.S.

TABLE 14 - Continued

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
2.5 mM Calcium 1.25 mM Magnesium	6	37.3 ± 4.5	1.01 ± 0.07	36.4 ± 2.4	<0.001
2.5 mM Calcium 1.50 mM Magnesium	3	24.3 ± 1.9	0.79 ± 0.02	31.2 ± 2.7	<0.001

[a]With control as 2.5 mM calcium, 1.0 mM magnesium, using supernatant activity, ng A I/mg cell protein.

TABLE 15

RELEASE OF RENIN FROM FUROSEMIDE PRE-TREATED[a] RKC CELLS IN RESPONSE TO SODIUM CHLORIDE MADE ISO-OSMOTIC WITH CHOLINE CHLORIDE

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein	p[b]
25 mM NaCl 125 mM Choline Cl	3	75.3 ± 2.2	1.13 ± 0.02	66.9 ± 3.0	N.S.
50 mM NaCl 100 mM Choline Cl	3	86.8 ± 1.1	1.34 ± 0.05	64.8 ± 3.0	N.S.
150 mM NaCl 0 mM Choline Cl (control)	3	113.05 ± 3.7	1.58 ± 0.03	71.8 ± 3.5	-
300 mM NaCl 0 mM Choline Cl	3	111.3 ± 3.9	1.38 ± 0.19	85.1 ± 5.6	N.S.

TABLE 15 - Continued

[a]Normal male New Zealand white rabbits were pretreated with 0.65 mg/kg Furosemide i.m. twice a day for two days.

[b]Control as 150 mM NaCl, using supernatant renin activity, ng A I/mg cell protein.

TABLE 16

RESPONSE OF RKC CELLS TO CATECHOLAMINES

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean $\pm$ S.E.M.	mg cell protein/5ml Mean $\pm$ S.E.M.	Supernatant Renin Activity ng A I/ml cell protein Mean $\pm$ S.E.M.	% of Control[a]
Control	12	44.9 $\pm$ 2.1	0.87 $\pm$ 0.02	51.8 $\pm$ 2.5	-
1-epinephrine 3×10^{-7} M	6	47.2 $\pm$ 1.3	0.85 $\pm$ 0.02	55.3 $\pm$ 1.4	107
1-epinephrine 3×10^{-6} M	6	45.9 $\pm$ 1.7	0.93 $\pm$ 0.03	49.5 $\pm$ 1.9	96
1-epinephrine 1.5×10^{-5} M	6	41.6 $\pm$ 1.7	1.05 $\pm$ 0.01	39.1 $\pm$ 1.6	75
Control	12	42.3 $\pm$ 1.7	0.87 $\pm$ 0.02	48.6 $\pm$ 2.0	-
1-norepinephrine 3×10^{-8} M	6	44.4 $\pm$ 2.0	0.83 $\pm$ 0.03	53.8 $\pm$ 2.3	111
1-norepinephrine 3×10^{-7} M	6	47.0 $\pm$ 1.2	0.89 $\pm$ 0.02	52.7 $\pm$ 1.3	108

TABLE 16 - Continued

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/ml cell protein Mean ± S.E.M.	% of Control[a]
l-norepinephrine 3×10^{-6} M	6	47.6 ± 1.4	0.91 ± 0.04	52.2 ± 2.1	107
l-norepinephrine 1.5×10^{-5} M	6	41.7 ± 1.8	1.05 ± 0.04	39.7 ± 1.9	82
Control	12	46.0 ± 1.2	0.85 ± 0.02	54.1 ± 1.4	-
Isoproterenol 3×10^{-7} M	6	40.5 ± 1.5	0.82 ± 0.01	48.9 ± 1.8	90
Isoproterenol 3×10^{-6} M	6	47.6 ± 2.1	0.83 ± 0.04	58.3 ± 2.8	107
Isoproterenol 1.5×10^{-5} M	6	45.7 ± 1.4	0.66 ± 0.02	69.7 ± 3.3	129

[a]Using supernatant renin activity, ng A I/mg cell protein.

TABLE 17

EFFECT OF EXOGENOUS CAMP, CAMP ANALOGUES AND SC 2964 ON RENIN RELEASE FROM RKC CELLS

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
Control	12	79.4 ± 3.0	0.87 ± 0.04	92.8 ± 3.7	-
CAMP, 5×10^{-7} M	6	75.3 ± 7.2	0.96 ± 0.05	77.6 ± 3.9	<0.05
CAMP, 5×10^{-5} M	6	85.1 ± 3.6	0.81 ± 0.03	104.6 ± 1.7	<0.05
Control	12	42.1 ± 1.1	0.77 ± 0.03	55.1 ± 1.7	-
Monobutyryl CAMP, 1×10^{-7}M	6	36.7 ± 1.3	0.93 ± 0.08	40.9 ± 2.6	<0.001
Monobutyryl CAMP, 1×10^{-5}M	6	29.6 ± 1.7	0.79 ± 0.01	37.5 ± 2.0	<0.001
Control	12	77.3 ± 4.5	0.68 ± 0.02	115.3 ± 6.3	-
Monobutyryl CAMP, 1×10^{-5}M	6	84.1 ± 4.7	0.61 ± 0.01	137.0 ± 6.8	<0.05
Control	6	25.5 ± 1.2	0.38 ± 0.02	60.5 ± 1.5	-
5' AMP 1×10^{-5}M	6	22.0 ± 1.2	0.38 ± 0.01	57.9 ± 1.5	<0.05
Control	12	20.8 ± 0.8	0.42 ± 0.01	50.5 ± 2.8	-

TABLE 17 - Continued

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
SC 2964 0.25 mM	6	22.5 ± 2.2	0.39 ± 0.01	57.1 ± 4.5	N.S.

[a]Appropriate control vs. treated supernatant renin activity, ng A I/mg cell protein.

TABLE 18

INHIBITION OF RENIN RELEASE FROM RKC CELLS BY ALDOSTERONE

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
Rabbit 1					
Control	4	62.5 ± 6.9	1.56 ± 0.05	39.7 ± 3.7	-
Aldosterone 0.002 pg/ml	4	38.8 ± 1.9	1.67 ± 0.08	23.3 ± 1.6	<0.01
Aldosterone 0.2 pg/ml	4	37.6 ± 1.0	1.83 ± 0.08	19.4 ± 0.8	<0.01
Rabbit 2					
Control	4	168.7 ± 4.4	1.25 ± 0.02	135.4 ± 4.7	-
Aldosterone 0.2 pg/ml	4	171.3 ± 5.7	1.49 ± 0.04	115.5 ± 5.4	<0.05
Aldosterone 2.0 pg/ml	4	162.2 ± 1.4	1.59 ± 0.03	102.2 ± 2.9	<0.001
Aldosterone 20 pg/ml	4	154.2 ± 10.9	1.57 ± 0.02	94.7 ± 6.6	<0.01

[a]Control compared to treated supernatant renin activity, ng A I/mg cell protein.

TABLE 19

EFFECT OF ARGENINE VASOPRESSIN ON RENIN RELEASE FROM RKC CELLS

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
Control	13	18.7 ± 1.5	0.38 ± 0.00	47.7 ± 3.8	-
Vasopressin 1 μU/ml	5	23.3 ± 1.8	0.38 ± 0.01	68.0 ± 2.4	<0.001
Vasopressin 10 μU/ml	6	27.5 ± 1.4	0.40 ± 0.01	68.7 ± 2.5	<0.001
Vasopressin 50 μU/ml	6	18.4 ± 1.6	0.42 ± 0.00	44.3 ± 4.2	N.S.
Vasopressin 100 μU/ml	6	19.8 ± 1.2	0.42 ± 0.00	47.3 ± 2.9	N.S.

[a]Control compared to treated supernatant renin activity, ng A I/mg cell protein.

TABLE 20

EFFECT OF PARATHYROID HORMONE WITH EGTA OR CALCIUM ON RELEASE OF RENIN FROM RKC CELLS

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p
2.5 mM EGTA Control	6	39.8 ± 0.5	0.63 ± 0.01	63.7 ± 1.5	-
2.5 mM EGTA + Parathyroid hormone 1 ng/ml	6	36.3 ± 5.5	0.62 ± 0.01	58.1 ± 8.2	N.S.[a]
2.5 mM EGTA + Parathyroid hormone 10 ng/ml	6	29.9 ± 1.3	0.63 ± 0.02	47.7 ± 2.2	<0.001[a]
2.5 mM Calcium Control	6	18.6 ± 1.4	0.72 ± 0.01	26.0 ± 2.3	<0.001[b]

TABLE 20 - Continued

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p
2.5 mM Calcium + Parathyroid hormone 1 ng/ml	6	19.5 ± 1.1	0.73 ± 0.01	26.5 ± 1.5	N.S.[c]
2.5 mM Calcium + Parathyroid hormone 10 ng/ml	6	15.7 ± 1.1	0.81 ± 0.00	19.3 ± 1.4	<0.05[c]

[a] 2.5 mM EGTA compared to 2.5 mM EGTA + parathyroid hormone-treated supernatant renin activity, ng A I/mg cell protein.

[b] 2.5 mM EGTA compared to 2.5 mM calcium, $p<0.001$.

[c] 2.5 mM calcium compared to 2.5 mM calcium + parathyroid hormone-treated supernatant renin activity, ng A I/mg cell protein.

TABLE 21

EFFECT OF INSULIN ON RENIN RELEASE FROM RKC CELLS WITH AND WITHOUT GLUCOSE

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p
Control 10 mM glucose	6	17.0 ± 0.7	0.80 ± 0.00	21.2 ± 0.9	-
Insulin 2.5 μU/ml 10 mM glucose	6	17.6 ± 1.8	0.83 ± 0.02	21.4 ± 2.3	N.S.[a]
Control 0 mM glucose	6	13.9 ± 0.9	0.79 ± 0.01	17.6 ± 1.2	<0.05[b]
Insulin 2.5 μU/ml 0 mM glucose	6	20.9 ± 2.8	0.80 ± 0.00	30.0 ± 1.2	<0.001[c]

[a] 10 mM glucose control compared to 2.5 μU insulin/ml and 10 mM glucose.

[b] 10 mM glucose compared to 0 mM glucose.

[c] 0 mM glucose control compared to 2.5 μU insulin/ml and 0 mM glucose.

TABLE 22

RELEASE OF RENIN FROM RKC CELLS WITH GLUCAGON

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
Control	6	17.9 ± 1.2	0.47 ± 0.01	37.9 ± 2.8	-
Glucagon 0.5 pg/ml	6	25.0 ± 1.6	0.52 ± 0.01	48.0 ± 2.6	<0.05
Glucagon 5.0 pg/ml	6	32.5 ± 0.6	0.51 ± 0.00	63.7 ± 0.7	<0.001

[a]Control as compared to glucagon-treated supernatant renin activity, ng A I/mg cell protein.

TABLE 23

EFFECT OF SEROTONIN ON RENIN RELEASE FROM RKC CELLS

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
Control	7	39.0 ± 2.2	0.58 ± 0.04	68.1 ± 4.0	-
Serotonin 0.8 ng/ml	7	35.4 ± 1.6	0.49 ± 0.02	71.9 ± 3.6	N.S.
Serotonin 8.0 ng/ml	7	27.7 ± 1.7	0.52 ± 0.01	53.5 ± 3.5	<0.02
Serotonin 80 ng/ml	7	24.3 ± 2.5	0.48 ± 0.01	50.6 ± 5.9	<0.05

[a]Control compared to serotinin-treated, supernatant renin activity, ng A I/mg cell protein.

TABLE 24

TIME COURSE OF PGE_2 ACTION ON RENIN RELEASE FROM RKC CELLS

Minutes	Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
0	Control	6	63.7 ± 3.0	0.68 ± 0.01	93.6 ± 3.2	-
	PGE_2 $3 \times 10^{-12}M$	6	65.2 ± 3.2	0.69 ± 0.01	94.2 ± 3.3	N.S.
5	Control	6	51.1 ± 2.4	0.60 ± 0.01	84.8 ± 3.8	-
	PGE_2 $3 \times 10^{-12}M$	6	56.5 ± 10.3	0.67 ± 0.02	83.4 ± 11.5	N.S.
15	Control	6	60.5 ± 3.2	0.65 ± 0.01	93.5 ± 5.4	-
	PGE_2 $3 \times 10^{-12}M$	6	73.9 ± 2.2	0.64 ± 0.01	113.4 ± 3.0	<0.02

TABLE 24 - Continued

Minutes	Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
30	Control	6	60.6 ± 3.3	0.65 ± 0.01	93.6 ± 5.5	-
	PGE_2 $3 \times 10^{-12}M$	6	80.2 ± 2.4	0.69 ± 0.01	121.6 ± 3.5	<0.01

[a]Appropriate control compared to treated supernatant renin activity, ng A I/mg cell protein.

TABLE 25

INFLUENCE OF CALCIUM OR EGTA ON THE DECREASE OF RENIN RELEASE FROM RKC CELLS WITH $PGF_{2\alpha}$

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean $\pm$ S.E.M.	mg cell protein/5ml Mean $\pm$ S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean $\pm$ S.E.M.	p[a]
Control 2.5 mM EGTA	6	23.4 $\pm$ 1.7	0.66 $\pm$ 0.01	35.7 $\pm$ 2.8	-
$PGF_{2\alpha}$, 3×10^{-12} M 2.5 mM EGTA	6	29.0 $\pm$ 4.0	0.69 $\pm$ 0.01	41.9 $\pm$ 5.2	N.S.
Control 1.5 mM $CaCl_2$	6	34.5 $\pm$ 2.6	0.82 $\pm$ 0.01	42.3 $\pm$ 2.5	-
$PGF_{2\alpha}$, 3×10^{-12} M 1.5 mM $CaCl_2$	6	28.8 $\pm$ 1.3	0.81 $\pm$ 0.01	35.8 $\pm$ 1.9	<0.05
Control 2.5 mM $CaCl_2$	6	22.2 $\pm$ 3.9	0.79 $\pm$ 0.01	28.3 $\pm$ 5.2	-

TABLE 25 - Continued

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p[a]
$PGF_2\alpha$, 3×10^{-12} M 2.5 mM $CaCl_2$	6	18.5 ± 2.9	0.80 ± 0.00	23.0 ± 3.6	N.S.

[a]Appropriate control compared to $PGF_2\alpha$-treated supernatant renin activity, ng A I/mg cell protein.

TABLE 26

EFFECT OF ARACHIDONIC ACID ON RENIN RELEASE FROM RKC CELLS AND THE ACTION OF PROSTAGLANDIN SYNTHESIS INHIBITORS ON THIS EFFECT

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p
Control	6	61.5 ± 1.7	0.45 ± 0.01	137.4 ± 6.9	-
Arachidonic acid 2.5 μg/ml	6	92.3 ± 2.0	0.38 ± 0.01	242.9 ± 3.0	<0.001[a]
Indomethacin 0.1 μg/ml	6	57.1 ± 2.2	0.43 ± 0.01	134.7 ± 7.8	N.S.[a]
Arachidonic acid 2.5 ug/ml and Indomethacin, 0.1 μg/ml	6	81.7 ± 1.2	0.39 ± 0.01	211.3 ± 6.4	<0.001[b]

TABLE 26 - Continued

Treatment	n	Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	p
Aspirin, 10 µg/ml	6	41.4 ± 1.6	0.49 ± 0.02	84.8 ± 4.3	$<0.001^{a}$
Arachidonic acid 2.5 ug/ml and Aspirin, 10 µg/ml	6	58.8 ± 3.9	0.43 ± 0.00	137.0 ± 8.0	N.S.[a] $<0.001^{b}$

[a]Control compared to treated supernatant renin activity, ng A I/mg cell protein.

[b]Aspirin-treated compared to arachidonic acid and aspirin.

TABLE 27

CELL PREPARATIONS AND RESPONSIVENESS TO CALCIUM[c]

mM Calcium		Supernatant Renin Activity ng A I/5ml Mean ± S.E.M.	mg cell protein/5ml Mean ± S.E.M.	Supernatant Renin Activity ng A I/mg cell protein Mean ± S.E.M.	Specific Activity Difference between a and b, p
0	a	34.79 ± 4.37	0.69 ± 0.00	51.47 ± 5.51	N.S.
	b	45.42 ± 4.95	0.71 ± 0.00	63.97 ± 6.99	
1.0	a	56.19 ± 4.95	0.71 ± 0.02	79.24 ± 2.33	<0.001
	b	80.71 ± 3.67	0.69 ± 0.02	96.26 ± 1.90	
1.5	a	50.73 ± 2.03	0.66 ± 0.00	76.51 ± 3.39	<0.001
	b	69.19 ± 1.15	0.70 ± 0.00	98.38 ± 0.94	
2.0	a	44.69 ± 0.71	0.69 ± 0.01	65.41 ± 1.44	<0.001
	b	61.91 ± 1.56	0.71 ± 0.02	87.40 ± 4.21	
2.5	a	47.48 ± 2.24	0.70 ± 0.03	67.83 ± 1.18	N.S.
	b	51.74 ± 1.49	0.72 ± 0.02	71.90 ± 1.78	
4.0	a	36.74 ± 3.31	0.67 ± 0.02	54.72 ± 5.34	<0.05
	b	48.28 ± 2.33	0.70 ± 0.00	69.31 ± 3.35	

TABLE 27 - Continued

a = collagenase isolated cells (n = 3).

b = citrate isolated cells (n = 3).

c 0.5 ml of cell suspension was added to 4.5 ml of Earles physiological buffer containing 0 - 4 mM calcium. The cells were incubated at 37°C for 10 minutes under 95% O_2 - 5% CO_2. Assays are described in analytical procedures.

TABLE 28

STIMULATION AND INHIBITION OF RENIN RELEASE

Maximum Stimulation	Renin Release % of Control
Arachidonic acid, 0.1 μg/ml	240
Acetylcholine, 1.5×10^{-5} M	169
Glucagon, 5 pg/ml	168
PGA_2, 3×10^{-12} M	163
PGE_2, 3×10^{-12} M	144
Vasopressin, 10 μU/ml	144
Histamine, 6 ng/ml	128
Isoproterenol, 1.5×10^{-5} M	129
Norepinephrine, 3×10^{-7} M	111
Epinephrine, 3×10^{-7} M	107
Maximum Inhibition	
Angiotensin I, 600 ng/ml	0
Aldosterone, 0.2 pg/ml	49
Angiotensin II, 600 ng/ml	63
$PGF_{2\alpha}$, 3×10^{-12} M	73
Serotonin, 80 ng/ml	74
Epinephrine, 1.5×10^{-5} M	75

APPENDIX B

ILLUSTRATIONS

Figure 1.--Time course of the reaction of cell protein with substrate.

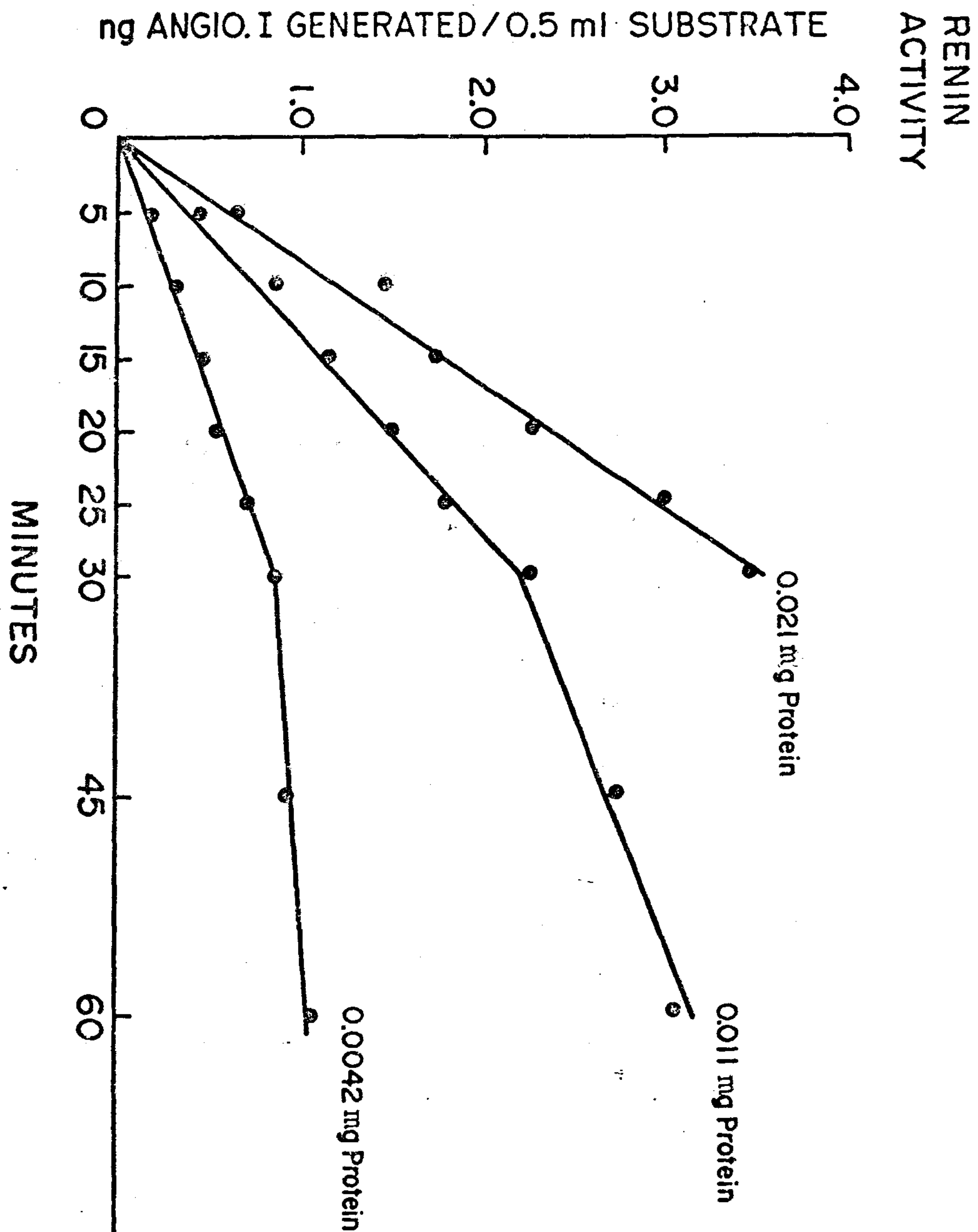

RENIN ACTIVITY
ng ANGIO. I GENERATED / 0.5 ml SUBSTRATE
4.0
3.0
2.0
1.0
0
5
10
15
20
25
30
45
60
MINUTES
0.021 mg Protein
0.011 mg Protein
0.0042 mg Protein

Figure 2. Time course of the reaction of supernatant (released from cells) protein with substrate.

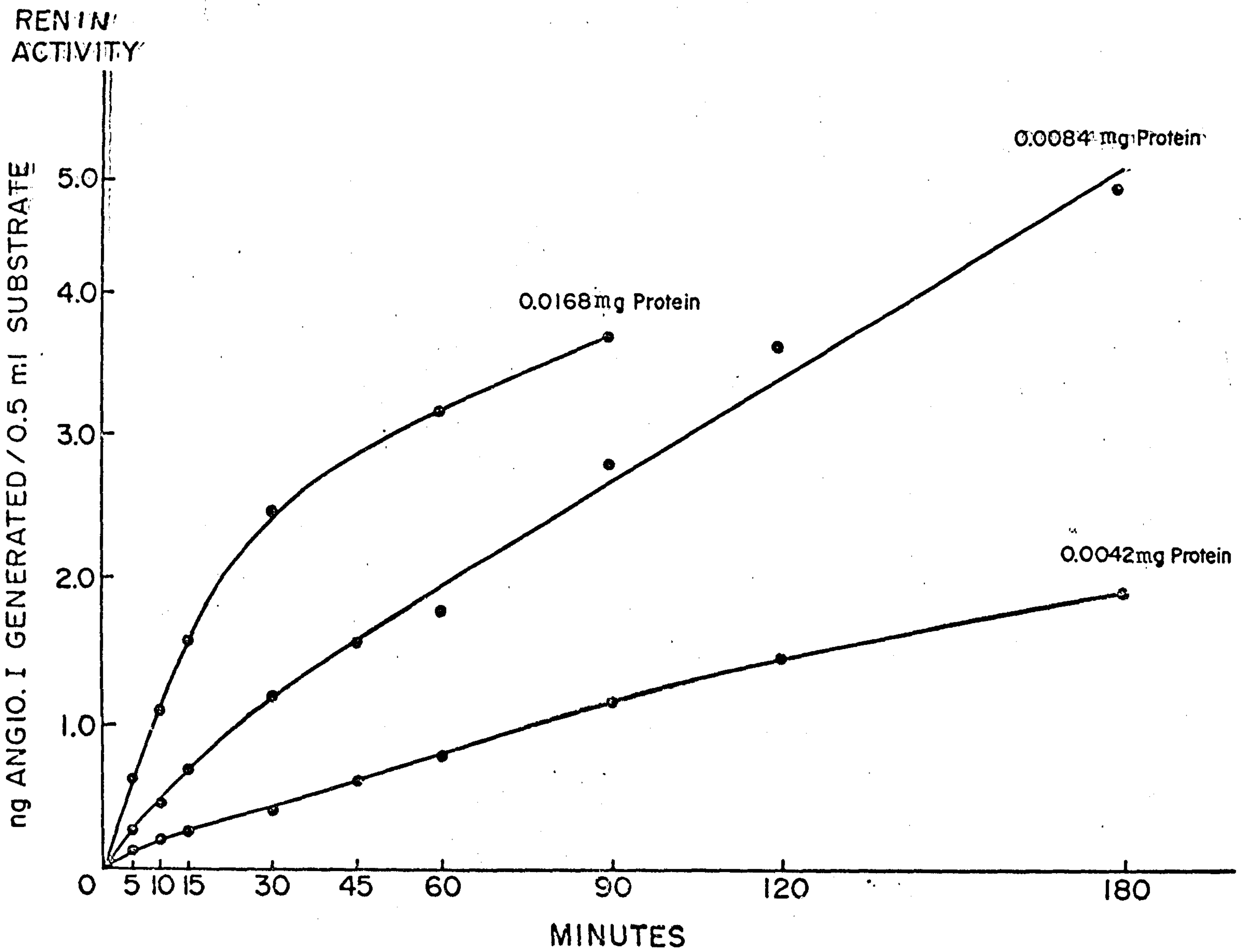
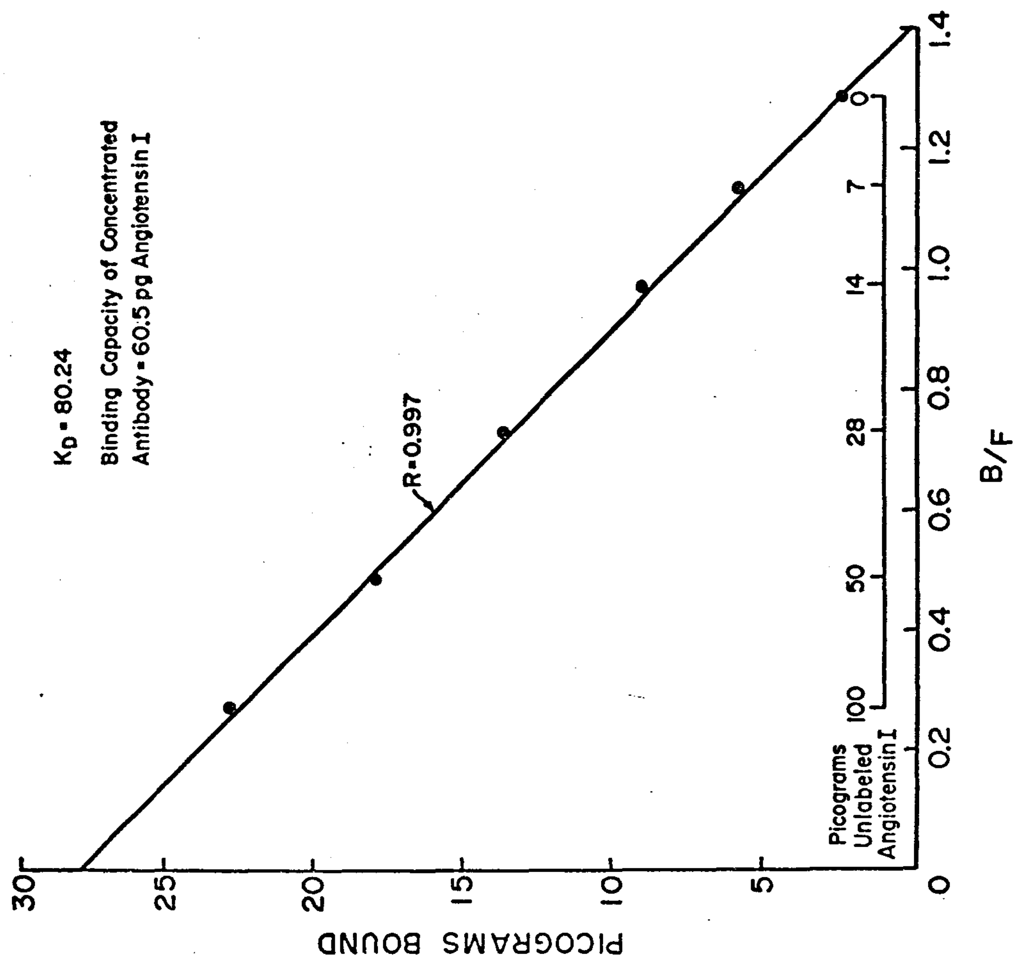

RENIN ACTIVITY
ng ANGIO. I GENERATED / 0.5 ml SUBSTRATE
5.0
4.0
3.0
2.0
1.0
0
5 10 15
30
45
60
90
120
180
MINUTES
0.0168 mg Protein
0.0084 mg Protein
0.0042 mg Protein

Figure 3.--Scatchard plot of renin radioimmunoassay standard curve.

This plot also shows physical constants of the antibody. K_D represents the dissociation constant of the antibody. R represents the regression coefficient, i.e., the slope of the line. B/F represents bound counts/free counts.

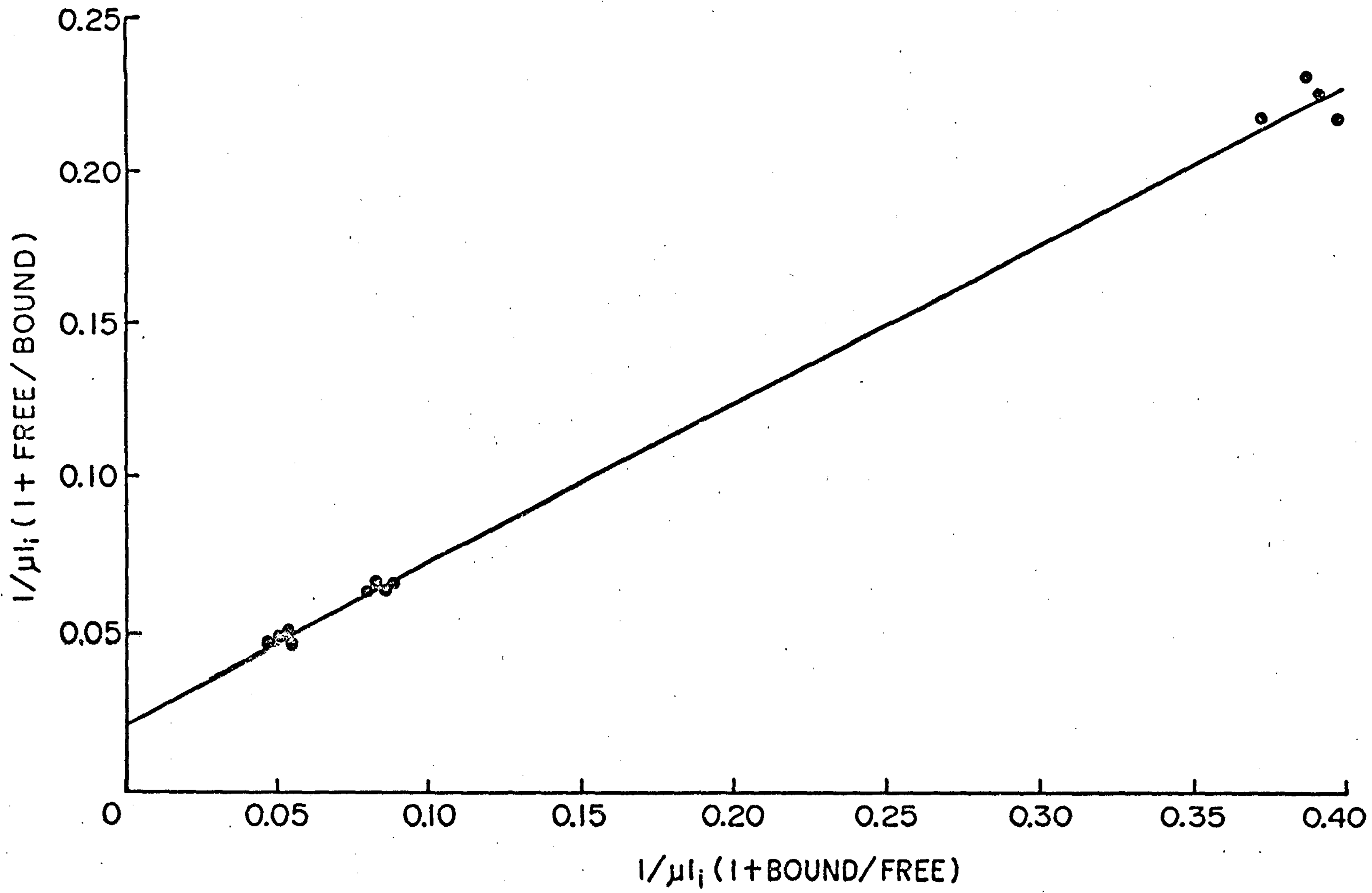


K_D = 80.24
Binding Capacity of Concentrated Antibody = 60.5 pg Angiotensin I
R = 0.997
PICOGRAMS BOUND
30
25
20
15
10
5
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
B/F
Picograms Unlabeled Angiotensin I
100
50
28
14
7
0

Figure 4.--Determination of ^{125}I-A I by isotope dilution.

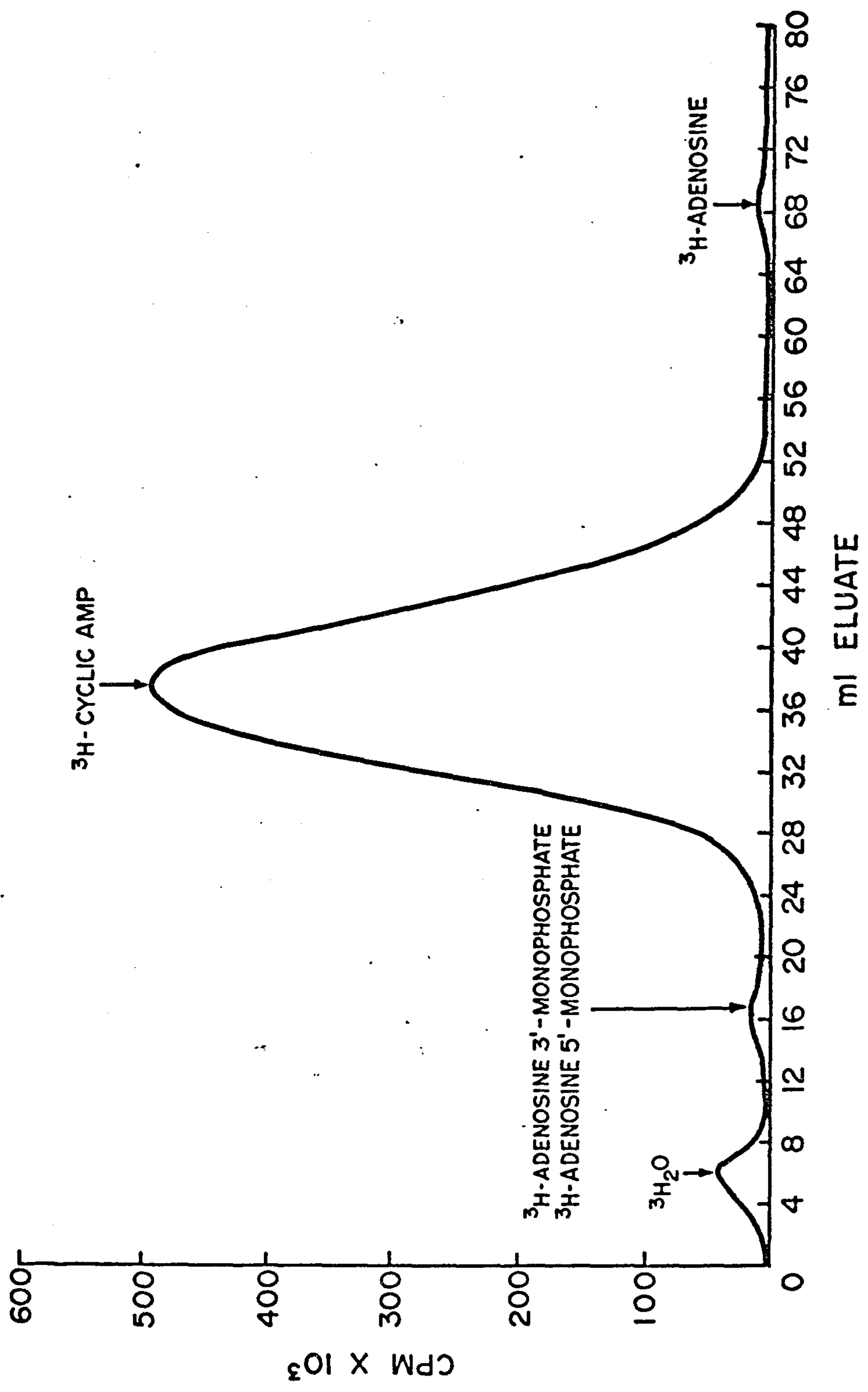
50 ul of antibody diluted 1:2 (v:v) was incubated at 4° C for 24 hours with 25 ul, 50 ul and 100 ul of ^{125}I-A I and pH 9.0 tris acetate buffer-0.25% BSA added to a final volume of 250 ul. The bound moiety was separated from free by charcoal in pH 9.0 barbital buffer and a 350 ul aliquot of the supernatant and the remaining charcoal pellet was counted in a Nuclear Chicago gamma well scintillation counter for 4 minutes.

$1/ul_i$ (1 + free/bound) and $1/ul_i$ (1 + bound/free) are explained in section III.A.3.e.



0.25
0.20
0.15
0.10
0.05
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
1/μI_i (1+FREE/BOUND)
1/μI_i (1+BOUND/FREE)

Figure 5.--Purification of commercial ^{3}H-CAMP.



CPM X 10^3
600
500
400
300
200
100
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
ml ELUATE
^3H_2O
^{3}H-ADENOSINE 3'-MONOPHOSPHATE
^{3}H-ADENOSINE 5'-MONOPHOSPHATE
^{3}H-CYCLIC AMP
^{3}H-ADENOSINE

Figure 6.--Calibration of Dowex 50W AG x 8 H^+ 0.7 x 20 cm column.

Elution of 1 mM ATP, ADP and AMP and ^{3}H-CAMP with 0.1N HCl.

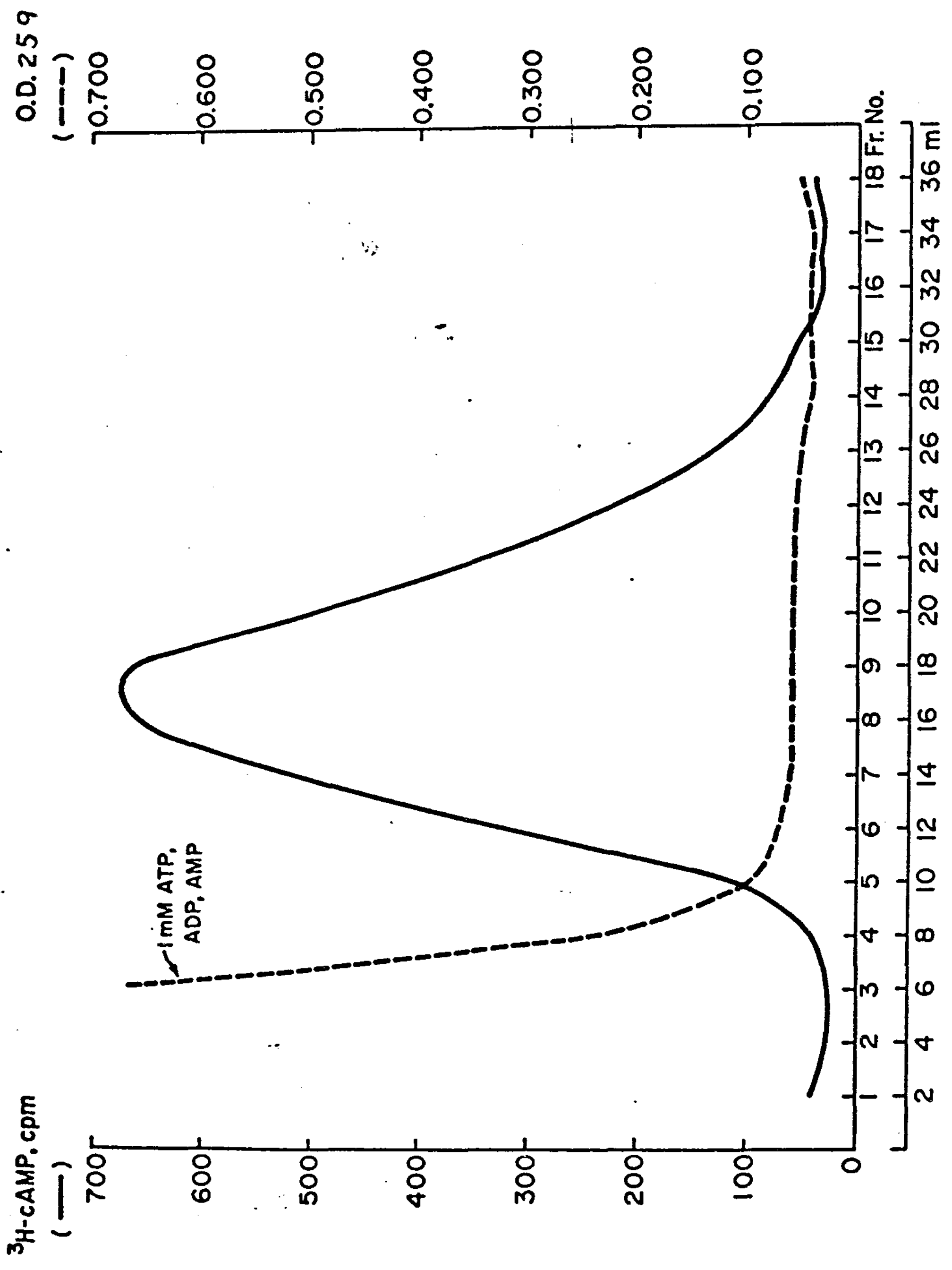
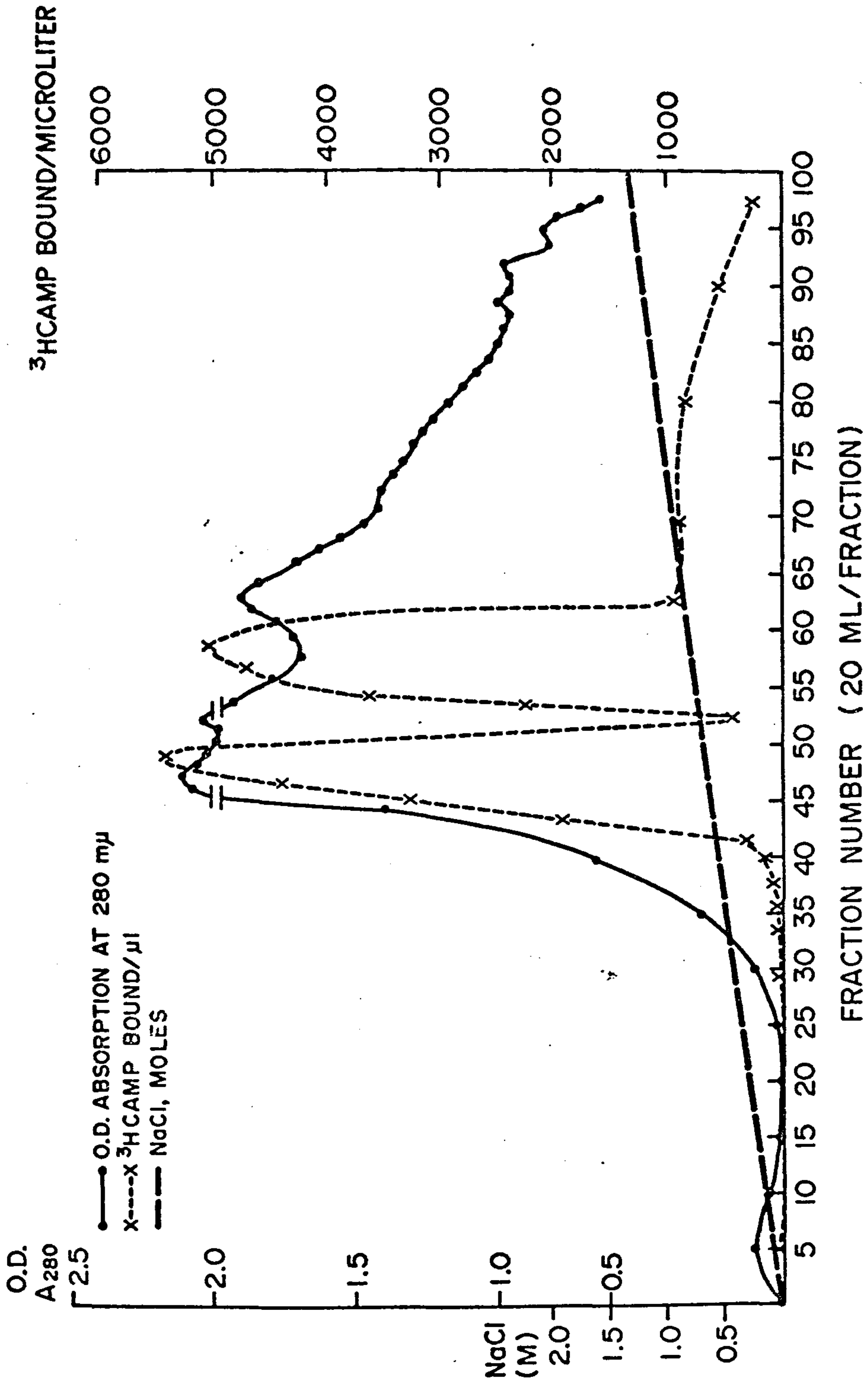

^{3}H-cAMP, cpm
(——)
700
600
500
400
300
200
100
0
O.D. 259
(- - -)
0.700
0.600
0.500
0.400
0.300
0.200
0.100
1 mM ATP,
ADP, AMP
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 Fr. No.
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 ml

Figure 7.--Purification of rabbit muscle protein kinase by protamine sepharose.

Kinase binding of ^{3}H-CAMP was highest from fractions 40-65.



³HCAMP BOUND/MICROLITER
6000
5000
4000
3000
2000
1000
O.D.
A280
2.5
2.0
1.5
1.0
0.5
NaCl
(M)
2.0
1.5
1.0
0.5
O.D. ABSORPTION AT 280 mμ
³HCAMP BOUND/μl
NaCl, MOLES
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
FRACTION NUMBER (20 ML/FRACTION)

Figure 8.--Continuous fractionation scheme for obtaining subcellular components from rat kidney.

R = residue, or pellet, Supt = supernatant, $Microsomes_H$ = heavy microsomes, $Microsomes_L$ = light microsomes.

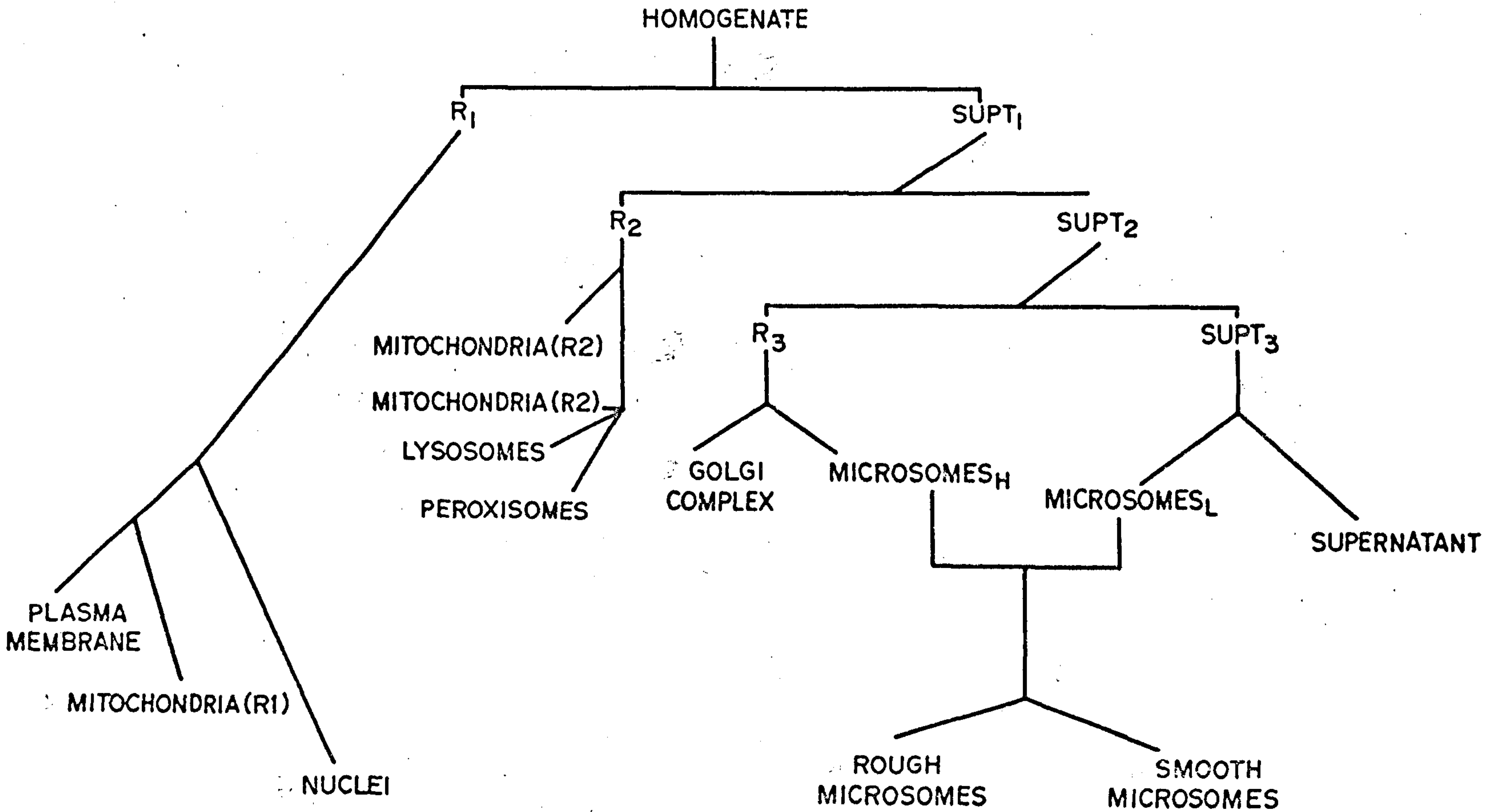
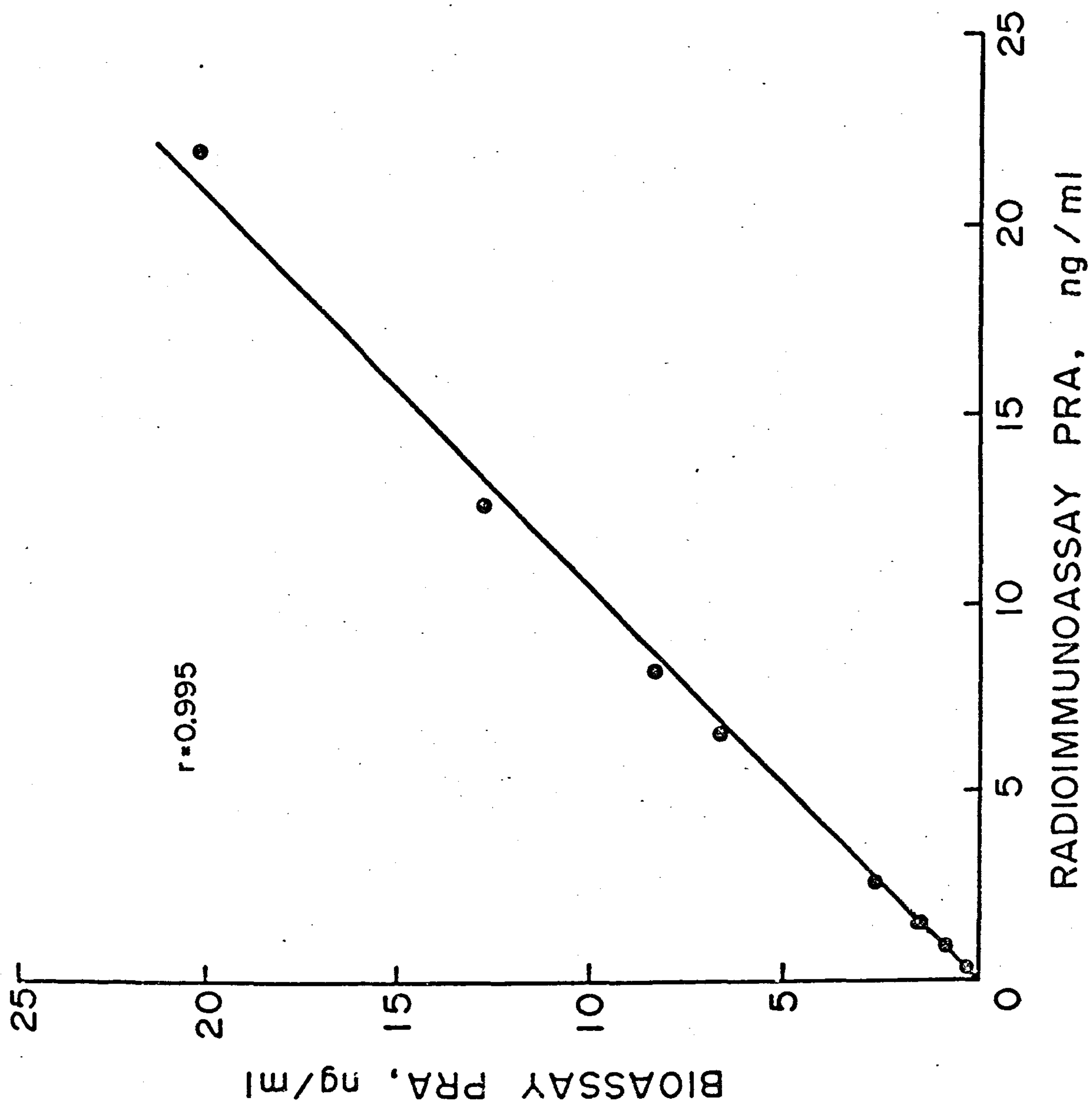

FRACTIONATION OF RAT KIDNEY
HOMOGENATE
R_1
$SUPT_1$
R_2
$SUPT_2$
MITOCHONDRIA (R2)
MITOCHONDRIA (R2)
LYSOSOMES
PEROXISOMES
R_3
$SUPT_3$
GOLGI COMPLEX
$MICROSOMES_H$
$MICROSOMES_L$
SUPERNATANT
PLASMA MEMBRANE
MITOCHONDRIA (R1)
NUCLEI
ROUGH MICROSOMES
SMOOTH MICROSOMES

Figure 9.--Correlation between human plasma renin (PRA) values obtained with bioassay and with the radioimmunoassay.

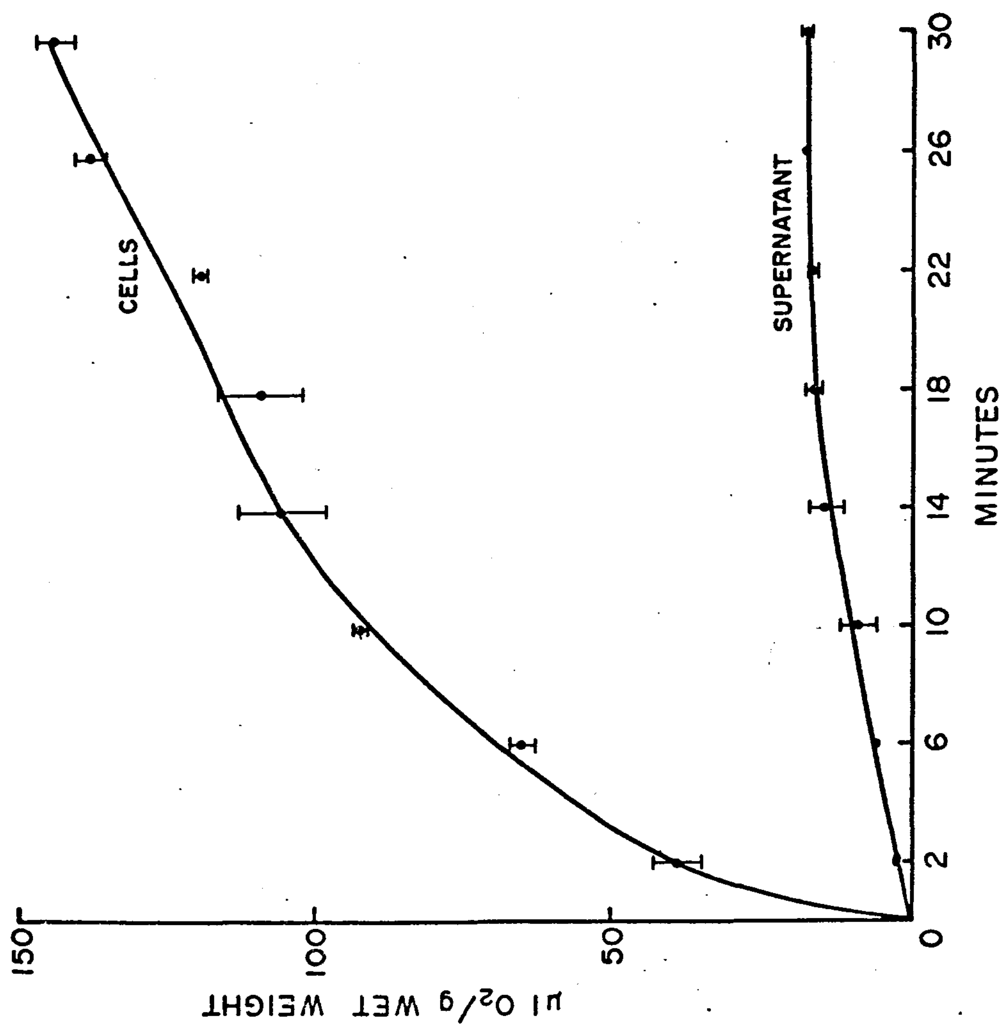
Circles represent the values obtained by each method for plasma samples. r represents the correlation coefficient.



r=0.995
BIOASSAY PRA, ng/ml
RADIOIMMUNOASSAY PRA, ng/ml
0
5
10
15
20
25

Figure 10.--Oxygen consumption of RKC cells and combined wash supernatants from collagenase-prepared cells.

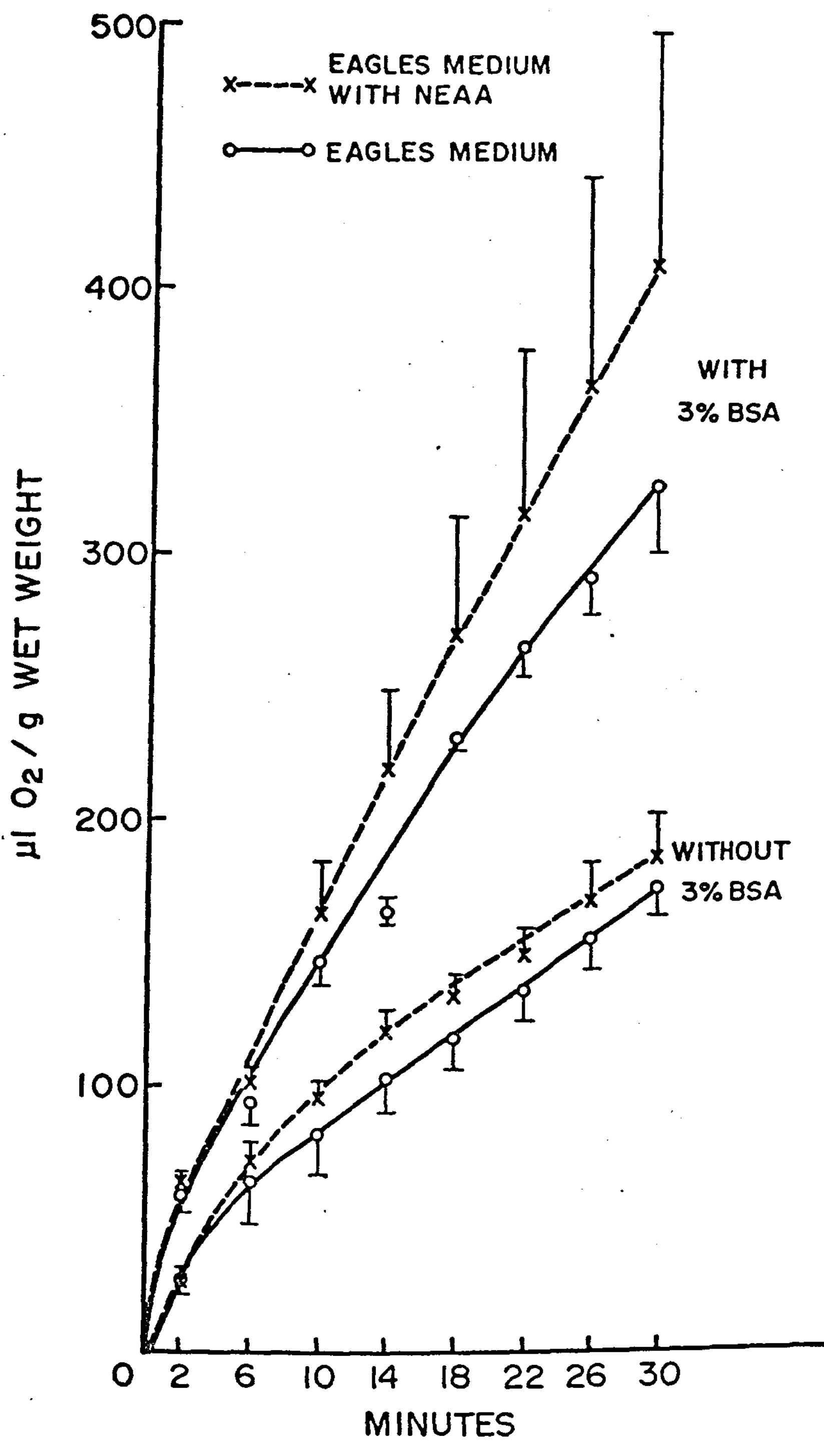
Dots represent the mean of duplicate Warburg flasks and brackets indicate S.E.M. 0.5 ml of cells (about 500 mg wet weight) or supernatants was added to 1.5 ml Eagles medium and equilibrated 10 minutes under 95% O_2-5% CO_2 at 36.5° C. Oxygen consumption was recorded every 4 minutes for 30 minutes.



µl O2/g WET WEIGHT
150
100
50
0
CELLS
SUPERNATANT
0
2
6
10
14
18
22
26
30
MINUTES

Figure 11.--Effect of 3% BSA and NEAA on oxygen consumption of isolated dog cortical cells.

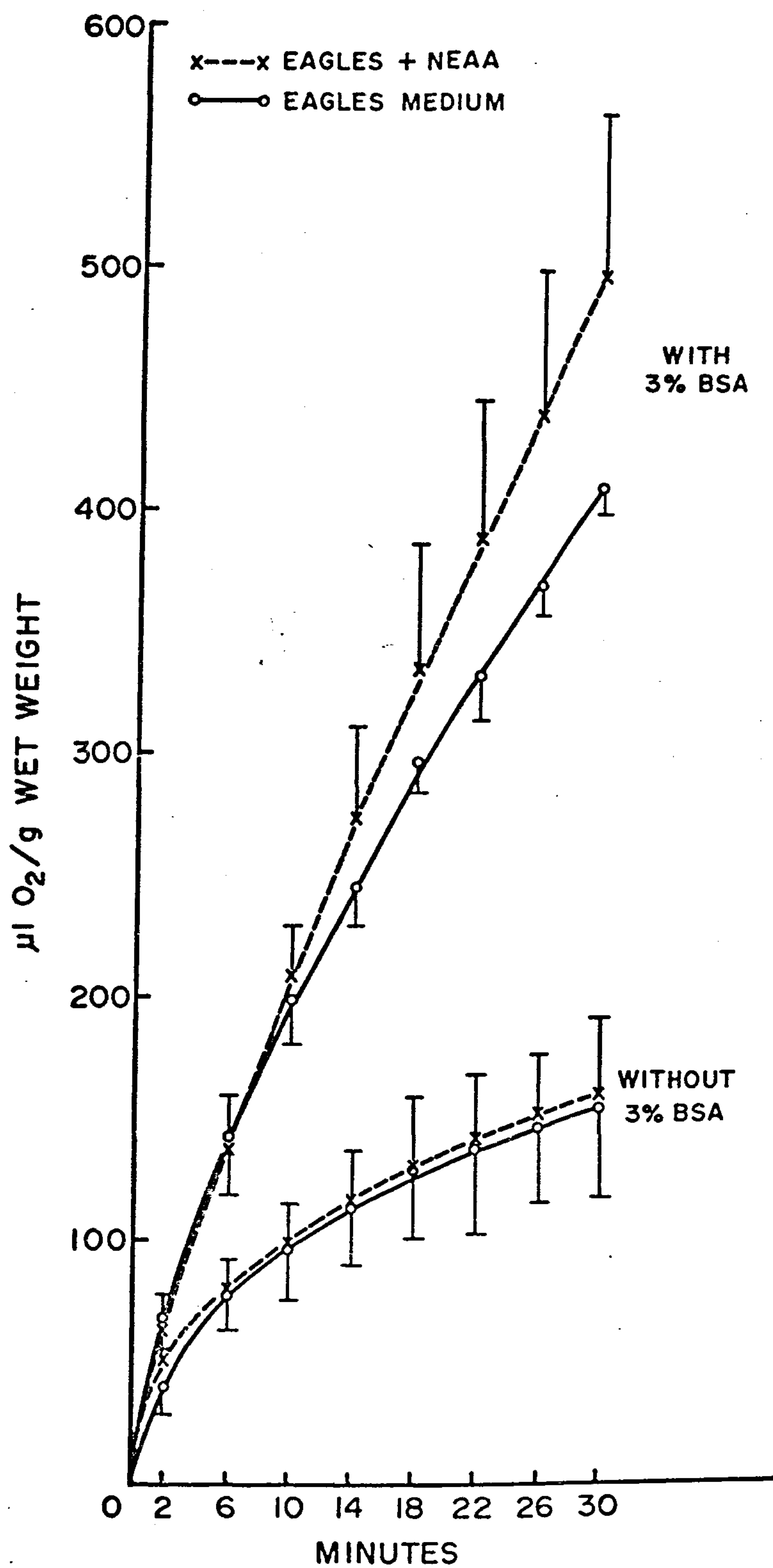
Each point represents the means of two separate experiments, using duplicate Warburg flasks for each experiment, and brackets indicate S.E.M. 0.5 ml of cells (approximately 100 mg wet weight) was added to 1.5 ml of medium and equilibrated 10 minutes under 95% O_2-5% CO_2 at 37.5° C. Oxygen consumption was recorded every 4 minutes for 30 minutes.



500
400
300
200
100
0
2
6
10
14
18
22
26
30
MINUTES
μl O_2 / g WET WEIGHT
EAGLES MEDIUM
WITH NEAA
EAGLES MEDIUM
WITH
3% BSA
WITHOUT
3% BSA

Figure 12.--Effect of 3% BSA and NEAA on oxygen consumption of isolated RKC cells.

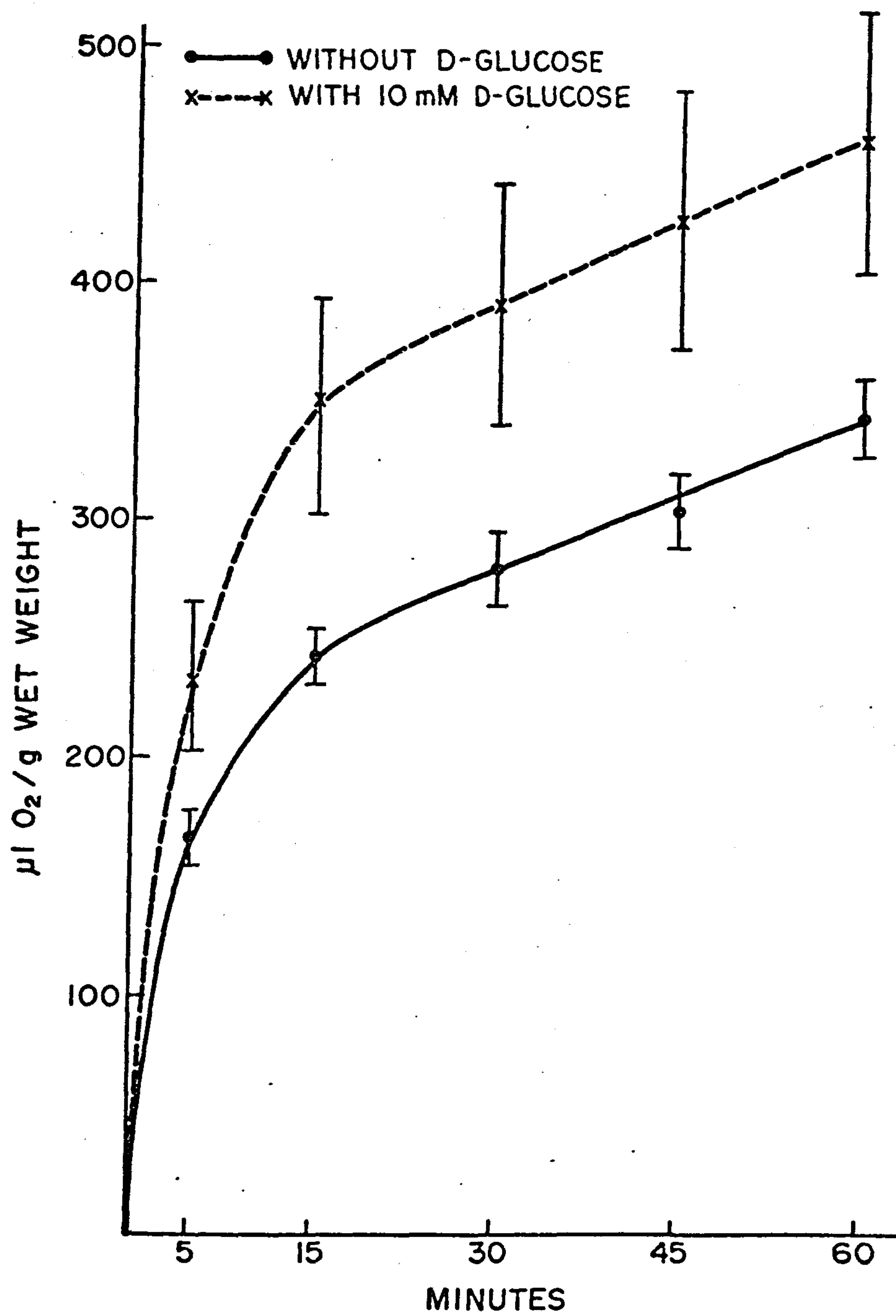
Each point represents the mean of two separate experiments, using duplicate Warburg flasks for each experiment, and brackets indicate S.E.M. 0.5 ml of cells (approximately 200 mg wet weight) was added to 1.5 ml of medium and treated as described in figure 11.



600
500
400
300
200
100
0
x----x EAGLES + NEAA
o——o EAGLES MEDIUM
WITH
3% BSA
WITHOUT
3% BSA
µl O_2/g WET WEIGHT
2
6
10
14
18
22
26
30
MINUTES

Figure 13.--Effect of 10 mM D-glucose on the oxygen consumption of dog kidney cortical cells.

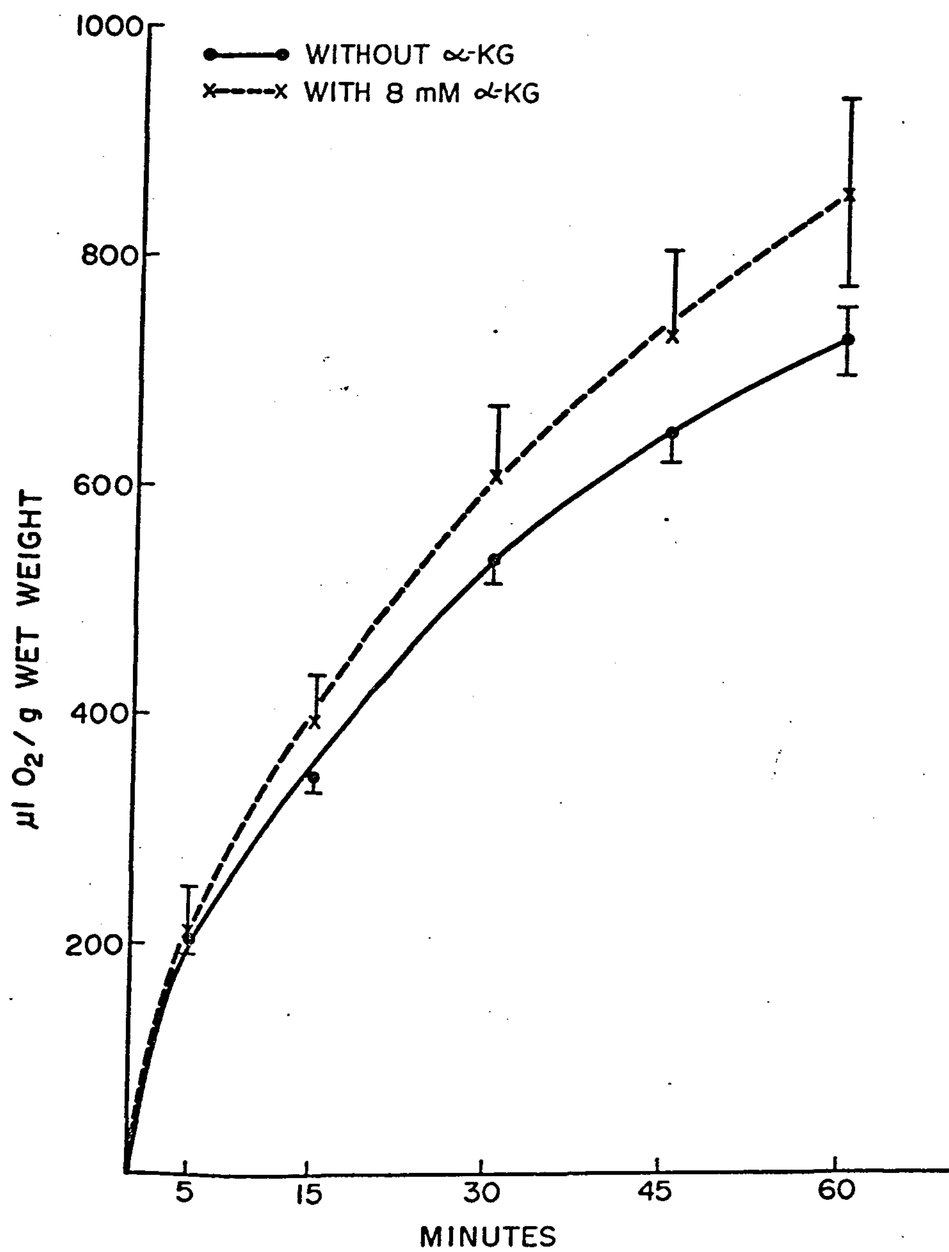
Each point is the mean of 3 Warburg flasks; brackets indicate S.E.M. The flasks, which contained 1.2 ml 10 mM potassium phosphate buffer, pH 7.5, 0.5 ml cells (about 150 mg wet weight) and 0.3 ml 10 mM D-glucose in the sidearms, were equilibrated for 10 minutes under 95% O_2-5% CO_2. The glucose was tipped into the main compartment and oxygen consumption was recorded for one hour at 15 minute intervals.



WITHOUT D-GLUCOSE
WITH 10 mM D-GLUCOSE
500
400
300
200
100
μl O₂ / g WET WEIGHT
5
15
30
45
60
MINUTES

Figure 14. Effect of 8 mM α-ketoglutarate on oxygen consumption of dog kidney cortical cells.

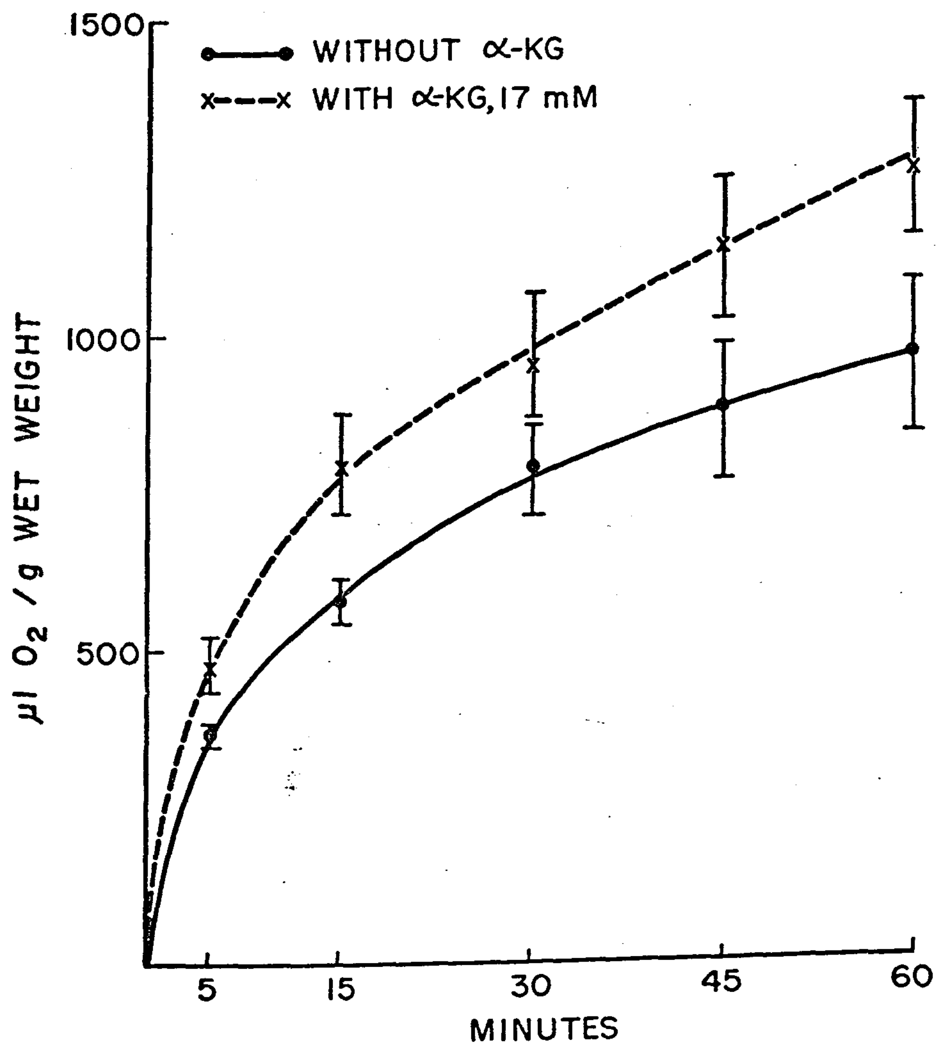
Each point for control represents the mean of 3 Warburg flasks, and for experimental, the mean of 4 flasks; brackets indicate S.E.M. The flasks, which contained 1.3 ml Eagles medium, 3% BSA, 0.67 mM ADP, 0.5 mM NAD^+ and 0.5 ml cells (about 85 mg wet weight), with 0.2 ml 8 mM α-KG in the sidearms were equilibrated with 95% O_2-5% CO_2 at 37° C for 10 minutes. After the substrate was tipped into the main compartment, oxygen consumption was measured as in figure 13.



1000
800
600
400
200
WITHOUT α-KG
WITH 8 mM α-KG
μl O2 / g WET WEIGHT
5
15
30
45
60
MINUTES

Figure 15.--Effect of 17 mM α-Ketoglutarate on oxygen consumption of dog kidney cortical cells.

Each point for control represents the mean of 3 Warburg flasks, and for experimental, the mean of 4 flasks; brackets indicate S.E.M. The flasks which contained 1.3 ml Eagles medium, 3% BSA, 0.67 mM ADP, 0.5 mM NAD^+ and 0.5 ml cells (about 50 mg wet weight), with 0.2 ml 17 mM α-kg in the sidearms, were treated as in figure 14.



WITHOUT α-KG
WITH α-KG, 17 mM
1500
1000
500
μl O_2 / g WET WEIGHT
5
15
30
45
60
MINUTES

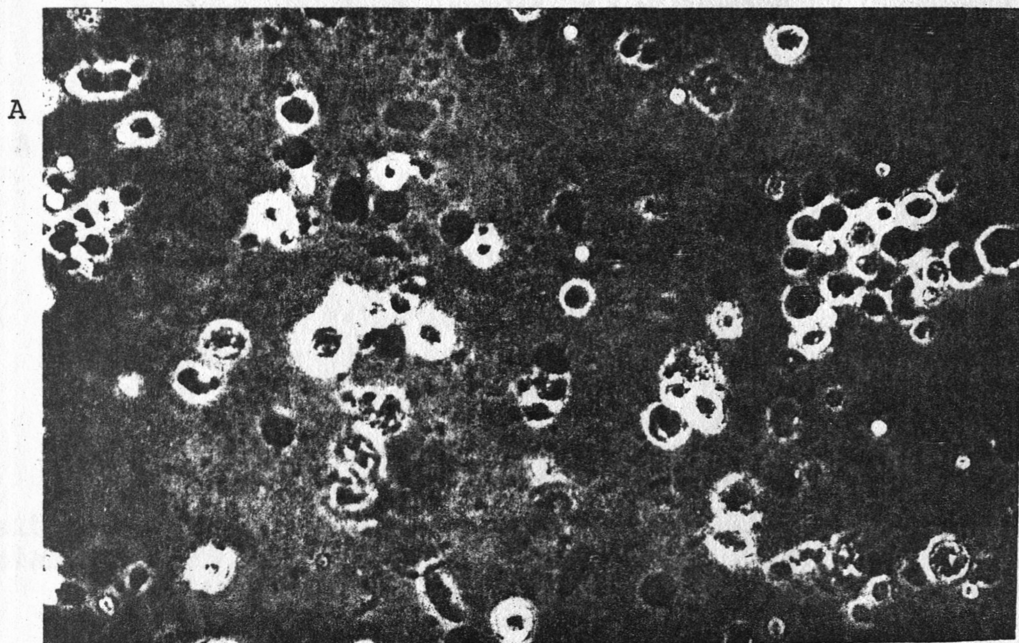
Figure 16.--Trypan blue uptake of cells dissociated from kidney cortex by the collagenase-mechanical method.

One drop of cell suspension was added to one drop 0.3% trypan blue in Earles buffer and photographed under a Zeiss phase-contrast microscope with a 600 mμ filter. Polaroid type 52 4 x 5 black and white film was used with a 0.5 second exposure, = 1.5.

A = x 160.

B = x 400.

A



B

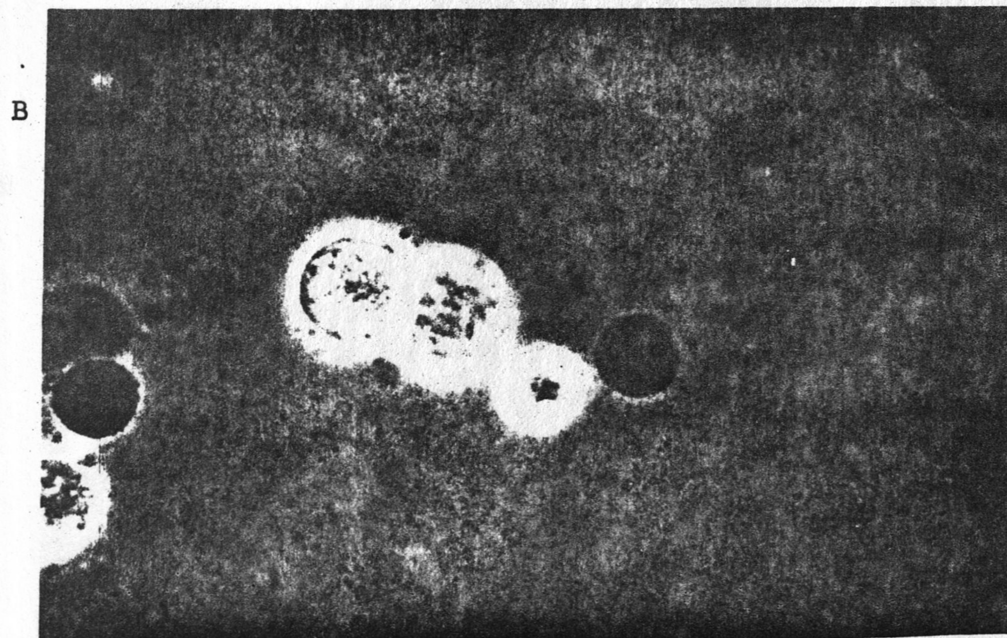


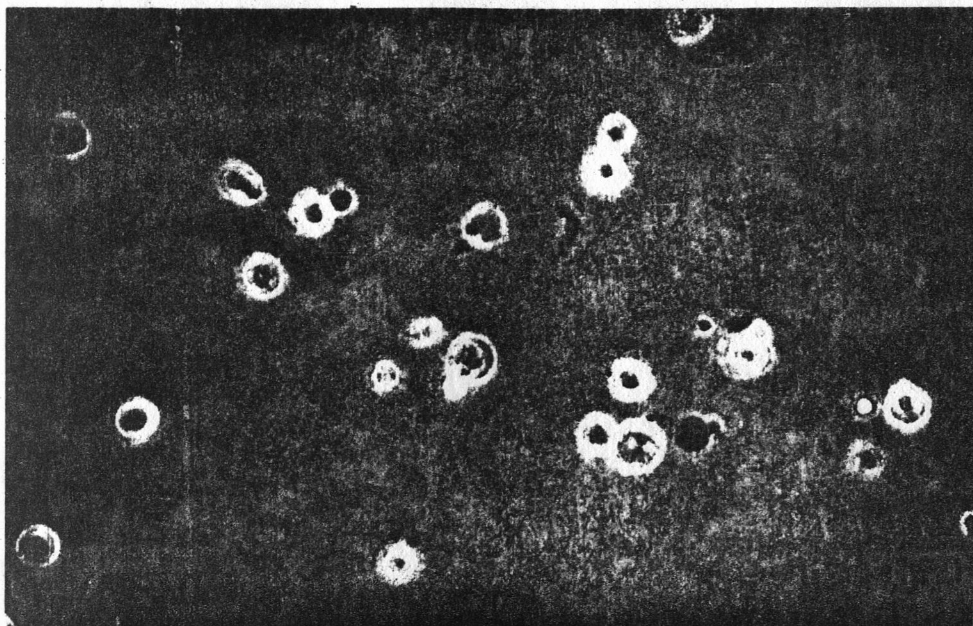
Figure 17.--Trypan blue uptake of cells dissociated from tissue by the sodium citrate-mechanical method.

Details are as described in figure 16.

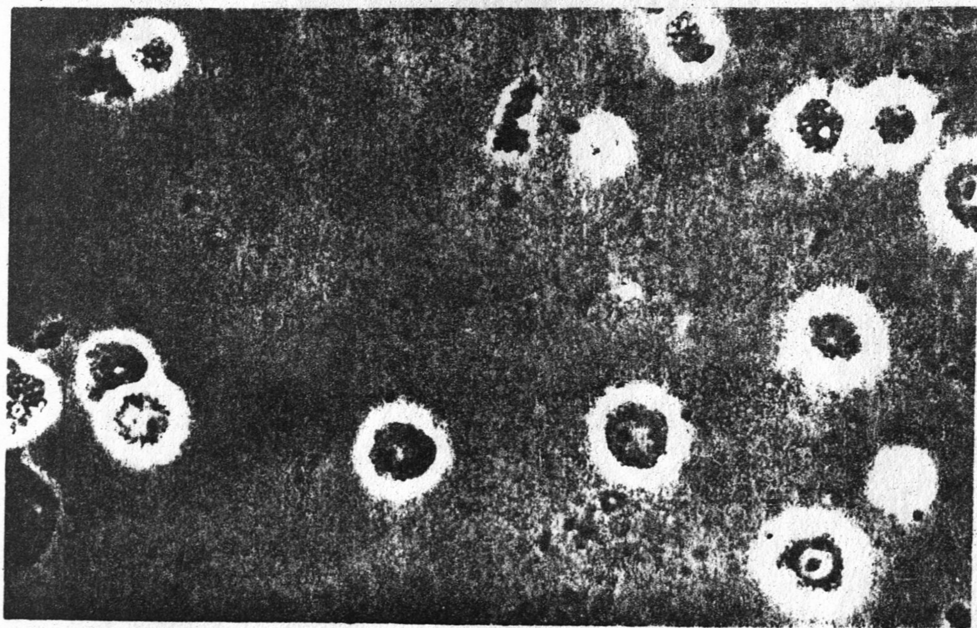
A = x 160.

B = x 400.

A



B



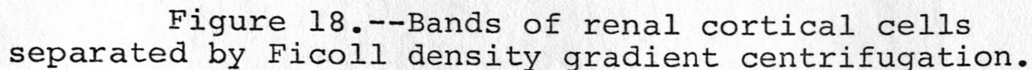
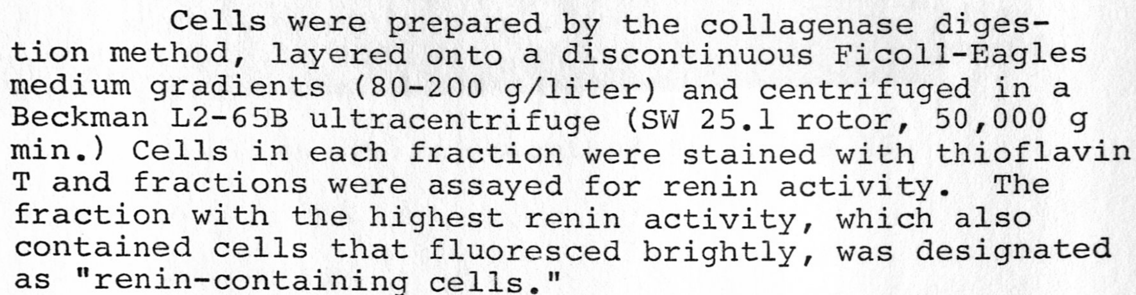


Figure 18.--Bands of renal cortical cells separated by Ficoll density gradient centrifugation.



Cells were prepared by the collagenase digestion method, layered onto a discontinuous Ficoll-Eagles medium gradients (80-200 g/liter) and centrifuged in a Beckman L2-65B ultracentrifuge (SW 25.1 rotor, 50,000 g min.) Cells in each fraction were stained with thioflavin T and fractions were assayed for renin activity. The fraction with the highest renin activity, which also contained cells that fluoresced brightly, was designated as "renin-containing cells."

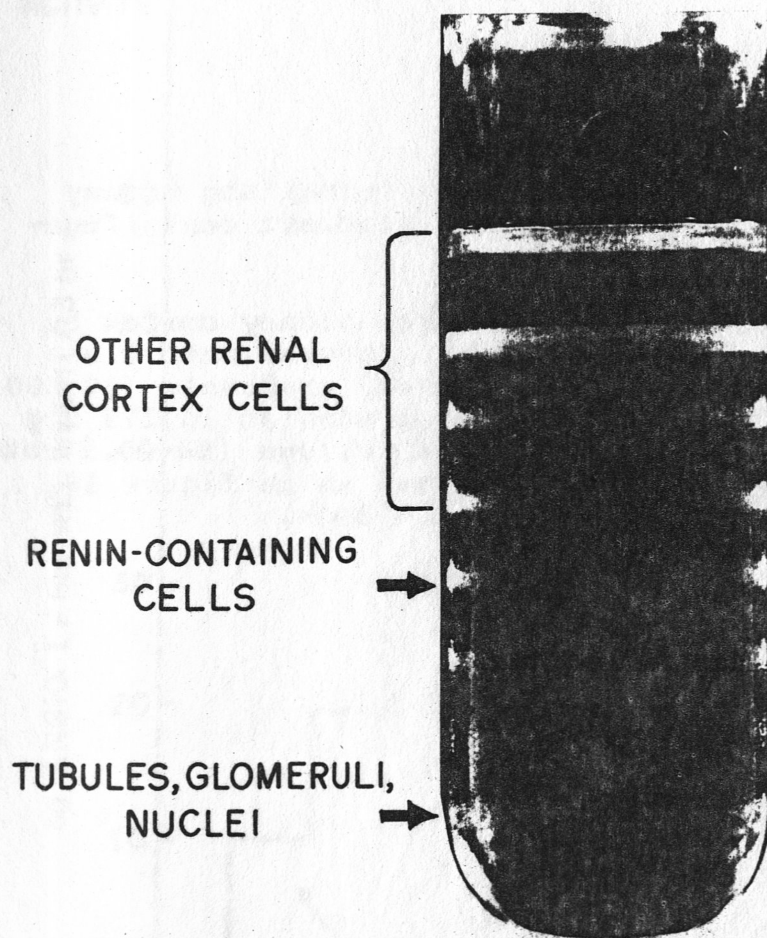

OTHER RENAL
CORTEX CELLS
RENIN-CONTAINING
CELLS
TUBULES, GLOMERULI,
NUCLEI

Figure 19.--Separation of normal dog kidney cortical cells by Ficoll density gradient centrifugation.

Cells were dissociated from kidney cortex by the collagenase digestion method, layered onto discontinuous Ficoll (in Eagles medium) gradients (80-200 g/liter) and centrifuged for 40 minutes at 10,173 x g (av.) in a Beckman L5-65B ultracentrifuge (SW 25.1 rotor). Cells in each fraction were treated as in figure 18. Circles represent the mean of five dogs.

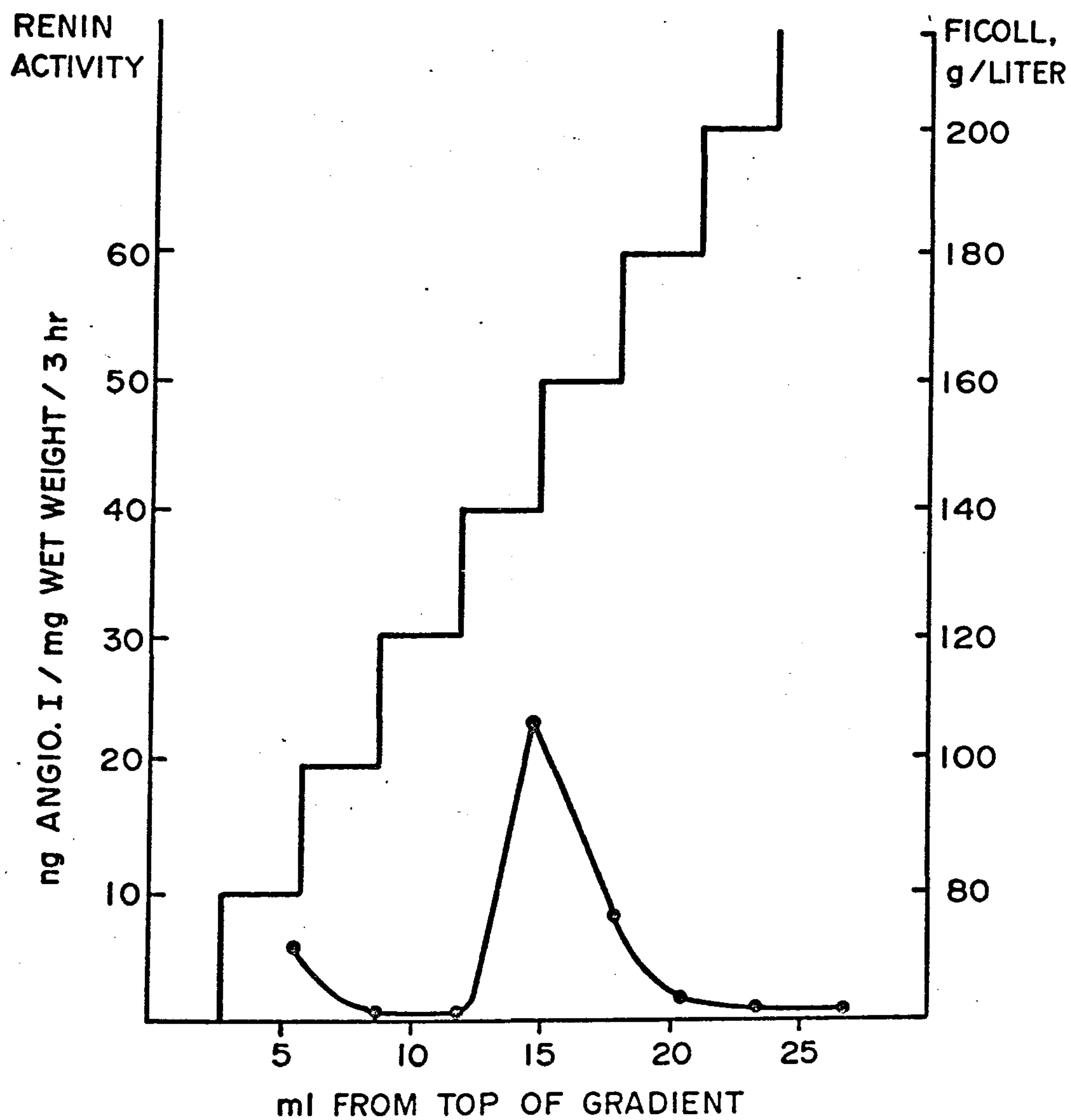

RENIN
ACTIVITY
ng ANGIO. I / mg WET WEIGHT / 3 hr
60
50
40
30
20
10
FICOLL,
g/LITER
200
180
160
140
120
100
80
5
10
15
20
25
ml FROM TOP OF GRADIENT

Figure 20.--Separation of kidney cortical cells from dogs pretreated with furosemide, 80 mg/day i.m for 2 days.

Cells were isolated and characterized as described in figure 19. Circles represent the mean of 5 dogs.

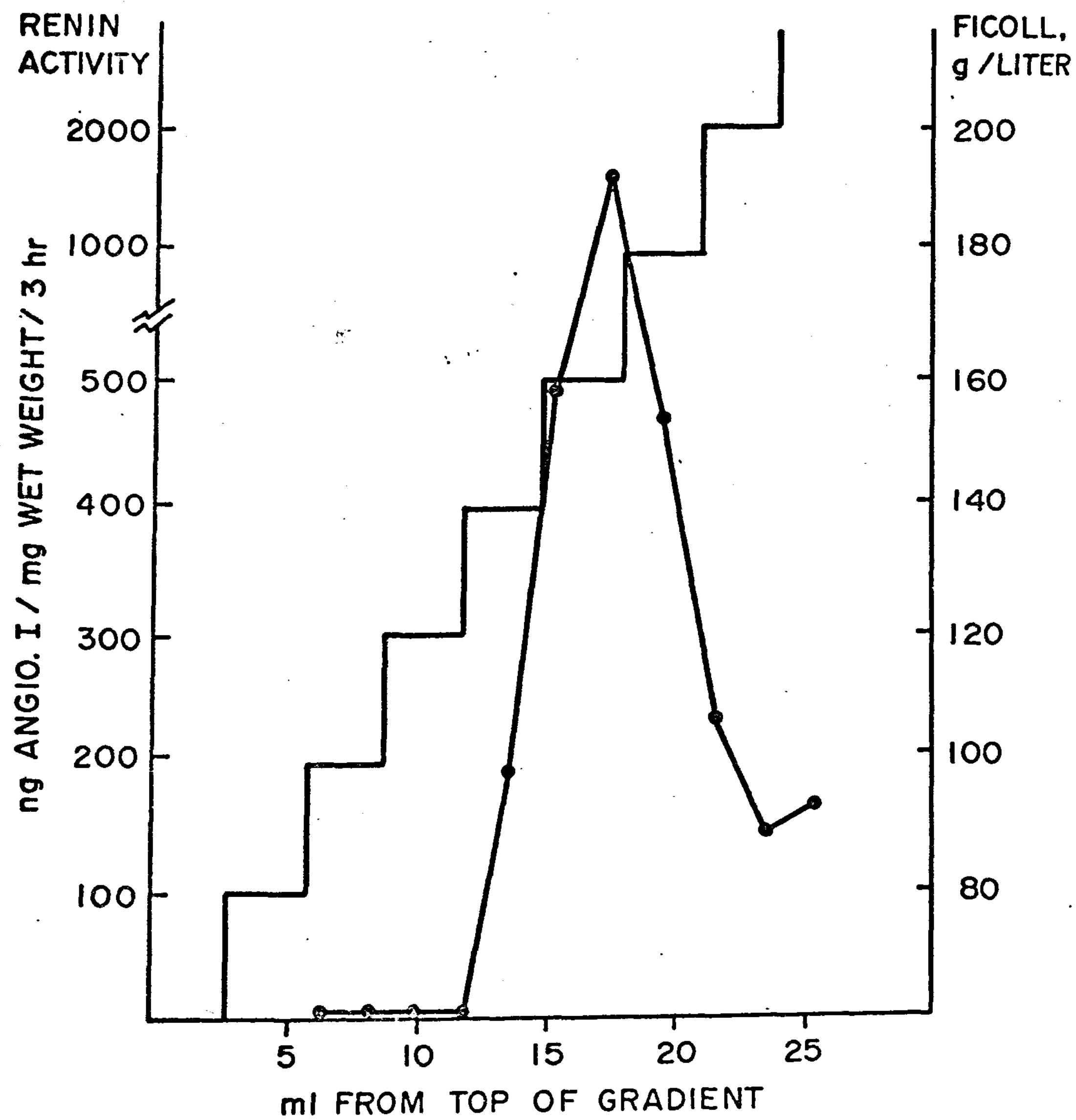
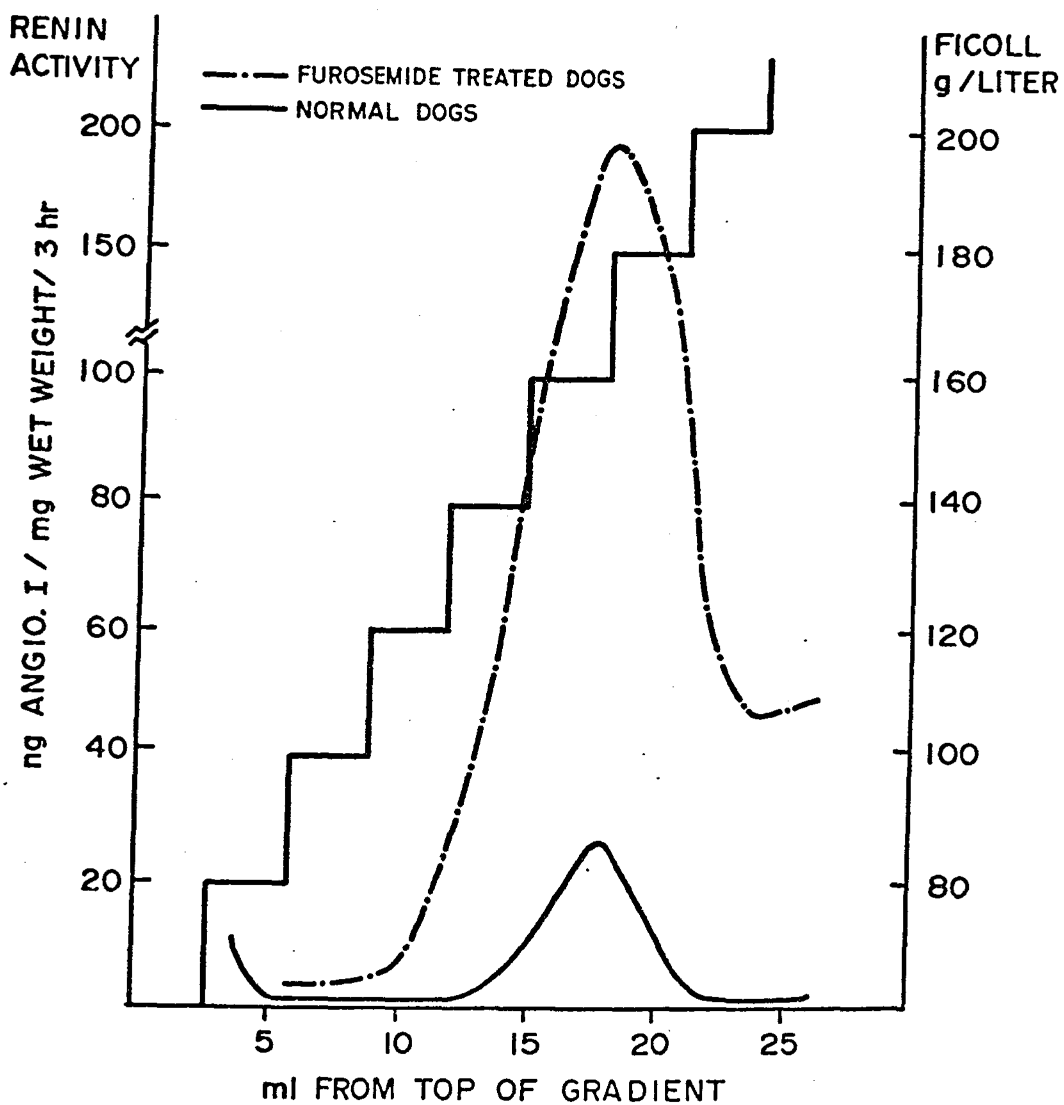

RENIN
ACTIVITY
ng ANGIO. I / mg WET WEIGHT / 3 hr
2000
1000
500
400
300
200
100
FICOLL,
g / LITER
200
180
160
140
120
100
80
5
10
15
20
25
ml FROM TOP OF GRADIENT

Figure 21.--Comparison of renin-containing cells obtained from five normal dogs and five dogs pretreated with furosemide, 40 mg/day i.m for 2 days.

The solid line represents the normal dogs and broken line represents furosemide-treated dogs. The discontinuous gradient is shown by steps of 80-200 g/liter Ficoll in Eagles medium.



RENIN ACTIVITY
ng ANGIO. I / mg WET WEIGHT/ 3 hr
FUROSEMIDE TREATED DOGS
NORMAL DOGS
FICOLL g/LITER
200
150
100
80
60
40
20
200
180
160
140
120
100
80
5
10
15
20
25
ml FROM TOP OF GRADIENT

Figure 22.--Separation of normal RKC cells by discontinuous Ficoll density gradient centrifugation.

Cells were prepared by the collagenase digestion method, layered onto 140-220 g/liter Ficoll, with a 500 g/liter cushion, centrifuged and characterized as described in figure 19. Circles represent the mean of 3 separate rabbit cell separations.

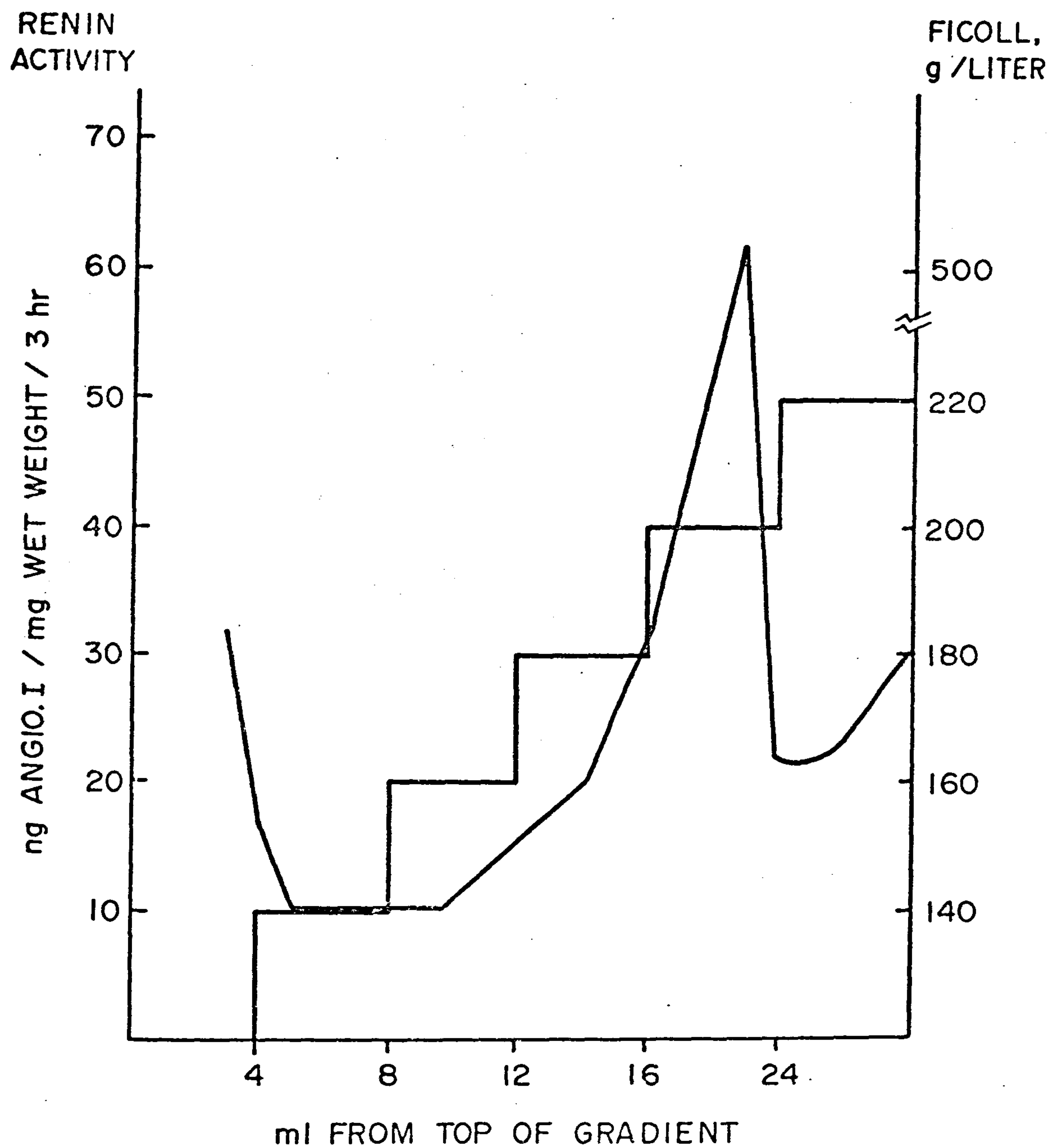

RENIN
ACTIVITY
FICOLL,
g /LITER
ng ANGIO.I / mg WET WEIGHT / 3 hr
70
60
50
40
30
20
10
500
220
200
180
160
140
4
8
12
16
24
ml FROM TOP OF GRADIENT

Figure 23.--Renin-containing cells from normal and sodium-deficient rabbits.

Cells were prepared and characterized as in figure 19. Bars represent the average amount of renin activity found in cell peaks from 3 normal rabbits and from 2 rabbits fed sodium deficient diet for 7 days and brackets indicate S.E.M.

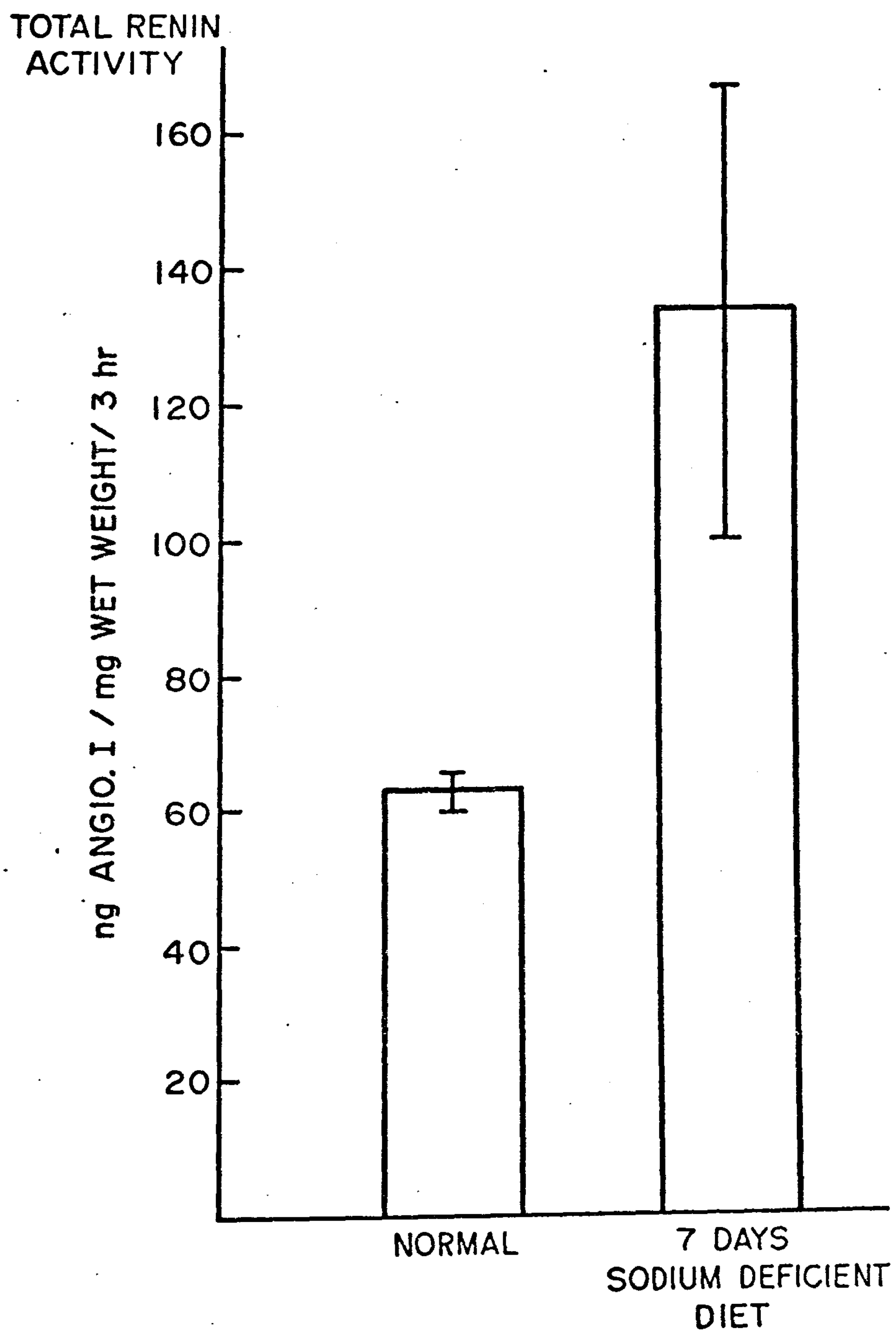
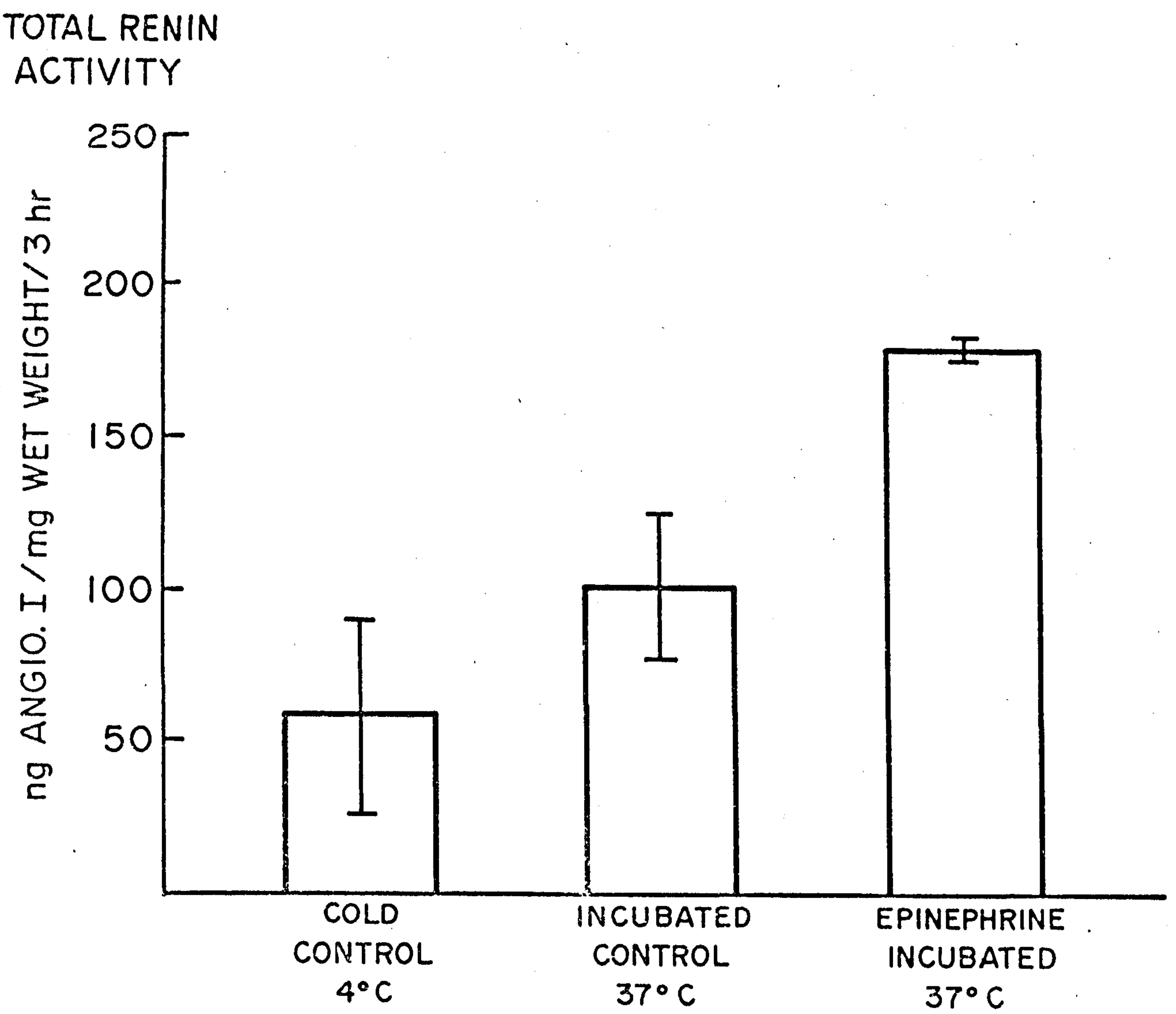

TOTAL RENIN
ACTIVITY
ng ANGIO. I / mg WET WEIGHT / 3 hr
160
140
120
100
80
60
40
20
NORMAL
7 DAYS
SODIUM DEFICIENT
DIET

Figure 24.--Responsiveness to epinephrine of renin-containing cells separated by Ficoll density gradient centrifugation.

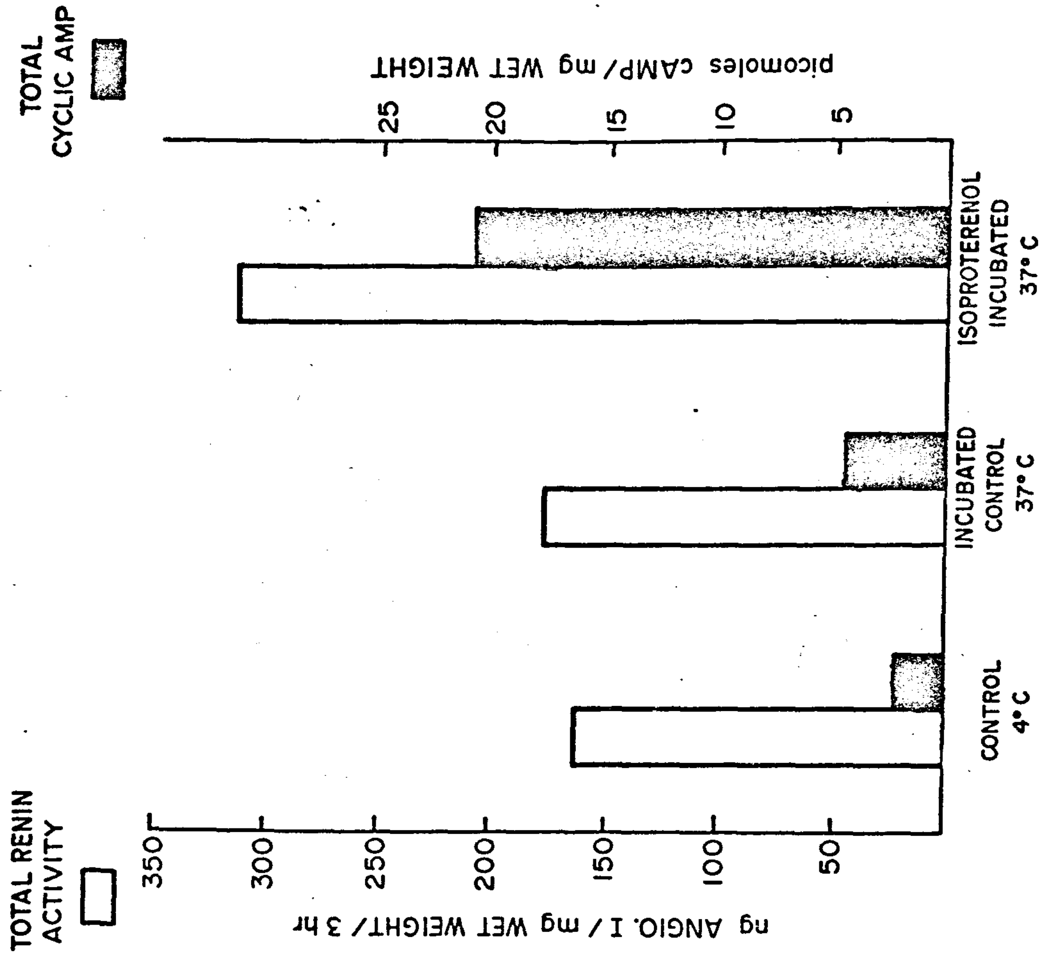
Kidney cells from rabbits kept 5 days on low sodium diet were dissociated by collagenase digestion and were centrifuged on a 160-260 g/liter Ficoll-Eagles density gradient for one hour at 10,173 x g (av.). Each fraction was washed free of Ficoll with freshly oxygenated Eagles medium and diluted to 3 ml with Eagles medium. 0.5 ml of each fraction was added to Eagles medium to a total volume of 2 ml and either kept at 4° C, as a starting total activity control, or incubated at 37° C under 95% O_2-5% CO_2. Either Eagles medium (control) or 2.5 ug/ml epinephrine was added every 5 minutes for 30 minutes. The total renin activity in the samples was analyzed by renin radioimmunoassay. Bars represent the mean of two separate rabbit renin-containing cell separations, and brackets indicate S.E.M.



TOTAL RENIN ACTIVITY
ng ANGIO. I / mg WET WEIGHT / 3 hr
250
200
150
100
50
COLD CONTROL 4°C
INCUBATED CONTROL 37° C
EPINEPHRINE INCUBATED 37° C

Figure 25.--Increase in renin activity and CAMP levels caused by isoproterenol in renin-containing cells separated by Ficoll density gradient centrifugation.

Kidney cortical cells from one rabbit kept on low sodium diet for 7 days were dissociated by collagenase digestion and were centrifuged on a 240-360 g/liter Ficoll-Eagles discontinuous density gradient for one hour at 10,173 x g (av.). 0.5 ml of each fraction was added to Eagles medium to a final volume of 2 ml and either kept cold, as a starting total activity control, or incubated at 37° C under 95% O_2-5% CO_2. Either Eagles medium (control) or 2.5 ug/ml isoproterenol was added every 5 minutes for 30 minutes. Perchloric acid was immediately added to the samples to be analyzed for CAMP and samples were frozen immediately in liquid nitrogen. Total renin activity was analyzed by renin radioimmunoassay.



TOTAL RENIN ACTIVITY
TOTAL CYCLIC AMP
ng ANGIO. I / mg WET WEIGHT/ 3 hr
picomoles cAMP/ mg WET WEIGHT
350
300
250
200
150
100
50
25
20
15
10
5
CONTROL 4° C
INCUBATED CONTROL 37° C
ISOPROTERENOL INCUBATED 37° C

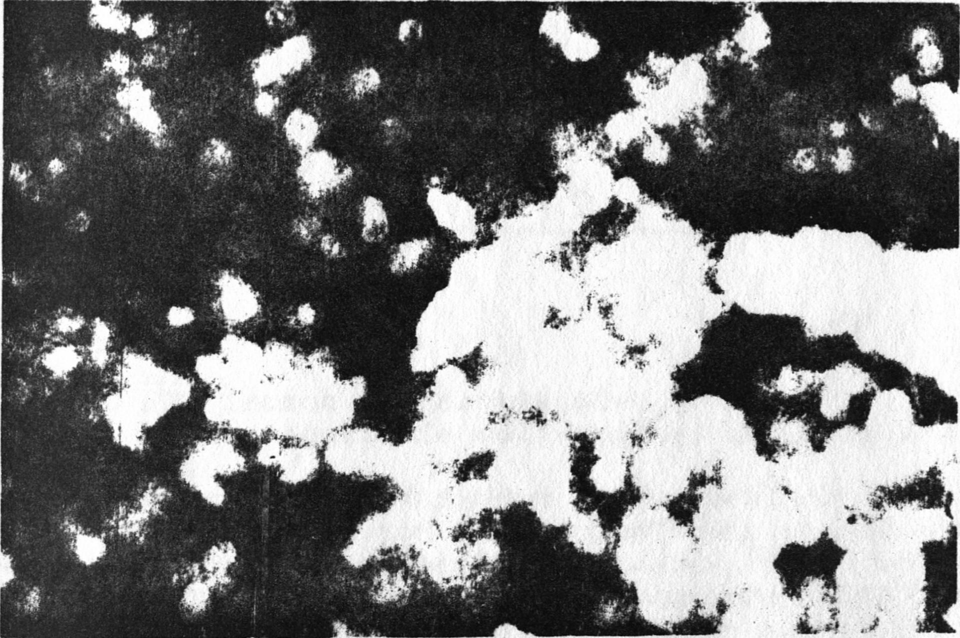
Figure 26.--Renin-rich fraction of RKC cells isolated by Ficoll density gradient centrifugation.

Cells were collected onto a clean glass slide by cytocentrifugation at 1,900 rpm 5 minutes, fixed in 95% ethanol and stained with thioflavin T according to the method in III E. 3. The cells were photographed under a Zeiss fluorescent microscope using a dark field condensor, BG 12/4 exciter filter and barrier filters 47 and 53. Photographs from the phase-contrast aperture had a 600 nm filter in the microscope. The film used was Polaroid high speed type 57, 4 x 5 black and white. A 2.5 second exposure was used.

A = Fluorescent cells, x 250.

B = The same field viewed with phase-contrast aperture, x 250.

A



B

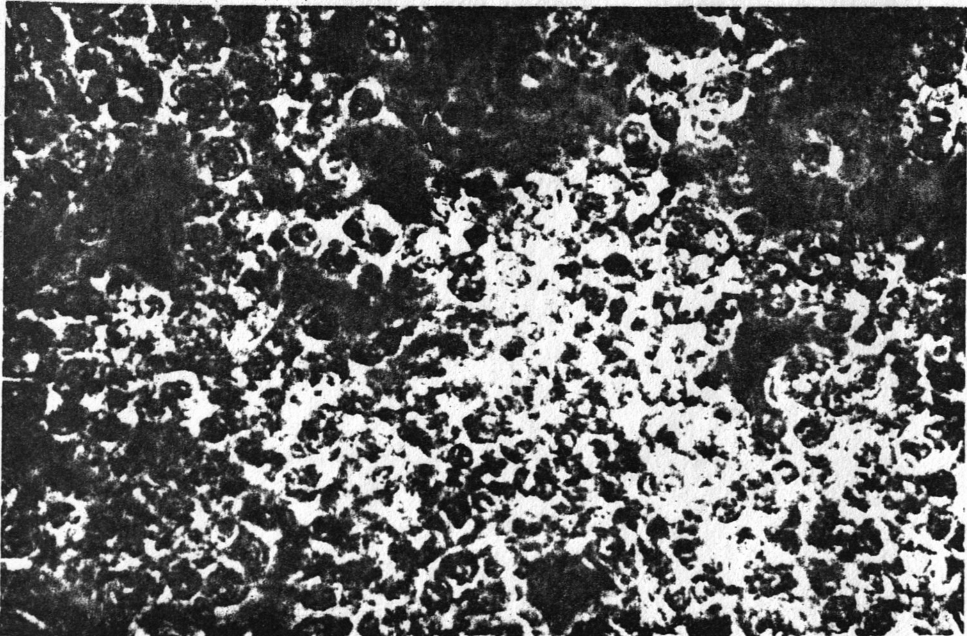
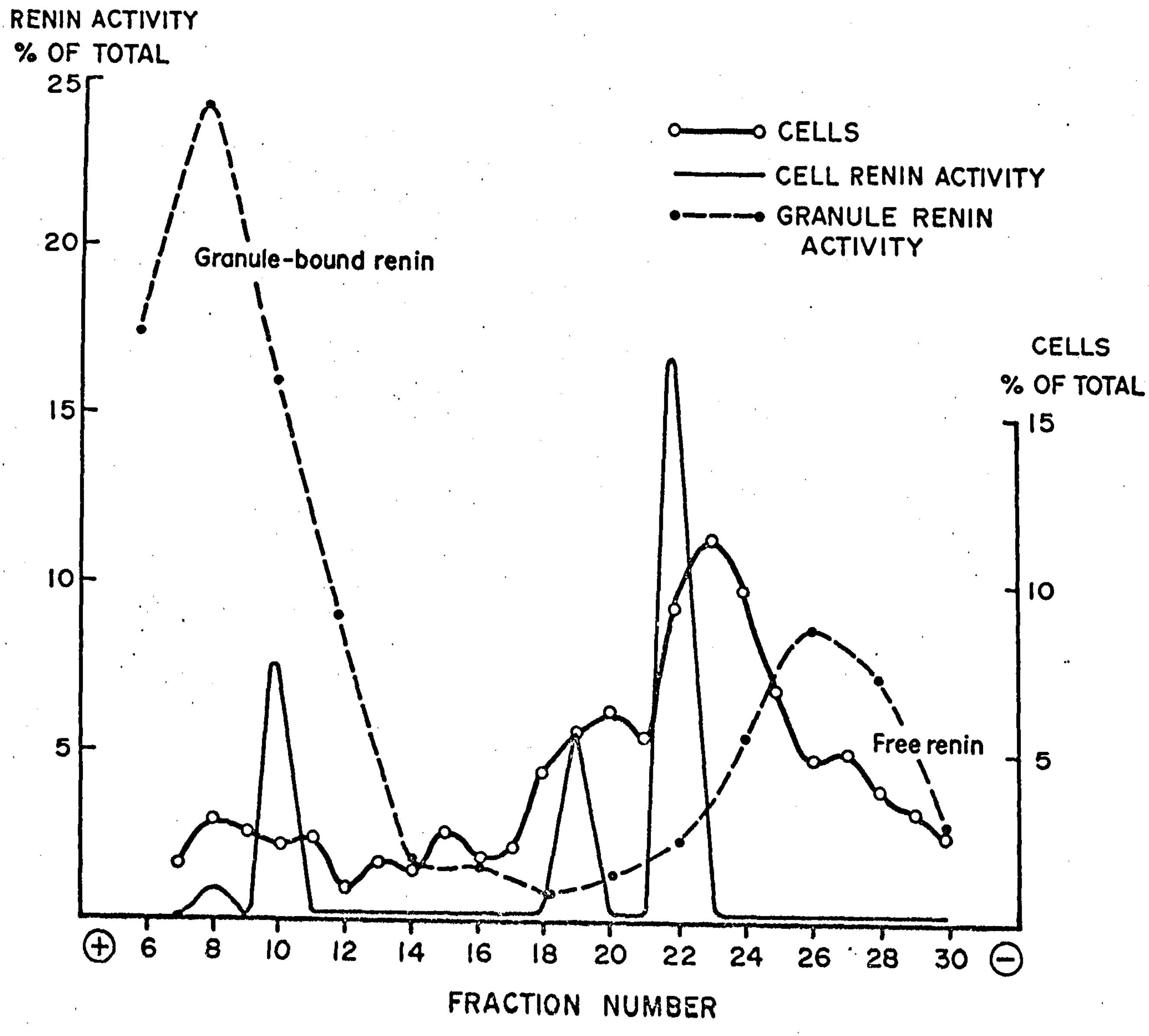


Figure 27.--Separation of normal RKC cells and renin granules by free-flow electrophoresis.

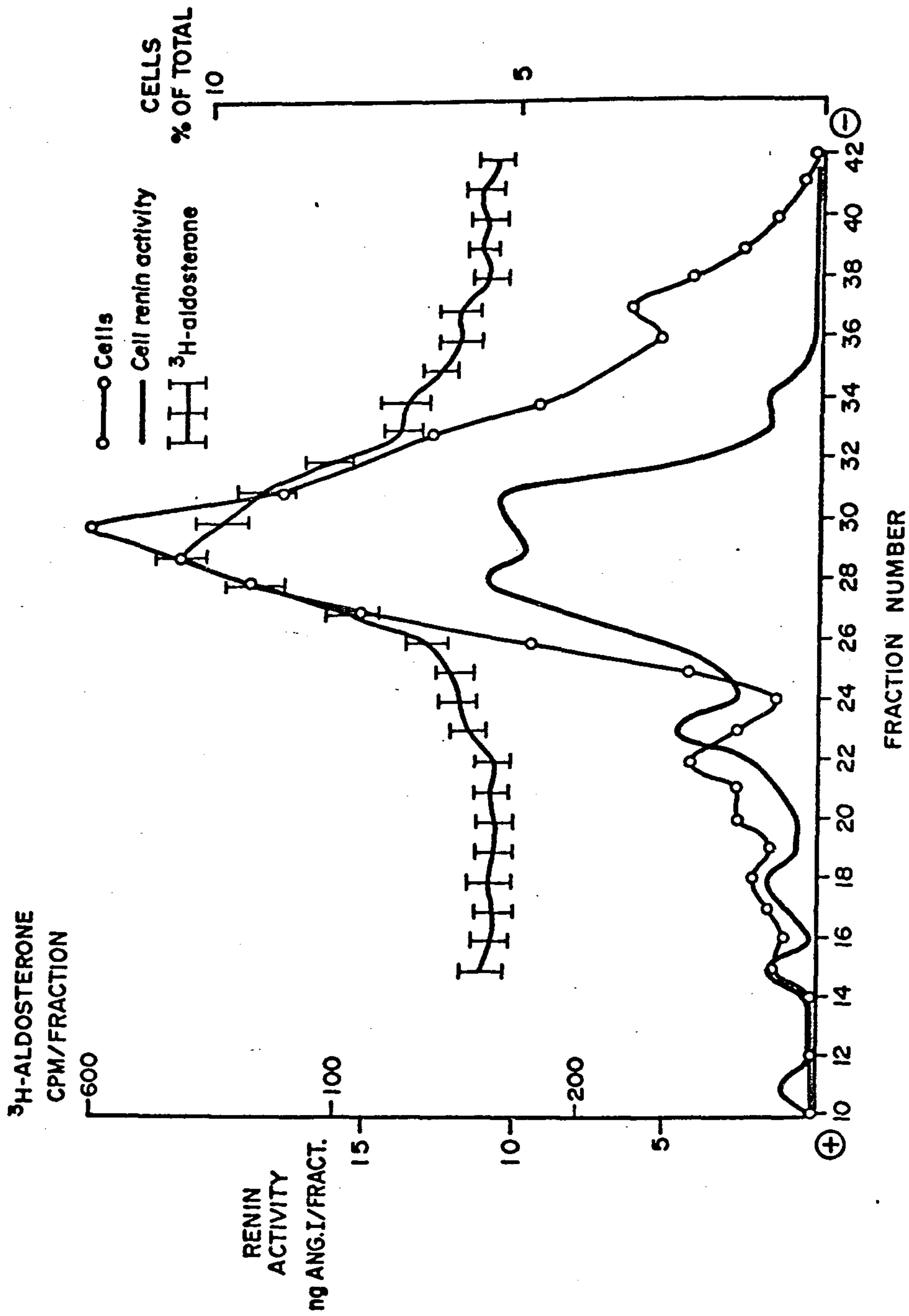
Cells from one rabbit kept on a normal diet were dissociated from kidney cortex by the combined enzymatic and mechanical dissociation method. Granules were prepared from the cell suspension supernatant by differential centrifugation at 6,500 x g (av.) for 5 minutes in a Beckman L5-65 ultracentrifuge using a SW 27 rotor. Cells and granules were resuspended in electrophoresis cell buffer, injected separately into the free-flow electrophoresis apparatus separated at 1150-1160 V, 200 mA, and collected into 30 fractions each. Cells in each fraction were counted with a hemocytometer and assayed for renin activity. Every two fractions of the granule separation were pooled and centrifuged in a Beckman ultracentrifuge, type 30 rotor, at 35,000 x g (av.) 20 minutes. "Granule-bound renin" and "free renin" were determined by centrifugation experiments.



RENIN ACTIVITY % OF TOTAL
25
20
15
10
5
CELLS
CELL RENIN ACTIVITY
GRANULE RENIN ACTIVITY
Granule-bound renin
CELLS % OF TOTAL
15
10
5
Free renin
+
6
8
10
12
14
16
18
20
22
24
26
28
30
−
FRACTION NUMBER

Figure 28.--Separation of ^{3}H-aldosterone pre-treated RKC cells by free flow electrophoresis.

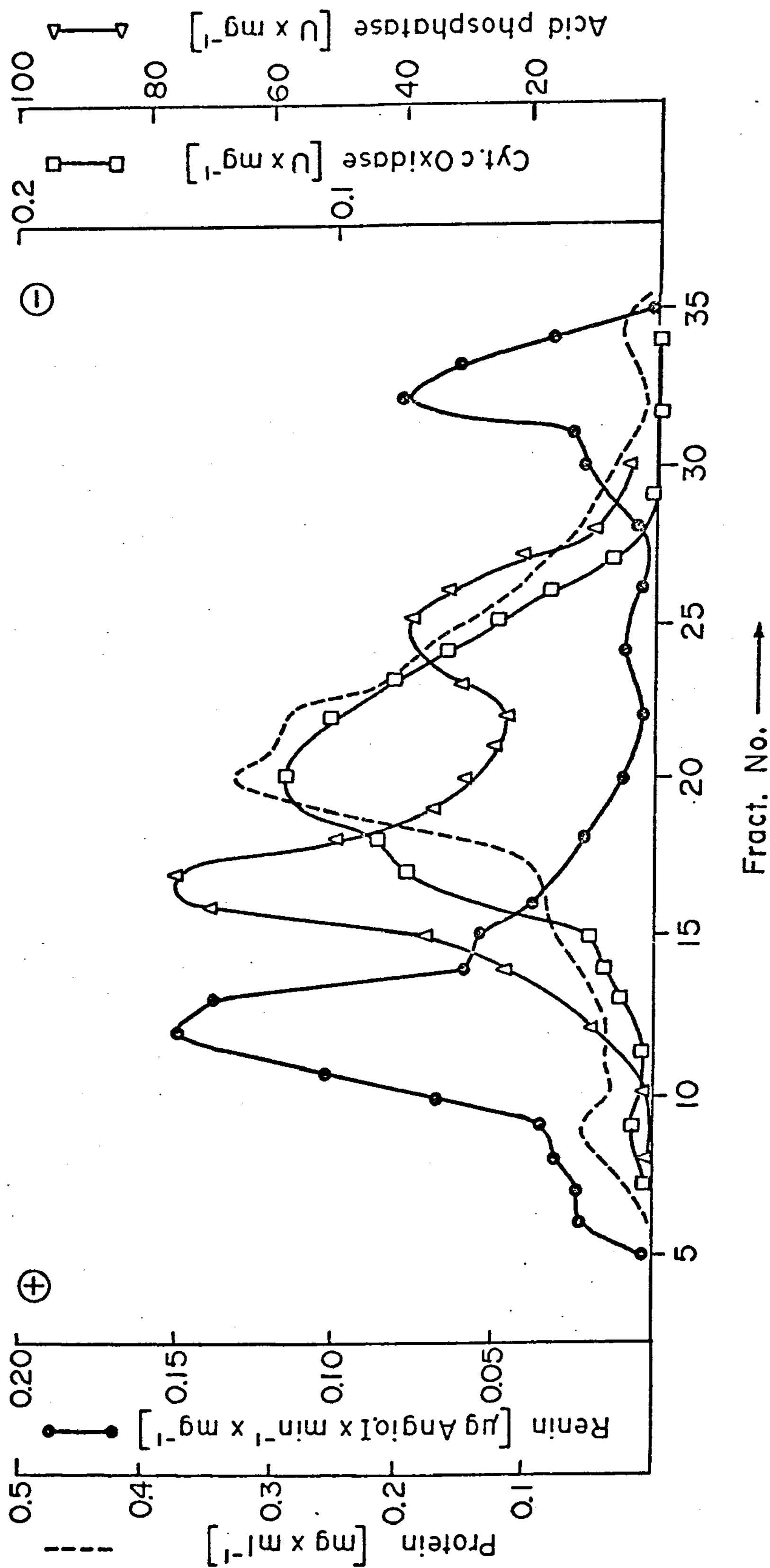
Kidneys from one rabbit, kept on a normal diet, were pressure-perfused with 3×10^{-8} M ^{3}H-aldosterone in 0.2% trypsin-free collagenase-Earles buffer. Cells were dissociated from the tissue by the combined enzymatic and mechanical method, resuspended in cell electrophoresis buffer and separated at 1090 V, 210 mA. Cells in each fraction were counted with a hemocytometer, analyzed for renin activity, and aliquots were counted in a 5 ml triton-toluene scintillation "cocktail" 20 minutes in a Packard Tri-Carb scintillation counter. The ^{3}H-aldosterone peak marks distal tubule cells and the renin peak marks renin-containing cells.



^{3}H-ALDOSTERONE
CPM/FRACTION
600
100
200
RENIN
ACTIVITY
ng ANG.I/FRACT.
15
10
5
CELLS
% OF TOTAL
10
5
Cells
Cell renin activity
^{3}H-aldosterone
FRACTION NUMBER
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42

Figure 29.--Electrophoretic separation of a heavy mitochondrial fraction from rabbit kidney cortex in a free-flow electrophoresis apparatus.

7 g of kidney cortex yielded 10-15 mg protein from a heavy mitochondrial fraction. Approximately 0.4 mg of granule protein could be found in electrophoresis fractions 9-13.



Protein [mg x ml⁻¹]
Renin [µg Angio.I x min⁻¹ x mg⁻¹]
Cyt.c Oxidase [U x mg⁻¹]
Acid phosphatase [U x mg⁻¹]
0.5
0.4
0.3
0.2
0.1
0.20
0.15
0.10
0.05
0.2
0.1
100
80
60
40
20
5
10
15
20
25
30
35
Fract. No.

Figure 30.--Electron micrograph of anodic fractions 13-15 obtained after electrophoresis and centrifugation at 4,500 x g in 5% BSA and OsO_4 1:1 (v:v).

Pellets were fixed in OsO_4. Magnification = x 18,000; 1.8 cm = 1.0 u.

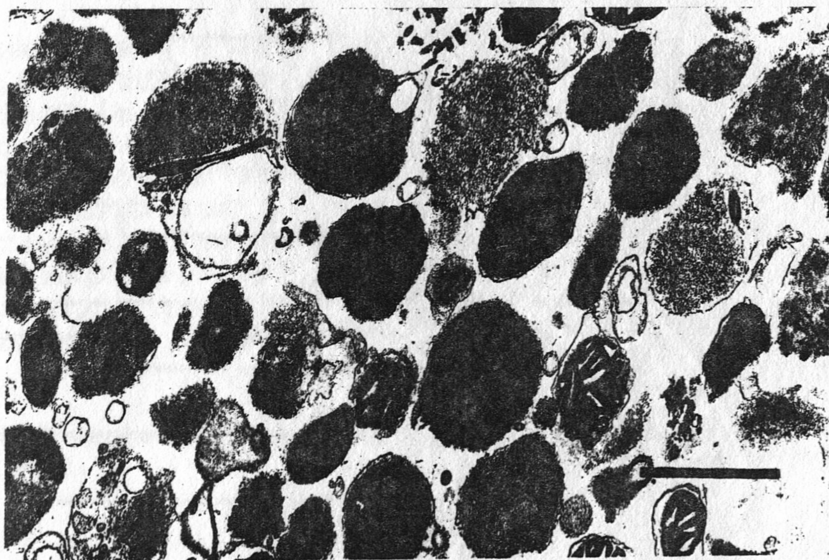


Figure 31.--Effect of changes in calcium concentration on renin release by isolated RKC cells.

Cells were dissociated from kidney tissue by the combined collagenase-mechanical method. 0.5 ml (0.7 mg protein) cell suspension in 4.5 ml Earles buffer with 6 different calcium concentrations was incubated under 95% O_2-5% CO_2 at 37° C for 10 minutes. The data represents a mean (bars) $\pm$ S.E.M. (brackets) of 2 separate experiments (2 kidneys/experiment) with triplicate incubations for each point, in the experiment. "Renin activity" is the amount of renin activity measured in the supernatant/mg protein measured in the cell pellet after incubation.

** = $p < 0.01$ and * = $p < 0.02$ with 2.5 mM calcium as control.

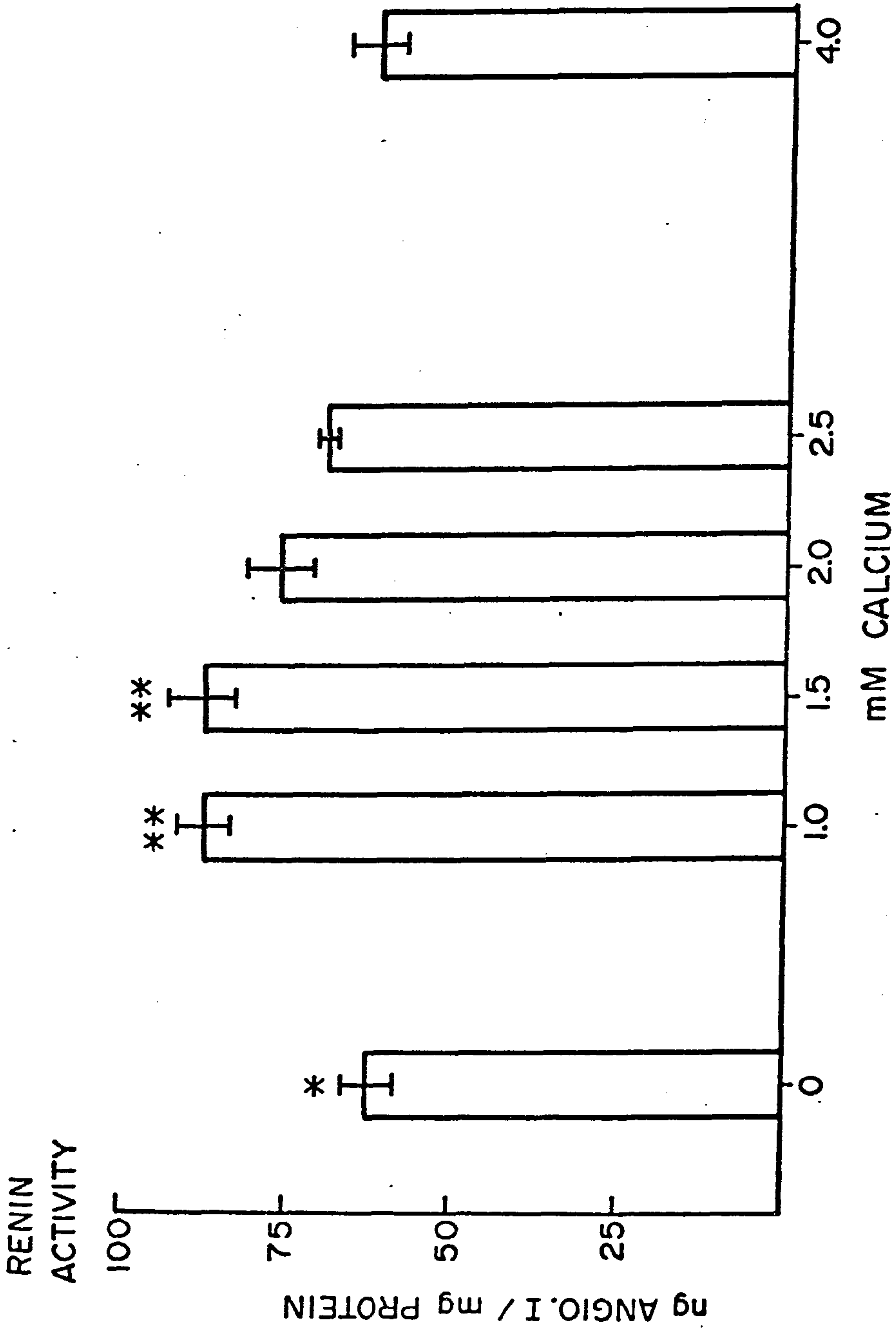

RENIN
ACTIVITY
100
75
50
25
ng ANGIO. I / mg PROTEIN
*
**
**
0
1.0
1.5
2.0
2.5
4.0
mM CALCIUM

Figure 32.--Effect of osmolarity change on renin release by RKC cells.

Cells were dissociated as in figure 31. 0.5 ml cell suspension (about 0.8 mg protein) was added to 4.5 ml Earles buffer with different concentrations of either sodium chloride or choline chloride and incubated as in figure 31. The data represents a mean (bars) $\pm$ S.E.M. (brackets) of 2 separate experiments, using 2 kidneys/experiment, with triplicate incubations for each point in the experiment. "Renin activity" is as described in figure 31.

** = $p < 0.001$ and * = $p < 0.01$ with 150 mM sodium or choline chloride as control.

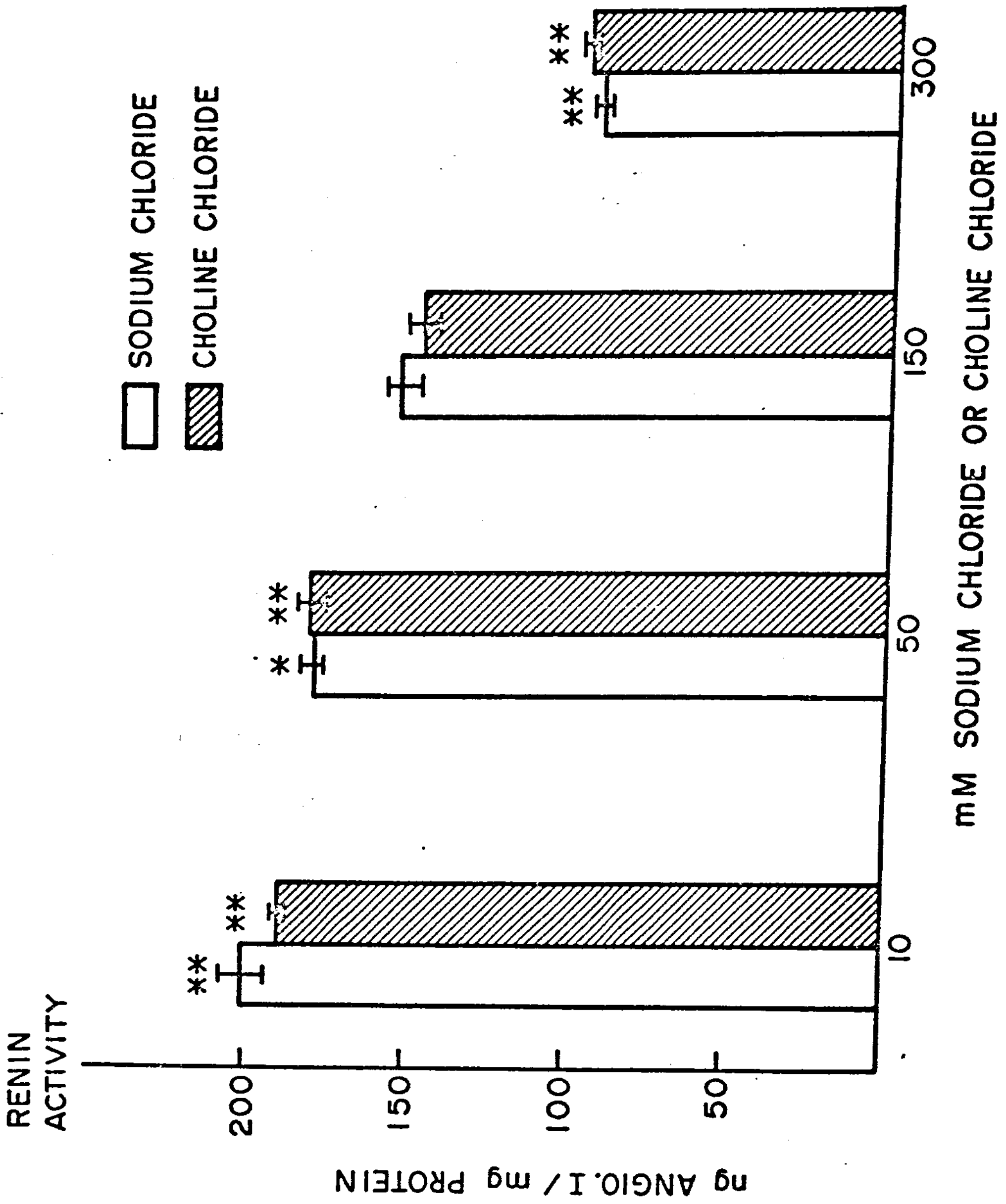

SODIUM CHLORIDE
CHOLINE CHLORIDE
RENIN ACTIVITY
ng ANGIO.I / mg PROTEIN
200
150
100
50
10
50
150
300
mM SODIUM CHLORIDE OR CHOLINE CHLORIDE

Figure 33.--Effect of variation in ratios of sodium to potassium on renin release from RKC cells.

Cells were dissociated as in figure 31. 0.5 ml (about 1.0 mg protein) cell suspension was added to 4.5 ml of various concentrations of NaCl and KCl in Earles buffer and incubated 30 minutes at 37° C under 95%-O_2-5% CO_2. "Renin activity" is as in figure 31. Bars are the mean of 2 separate experiments using 2 kidneys per experiment with triplicate incubations for each point in the experiment and brackets are S.E.M. Data was analyzed with 5.0 mM KCl - 150 mM NaCl as a control.

* = $p < 0.05$ and ** = $p < 0.001$.

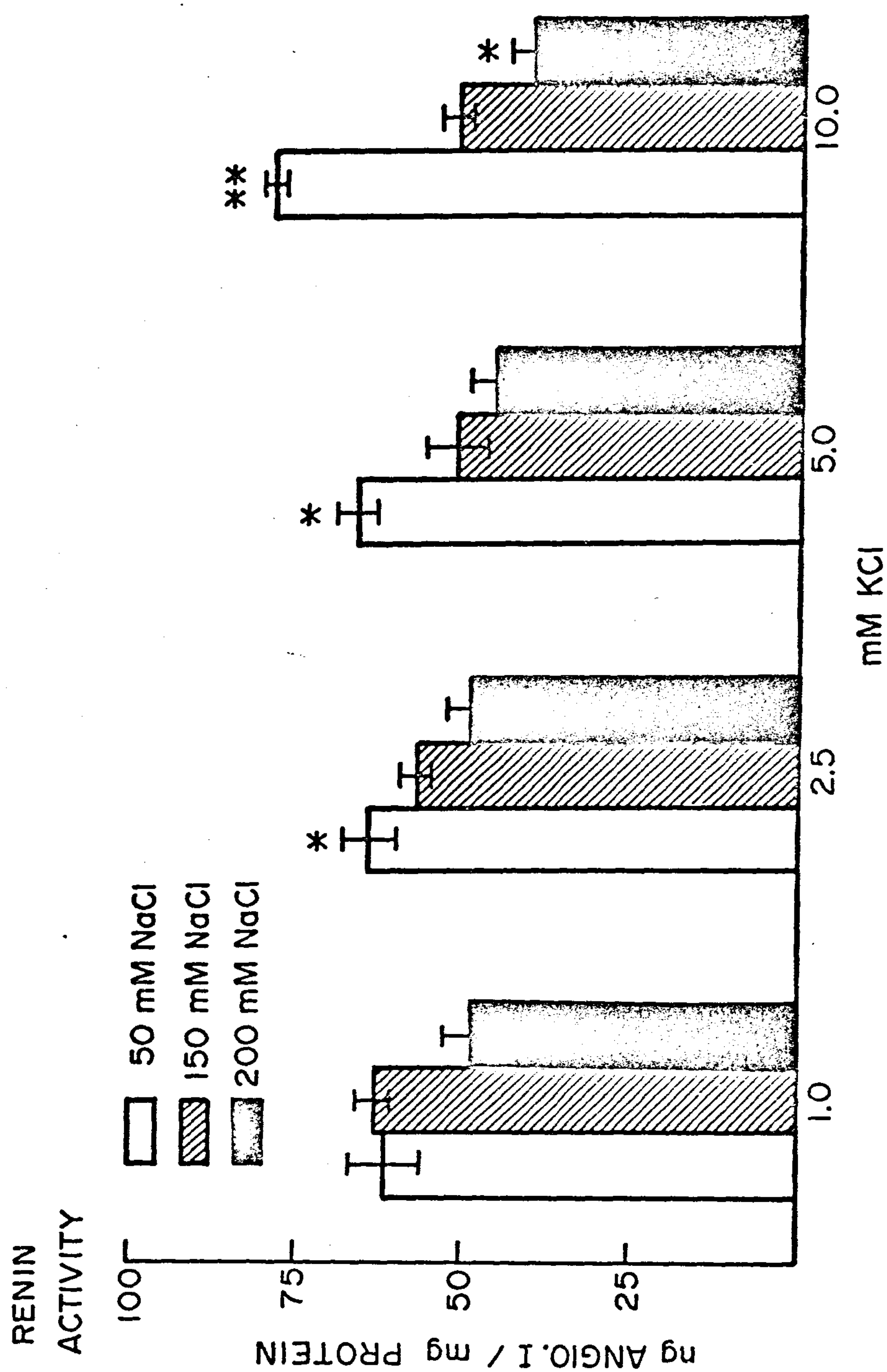

RENIN
ACTIVITY
ng ANGIO. I / mg PROTEIN
100
75
50
25
50 mM NaCl
150 mM NaCl
200 mM NaCl
1.0
2.5
5.0
10.0
mM KCl

Figure 34.--Increase in renin release from RKC cells with epinephrine, norepinephrine, or isoproterenol, and specific blockade of this release by α or β blockers.

Cells were prepared by sodium citrate and mechanical dissociation of kidney cortex. 0.5 ml of cell suspension (about 0.6 mg protein) was added to 4.5 mg Earles buffer containing physiologically normal ionic concentrations and incubated 35 minutes at 37° C under 95% O_2-5% CO_2. Antagonists were added before the cell suspension and cells were incubated in the presence of antagonists 15 minutes. Then agonist was added every 10 minutes and just before centrifugation. Total concentrations used were: phentolamine 5×10^{-7} M, propranolol 1×10^{-4} M; norepinephrine 3×10^{-8} M, epinephrine 3×10^{-8} M, isoproterenol 5×10^{-7} M. Control samples were treated similarly using Earles buffer. The data represent a mean (bars) $\pm$ S.E.M. (brackets) of 6 samples per point. "Renin activity" is as described in figure 31.

* = $p < 0.05$ and ** = $p < 0.001$.

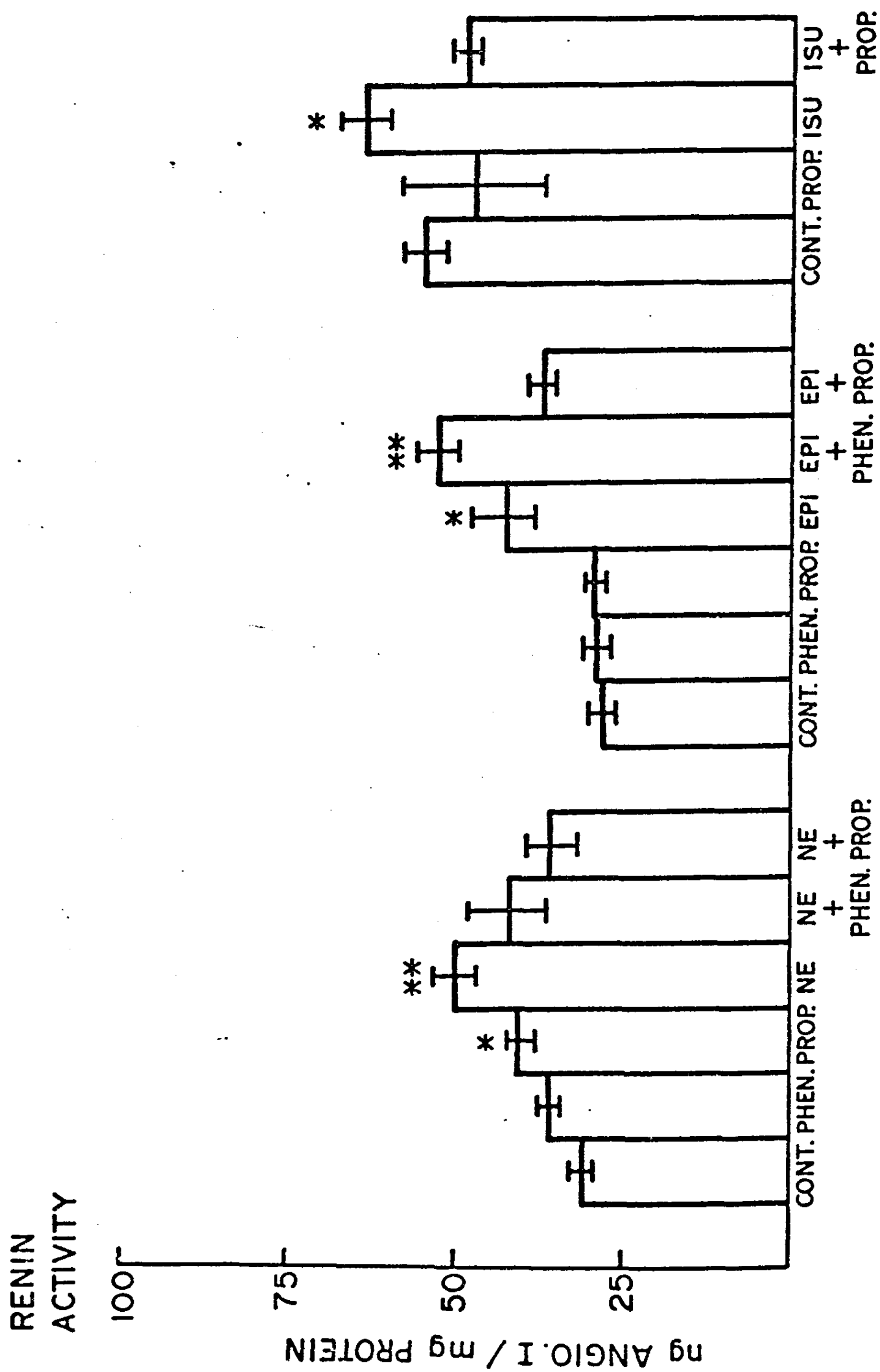
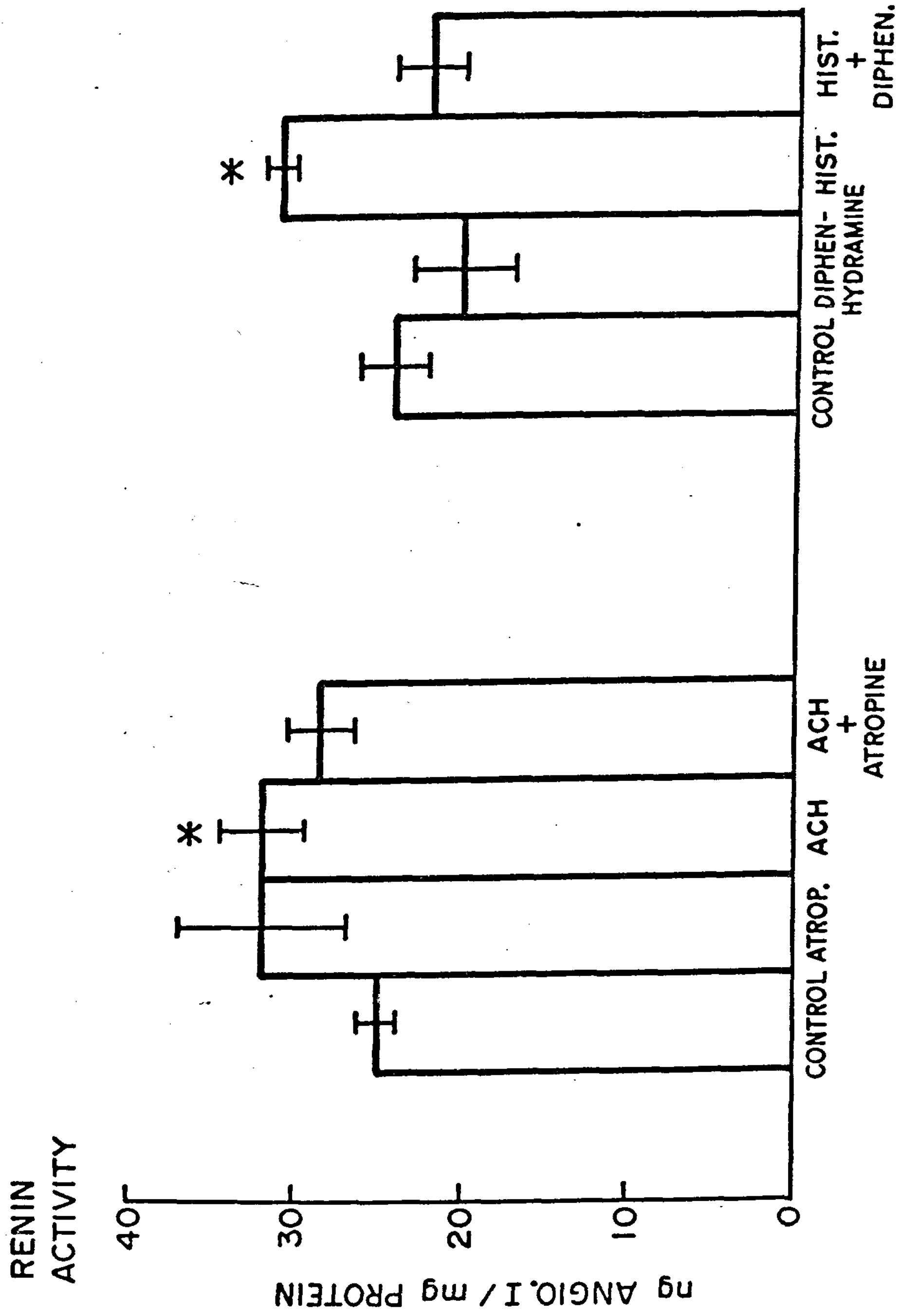

RENIN
ACTIVITY
ng ANGIO. I / mg PROTEIN
100
75
50
25
CONT.
PHEN.
PROP.
NE
NE + PHEN.
NE + PROP.
CONT.
PHEN.
PROP.
EPI
EPI + PHEN.
EPI + PROP.
CONT.
PROP.
ISU
ISU + PROP.

Figure 35.--Effect of acetylcholine and histamine on renin release from RKC cells and blockade of the response by atropine and diphenhydramine.

Cells were prepared as described in figure 34. 0.5 ml cell suspension (about 0.4 mg protein) was added to 4.5 ml normal Earles buffer and incubated as in figure 34. Concentrations were: atropine 2×10^{-5} M, diphenhydramine 6 ng/ml, acetylcholine 1.5×10^{-5} M, histamine 6 ng/ml. Data represents a mean (bars) $\pm$ S.E.M. (brackets) of 12 samples for controls and 6 samples for each dose point. "Renin activity" is as described in figure 31.

$* = p < 0.05$.



RENIN ACTIVITY
ng ANGIO. I / mg PROTEIN
40
30
20
10
0
CONTROL
ATROP.
ACH
ACH + ATROPINE
CONTROL
DIPHEN- HYDRAMINE
HIST.
HIST. + DIPHEN.
*
*

Figure 36.--Renin release from RKC cells in response to angiotensin I and angiotensin II.

Cells were prepared as in figure 34. 0.5 ml of cell suspension (about 0.5 mg protein) was added to 4.5 ml normal Earles buffer and incubated 20 minutes at 37° C under 95% O_2-5% CO_2. A I and A II were added before cells, every 10 minutes during incubation and before centrifugation, making the total concentrations shown. Earles buffer was added in a similar manner as a control. The data represent a mean (bars) $\pm$ S.E.M. (brackets) of 12 samples for controls and 6 samples for each dose. "Renin activity" is as in figure 31.

* = $p < 0.05$ and ** = $p < 0.001$.

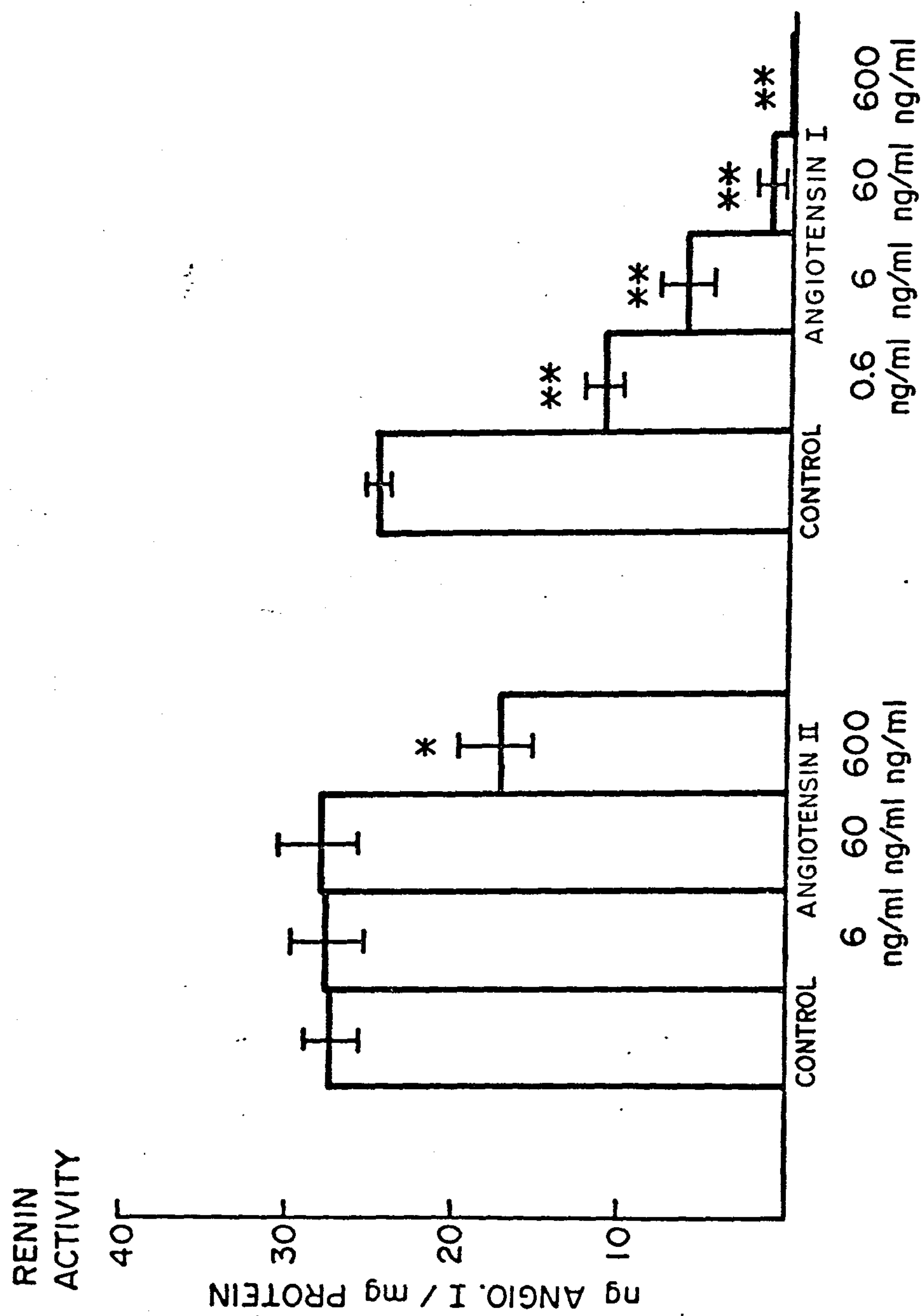

RENIN ACTIVITY
ng ANGIO. I / mg PROTEIN
40
30
20
10
CONTROL
6 ng/ml
60 ng/ml
600 ng/ml
ANGIOTENSIN II
*
CONTROL
0.6 ng/ml
6 ng/ml
60 ng/ml
600 ng/ml
ANGIOTENSIN I
**
**
**
**

Figure 37.--Formation of PGE_2 and PGF_2 from arachidonic acid, and structures of important prostaglandin compounds.

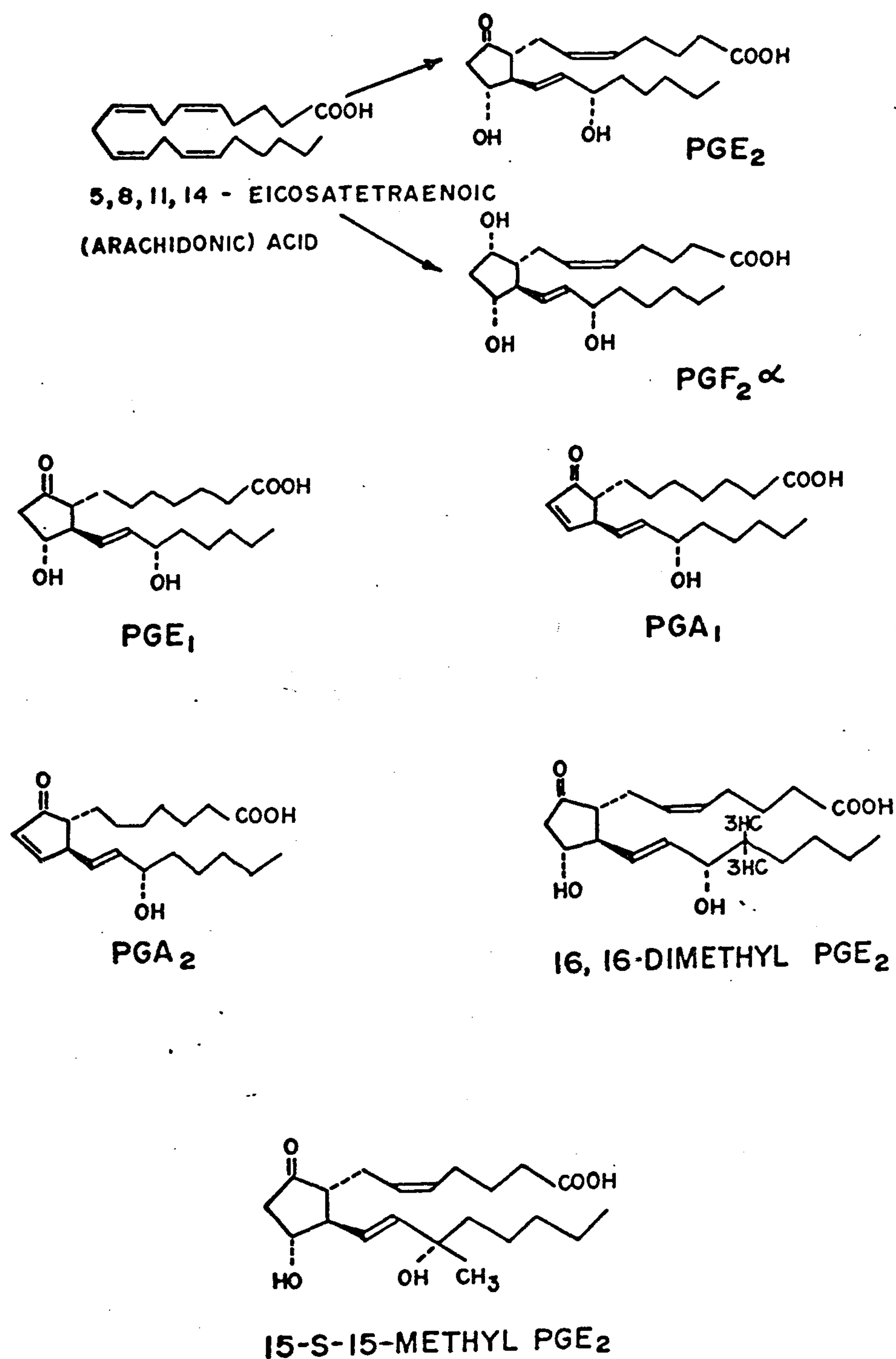

COOH
5,8,11,14 - EICOSATETRAENOIC
(ARACHIDONIC) ACID
O
COOH
OH
OH
PGE2
OH
COOH
OH
OH
PGF2α
O
COOH
OH
OH
PGE1
O
COOH
OH
PGA1
O
COOH
OH
PGA2
O
COOH
3HC
3HC
HO
OH
16, 16-DIMETHYL PGE2
O
COOH
HO
OH
CH3
15-S-15-METHYL PGE2

Figure 38.--Release of renin from RKC cells in response to various levels of prostaglandins or oleic acid.

0.5 ml (about 1.0 mg protein, 0.5 mg protein for oleic acid) of the cell suspension, prepared as in figure 34, was added to 4.5 ml normal Earles buffer and incubated 20 minutes under 95% O_2-5% CO_2 at 37°C. The compounds, or 80% ethanol as control, were added before the cells, every 10 minutes during the incubation, and before centrifugation to make the total doses shown in the graph. The data is expressed as percentage of control and each dose level represents the mean (bars) and S.E.M. (brackets) of 6 samples per treatment and 12 control samples for PGA_1, PGA_2 and oleic acid.

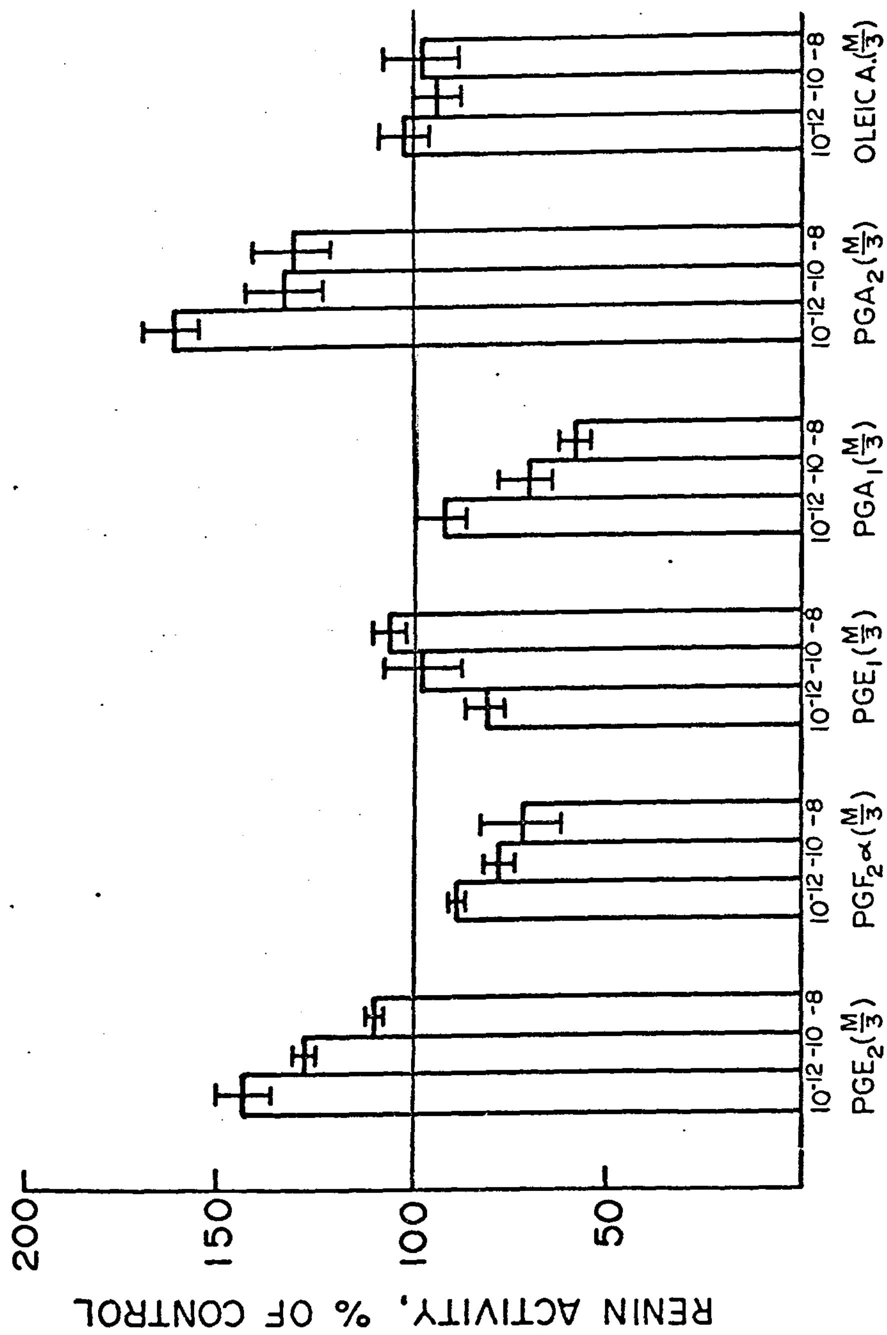

RENIN ACTIVITY, % OF CONTROL
200
150
100
50
10-12 -10 -8
PGE2(M/3)
10-12 -10 -8
PGF2α(M/3)
10-12 -10 -8
PGE1(M/3)
10-12 -10 -8
PGA1(M/3)
10-12 -10 -8
PGA2(M/3)
10-12 -10 -8
OLEIC A.(M/3)

Figure 39.--Release of renin from RKC cells in response to the E- and F-type prostaglandins.

Cells were prepared as in figure 34, and 0.5 ml of cell suspension (about 1.0 mg protein, and 0.5 mg protein for the 15-S-15-methyl PGE_2) was added to 4.5 ml normal Earles buffer and incubated 20 minutes at 37° C under 95% O_2-5% CO_2. Prostaglandins, or 80% ethanol (or Earles buffer for $PGF_{2\alpha}$) control, were added as described in figure 38, making the total concentrations on the graph. Data is expressed as percentage of control specific activity, and each point represents the mean (dots) $\pm$ S.E.M. (brackets) of 6 samples, and in the 15-S-15-methyl PGE_2 experiment, 12 samples for control and 6 samples for each treatment.

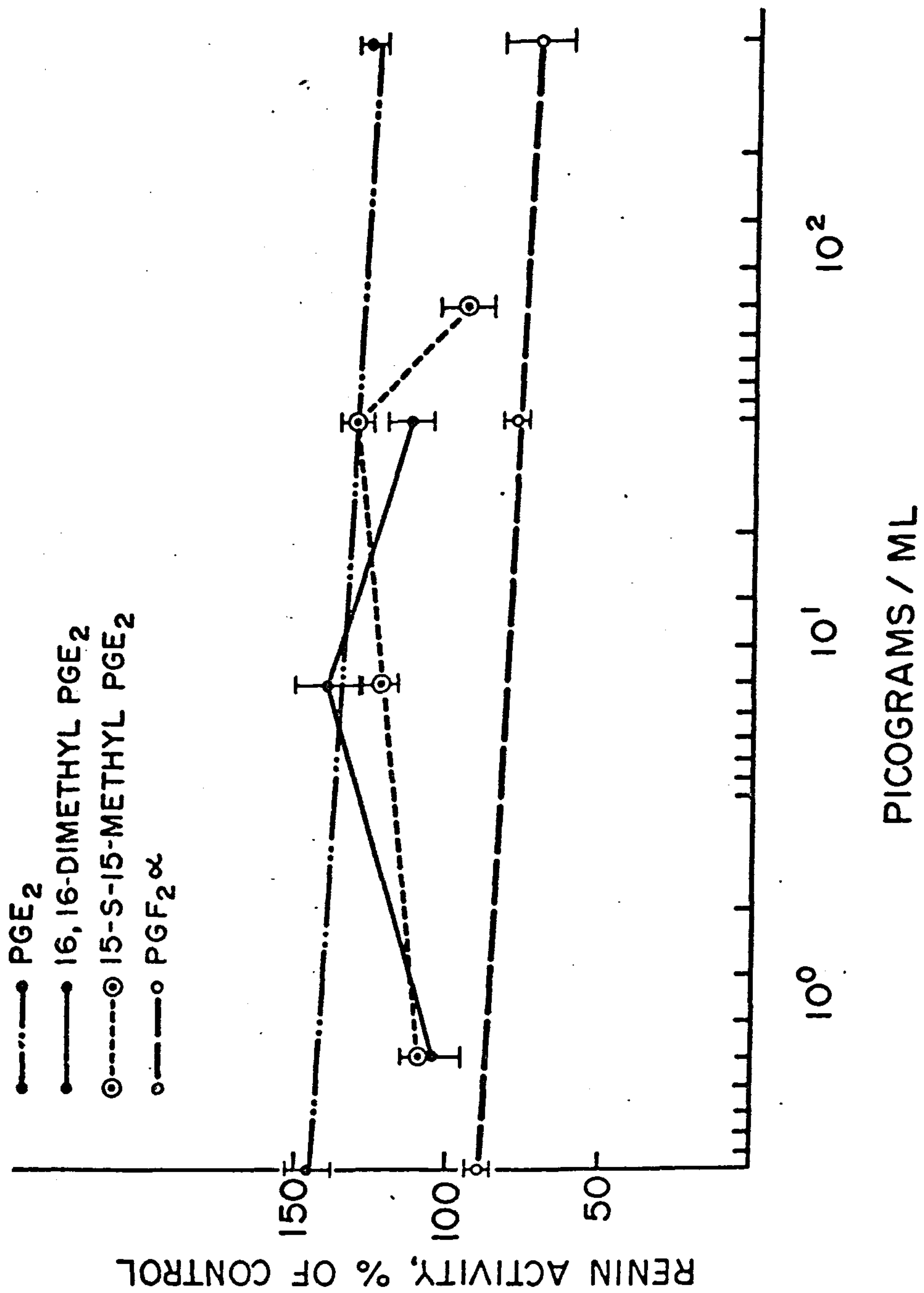

PGE$_2$
16, 16-DIMETHYL PGE$_2$
15-S-15-METHYL PGE$_2$
PGF$_2$α
RENIN ACTIVITY, % OF CONTROL
150
100
50
PICOGRAMS / ML
10^0
10^1
10^2

Figure 40.--The effect of PGE_2 on renin release from RKC cells with and without calcium.

0.5 ml (about 0.6 mg protein) cell suspension, prepared as in figure 34, was added to 4.5 ml Earles buffer containing 2.5 mM EGTA, 1.5 mM or 2.5 mM $CaCl_2$. The cells were incubated 20 minutes under 95% O_2-5% CO_2 at 37°C, and PGE_2 or 80% ethanol control was added as in figure 38, making a total concentration of 3×10^{-12} M. Data represents a mean (bars) $\pm$ S.E.M. (brackets) of 6 samples per treatment. "Renin activity" is as described in figure 31.

$* = p < 0.05$ and $** = p < 0.01$.

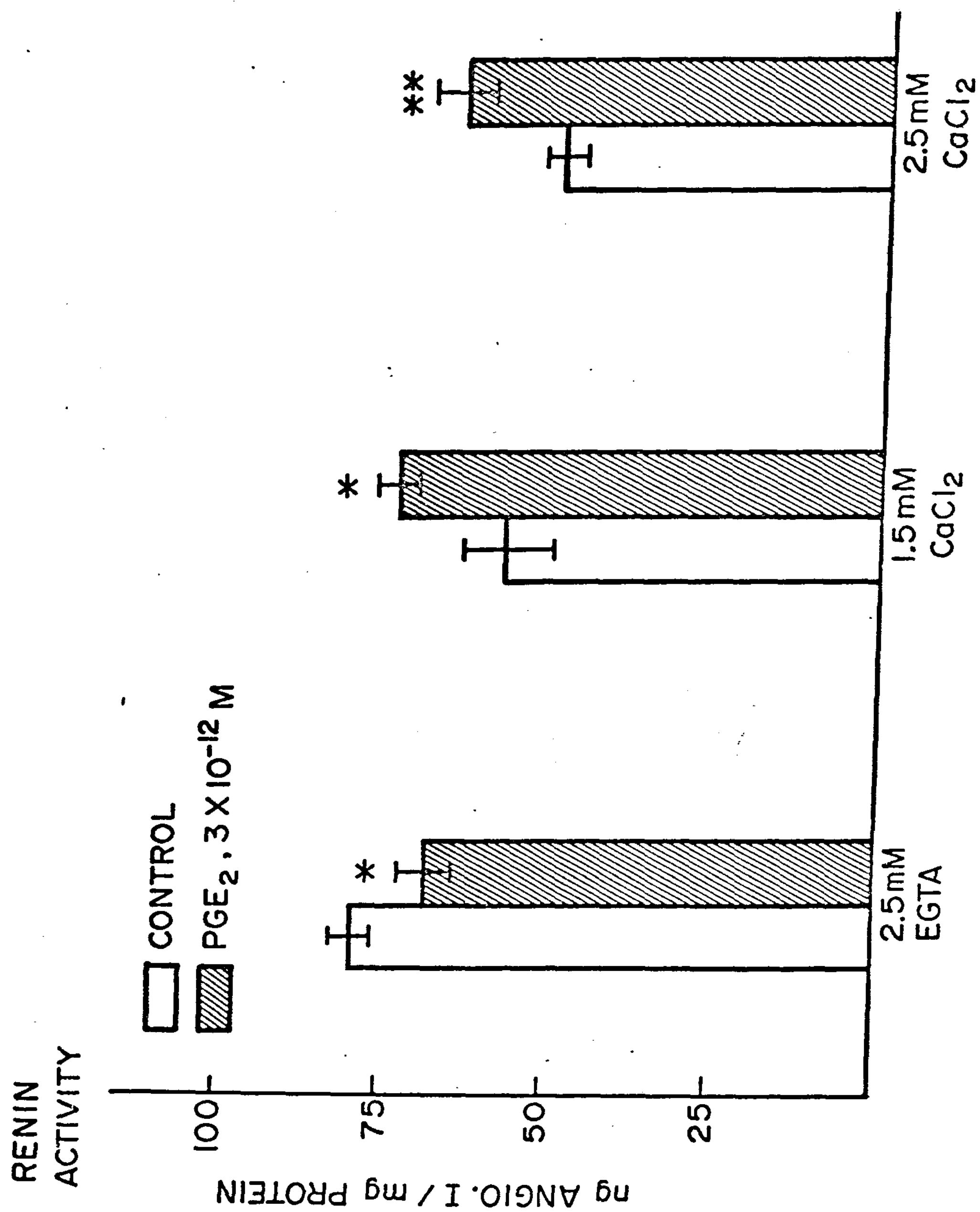

RENIN ACTIVITY
ng ANGIO. I / mg PROTEIN
100
75
50
25
CONTROL
PGE2, 3 X 10-12 M
*
*
**
2.5 mM EGTA
1.5 mM CaCl2
2.5 mM CaCl2

Figure 41.--Effect of arachidonic acid and indomethacin on the release of renin and formation of PGE_2 in RKC cells.

Cells were dissociated from tissue as in figure 34, and 0.5 ml cell suspension (about 0.4 mg protein) was added to 4.5 ml normal Earles buffer which contained 0.1 ug/ml arachidonic acid salt, 1.0 ug/ml indomethacin or the same volume of 80% ethanol as control. The samples were incubated 30 minutes at 37°C under 95% O_2-5% CO_2 and at the end of incubation the samples were centrifuged and supernatants analyzed for renin activity. The cell pellets were resuspended in BES-saline and an aliquot of each was analyzed for protein. All of the samples for each treatment were pooled and PGE_2 extracted as outlined in III, A.7. The data for renin activity represents the mean (bars) and S.E.M. (brackets) of 7 samples for control, 5 samples for arachidonic acid and 6 samples for other treatments. "Renin activity" is as described in figure 31. The PGE_2 activity was corrected for recovery of labelled PGE_2 in the samples, and is expressed as ng PGE_2 extracted from the cell pellet/mg protein in the cell pellet.

** = $p < 0.001$.

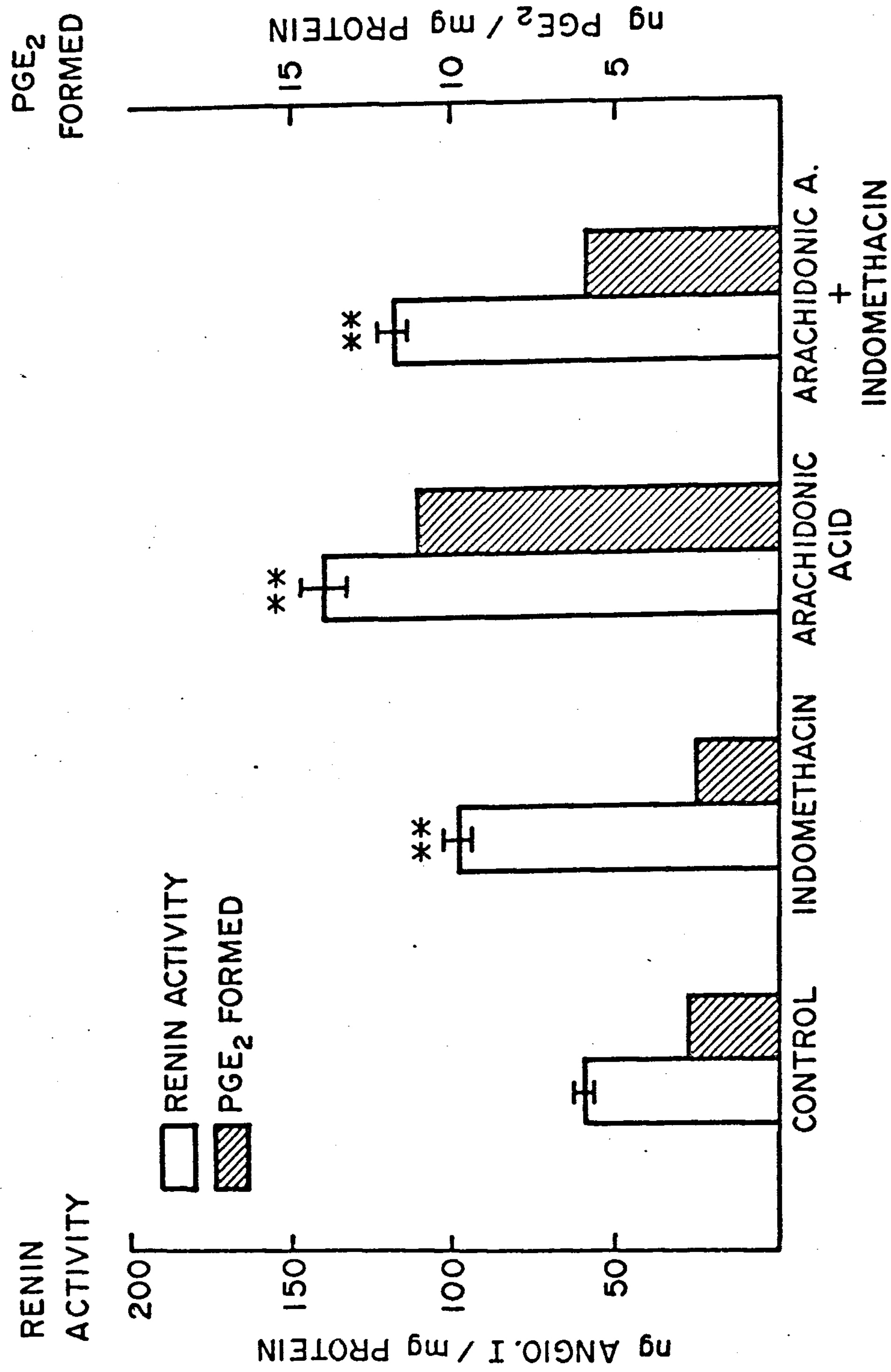
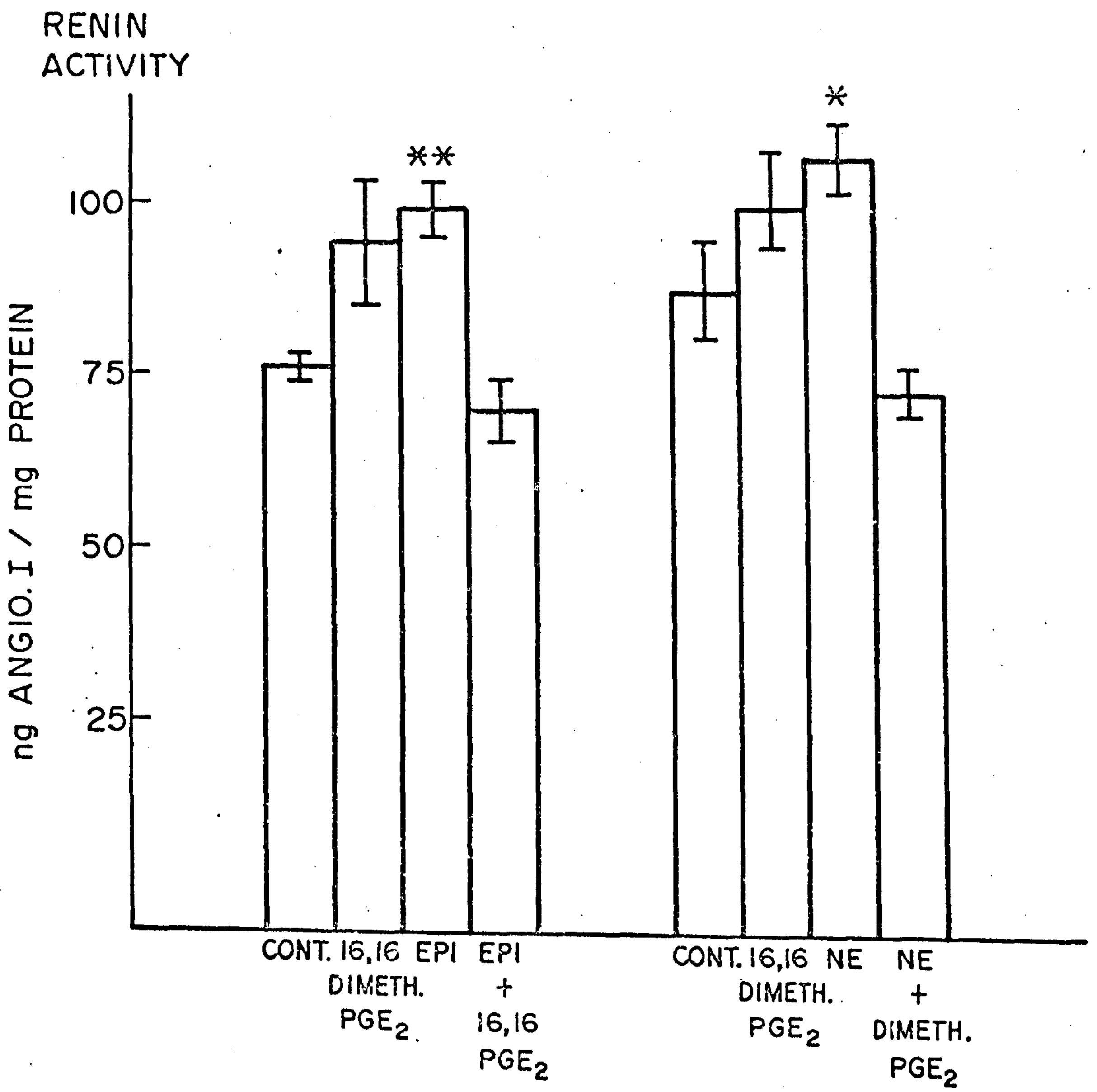

RENIN ACTIVITY
ng ANGIO. I / mg PROTEIN
200
150
100
50
PGE$_2$ FORMED
ng PGE$_2$ / mg PROTEIN
15
10
5
RENIN ACTIVITY
PGE$_2$ FORMED
**
**
**
CONTROL
INDOMETHACIN
ARACHIDONIC ACID
ARACHIDONIC A. + INDOMETHACIN

Figure 42.--Interaction of catecholamines and prostaglandin on the release of renin from RKC cells.

Cells were dissociated from tissue as in figure 34. 0.5 ml cell suspension (about 0.5 mg protein) was added to 4.5 ml Earles buffer and incubated under 95% O_2-5% CO_2 at 37°C. 5.0 pg/ml of 16,16-dimethyl PGE_2 was added before the cells and was not added again. The catecholamines were added as in figure 38 to make total concentrations of 3×10^{-8} M. The control samples were treated in the same way, but with 80% ethanol and Earles buffer added instead of prostaglandin and catecholamines. The data represents the mean (bars) and S.E.M. (brackets) of 12 samples for control and 8 samples for treated.

* = $p < 0.05$ and ** = $p < 0.001$.



RENIN ACTIVITY
ng ANGIO. I / mg PROTEIN
100
75
50
25
**
*
CONT.
16,16 DIMETH. PGE2
EPI
EPI + 16,16 PGE2
CONT.
16,16 DIMETH. PGE2
NE
NE + DIMETH. PGE2

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