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THE RENIN-ANGIOTENSIN-ALDOSTERONE HORMONAL SYSTEM

Jean E. Sealey, B.Sc.

Research Associate in Medicine
Columbia University
College of Physicians and Surgeons
630 West 168th Street
New York, New York 10032

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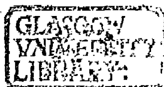

The Renin-Angiotensin-Aldosterone Hormonal System

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Summary

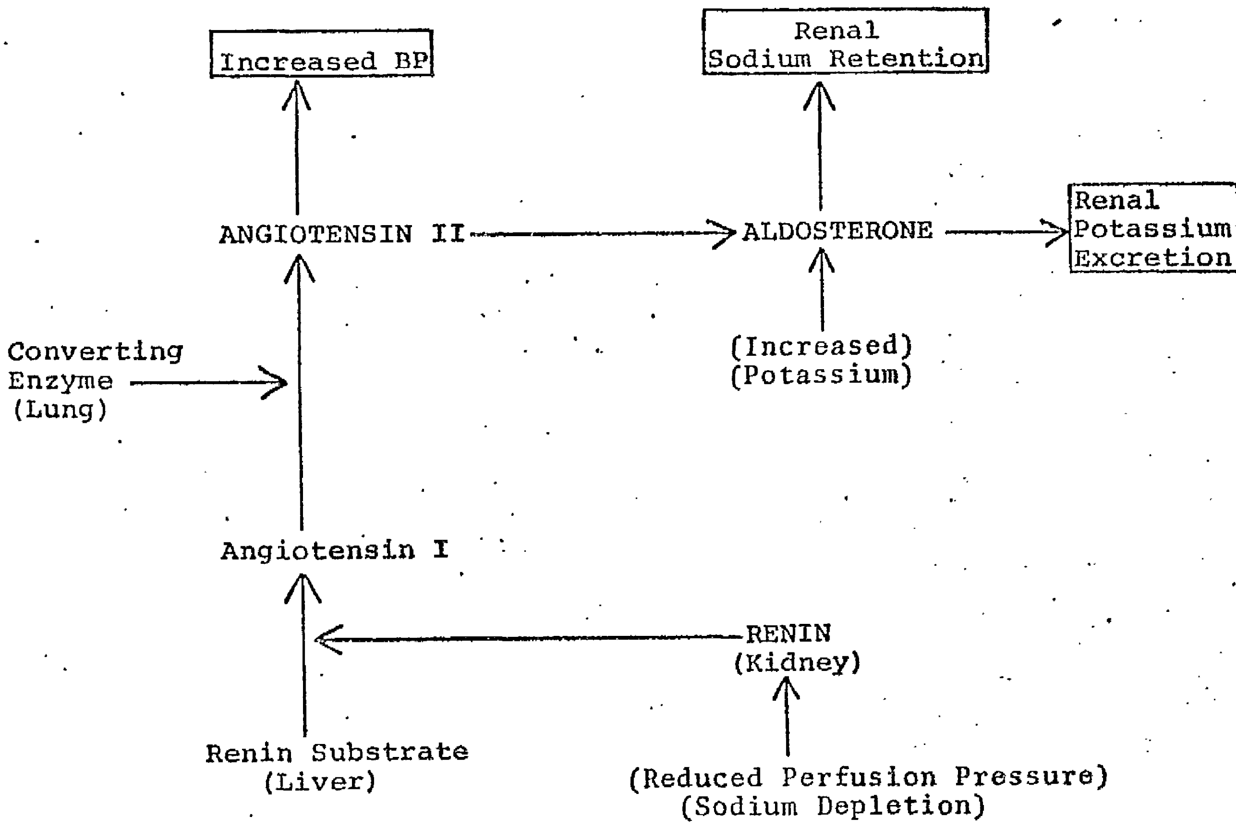
During the last fifteen years there has been increasing awareness that the renin-angiotensin-aldosterone hormonal system affects sodium and potassium balance and blood pressure homeostasis (1-4). The system appears to function as follows: renin is released from the kidney under situations in which its perfusion pressure is compromised, such as sodium depletion, hemorrhage, or a fall in mean arterial pressure (5). Renin, released into the circulation, hydrolyses plasma renin substrate (angiotensinogen) to yield a decapeptide, angiotensin I (Figure 1). Plasma angiotensin I is then rapidly hydrolysed by converting enzyme to angiotensin II primarily during passage through the lungs.

Neither renin nor angiotensin I has any well documented physiological effect. Angiotensin II however has at least two major physiological actions. As the most powerful vasoconstrictor substance known, it constricts arteriolar smooth muscle thus increasing arterial pressure, while at the same time it is a potent stimulus for aldosterone biosynthesis. Aldosterone is the second effector hormone of the system. Its adrenal secretion is governed by two major stimuli, the plasma levels of potassium and angiotensin II. Aldosterone, in turn, increases renal potassium excretion and promotes renal sodium conservation (Figure 1).

From this brief outline, it can be appreciated that the renin-angiotensin-aldosterone system reacts to changes in sodium and potassium balance or blood pressure, and these three parameters in turn affect the hormonal system.

Figure 1

THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM



The present work was begun in a setting in which the gross physiology of the three hormones had been worked out, but the role and interactions of the system in the minute by minute maintenance of sodium and potassium balance and blood pressure was still in question. Further elucidation of the nuances of the hormonal interactions required, first of all, refinement of the methods for measurement of the hormones. Accordingly, methods are presented which were developed or improved upon for measurement of plasma renin activity (6-8), plasma renin substrate (6) and urinary (9-11) and plasma aldosterone (12). These methods are simpler, more accurate and more sensitive than those previously utilized, providing the advantage that subnormal values can be detected with confidence, thus allowing investigation of the system over a wide spectrum of activity (13, 14).

Angiotensin II is the effector hormone of the renin limb of the hormonal system. Thus its blood level would be expected to be the most meaningful indicator of changes in physiological activity of the system. However, although methods for measurement of angiotensin II were available they were not sufficiently sensitive to detect with confidence low levels of the hormone. Accordingly, plasma renin "activity" measurements were utilized instead since all evidence suggests that angiotensin I generation closely reflects angiotensin II levels, there being no evidence that converting enzyme activity is ever rate limiting.

Since the production rate of angiotensin in plasma is

dependent on both plasma renin and substrate concentrations, studies were carried out to determine if a change in renin secretion is the dynamic component which maintains the rate of generation of angiotensin at an appropriate level. First, the importance of changes in plasma renin substrate on the measurement was examined (6,15,16). Then the characteristics of metabolic clearance rate of renin were evaluated over a wide range of plasma renin levels, allowing a more precise evaluation of the relationship of renal renin secretion to peripheral renin measurements (16).

Although it has been generally accepted that renin and aldosterone are important for regulation of sodium excretion at low and normal sodium intakes, the role of the hormonal system in regulating salt balance at high levels of sodium intake has not been clarified. To study this question, normal subjects were evaluated during sodium loading (11,17). Daily measurement of renin and aldosterone revealed that the two hormones continue to have a regulating influence on urine sodium excretion, even at high sodium intakes (18).

The hormonal responses to extreme changes in sodium and potassium balance were studied in parallel in normal man and in rats. A new coordinate of the renin-angiotensin-aldosterone system was exposed when it was demonstrated that changes in potassium balance directly affect renin secretion (19,20). Sodium depletion has been shown to induce moderate potassium retention. The influence of this increase in potassium balance on the relationship of renin to aldosterone during sodium

depletion was studied in normal man (17,18). It was found that both plasma renin activity (i.e., angiotensin II) and potassium are important trophic stimuli for aldosterone secretion during sodium deprivation in normal subjects with the subtly positive potassium balance working to attenuate the rise in renin secretion.

Aldosterone promotes both renal sodium retention and potassium excretion. On the face of it these two simultaneous actions would be expected to produce wide and inappropriate fluctuations in the balance of one of the two ions in defending changes in dietary intake of the other. However, it will be demonstrated that when aldosterone secretory changes are considered together with concurrent changes in intrarenal physical forces such as glomerular filtration rate, and proximal tubule sodium reabsorption, a system can be proposed which accounts for the simultaneous appropriate regulation of both ions (18).

A question that has intrigued many investigators who study the factors involved in maintenance of sodium balance, is whether another hormone exists which promotes sodium elimination (i.e. a natriuretic hormone). This possibility was suggested initially by a study by de Wardener and co-workers (21) who demonstrated that urine sodium excretion could be regulated appropriately under conditions in which glomerular filtration and aldosterone were unchanged or inappropriate. To investigate the existence of a natriuretic hormone, extraction, concentration and purification proce-

dures were developed for the fractionation of urine and plasma on the basis of molecular weight and a new biological assay was elaborated for detection and quantitation of natriuretic extracts (22). We found a natriuretic substance in urine and plasma which appears to have physiological relevance since it could be detected in urine and plasma from salt-loaded subjects but not in extracts derived from subjects who were sodium deprived. Evidence suggests that the substance may participate in regulation of sodium reabsorption in a distal portion of the nephron, beyond the distal tubular potassium secretory site (22-24).

The renin-angiotensin-aldosterone hormonal system is also involved in blood pressure homeostasis. Variations in pressor sensitivity to angiotensin II are known to occur with changes in sodium balance. To investigate the physiological basis for these differences, angiotensin II antibodies were injected into rats on three different sodium intakes. It was possible to characterise specific angiotensin vascular receptors which exhibit significant variation in their affinity for angiotensin depending on the state of salt balance (25).

The interdependence of sodium balance and angiotensin II in the maintenance of arterial pressure was also studied in experimental and human renovascular hypertension. When we blocked angiotensin II activity in two different experimental models of renovascular hypertension, blood pressure fell only in the two-kidney model in which plasma renin activity was elevated (26). Other investigators of our group then demon-

strated that when arterial filling was reduced in the non-responsive model by sodium depletion, blood pressure fell in this model too in response to angiotensin II blockade (27). We conclude that, depending on the availability of sodium, experimental renal hypertension may be maintained by either excess renin (angiotensin II) or alternatively by inappropriately excessive arterial filling (sodium).

A new approach to analysis, understanding and identification of curable forms of human renovascular hypertension emerged when these studies of animal models were analysed in conjunction with another study of renal vein renin measurements in patients with essential hypertension. Clinical counterparts of the two different animal models of renovascular hypertension appear to exist in man. It will be demonstrated in the final section of this memoir that the two forms can be distinguished on the basis of peripheral and renal vein renin measurements alone (16,28). Since only the renin dependent form can be cured by unilateral renal surgery, this new understanding is important for evaluation of patients with renovascular hypertension.

Altogether, therefore, this memoir is concerned with the renin-angiotensin-aldosterone hormonal system, the methods for measurement of its components, the interpretation of these measurements in physiological and pathological settings, and an evaluation of the relative importance of the systems in the overall maintenance of sodium and potassium balance, and blood pressure homeostasis.

PART I:

METHODOLOGY OF RENIN, ALDOSTERONE AND NATRIURETIC HORMONE

I. Measurement of the Components of the Renin System

Renin was discovered in 1898 by two medical students, Robert Tigerstedt and Per Bergman, who noted an increase in the blood pressure of anesthetized rabbits when saline extracts of kidney were injected (29). These observations remained in dispute until 1938, when three groups confirmed this earlier finding (30-32). Meanwhile, other studies had implicated the kidney in the hypertensive process (33) and in 1934 Goldblatt produced chronic experimental hypertension for the first time by placing a clip around the renal artery of a uninephrectomized dog (34).

In 1939, Page (35) and Braun-Menendez and coworkers (36) reported that renin was not by itself a pressor substance but acted in plasma as an enzyme to release a pressor peptide. Angiotensin is released from a circulating plasma globulin (37-39) and has been shown by Peart and also Skeggs and coworkers to be present in plasma in two forms, a decapeptide and an octapeptide (40-43). Skeggs and colleagues in 1956 (44) isolated a converting enzyme from horse plasma which splits the decapeptide angiotensin I to angiotensin II. These workers were able to identify angiotensin II and the dipeptide, histidyl-leucine as products of reaction.

Measurement of the components of the renin system was not a simple task. Circulating levels of angiotensin are so minute that, in the early assays, excessive amounts of blood had to be collected (45,46). However, in 1963 Helmer

and Judson (47) developed a bioassay method for measuring plasma renin which utilized the fact that renin reacts with plasma renin substrate to generate angiotensin. By incubating the plasma for a fixed period of time, prior to bioassay, sufficient angiotensin was generated to enable detection and quantitation in a small volume of plasma.

Most methods which have been developed for measurement of plasma renin are based on this principle and differ mainly in techniques used for increasing sensitivity of the assay and for inactivation of angiotensinases which destroy the angiotensin formed during incubation. The first method to be developed which successfully eliminated the angiotensinase problem, and thus could be used to accurately measure plasma renin, was developed by Lever and coworkers (48). In this method addition of exogenous substrate helped to increase the sensitivity of the measurement since substrate concentration in plasma is normally rate limiting. Angiotensinases were separated chemically from renin. Other techniques for angiotensinase inhibition include adsorption of the formed angiotensin onto a resin (45), dialysis (49-51), inactivation by heat at pH 4.5 (51,52) and addition of inhibitors (6,7, 49,50,53-57).

The use of plasma renin rather than angiotensin II measurements in assessing the physiological role of the renin system has introduced some complications. Since the plasma concentration of renin substrate is normally rate limiting, the capacity of plasma to generate angiotensin is a function of both

renin and substrate concentrations. Thus methods for measurement of plasma renin differ in whether they measure plasma renin concentration or plasma renin activity. In the first approach, a fixed amount of renin substrate (angiotensinogen) is added to the incubation medium so that the measurement reflects only differences in enzyme concentration (51,52,55,58). However, when plasma renin activity is measured, endogenous plasma angiotensinogen is utilized as the substrate in the incubation step and the measurement reflects the net capacity of plasma to generate angiotensin (6,45,49,51,53,54,56,57). Thus the measurement is a reflection of a first order reaction between the enzyme and its substrate and for this reason is referred to as plasma renin activity, and not plasma renin concentration.

Plasma renin substrate is measured by its capacity to generate angiotensin I during incubation in the presence of an excess of renin (6,50,53,59). In addition, the capacity of plasma to generate angiotensin can be evaluated by measuring the response of plasma to addition of a fixed amount of endogeneous renin (6, 49). This latter measurement allows analysis of the effects of activators and inhibitors on the renin reaction.

At first a rat bioassay was used to quantitate the angiotensin generated during the incubation step (6,45,49-51,53,58, 60). More recently, with the development of radioimmunoassay techniques by Yalow and Berson (61), the rat pressor bioassay has been replaced by a more sensitive radioimmunoassay of formed

angiotensin I (6,52,54-57).

In this section, the methods developed in our laboratory for measurement of plasma renin activity, renin substrate and the reactivity of plasma to renin will be presented in detail. They comprise a basic incubation step during which angiotensin I is generated followed by quantitation of the angiotensin by radioimmunoassay.

Incubation Steps:

(a) Plasma renin activity: (Table 1, Figure 2)

Since renin is measured by its capacity to form angiotensin I during a fixed incubation time, generation of angiotensin I prior to incubation must be inhibited. For this reason the plasma is chilled immediately after collection. In addition, conversion of angiotensin I to angiotensin II or to break-down products must be inhibited. Three factors operate to destroy angiotensin I: converting enzyme, angiotensinases and bacteria (Figure 2). These must all be inhibited during the incubation step.

EDTA, added during collection of blood, acts as anticoagulant. In addition, the concentration used as anticoagulant (0.003M) also inhibits converting enzyme (44) and, to some extent, angiotensinases during the incubation procedure (49). DFP (diisopropylfluorophosphate), added to plasma, inhibits remaining angiotensinases (49) and addition of neomycin sulphate helps to retard bacterial growth (6).

Prior to incubation, the plasma is adjusted to the pH

FIGURE 2

PLASMA RENIN ACTIVITY MEASUREMENT: INCUBATION OF PLASMA FOR GENERATION OF ANGIOTENSIN I

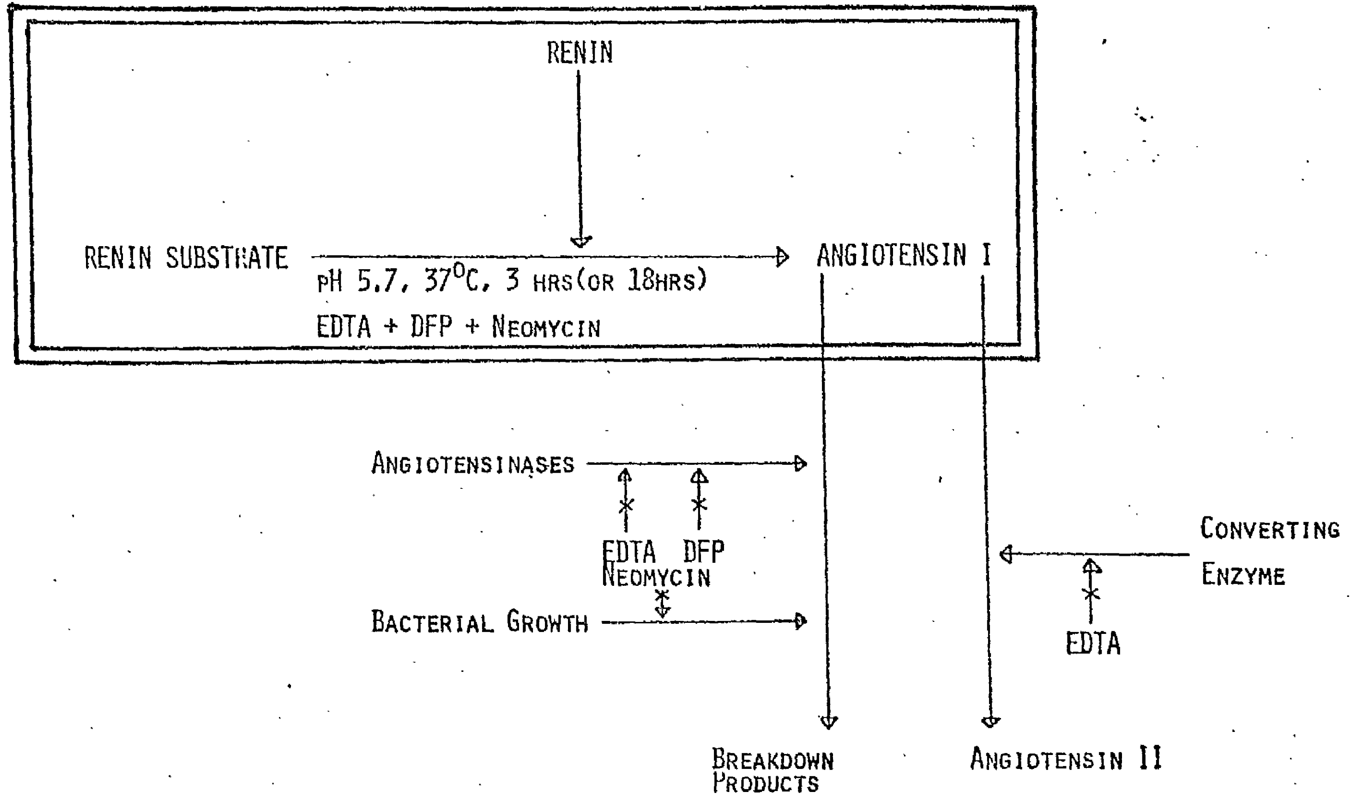


TABLE 1

INCUBATION FOR GENERATION OF ANGIOTENSIN I

(20 or more samples can be incubated as one batch)

Combine	2 ml plasma containing .003M EDTA* 25 ul 10% neomycin sulphate 2 drops (40 ul) DFP (1/20 dilution in isopropyl alcohol)‡
Adjust pH to 5.7** with 1N, 0.5N, or 0.1N HCl	
Divide into 3 portions	
Incubate for 3 or 18*** hours at 37°C	
Freeze	
Radioimmunoassay one of the portions (the other 2 are for repeats, if necessary)	

* Blood collected into potassium EDTA vacutainers

** Acceptable range 5.6 - 5.7

*** Entire incubation step is repeated for 18 hours if PRA is less than 1 ng/ml/hr. Samples are incubated overnight

‡ Toxic! Wear disposable gloves

TABLE 2

INCUBATION FOR RENIN SUBSTRATE

20 ul plasma

400 ul human renin: 0.034 GU/ml in 0.1 M phosphate buffer, pH 5.7, containing 0.003 M EDTA and 200 mg/100 ml neomycin

One drop DFP (1:20 dilution)

Incubate 1 hour at 37°C

Freeze

Assay 10 and 20 ul at 1:10 dilution

INCUBATION FOR SUBSTRATE REACTIVITY

0.9 ml plasma containing 0.003 M EDTA

0.1 ml human renin 0.0085 GU/ml in 0.1 M phosphate buffer, pH 5.7

One drop DFP

Incubate 2 hours at 37°C

Freeze

Assay 10 and 20 ul at 1:20 dilution

optimum, 5.7, with hydrochloric acid. This step increases by two-fold the rate of angiotensin generation as compared with pH 7.4, and it also potentiates the actions of EDTA and DFP to inhibit angiotensinase and converting enzyme activities. Incubation is carried out for three hours at 37°C in a shaker water bath. However, if the rate of angiotensin generation is found to be less than 1 ng/ml/hour, the incubation step is repeated for 18 hours (8).

(b) Plasma renin substrate: (Table 2).

Plasma renin substrate is measured by its capacity to yield angiotensin I in the presence of excess renin. Highly purified human renin is added to plasma containing EDTA, DFP and neomycin, in amounts sufficient to exhaust the substrate during a one hour incubation. The generated angiotensin is then quantitated by radioimmunoassay. Plasma renin substrate is reported as ng angiotensin I generated per ml plasma.

(c) Substrate reactivity: (Table 2)

This index describes the capacity of a given plasma to generate angiotensin in response to addition of a fixed amount of highly purified human renin and can be used to detect inhibitors and activators in plasma, or changes in renin activity due to changes in plasma renin substrate (6,49,62). The added renin is sufficient to generate about 50 ng angiotensin I per ml plasma per hour of incubation assuming normal levels of renin substrate. In the calculation of substrate reactivity endogenous plasma renin activity is subtracted so that only

the angiotensin I generated in response to the added renin is reported.

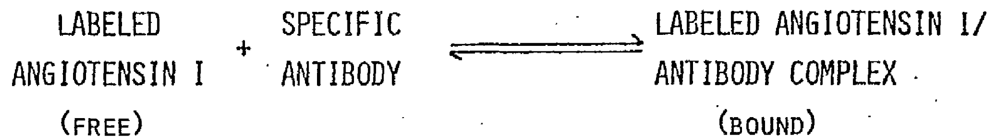
Quantification of Angiotensin I by Radioimmunoassay:

1) Principles of radioimmunoassay (63): The angiotensin I generated during the incubation step is quantitated by utilizing (a) its affinity for highly specific antibodies and (b) competition between radioactive and unlabelled angiotensin I for binding sites on the antibodies (Figure 3) (63). The angiotensin to be quantitated is added to a mixture of radioactive angiotensin and antibody in Tris buffer. The proportions of these two components are such that half of the radioactive angiotensin is bound to antibody and the other half remains free. The extent to which radioactive angiotensin I is displaced from the antibody by the unknown sample is a measure of the amount of angiotensin in the sample. The angiotensin I content of the unknown sample is determined precisely by comparing it to the displacement caused by addition of known amounts of unlabelled angiotensin I.

In practice, a mixture of antibody and labelled angiotensin is added to a series of tubes containing known amounts of angiotensin or unknown samples. After 18 hours the bound angiotensin is separated from the free by adsorption of the free angiotensin onto dextran coated charcoal. After centrifugation the bound angiotensin is decanted and counted in a gamma spectrometer, while the free (charcoal plug) is discarded. From the counts bound the amount of angiotensin in

FIGURE 3

PRINCIPLES OF RADIOIMMUNOASSAY



+

UNLABELED
ANGIOTENSIN I
(IN KNOWN STANDARD SOLUTIONS OR UNKNOWN SAMPLES)

UNLABELED
ANGIOTENSIN I/ANTIBODY
COMPLEX

the unknown sample can be calculated (6,8).

2) Radioimmunoassay of angiotensin I: A flow-sheet of the radioimmunoassay is presented in Table 3. 0.1M Tris buffer, pH 7.5, is used throughout the procedure. Protein is added to the buffer to inhibit adsorption of angiotensin onto glassware and plastic and to increase the stability of the diluted antibody. Considerable adsorption to plastic has been observed when the protein concentration is reduced below 0.4%. Neomycin and phenylmecuric acetate are added to retard bacterial growth.

A unique feature of this particular radioimmunoassay is the prior mixing of radioactive angiotensin with antibody. This simplifies the procedure considerably and reduces inaccuracies caused by multiple pipettings.

Prior to radioimmunoassay the samples are diluted in Tris buffer if the renin value is expected to be above the normal range. However, for most samples no pre-dilution is necessary. 10 tubes in duplicate are included in the standard curve. Different amounts of angiotensin I are added to seven pairs of tubes. Tubes are also included in duplicate for measurement of the total counts, the binding of ^{125}I angiotensin in the absence of antibody to proteins in the buffer solution (non-specific binding) and the binding of radioactive angiotensin to antibody in the absence of unlabelled angiotensin I.

Tubes are prepared in duplicate for about 20 plasma samples and for 2 standard plasma samples. The

TABLE 3

RADIOIMMUNOASSAY

1. Dilution of Samples

If the expected value for PRA is high, the samples should be diluted prior to radioimmunoassay, or if in a previous radioimmunoassay, the values fell outside the standard curve, they should be repeated after appropriate dilution.

The samples can be diluted 1:2, 1:5, 1:25, 1:100, depending on the expected value. All dilutions are carried out on the chilled samples using tris buffer.

2. Radioimmunoassay

(a) Standard Curve:

Tube #	A I Standard ng/ml	Volume μl	A I ng
1,2	1	10	.010
3,4	1	20	.020
5,6	4	10	.040
7,8	4	20	.080
9,10	15	10	.150
11,12	15	20	.300
13,14	20	20	.400
15,16	total counts, no charcoal (T)		
17,18	non-specific binding; no antibody		
19,20	no added angiotensin (B_0)		

(b) Unknown Samples:

10 and 20 ul of each unknown sample and of 2 standard plasma samples are added to small test tubes.

(c) Addition of ^{125}I Angiotensin I/Antibody Mixture:

i. 2 ml mixture are added to each tube from (a) and (b) except #17,18

ii. 2 ml mixture without antibody are added to tubes #17,18

iii. Mix well

iv. Place in refrigerator for 18 hours (overnight)

3. Addition of Charcoal

(a) Place rack of tubes in ice bath
(b) Add 0.5 ml chilled tris buffer to tubes #15,16
(c) Add 0.5 ml chilled charcoal mixture to all other tubes

4. Centrifugation

Centrifuge 20 minutes at 2000 rpm. All tubes should be centrifuged together as one batch.

5. Counting

Decant supernatant into appropriately labeled test tubes.
Count for 10 minutes each with background subtract.

6. Calculations

(a) Calculate standard curve
(b) Plot standard curve (Figure 4)
(c) Calculate standard plasma samples and derive ng from standard curve
(d) If error is less than 20% calculate unknowns
(e) If duplicates of unknowns vary by less than 15%, answer is acceptable

latter are included for quality control purposes. 10 and 20 ul of each sample are assayed. 2 ml of the radioactive angiotensin/antibody mixture is added to all tubes except 17 and 18 (Table 3). 2 ml of mixture without antibody is added to tubes 17 and 18 for measurement of non-specific binding.

After 18 hours at 4°C for equilibration of angiotensin with antibody, the angiotensin bound to antibody is separated from the free by adsorption of free angiotensin onto dextran-coated charcoal. Because of the tendency of the charcoal to slowly adsorb small amounts of bound angiotensin (64) the duplicates of the standard curve are divided so that charcoal is added to one half at the beginning and to the other half at the end of the series. After centrifugation the supernatant from each tube is counted.

Plasma renin activity is calculated as illustrated in Tables 3 and 4. The relationship of bound to free angiotensin I is calculated first, using a modified logit plot (Figure 4)(65). The counts bound in the standard tubes are divided by the mean counts bound in tubes 19 and 20 to which no unlabelled angiotensin was added (B/B_O). $\frac{B/B_O}{1-B/B_O}$ is then calculated and plotted against the amount of angiotensin I for each tube of the standard curve. When log/log paper is used a straight line relationship is achieved.

The amount of angiotensin in unknown samples is derived from the standard curve and then plasma renin activity is is calculated taking into account (a) the volume of plasma

Renin: RIA

TABLE 4

Sample #	Counts bound (B)	B/B_o	$\frac{B/B_o}{1-B/B_o}$ (=Y)	angio ng	chem DF	RIA DF	ng AI /ml/3hr	mean	error %	PRA ng AI /ml/hr
Standard Curve										
1	12789	.96	24.0	.010						
2	12995	.98	48.0	.010						
3	12262	.92	11.5	.020						
4	12238	.92	11.5	.020						
5	11448	.86	6.1	.040						
6	11328	.85	5.7	.040						
7	9579	.72	2.57	.080						
8	9785	.74	2.85	.080						
9	7549	.57	1.33	.150						
10	7660	.57	1.33	.150						
11	5547	.42	0.72	.300						
12	5411	.41	0.69	.300						
13	4718	.35	0.54	.400						
14	4888	.37	0.59	.400						
15	148									
16	163	1% non specific binding								
17	24314									
18	24739	total counts								
19 (B_o)	13162									
20	13462	54% binding								

Standard Plasma Samples

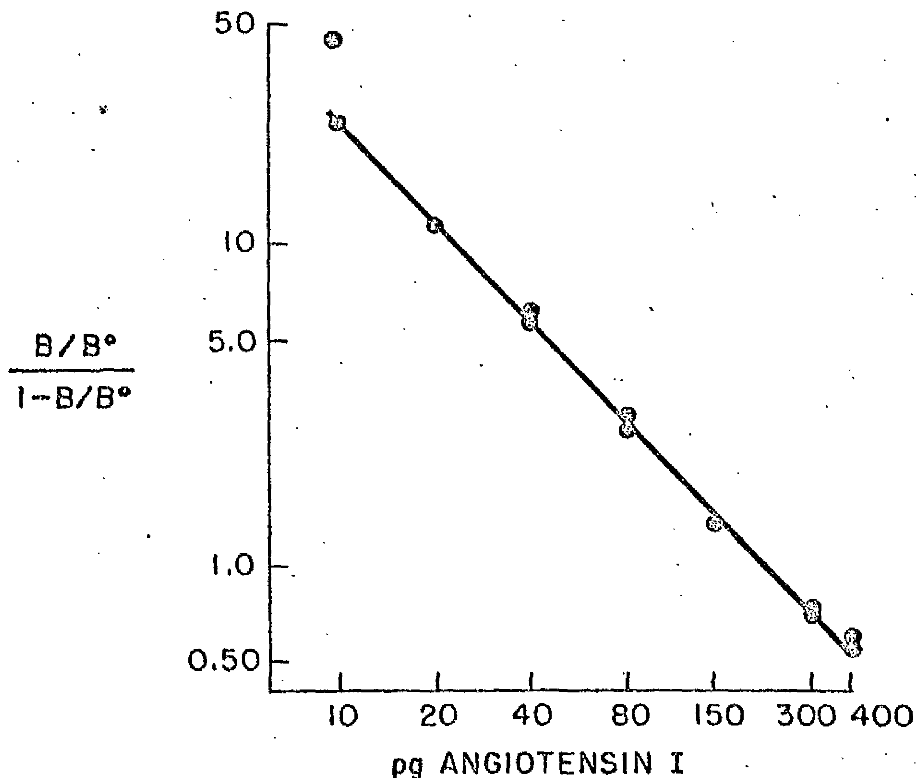
Sample #	Counts bound (B)	B/B_o	$\frac{B/B_o}{1-B/B_o}$ (=Y)	angio ng	chem DF	RIA DF	ng AI /ml/3hr	mean	error %	PRA ng AI /ml/hr
X(30)**	11841	.89	8.1	.029(-3%)						
X(60)	10574	.79	3.76	.060(0%)						
Z(150)	8020	.60	1.50	.139(-7%)						
Z(300)	5710	.43	0.75	.270(-10%)						
Unknown Samples										
379	10153	.76	3.2	.070	1.06	100	7.4	7.3	1%	2.4
379'	8289	.62	1.6	.135		50	7.2			
380	10854	.82	4.6	.050	1.06	100	5.3	5.2	2%	1.7
380'	9315	.70	2.3	.095		50	5.0			
381	12250	.92	11.5	.021	1.08	100	2.3	2.2	5%	0.7*
381'	11510	.86	6.1	.038		50	2.1			
384	8420	.63	1.7	.130	1.06	100	13.8	13.3	4%	4.4
384'	6337	.48	0.92	.240		50	12.7			

*Repeat incubation for 18 hours.

Chem DF = dilution factor due to addition of DFP, neomycin and acid for pH adjustment.
RIA DF = dilution factor for volume of plasma added to radioimmunoassay and if plasma was diluted before radioimmunoassay.

**Numbers in parenthesis = mean pg value for standard plasma samples.

FIGURE 4



Picograms of angiotensin I added to each tube of the standard curve plotted on log/log paper against a function of the change in the counts bound caused by addition of unlabelled angiotensin II.

assayed (10 or 20 ul), (b) dilution of plasma due to addition of DFP, neomycin and acid to adjust the pH, (c) dilution of incubated plasma prior to radioimmunoassay, (if any) and (d) incubation time (3 or 18 hours). Plasma renin activity is expressed as ng angiotensin I generated per ml per hour.

3) Problems encountered in the radioimmunoassay: Radioimmunoassay involves many steps and when variability occurs it can be ascribed to many different problems. Addition of charcoal for separation of bound from free is often a source for error. The charcoal should be stirred vigorously and every effort should be made to add it as quickly and consistently as possible. A useful guide to detection and identification of changes in the radioimmunoassay is the non-specific binding (Table 4, tubes 15 and 16) (66). If the duplicates of these tubes vary, erratic addition of charcoal may be the cause. When changing to a new batch of charcoal the non-specific binding may differ. Different batches of charcoal often have different activity and the optimum amount should be calculated for each batch. If too much is added, adsorption of antibody-bound angiotensin will increase to unacceptable levels.

If the non-specific binding gradually and consistently increases with each set -- it should be 2-3% of the total counts -- this is a sign that the radioactive angiotensin is deteriorating and fresh label should be acquired.

Another guide to adequacy of radioactive angiotensin I is the variability of the standards at the extremes of the

standard curve. As the label ages the sensitivity of the standard curve decreases and the lowest point often fails to fall on the straight line (Figure 4). Also if the antibody binds considerably greater or less than 50% of the iodinated angiotensin the points at the extremes of the standard curve may not fall on the straight line. Only that portion of the standard curve which falls on the straight line should be used in the assay. Samples which fall outside of this range should be repeated.

Because angiotensin II is not stable when stored in dilute solution, the standards used in the radioimmunoassay are prepared fresh daily from concentrated solutions (6). To eliminate this as a potential source of error two standard plasma samples are run with each set. If these do not fall within a predetermined range the set is discarded and repeated.

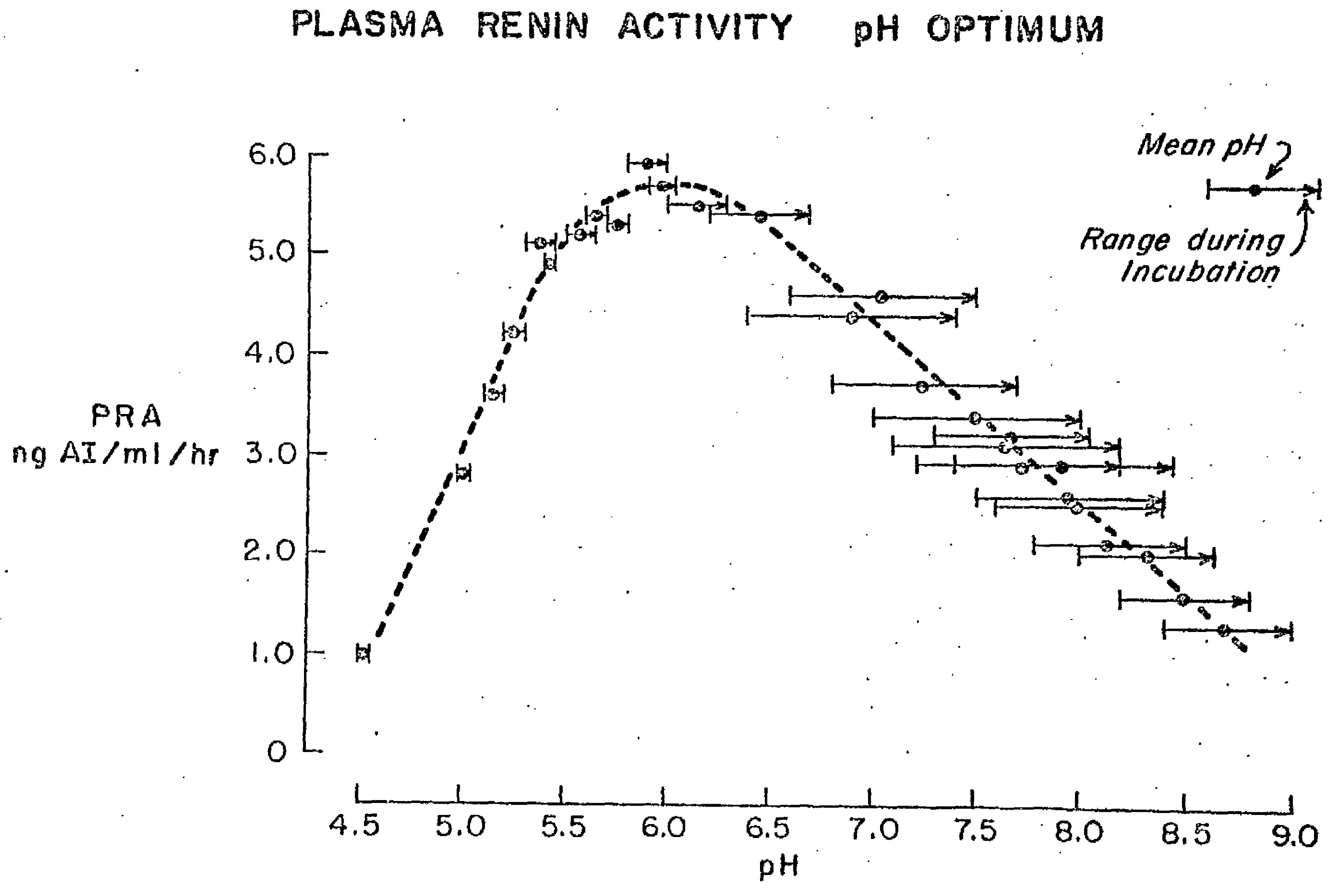
Validation of Method:

A. Incubation:

1. pH Optimum:

The pH optimum for human renin with plasma renin substrate was found to be between pH 5.5 and 6.5 (Figure 5)(7,8) confirming previous reports (49,56,67,68). The rate of angiotensin generation fell steeply below pH 5.5 and declined less steeply above 6.5. At pH 7.4, the rate of angiotensin generation was almost half that found at the pH optimum. In addition, the pH of plasma did not remain stable during

FIGURE 5



pH optimum of human renin with human renin substrate. The same sample was incubated at different pH levels for 3 hours with EDTA, DFP and neomycin. Arrows represent the change in pH during incubation. The pH remains quite stable below pH 6.0 but can increase by almost 1 pH unit during incubation at alkaline pH.

incubation above pH 6.0 and in some samples it drifted high enough to almost stop the reaction (7,68). In this study buffers were not used to adjust the pH on the assumption that the endogenous plasma buffers would hold the pH constant. This had previously been confirmed for pH 5.7. The gradual increase in pH during incubation in samples adjusted to above pH 6.0 may have been due to slow elimination of carbon dioxide from plasma. The lack of change in pH below 6.0 may have been caused by prior elimination of CO_2 during addition of acid to adjust the pH.

The use of the pH optimum for incubation precludes the need for addition of strong buffer to hold the pH constant and leads to a two-fold increase in the rate of angiotensin generation. In addition, if perchance the pH does change slightly during incubation, the rate of generation of angiotensin will not vary since the flat portion of the pH optimum curve is exploited.

2. EDTA, DFP and Neomycin as Protective Agents: The effectiveness of EDTA as both a converting enzyme and an angiotensinase inhibitor is well documented (44,49). The action of EDTA to inhibit converting enzyme may be more effective at pH 5.7 than at alkaline pH (57) because the pH optimum for converting enzyme is around 7.4 (50) and its action at pH 5.7 may be somewhat retarded. DFP does not inhibit converting enzyme (68), but it has been shown to be an effective angiotensinase inhibitor, especially at acid pH (69).

To evaluate its importance, DFP was omitted from the incubation medium in 15 plasma samples (7)(Table 5). After 3 hours incubation with DFP 23.6 ng/ml angiotensin I was found and this fell to 11.4 ng/ml (48%) when DFP was omitted. The activity of low renin samples, incubated for 18 hours, fell from 15.8 to 1.3 (8%) when DFP was omitted.

The potency of DFP as an angiotensinase inhibitor was also evaluated at pH 7.4. During 18 hours incubation, the activity of low renin samples increased from 2.63 to 5.88 ng/ml (224%) when DFP was added to the incubation medium. However, the effect of addition of DFP to the incubation medium was less apparent during a 3 hour incubation period (Table 5). The data suggested that angiotensinase blockade by DFP was incomplete during 18 hours' incubation at pH 7.4, since the renin value was only 37% of that found at pH 5.7, whereas for the 3 hour incubation the renin value was 46% of that found at pH 5.7.

BAL(dimercaprol) and 8-hydroxyquinoline have also been recommended as protective agents during incubation at pH 7.4 (57). In our studies they were found to be less effective than DFP both at pH 5.7 and 7.4 (7) (Table 5). During 3 hours incubation at pH 5.7, 12.9 ng/ml were found compared to 23.6 ng/ml with DFP. During 18 hours incubation at pH 7.4, 3.0 ng/ml were found compared with 5.88 ng/ml with DFP.

Addition of neomycin to the incubation medium has been shown by us to increase net production of angiotensin (6). In another study in which neomycin was omitted and the water

TABLE 5

COMPARISON OF THE EFFECT OF DFP OR BAL AND 8-HYDROXYQUINOLINE ON INCUBATION FOR GENERATION OF ANGIOTENSIN I

	pH 5.7			pH 7.4		
	DFP	BAL etc	None	DFP	BAL etc	None
3 hour incubation: normal and high renin samples (N=10)						
Angiotensin I*(ng/ml)	23.6	12.9	11.4	10.9	10.2	10.3
	(6.8)	(3.6)	(2.9)	(3.0)	(3.0)	(2.7)
%AI found at pH 5.7 with DFP		55%	48%	46%	43%	44%
18 hour incubation: low renin samples (N=5)						
Angiotensin I*(ng/ml)	15.8	2.8	1.3	5.9	3.0	2.6
	(2.1)	(0.9)	(0.6)	(1.4)	(0.5)	(0.7)
%AI found at pH 5.7 with DFP		18%	8%	37%	19%	17%

* Angiotensin I generated during incubation. The 3 hour incubation was carried out using different samples from those used in the 18 hour incubation. Figures in parenthesis represent the standard error of the mean.

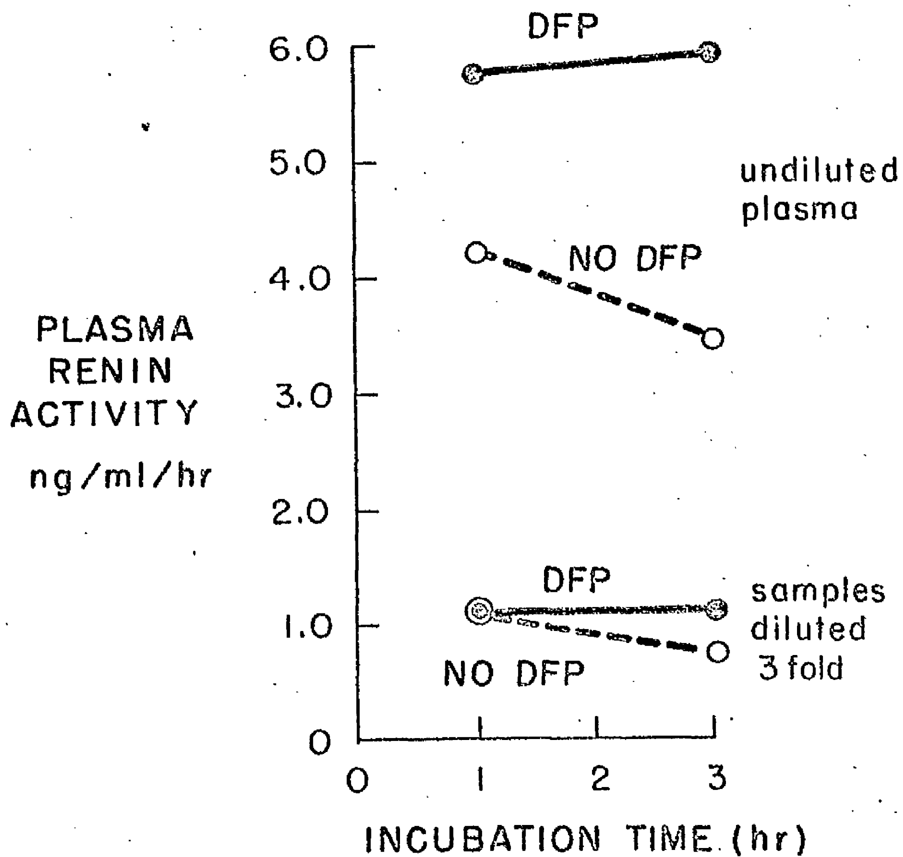
bath was not shaken during incubation, the amount of angiotensin I found was reduced by 42% (7).

Several publications have presented evidence that BAL and 8-hydroxyquinoline together with EDTA are effective in completely inhibiting converting enzyme and angiotensinase activities (57,68,70,71). The discrepancy between those observations and our own are based on three differences in design of the studies: In our studies (1) the incubation time is prolonged (2) dilution of plasma is kept to a minimum and (3) pH 5.7 is used for incubation. Since EDTA is effective in completely inhibiting converting enzyme activity, the discrepancies appear to be related to differences in effectiveness of inhibition of angiotensinases.

Incubation time: Plasma angiotensinases are slow acting (68). In the studies in which neither DFP or BAL and 8-hydroxyquinoline were added, destruction of angiotensin I during 18 hours incubation was fractionally greater than during 3 hours incubation (7). This may be due to the slow rate of destruction of formed angiotensin and because during prolonged incubations larger amounts of angiotensin are generated. Thus, studies which utilize a short incubation time are less likely to reveal the limited effectiveness of protective agents.

Dilution of plasma prior to incubation reduces the concentration of angiotensinases in the medium and thus reduces their activity. The amount of angiotensin found after one hour incubation of 3-fold diluted plasma was not increased by addition of DFP and was only slightly increased during 3 hours incuba-

FIGURE 6



When plasma was incubated after three-fold dilution with maleate buffer, pH 5.7, addition of DFP to the incubation medium did not enhance the amount of angiotensin measured after one hour of incubation. However, a more prolonged period of incubation, or incubation of undiluted plasma, required the presence of DFP for complete angiotensinase inhibition.

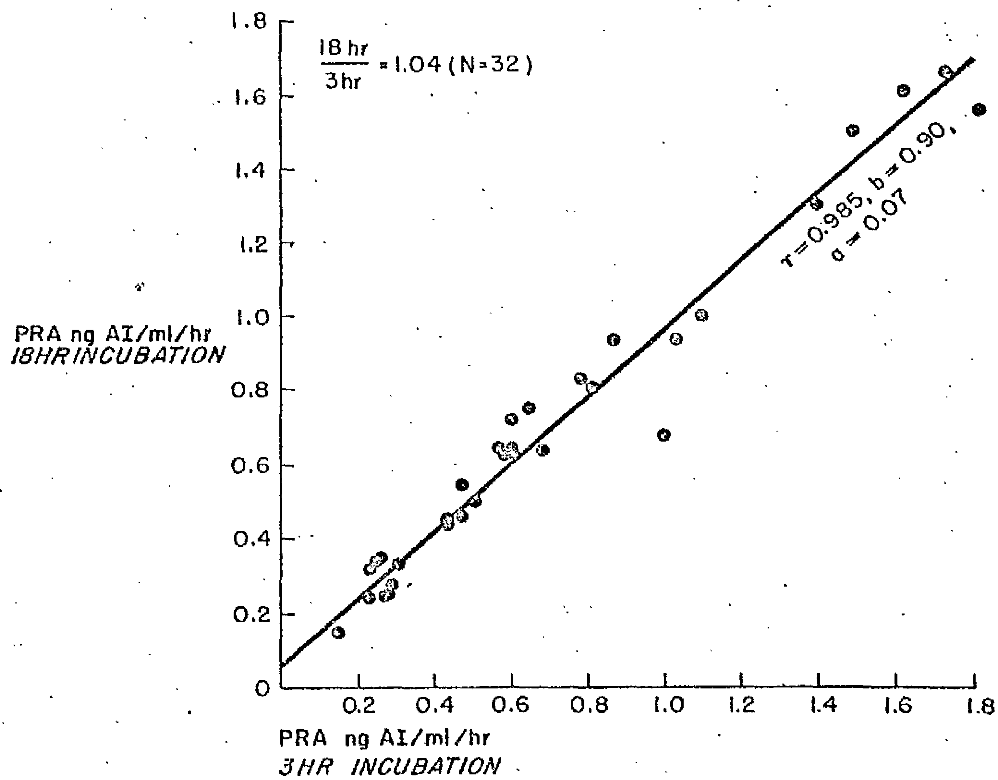
tion (Figure 6) (7), suggesting that angiotensinase activity was minimal. However, addition of DFP markedly increased the amount of angiotensin found after incubation of undiluted plasma. Therefore, a more prolonged period of incubation, or incubation of undiluted plasma requires the presence of DFP for complete angiotensinase inhibition.

pH of incubation: The angiotensinase inhibited by DFP has greatest activity at acid pH (49,69) so that incubations in alkaline media would be expected to exhibit less angiotensinase activity.

Therefore, by incubating diluted plasma at alkaline pH for a short incubation time, other protective agents will appear to be as effective as the ones we have chosen. Nonetheless, maximum generation of angiotensin is very important in detecting accurately low renin samples. Since complete inhibition of angiotensinase and converting enzyme activity for 18 hours can only be accomplished when the incubation is carried out at pH 5.7 in the presence of EDTA, DFP and neomycin, we have chosen to use this combination routinely. In addition, stability of pH is ascertained without addition of strong buffers and without dilution of plasma (7).

3. Linearity in the Rate of Angiotensin Generation: The effectiveness of EDTA, DFP and neomycin as protective agents is confirmed in studies in which samples were incubated for both 3 and 18 hours (8). Samples with low plasma renin activity were used in this study to minimize an effect of substrate utilization on the rate of angiotensin genera-

FIGURE 7



No difference was found in the hourly rate of angiotensin generation when samples were incubated for three or eighteen hours.

tion. 26 different plasmas were incubated for both 3 and 18 hours and the hourly rates of angiotensin generation were compared (Figure 7). There was a direct relationship between the two measurements over the entire range of activity and the best fit was a line of slope 0.9 and intercept 0.07, demonstrating an equal rate of angiotensin generation for the two time periods. The ratio of the 18 hour rate of angiotensin generation to the 3 hour rate was 1.04. The correlation coefficient of 0.985 was very high, which emphasizes the accuracy of the two measurements, even at low levels of plasma renin activity.

B. Radioimmunoassay:

Adequacy of the renin assay is determined in part by the sensitivity and specificity of the antibody used in the radioimmunoassay step. Unlike most other radioimmunoassay procedures, such as the angiotensin II assay, sensitivity of the radioimmunoassay system need not be a problem because the amount of angiotensin generated can be increased by prolonging the incubation time until there is sufficient to be measured accurately. Few methods take advantage of this great potential. Thus, even though our radioimmunoassay step has a sensitivity similar to other methods, we can detect much lower levels by prolonging the time of incubation to 18 hours and optimizing the conditions of the incubation step (7).

(1) Blank subtraction: The ability to prolong the incubation time also eliminates a problem inherent to radio-

immunoassay. Most antibodies to angiotensin I cross-react with non-specific substances in plasma (72). Since renin activity is expressed as the rate of angiotensin generation, if angiotensin or other immuno-reactive substances are present in unincubated plasma the value will be falsely high unless the blank is subtracted. By prolonging the incubation time it is possible to generate so much angiotensin that the amount present in unincubated plasma can be ignored (8,66). Thus, in the method just described, no blank subtraction is included so that the number of samples for assay is reduced by half.

(2) Volume of plasma added to radioimmunoassay: Many methods recommend addition of 50 ul or more of plasma to 1 ml of radioimmunoassay mixture (57,68,70). In our method a maximum volume of 20 ul is added to 2 ml of radioactive angiotensin/antibody mixture. This small volume of plasma was chosen because when it was increased to 50 ul the values for plasma renin activity fell significantly. Thus mean values for plasma renin activity for 10 samples fell from 0.68 ng/ml/hr. (10 ul sample) to 0.50 ng/ml/hr. (50 ul sample) ($p < .01$).

In our original radioimmunoassay method (6), instead of reincubating plasmas with low renin activity for 18 hours, 50 and 100 ul of the 3 hour incubated samples were assayed in a special radioimmunoassay in which charcoal extracted pooled plasma was added to each tube so that the volume of plasma in each tube was 0.1 ml (5% plasma)(6,7).

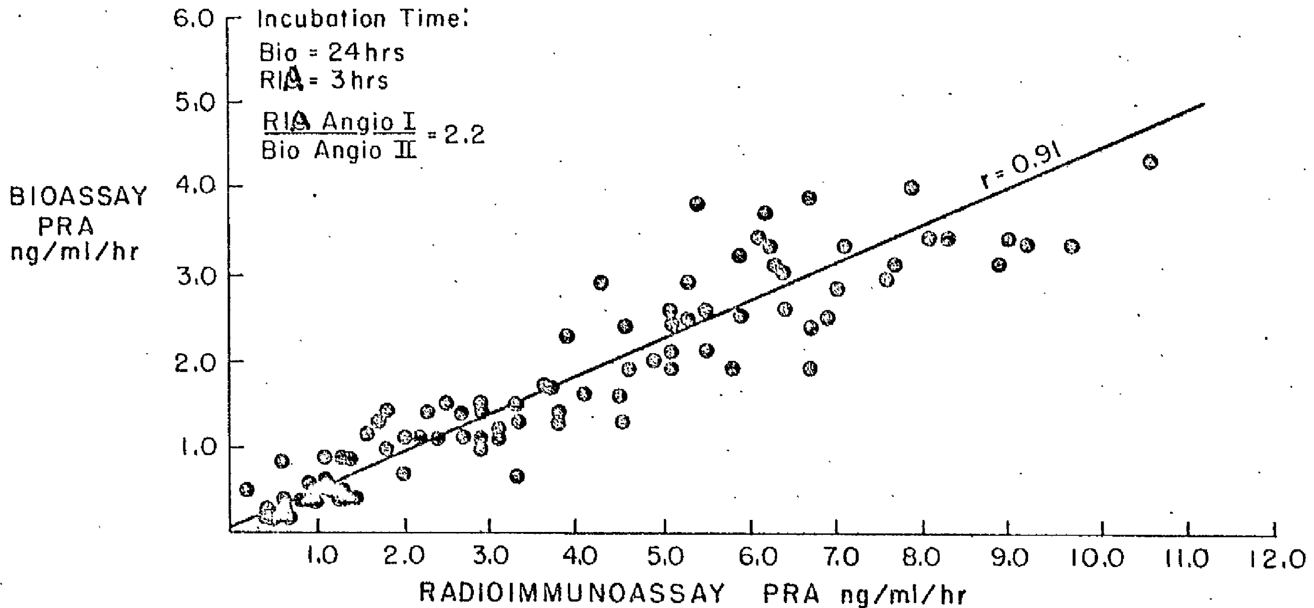
This addition of plasma to each tube, including the standard curve, eliminated the necessity of subtracting a blank from each result, and interference caused by addition of a large volume of plasma only to the unknown samples was eliminated. However, this approach was replaced by the longer incubation when we demonstrated that the 18 hour incubation afforded greater reproducibility (7).

C. Relationship of Bioassay to Radioimmunoassay:

When bioassay of angiotensin was replaced by radioimmunoassay, it was found that samples measured by radioimmunoassay were consistently 2.2 times higher than those measured by rat pressor bioassay (Figure 8)(6). Studies were carried out to identify the source of the discrepancy.

Two changes were introduced when the assay was switched. For radioimmunoassay the incubation time for generation of angiotensin I was reduced for most samples from 18 to 3 hours. Secondly, the reference standard was changed from angiotensin II (bioassay) to angiotensin I. Angiotensin II had been used as reference standard in the bioassay because it had been assumed that angiotensin II was the final product in the incubation. However, in the presence of EDTA, conversion of angiotensin I to angiotensin II is inhibited (44). Angiotensin I can be measured by rat pressor bioassay only because it is converted to angiotensin II by the rat's own converting enzyme. When the potency of angiotensin I was compared with angiotensin II in the

FIGURE 8



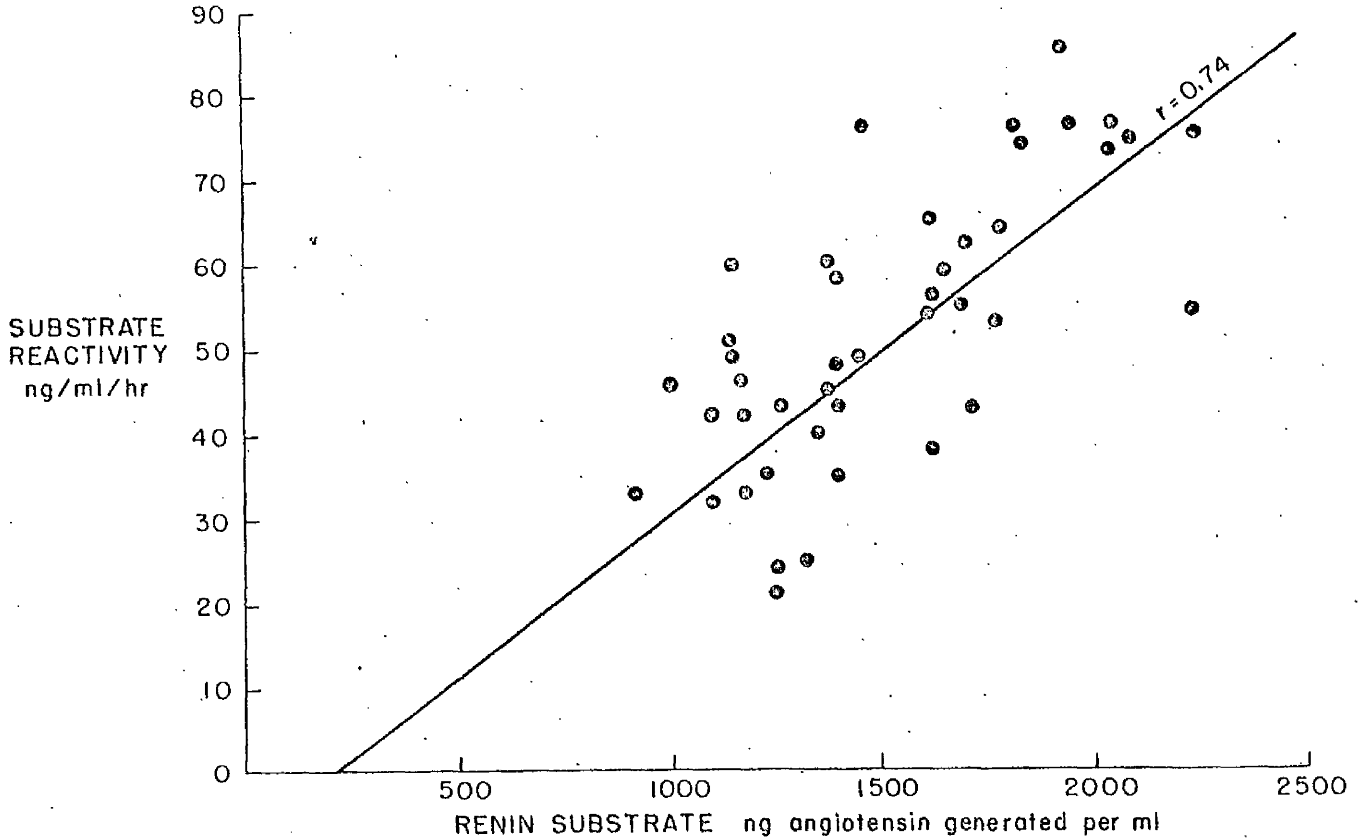
Measurement of plasma renin activity by radio-immunoassay resulted in values 2.2 times higher than those measured by bioassay. The correlation of the two values was high (r = 0.91) over a wide range of plasma renin measurements. The difference in values could be ascribed to the inappropriate use of angiotensin II instead of angiotensin I as reference standard in the bioassay and substrate utilization during the more prolonged incubation time used for the bioassay.

rat assay it was found that, on a weight basis, it had only 50% of the activity. This observation was supported by a study in which the bioassay was compared with the radioimmunoassay using angiotensin I as reference standard in both assays. In this comparison study the values differed by only 20%. Thus the incorrect use of angiotensin II as reference standard in the bioassay had resulted in falsely low estimates of plasma renin activity. This also led to falsely low estimates of plasma renin substrate (6,73).

The 20% difference in the results by radioimmunoassay and bioassay in the preceding experiment may be explained by substrate utilization during incubation. In that experiment samples assayed by radioimmunoassay were incubated for 3 hours whereas those assayed by bioassay had been incubated for 18 hours. As illustrated in Figure 9, the rate of angiotensin generation is dependent on substrate concentration at levels which span the physiological range (6). A gradual reduction in substrate concentration during the 18 hour incubation utilized for bioassay may have gradually but significantly lowered the rate of generation during the incubation, especially in samples with high plasma renin activity. This possibility was supported by the observation that the ratio of assays of samples incubated for the same time period (18 hours) but assayed against different reference standards, was 1.9 in contrast to the ratio of 2.2 which was found when different incubation times and reference standards were used.

Using a correction factor of 2.2 for measurements of

FIGURE 9



Direct relationship between plasma renin substrate concentration and the amount of angiotensin generated in response to addition of a fixed amount of human renin (substrate reactivity) in 19 normal subjects.

plasma renin activity and one of 2.0 for the measurement of renin substrate concentration, data derived from bioassay and radioimmunoassay become completely interchangeable. The reasons for the discrepancies in the data have been accounted for by differences in the reference standard and substrate utilization during the incubation for plasma renin activity.

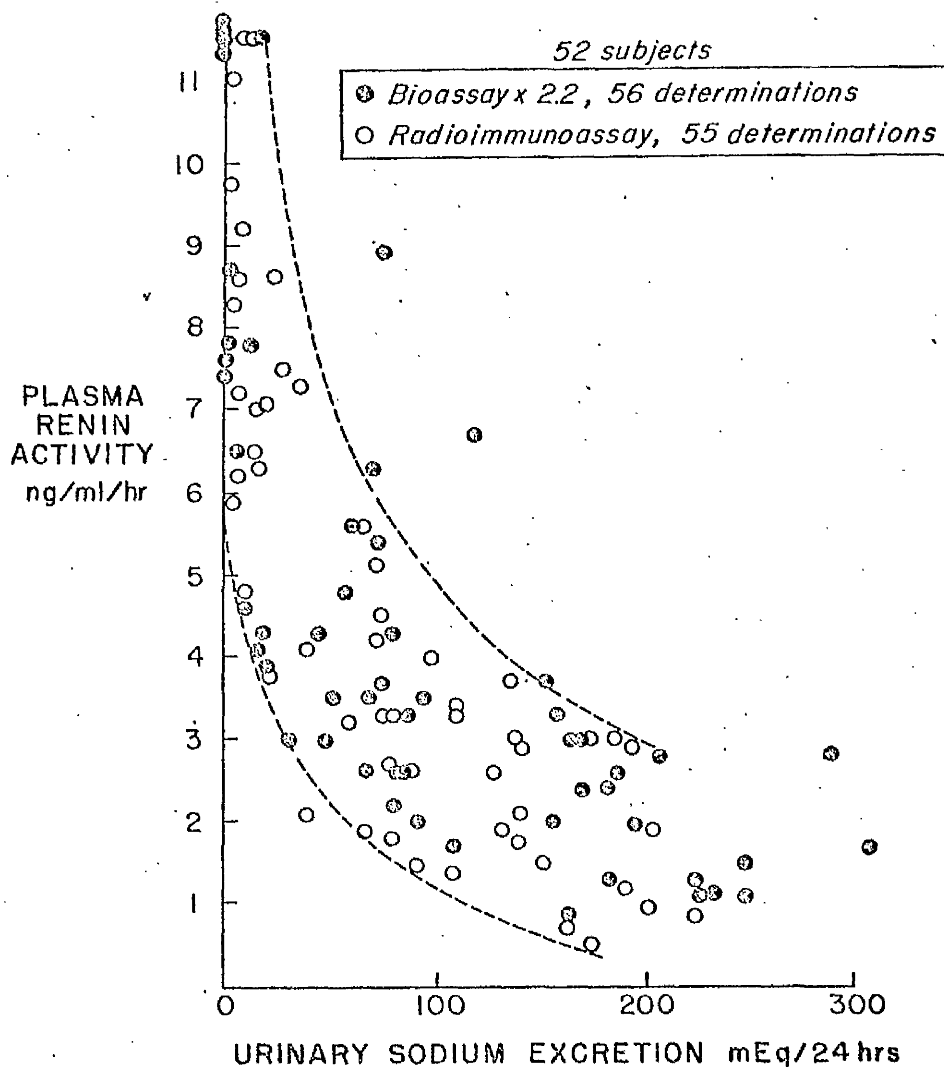
When plasma renin activity is related to the concurrent 24-hour urine sodium excretion in normal man a hyperbolic relationship is found (Figure 10). Using a corrective factor of 2.2, data from bioassay and radioimmunoassay are superimposable over the entire range of plasma renin activity.

D. Effect of Changes in Plasma Renin Substrate on Plasma Renin Activity Measurements:

In a study of eleven hypertensive patients on oral contraceptive medication, renin substrate (measured by bioassay) was found to range from 1980 to 8650 ng/ml, as compared to approximately 1000 ng/ml found in normal subjects (15,62). In two normal subjects and one male hypertensive patient entirely similar effects were produced by treatment with the estrogen-progestogen combination. The increase in substrate in these patients was not surprising since renin substrate had previously been shown to be elevated during pregnancy (49,59,74) and in animals treated with estrogen (75).

To investigate whether the observed increases in substrate concentration could increase the rate of angiotensin

FIGURE 10



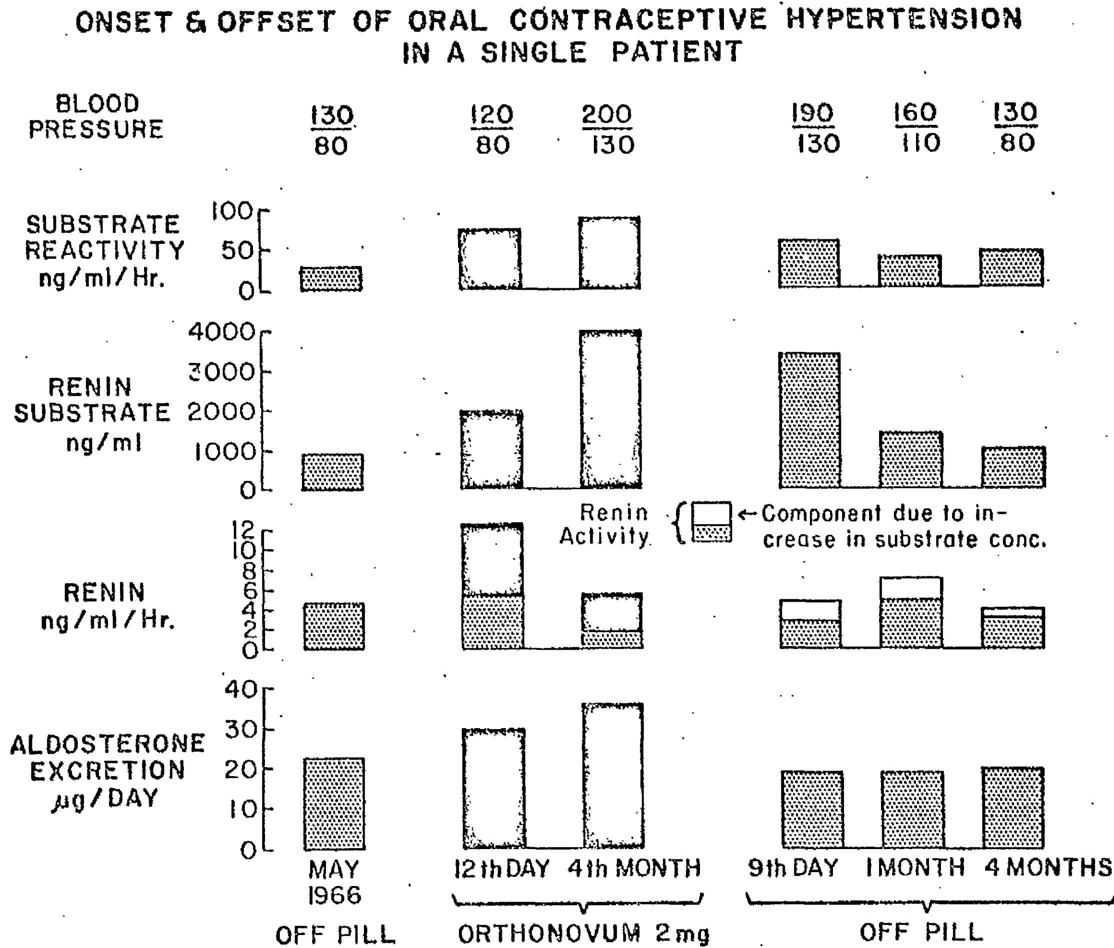
Hyperbolic relationship of plasma renin activity to the 24 hour urine sodium excretion in 52 normal subjects. The data derived from bioassay and radioimmuno-assay are interchangeable when a correction factor of 2.2 is applied. Urine sodium excretion is taken as an indicator of the state of sodium balance.

generation, the capacity of plasma to form angiotensin was measured in an in vitro system after addition of a fixed amount of renin to the individual plasma. Significant increases in plasma reactivity were consistently observed (Figures 11,12). These increases were directly related to the corresponding induced rise in renin substrate concentration. Reactivity increased as the substrate concentration rose to the region of 2000 ng/ml. Above this concentration, further increases in substrate induced lesser increments in reactivity. These results suggest that changes in concentration of substrate account for the increased "reactivity" to renin and that changes in substrate concentration can markedly affect measurements of plasma renin activity.

However, it is interesting to note that in the study illustrated in Figure 11, increased substrate concentration only transiently led to increased plasma renin activity. Renin secretion apparently slowly fed-back so that eventually plasma renin activity returned to normal levels. In addition, in normal subjects the range of renin substrate is quite wide (Figure 9) and yet no correlation was found between plasma renin activity and plasma renin substrate concentrations (6). Thus, physiologically, changes in substrate concentration are not normally determinants of plasma renin activity under steady state conditions since renin secretion appears to feed back to maintain renin activity constant.

Previous studies had suggested that under normal conditions substrate concentration is not rate-limiting so that the

FIGURE 11



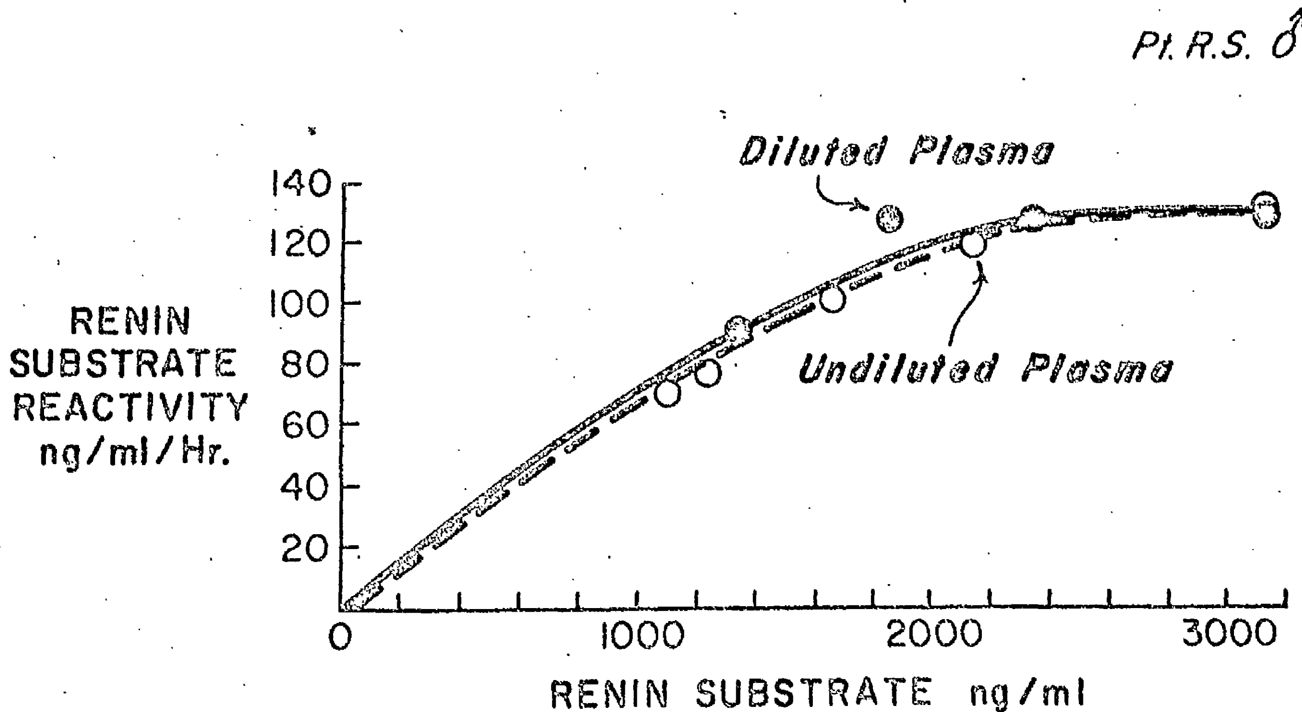
Administration of oral contraceptive therapy was associated with increases in renin substrate and substrate reactivity to renin. The data illustrate that the observed transient increase in plasma renin activity on the 12th day was largely due to an increase in renin substrate concentration. Renin secretion appeared to feed-back in response to the increase in substrate concentration.

reaction velocity for a given amount of renin is near maximum (49,76). Because of these reports, the possibility was raised that increases in reactivity to renin might have resulted from changes in the plasma concentration of an activator or inhibitor. To test this possibility, a plasma sample with substrate concentration of 3,000 ng/ml was diluted and the reactivity of various dilutions of this sample was compared with that of other samples drawn from the same patient as the substrate concentration was rising in response to oral contraceptive therapy (Figure 12). The two curves of reactivity as related to substrate concentration were superimposable. This result provides no positive evidence for any activator or inhibitor and instead suggests that the increased reactivity to renin merely results from an increased substrate concentration.

Effect of dilution of plasma renin substrate on plasma renin measurements: Since the concentration of renin substrate in normal plasma is rate limiting, dilution of plasma prior to incubation for generation of angiotensin I is likely to reduce the rate of angiotensin generation and produce falsely low measurements of plasma renin activity. A study was carried out to investigate the magnitude of the effect (7).

When plasma was diluted by 1.5, 2 or 3-fold the rate of angiotensin generation was considerably reduced. Two-fold dilution reduced the amount of angiotensin generated to only

FIGURE 12



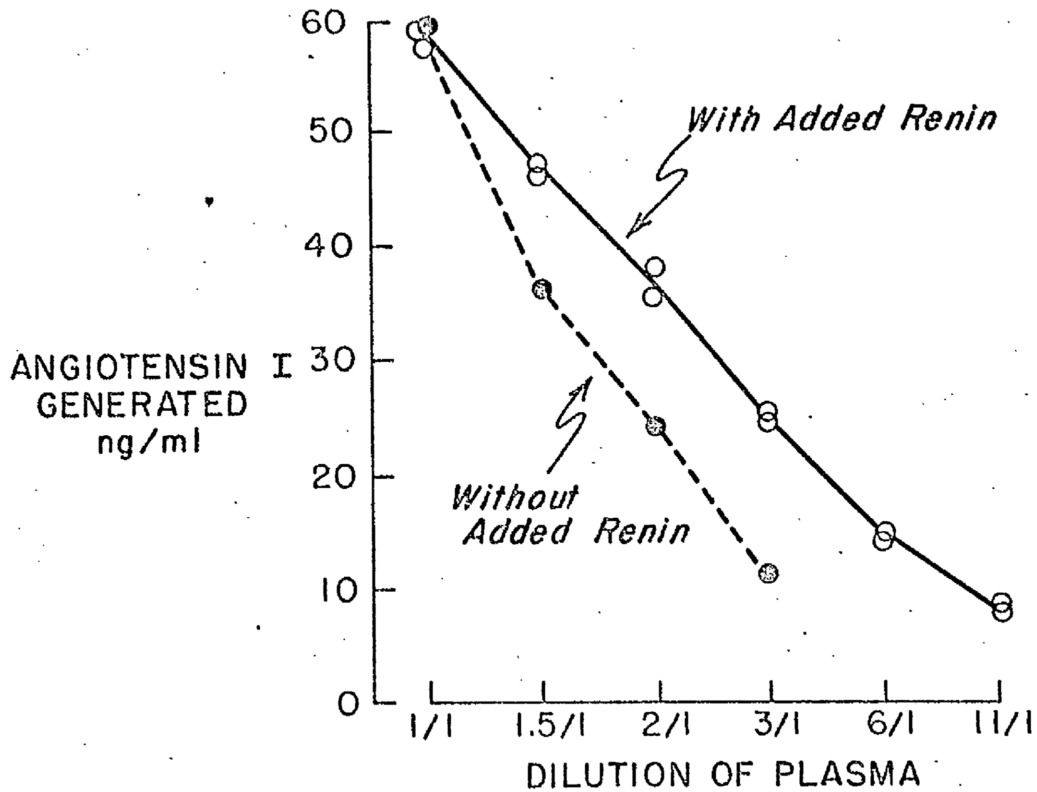
Effect of dilution of renin substrate on the rate of generation of angiotensin compared with undiluted samples of various substrate concentrations. These latter samples were obtained at different times in the course of oral contraceptive therapy. It is apparent that increases in substrate above normal (i.e., 1000 ng/ml; bioassay) can markedly increase the capacity for angiotensin generation. Changes in the capacity of plasma to release angiotensin appear related to changes in substrate concentration rather than the result of variation in concentration of an activator or inhibitor.

40% of the amount formed in undiluted plasma and this fell to 19% in 3-fold diluted plasma (Figure 13).

In a second study (Figure 13), a fixed amount of renin was added to the diluted and undiluted plasma so that only dilution of substrate, and not differences in renin concentration could affect the rate of angiotensin generation. Two-fold dilution of renin substrate reduced the rate of generation of angiotensin to 62% and only 14% of the activity was found in 11-fold diluted plasma. Thus dilution of plasma renin substrate per se can markedly affect the rate of angiotensin generation and plasma renin activity measurements.

Since different plasmas may have quite different substrate concentrations (the normal range is from 900 to 2200 ng/ml), 50% dilution of substrate may lead to considerable reduction in the rate of angiotensin generation when substrate concentration is low. However, if the substrate concentration is quite high, dilution of substrate may cause only slight reduction in the rate of angiotensin generation. There is no simple formula to correct for the reduction in angiotensin production due to substrate dilution unless the rate of angiotensin generation in response to addition of a fixed amount of renin is also measured. For this reason, dilution of plasma prior to incubation for generation of angiotensin is avoided in the methods for measurement of plasma renin activity and substrate reactivity.

FIGURE 13



When plasma was diluted up to three-fold, the rate of angiotensin generation was reduced to less than 20%. Addition of a fixed amount of renin to each dilution of plasma revealed that reduction in plasma renin substrate concentration considerably reduced the angiotensin production rate.

E. Effect of Competitive Inhibitor (Heparin) on Plasma Renin Activity.

Plasma renin substrate concentration has been shown to be rate limiting in normal plasma so that changes in its concentration can affect the rate of angiotensin generation. Many studies have been carried out to determine whether or not activators or inhibitors are present in plasma which also modify the rate of the reaction (49,50,77-79). Although several studies claimed to have demonstrated activators or inhibitors (77-79) the physiological relevance of such substances has yet to be established.

In an early method for measurement of plasma renin activity in our laboratory, heparin was used as anticoagulant and the plasma was dialysed for 24 hours against 0.003M EDTA (49, 53). After another dialysis for 24 hours against distilled water, incubation was carried out for generation of angiotensin at pH 5.7 in the presence of DFP. We found that, under these conditions, the heparin used as anticoagulant acted as a competitive inhibitor of the reaction (80).

To characterize the effect of heparin, blood was collected from nine normal subjects using either heparin (three different concentrations) or EDTA as anticoagulant or the blood was allowed to clot and the serum was assayed. As illustrated in Table 6, the presence of 5.2×10^{-6}M (10 U/ml) heparin reduced angiotensin formation by a mean of 41% and 2.6×10^{-5}M (50 U/ml) produced an 81% reduction in the mean

TABLE 6

ANGIOTENSIN GENERATION IN VITRO IN SERUM AND IN EDTA AND HEPARINIZED PLASMA

	SERUM	EDTA 3.5×10^{-3} M	HEPARIN 5.2×10^{-6} M (10 u/ml)	1.3×10^{-5} M (25 u/ml)	2.6×10^{-5} M (50 u/ml)
Angiotensin ng/ml 4 h Mean Values	7.5	6.3	4.4	4.0	1.4
Standard Deviation ±	(2.2)	(1.4)	(1.4)	(1.3)	(0.6)
p Value*		$>.05$	$<.05$	$<.02$	$<.001$

Blood samples taken at a single venepuncture in each of 9 normal subjects.

* p Value represents the statistical significance of the difference between the serum as compared with anticoagulated plasma.

yield of angiotensin as compared with serum or samples collected in EDTA.

The effects of heparin were consistently observed whether it was added to the samples before or after dialysis. No differences were detected in the inhibitory action of similar concentrations of heparin added to serum, plasma or whole blood, indicating that factors released or removed by the clotting process were not involved in the inhibition.

Certain of the data suggested that the degree of inhibition of renin by heparin might be related to renin substrate concentration. This relationship was documented by a study in which heparin was added to normal serum which had been diluted to obtain various substrate concentrations (Table 7). Substrate concentration ranged from 0.38 to 3.04 µM (380-3040 ng/ml) in the presence of a constant heparin concentration. At the lowest substrate concentration there was a 93% reduction in angiotensin formation. As the substrate concentration was raised, the effect of heparin was reduced so that, at a concentration of 3.04 uM, the angiotensin yield was reduced by only 43%. However, when the concentration of either substrate of inhibitor was altered by a factor of eight, while maintaining a constant ratio of one to the other, the degree of inhibition of the rate of angiotensin formation did not vary.

Effect of heparin on enzyme kinetics. The effect of varying the renin substrate concentration on the rate of angiotensin formation in the presence or absence of heparin is illus-

TABLE 7

RELATION OF HEPARIN INHIBITION TO RENIN SUBSTRATE CONCENTRATION

NORMAL SERUM

Substrate (S) µM	Heparin (I)	$\frac{S}{I}$	Reduction in Angiotensin Formation
0.38	26	0.015	93%
0.76	26	0.029	83%
1.52	26	0.058	63%
2.20	26	0.085	55%
3.04	26	0.117	43%
0.38	13	0.029	89%
0.76	26	0.029	83%
1.52	52	0.029	86%
3.04	104	0.029	78%

trated in Figure 14. Data from unheparinized samples indicate that this enzyme system can be described in terms of the Michaelis-Menten equation since a linear relationship was observed between the reciprocals of the substrate concentration and the corresponding reaction velocity.

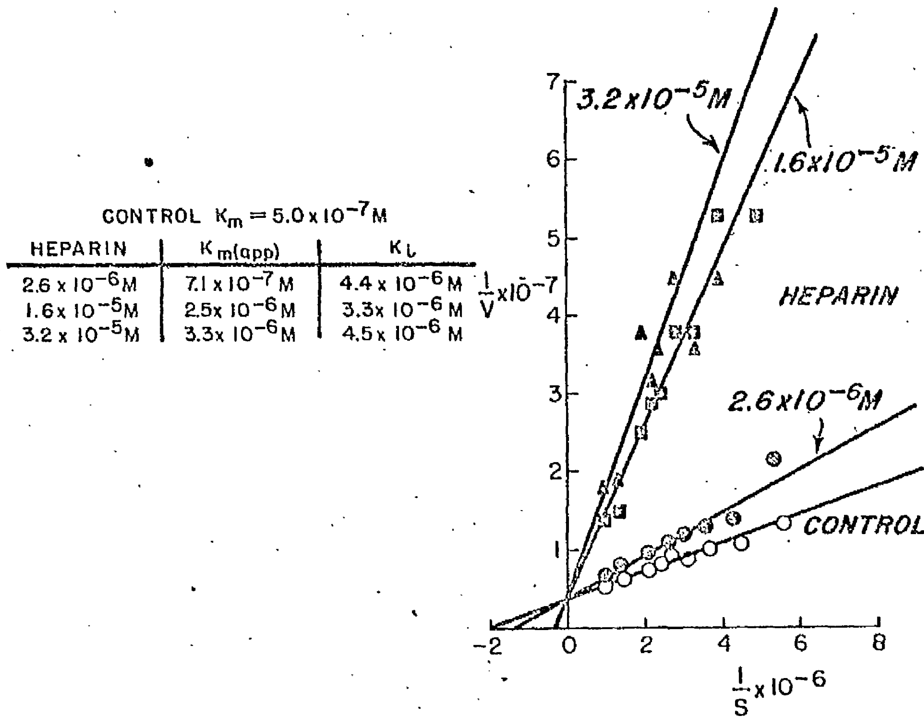
Increasing the concentration of heparin progressively increased the value of the Michaelis constant (K_m) without altering the maximum velocity (V_{max}). The apparent K_m produced in the presence of heparin was measured at three different heparin concentrations (Figure 14). This allowed calculation of K_i, the binding constant of the enzyme for heparin. The mean value was 4.1×10^{-6}M with a standard deviation of 0.55×10^{-6}M. The Michaelis constant for the natural substrate was shown to be 5.0×10^{-7}M, a value less than one order of magnitude different from the K_i for heparin. The behavior of heparin in these systems is thus that of a reversible competitive inhibitor, whose effects are consistently related to the concentration of available substrate.

Most of our experiments were carried out at pH 5.7, since this was previously shown to be near the pH optimum for the *in vitro* reaction. Inhibition by heparin was also demonstrated at pH 7.4. The per cent inhibition of angiotensin formation was independent of the concentration of renin.

It has been reported that the velocity of angiotensin formation is reduced during incubation of plasma when saline

FIGURE 14

HEPARIN AS A REVERSIBLE COMPETITIVE INHIBITION OF RENIN
Water Incubation
pH 5.7



CONTROL $K_m = 5.0 \times 10^{-7}$ M

HEPARIN	$K_{m(app)}$	K_i
2.6×10^{-6} M	7.1×10^{-7} M	4.4×10^{-6} M
1.6×10^{-5} M	2.5×10^{-6} M	3.3×10^{-6} M
3.2×10^{-5} M	3.3×10^{-6} M	4.5×10^{-6} M

Lineweaver-Burk plot of the relationship between the reciprocals of substrate concentration and reaction velocity. Three different concentrations of heparin significantly and proportionately increased the Michaelis constant. The binding constant, K_i, for heparin is within an order of magnitude of the K_m for the natural substrate.

is removed from the incubation medium (81). However, the differences observed may have been due in part to the use of heparin as anticoagulant because in the present study the inhibiting action of heparin was consistently greater in the absence of saline. Sodium chloride therefore protects renin from the effects of heparin.

In Summary. Heparin inhibits the *in vitro* production of angiotensin by renin. The inhibition of renin is much greater in water than in saline incubations, and is dependent on the concentration of the renin substrate. Heparin inhibition thus occurs as a consequence of reversible competition with the renin substrate for the active site of the enzyme. The data indicate that, in salt-free incubations, this inhibition may be quite specific because the binding constant for renin with heparin is nearly of the same order of magnitude as that observed for renin with its natural substrate.

Since plasma from patients may contain various drugs or other agents, this study points to the importance of evaluating the effect of them on the renin system before concluding that they act to affect renin secretion.

F. Searching Out Low Renin Patients: Evaluation of Commercial Kits.

Recent studies have revealed an important subgroup of patients with essential hypertension who have subnormal plasma renin activity (9,13,14,82,83). It is important to accurately identify these patients since specific antihypertensive therapy

can be applied to them (84-87) and, in addition, these patients appear to be protected from heart attack and stroke relative to other patients with essential hypertension(88).

For these patients the renin value is the only consistent feature which distinguishes them from patients with other forms of essential hypertension. Methods of the greatest sensitivity are therefore required so that truly low values can be discriminated from those falling in the normal range.

In order to evaluate the accuracy and sensitivity of other methods commonly used for identifying patients with subnormal plasma renin activity, three commercial kits were purchased and the same 15 samples were assayed four ways (7). All kits used EDTA with BAL and 8-hydroxyquinoline as protective agents and were based on the method of Haber et al (57). The Squibb and Schwarz/Mann incubation procedures were identical, incubating plasma with unadjusted pH for 3 hours; New England Nuclear recommended pH 6.0 and 1 hour incubation. The pH in this latter kit was adjusted by a three-fold dilution with buffer. No attempt was made to analyze the stability or the reproducibility of various batches of the reagents supplied with the kits.

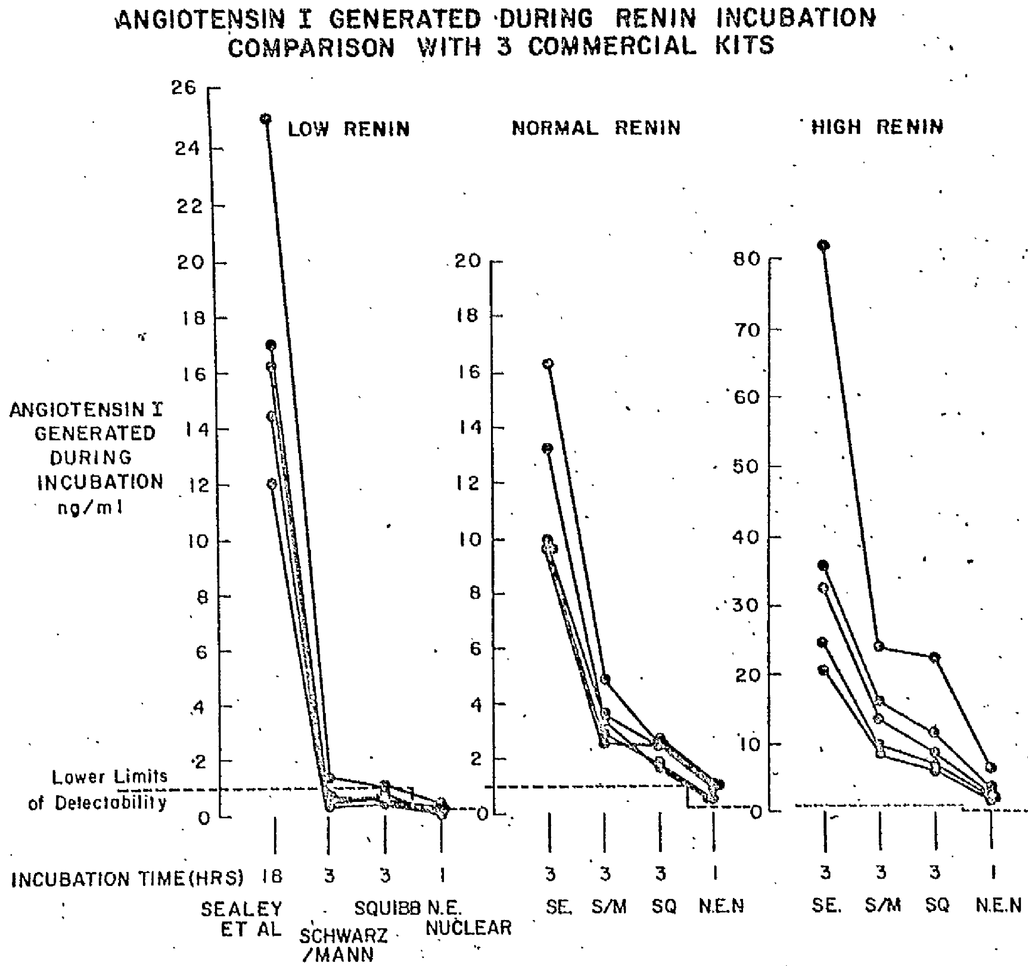
Values for plasma renin activity utilizing any of the three kits ranged from 17 to 70% of those found using our method (Table 8). Since pH 6.0 was used, data using the New England Nuclear kit were closest to our own. However the values using this kit remained lower than ours because of dilution of substrate during the incubation for which no consis-

TABLE 8

PLASMA RENIN ACTIVITY IN FIFTEEN SAMPLES USING THREE COMMERCIAL KITS

Sample #	Sealey	New England Nuclear	Schwarz/Mann	Squibb
LOW RENIN SAMPLES				
1	1.4	1.1	0.45	0.35
2	0.80	0.60	0.23	0.18
3	0.67	0.27	0.10	0.14
4	0.94	0.60	0.19	0.24
5	0.90	0.54	0.16	0.19
mean	0.94	0.62	0.23	0.22
NORMAL RENIN SAMPLES				
6	3.2	1.8	0.83	0.55
7	5.4	3.1	1.6	0.87
8	3.2	1.6	0.96	0.61
9	3.3	1.5	1.1	0.53
10	4.4	2.6	1.2	0.81
mean	3.9	2.1	1.1	0.67
HIGH RENIN SAMPLES				
11	10.8	7.4	4.5	2.8
12	11.9	9.5	5.4	3.8
13	27.3	18.1	8.1	7.5
14	6.9	4.7	2.7	2.0
15	8.2	6.1	3.2	2.3
mean	13.0	9.2	4.8	3.7

FIGURE 15



Comparison of angiotensin I generated in low, normal and high renin samples using our own method with those recommended by three commercial kits. Most values for low renin samples were undetectable using the kits. The amounts of angiotensin generated in low renin samples using and 18 hour incubation (Sealey) were almost identical with the yield from normal renin samples incubated for 3 hours so that, using the prolonged incubation times recommended by our own method, low and normal renin samples can be evaluated with equal accuracy.

tent correction factor can be applied. It was found that unadjusted plasma could end up as high as pH 8.4, and the use of unadjusted pH in both the Squibb and Schwarz/Mann kits resulted in plasma renin values only approximately 25% of those derived from our method.

The most dramatic difference between our method and the kits was in the ability to measure the low numbers. Even the advantage of using the pH optimum in the New England Nuclear kit was completely eliminated by their three-fold dilution of the plasma prior to incubation and by the use of only a one hour incubation. The amounts of angiotensin generated from low renin samples were below the lowest limit of immunoassay detectability for all three kits (Figure 15).

The study suggested that the major problem with the kits involves not the standard or the antibodies provided for the radioimmunoassay but the conditions recommended for the renin incubation step. The use of unadjusted pH or of diluted plasma seriously reduces the sensitivity of the assay. Also, it is essential to subtract a blank from each sample, thus reducing accuracy. This in turn doubles the number of samples to be assayed.

Moreover, no bacteriostatic agents were added to the incubation medium and the manufacturers of the kits do not recommend that the samples be shaken during incubation. These omissions may well account for lower values with the kits than we would have expected due to the use of unadjusted plasma.

Accordingly, if commercial kits are used, the following steps should be incorporated into the procedure. (1) The incubation step changed to pH 5.7 in the presence of DFP and neomycin, as well as EDTA. (2) For samples with low plasma renin activity, the incubation step should be repeated for 18 hours. (3) Minimum dilution of plasma during pH adjustment. (4) Angiotensin I generation should be prolonged until the necessity for blank subtraction is eliminated. (5) A minimum volume of plasma should be used in the radioimmunoassay. (6) A large source of antibody should be purchased so that variability in antibody characteristics does not become a problem. (7) For routine laboratories which have little control over the temperature of the blood after collection (it should be chilled) assay of the blank should not be discontinued since high blank values will expose those samples which have been allowed to sit at room temperature for an extensive period of time. (8) Strict quality control is essential in any radioimmunoassay. The same two standard plasma samples should be run with each set and if they vary by more than $\pm$ 15%, the set should be discarded and then repeated.

Summary:

A radioimmunoassay method for measurement of plasma renin activity has been described in which angiotensin I, generated by reaction of plasma renin with endogenous renin substrate during either 3 or 18 hours incubation, is quantitated. The incubation step is carried out in undiluted

plasma at the pH optimum (pH 5.7) and can be extended up to 18 hours because angiotensinases and converting enzyme are completely inhibited by EDTA and DFP. Blank subtraction is eliminated and accuracy enhanced by prolongation of the incubation time until the blank comprises only a minor fraction of the generated angiotensin.

Incubation at alkaline pH was found to be disadvantageous because of lower rates of generation of angiotensin I, inability to maintain constant pH without addition of buffer and because the incubation step could not be prolonged beyond 3 hours. It was also found, in both acid and alkaline incubations, that dimercaprol (BAL) and 8-hydroxyquinoline are considerably less effective than DFP in protecting against angiotensinase activity.

The capacity of plasma to form angiotensin I during the incubation step was found to vary directly with plasma renin substrate concentration. Therefore, dilution of plasma prior to incubation was avoided because of the slower reaction rate, due to dilution of both enzyme and substrate, with an inability to correct for the effect of substrate dilution.

The radioimmunoassay is sensitive, accurate and reproducible. By assaying a small volume of plasma, distortion of the standard curve by plasma is not a problem. Complete interchangeability of results derived from bioassay or radioimmunoassay can be achieved by application of a correction factor based on the observed lower pressor activity of angiotensin I and utilization of substrate during incubation.

The improved assay sensitivity by radioimmunoassay is important because approximately 30% of patients with essential hypertension have subnormal plasma renin activity and the renin measurement is the only consistently distinguishing feature of these patients. In a study using three different commercial kits, most low renin samples were undetectable and could not be discriminated with confidence from normal renin samples. However, by using an 18 hour incubation step, low renin samples could be detected with the same degree of accuracy as those with normal plasma renin activity.

II. Measurement of Aldosterone

Aldosterone was isolated, identified and synthesized in a series of important studies in the 1950's by Simpson, Tait and their coworkers (89,90). In normal subjects, the concentration of aldosterone in plasma is approximately 10 ng% and the adrenal secretion rate is about 100 µg/day. Because of the low concentration of aldosterone in plasma, until recently, methods for measurement of physiological and pathological variations utilized urine rather than blood (9,10,91-95). Although aldosterone is excreted into urine in small amounts (less than 1 µg/day) large and fairly constant fractions of the total adrenal secretion are normally excreted as two quantifiable metabolites. About 40% of secreted aldosterone is converted to 3,5 tetrahydroaldosterone (3a,11b,21 trihydroxy-18-oxopregnane-20-one)(96) and another 10% is excreted as the acid-labile conjugate (C-18-glucuronide)(97). This substance can be converted back to aldosterone by acid hydrolysis.

A technical problem in aldosterone measurements is the presence in blood and urine of other steroids in concentrations several orders of magnitude greater than that of aldosterone. Hence, aldosterone must be rigorously purified prior to quantitation.

Aldosterone was originally quantitated by bioassay and later by a fluorometric method (98). However, the first sensitive methods utilized a double isotope derivative

technique for measurement of the 24-hour adrenal secretion rate (91,92) and were based on a method developed by Ayres and coworkers (97). A tracer dose of tritiated aldosterone was injected into the patient. The tritiated aldosterone was diluted by the patient's own hormone and the specific activity of a urinary metabolite of aldosterone was calculated and used to estimate the secretion rate of the hormone. A similar double isotope dilution assay was later applied to measurement of the urinary excretion rate of aldosterone (9,10,94,95). In these later methods the radioactive tracer was added to the urine and the specific activity of the acid-labile conjugate of aldosterone was measured. This metabolite was chosen because tritiated aldosterone could be used as a marker since this metabolite is converted to aldosterone by acid hydrolysis.

Double isotope derivative methods for measurement of aldosterone in blood were also developed (99-103) but sensitivity was a problem since normal values fell at the lower limit of detection of the method.

The induction in animals of antibodies to steroids (104) and the development of radioimmunoassay by Yalow and Berson (61) introduced the potential for a new and more sensitive approach to aldosterone measurement. Several radioimmunoassay methods for measurement of aldosterone in plasma (12,105-109), and urine (11,108,110,111) have been developed. These new methods are faster and more sensitive but there still remains the problem that other steroids interfere with the assay. Thus, extraction and chromatographic procedures must be used

prior to quantitation by radioimmunoassay.

In the following section the principles of double isotope derivative assay will be reviewed and followed by a description of a method utilizing this technique, which was developed in our laboratory. A detailed account will follow of the radioimmunoassay procedures for urine and plasma which were developed and are in current use in our laboratory.

A. Double Isotope Derivative Assay

1. Principles of assay

(a) Isotope dilution: (112)

The technique of isotope dilution is applicable when quantitative isolation of the substance to be measured is not possible, but quantitation of an aliquot can be carried out.

In the method to be described, radioactive aldosterone of known specific activity is added to the pool to be measured, i.e. it is either injected into the body (secretion rate) or added to the urine (excretion rate). The specific activity (s.a.) of aldosterone in a urine aliquot relative to the specific activity of the added aldosterone is a function of the amount of aldosterone in the original pool. Quantitative recovery is not necessary and the fraction which is finally quantitated need not be known.

If a = µmoles of ^{3}H aldosterone added

b = µmoles in pool (to be measured)

c = µmoles in aliquot

n_1 = cpm in 'a'

n_2 = cpm in 'c'

Then:

$$\frac{n_2}{c} = \frac{n_1}{(a+b)}, \text{ and thus } b = \frac{n_1 c - n_2 a}{n_2}$$

In the aldosterone assay, 'a' is negligible relative to "c"; thus

$$b = \frac{n_1 c}{n_2} = \frac{n_1}{n_2/c} = \frac{\text{counts per minute } ^3\text{H added}}{\text{s.a. of isolated portion}} \qquad (1)$$

Thus under most conditions the specific activity of the radioactive aldosterone added to the pool is not crucial.

For application of the derived equations for measurement of the amount of aldosterone in a given volume of urine, a known amount of radioactive aldosterone is added to the urine (n_1). After extractions and chromatography the specific activity of an aliquot is determined (n_2/c). From this value using equation (1) the amount of aldosterone in the original urine (b) can be calculated.

(b) Isotope derivative assay:

The second stage of the double isotope dilution technique involves quantitation of 'c', that is, measurement of the amount of aldosterone in the isolated portion. To this end, a radioactive derivative of aldosterone is prepared using a reagent of known specific activity(113). In the method to be described aldosterone is acetylated with ^{14}C acetic anhydride. The amount of ^{14}C incorporated into aldosterone diacetate becomes a measure of the amount of aldosterone in the aliquot.

Aldosterone: Isotope dilution

$$'c' = \frac{cpm\,^{14}C \text{ (in 'c')}}{s.a. \text{ acetic anhydride}}$$

Only one of the two ^{14}C atoms in each molecule of acetic anhydride is incorporated into aldosterone diacetate. However, since two acetates are incorporated into each aldosterone molecule, the equation remains unchanged.

(c) Double isotope derivative assay:

The combined equation now becomes:

$$b = \frac{n_1}{n_2/(cpm\,^{14}C/s.a.\ ac.an.)} = \frac{cpm\,^{3}H \text{ aldo added}}{^{3}H/^{14}C^{*} \times s.a.\ ac.an.}$$

The ratio of ^3H to ^{14}C in the final aliquot, and not the absolute amount of either source of radioactivity, is used in the calculation. Thus quantitative conversion of aldosterone to the diacetate is not essential and quantitative isolation of the aldosterone derivative need not be achieved.

The specific activity of acetic anhydride is technically difficult to measure accurately because the compound is quite volatile. Instead, ^{14}C desoxycorticosterone acetate is prepared and the specific activity of ^{14}C DOCA is calculated with each set. The specific activity of acetic anhydride is twice the specific activity of DOCA since there is only one acetate in DOCA. In addition, since the molecular weight of DOCA and aldosterone are similar (372 c.f. 360) 'b' can be expressed as micrograms without further modification of the equation.

Therefore

$$b = \frac{cpm\,^{3}H \text{ aldo added}}{^{3}H/^{14}C \times s.a.\ DOCA^{\dagger} \times 2} (ug)$$

* $^3H/^{14}C$ ratio in the isolated aliquot

† = cpm per ug

2. Requirements for application of isotope dilution techniques: (9,91).

When measuring the excretion rate in urine of the acid-labile conjugate the metabolite must first be hydrolyzed to re-form the original steroid and recovery of the tracer does not account for variability in conversion to aldosterone. Hence hydrolysis must be carried out very systematically. The pH should be adjusted to exactly pH 1.0 and the time period for hydrolysis should be strictly controlled.

For measurement of aldosterone secretion rate the tritiated aldosterone is injected into the patient and must mix rapidly with endogenous aldosterone. Complete mixing of radioactive aldosterone with endogenous aldosterone is essential and it should be metabolized in identical fashion (9,91). The choice of metabolite for measurement of either secretion or excretion rates is not crucial as long as it is derived solely from aldosterone. All of the labelled metabolite destined for urinary excretion must be excreted during the collection period. The fraction of secreted aldosterone converted to the metabolite is not critical, but it should be constant.

Labelled and unlabelled hormone must react similarly in the purification and extraction procedures. This will be discussed further under the topic "isotope fractionation" (9,10).

3. Method for measurement of the urinary excretion rate of aldosterone: (Table 9)(9,10)

A flow sheet of the method is presented in Table 9. The tritiated aldosterone which is added to urine in the first step should be checked for radiochemical purity. Both a Bush B5 system and the first chromatography system (Step 4, Table 9) can be used. If the latter chromatogram is run for both 6 and 40 hours, impurities remaining at the origin and running with the solvent front can be detected.

After addition of tritiated aldosterone, the acid-labile conjugate of aldosterone is hydrolyzed for 24 hours at pH 1.0 to form free aldosterone which is then extracted into methylene chloride. The extract is washed with sodium hydroxide and then the pH is returned to neutrality by washing consecutively with acetic acid and water. Prior to acetylation the extract undergoes preliminary purification on descending paper chromatography. The aldosterone is eluted then acetylated with ^{14}C acetic anhydride. ^{3}H aldosterone ^{14}C diacetate is purified on two more descending chromatography systems before the final step of celite column chromatography. The combined $^{3}H/^{14}C$ ratio of the peak tubes from the column is used to calculate the amount of aldosterone in the original urine using the calculations derived previously.

The first chromatography system removes both hydrocortisone and corticosterone prior to acetylation. In this way the amount of ^{14}C acetate incorporated into the extract, and not associated with aldosterone, which has to be removed

TABLE 9

FLOW SHEET FOR MEASUREMENT OF THE
EXCRETION RATE OF ALDOSTERONE BY DOUBLE ISOTOPE DILUTION

1. Complete 24 hour urine collection

2. Hydrolysis

150 ml urine aliquot:	Add 25,000 cpm ^3H aldosterone
	Adjust to pH 1.0 (0.95-1.1) with 50% H_2SO_4
After 24 hours (23.5-24.5):	Extract with 500 ml ethyl acetate

3. Ethyl acetate extract

Wash with:	2 x 50 ml 0.1N NaOH
	50 ml 0.1N HAc
	50 ml distilled water
Add:	10 ml 95% ethanol
Evaporate to dryness	

4. 1st Chromatography (count 1/50 aliquot prior to chromatography)

Paper:	7.5 cm Whatman #1
Stationary Phase:	22% propylene glycol in acetone
	(Dip paper prior to application of sample)
Mobile Phase:	Toluene
Marker:	Isatin
Development:	40 Hours
Location:	^3H aldosterone located by strip scanner
Elution:	5 ml methanol

5. Partition (count 1/50 aliquot prior to partition)

Evaporate to dryness	
Add:	5 ml methylene chloride
Wash with:	3 x 1 ml distilled water
Evaporate to dryness	
Add:	2 x 5 ml benzene
Evaporate to dryness	

6. Acetylation (in well ventilated hood)

Add:	0.03 ml pyridine
Add:	0.03 ml ^{14}C acetic anhydride
After 18 hours add:	1 drop distilled water
Add:	2 x 5 ml benzene/ethanol (2/1;v/v)
Evaporate to dryness	
Add:	0.1 ml unlabelled aldosterone diacetate (50ug)
Add:	2 drops rhodamine B
Evaporate to dryness	

TABLE 9 CON'T

7. 2nd Chromatography

Paper:	3 cm Whatman #1
Stationary Phase:	55% formamide/methanol (Dip paper prior to application of sample)
Mobile Phase:	50% methylcyclohexane/toluene
Markers:	Aldosterone diacetate; rhodamine B
Development:	16 hours
Location:	UV
Elution:	5 ml methanol

8. 3rd Chromatography (count 1/50 aliquot prior to chromatography)

Add:	0.1 ml aldosterone diacetate
Add:	2 drops rhodamine B
Evaporate to dryness	
Paper:	3 cm Whatman #1
Reagents:	Mesitylene/methanol/water (3/2/1)
Stationary Phase:	mesitylene (Dip paper after application of sample)
Mobile Phase:	Methanol/water
Markers:	Aldosterone diacetate and rhodamine B
Development:	8 hours
Location:	UV
Elution:	5 ml methanol

9. 4th Chromatography (count 1/50 aliquot prior to column)

Add:	2 drops Sudan IV
Evaporate to dryness	
Reagents:	Isooctane/$MeCl_2$/methanol/water (1500/450/900/158)
Support:	Acid washed celite (12g) in 40 x 1.0 cm column
Stationary Phase:	7 ml methanol/water
Mobile Phase:	Isooctane/methylene chloride
Flow rate:	0.3-0.5 ml/min
Marker:	Sudan IV (void volume)
Location:	About 80 ml (2 ml fractions)
Evaporate to dryness	
Count to locate peak	
Peak eluted in 8 to 10 tubes	

10. Calculation:

Count for 2 x 10 minutes:	Each peak tube from column
Count for 2 x 10 minutes:	Aliquot of ^3H aldosterone (added to urine)
Count for 2 x 10 minutes:	^{14}C DOCA (for estimation of specific activity of acetic anhydride)

$$\text{Aldosterone excretion rate (ug/day)} = \frac{\text{cpm } ^3H \text{ aldo added} \times \text{urine total volume}}{^3H/^{14}C \times \text{s.a.DOCA} \times 2 \times 150}$$

subsequently is considerably reduced. After acetylation, aldosterone diacetate is purified until radiochemical purity is achieved.

Zaffaroni paper chromatographic systems were chosen, rather than Bush systems, because of their high capacity. Chromatography #3 (99,114) is a reverse phase system in which the relative running rates of various steroids are switched. Compounds which run ahead of aldosterone in more conventional systems are more polar in this system and are less likely to merge with the main aldosterone diacetate peak. Another approach to rapid purification which was investigated was mild hydrolysis to form aldosterone monoacetate. This approach was discarded because of variability in the yield of monoacetate and because two additional steps are required (hydrolysis of the acetate and an additional chromatography).

Column chromatography was chosen for the final step because constancy of the $^{3}H/^{14}C$ ratio in successive tubes can be taken as an indication of radiochemical purity. A constant $^{3}H/^{14}C$ ratio in successive paper chromatography systems can also provide similar proof, but at least one additional system is necessary. Another reason for choosing column chromatography was the accurate identification of the peak. When using paper chromatography the area to be eluted is often less simple to define. This is not a major problem in the first three chromatographic systems because rather generous amounts can be eluted and associated impurities can be removed on subsequent systems. However, in the last chromatography,

only the narrow band containing aldosterone diacetate should be eluted.

The acetylation step is a potential source of problems. Although 100% yield is not essential, maximum recovery at each step is required in order to maintain adequate overall recovery of the method. Any water associated with the extract during acetylation will reduce the yield of aldosterone diacetate because of rapid hydrolysis of acetic anhydride to acetic acid. Therefore, the extract is dried thoroughly prior to acetylation by refluxing in the presence of benzene. Propylene glycol present during acetylation also reduces the yield of aldosterone diacetate. Trace amounts can be carried over from the first chromatography system and must be removed prior to acetylation by partition between methylene chloride and water.

The specific activity of the acetic anhydride used for acetylation is measured by preparation of ^{14}C DOCA (see Principles of Assay). DOC is acetylated (Step 6, Table 9) and the ^{14}C DOCA is then crystallized a minimum of three times to constant specific activity. The specific activity of both crystals and mother liquor must be identical.

At least three sources of acetic anhydride of different specific activity should be available so that urine samples with different aldosterone content can be acetylated to yield similar $^{3}H/^{14}C$ ratios. The optimum ratio of $^{3}H/^{14}C$ is between 2 and 10. During simultaneous detection of ^{3}H and ^{14}C, not much ^{3}H is picked up by the ^{14}C channel (less than 1%). However, about 40% of the ^{14}C is detected by the ^{3}H

channel and accurate discrimination of the two isotope requires that tritium is present in slightly higher amounts.

An aliquot of the urine extract is routinely counted after each step to keep track of the recovery of tritiated aldosterone. In this way the source of any problem may be identified quickly. Since three weeks are required to complete one assay, rapid identification of the source of error is essential. Another check of the accuracy of the method is the inclusion in each set of known amounts of aldosterone.

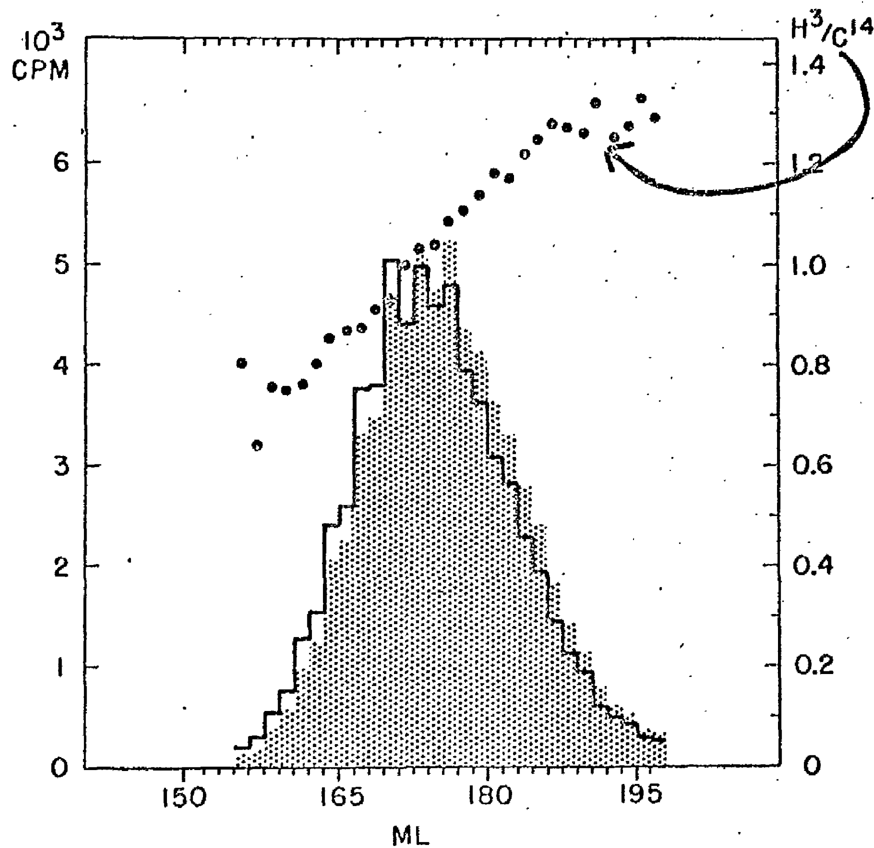
Despite the time needed for analysis and the number of steps involved, the double isotope derivative assay is an accurate method. As long as radiochemical purity is achieved in the final step there is little source for error. The use of tritiated aldosterone as a marker accounts for losses at each step, except the initial conversion of the acid-labile conjugate to aldosterone, and the preparation of a radioactive derivative is a precise method of quantitation.

4. Isotope fractionation: (9,10)

In the double isotope derivative method for measurement of aldosterone just described, radiochemical purity of aldosterone diacetate in the final step can be confirmed by constancy of the ratio of $^{3}H/^{14}C$ in successive fractions eluted from the column. During development of the method many different systems for the final column were investigated but in all of them the ratio of ^{3}H to ^{14}C rose progressively in the peak tubes. Because of this, to change the relative run-

FIGURE 16

ISOTOPE FRACTIONATION DURING COLUMN CHROMATOGRAPHY



^{3}H aldosterone^{14}Cdiacetate elution pattern from a celite partition column chromatography system. The ^{3}H/^{14}C ratio gradually and consistently increased across the peak. Mean displacement of the two isotopes was calculated to be 0.6%.

ning rates of possible impurities and thus improve purification, aldosterone diacetate was converted to the monoacetate. However a constant ratio of ^{3}H to ^{14}C could not be achieved. Finally pure standards were acetylated with ^{14}C acetic anhydride and when, under these ideal conditions, a constant ratio could not be demonstrated in several different columns, the possibility of isotope fractionation was considered.

There are six possible moieties in the aldosterone diacetate mixture which can differ in molecular weight by only 4 (10%): (1) ^{3}H aldosterone diacetate (2) aldosterone ^{14}C diacetate (3) aldosterone $^{14}C_2$ diacetate (4) ^{3}H aldosterone ^{14}C diacetate (5) ^{3}H aldosterone $^{14}C_2$ diacetate and (6) aldosterone diacetate. It is conceivable that these slightly different compounds might run differently on various chromatographic systems.

To investigate whether or not isotope fractionation occurs in partition column chromatography, pure ^{3}H aldosterone ^{14}C diacetate was prepared and applied to a 20 g celite partition column (Step 9, Table 9, p. 75). 1.5 ml fractions were collected so that the peak was divided into about 30 fractions (Figure 16). The $^{3}H/^{14}C$ ratio rose consistently across the peak. Mean displacement of the two isotopes, calculated by probit analysis, and corrected for differences in dispersion of the two peaks, was approximately 0.6%.

In another study isotope fractionation was analyzed using paper chromatography (Step 7, Table 9, p. 75). The radioactive peak was cut into multiple zones. In this system fractionation was less, but still identifiable (approximately 0.3%).

In a third study, pure ^{3}H aldosterone ^{14}C diacetate was again run on paper chromatography and the peak was cut into four zones, each of which was then applied separately to celite partition columns. Isotope fractionation was readily identifiable in both systems (Fig. 17) and the combination of the two steps increased the displacement of the isotopes to almost 1%.

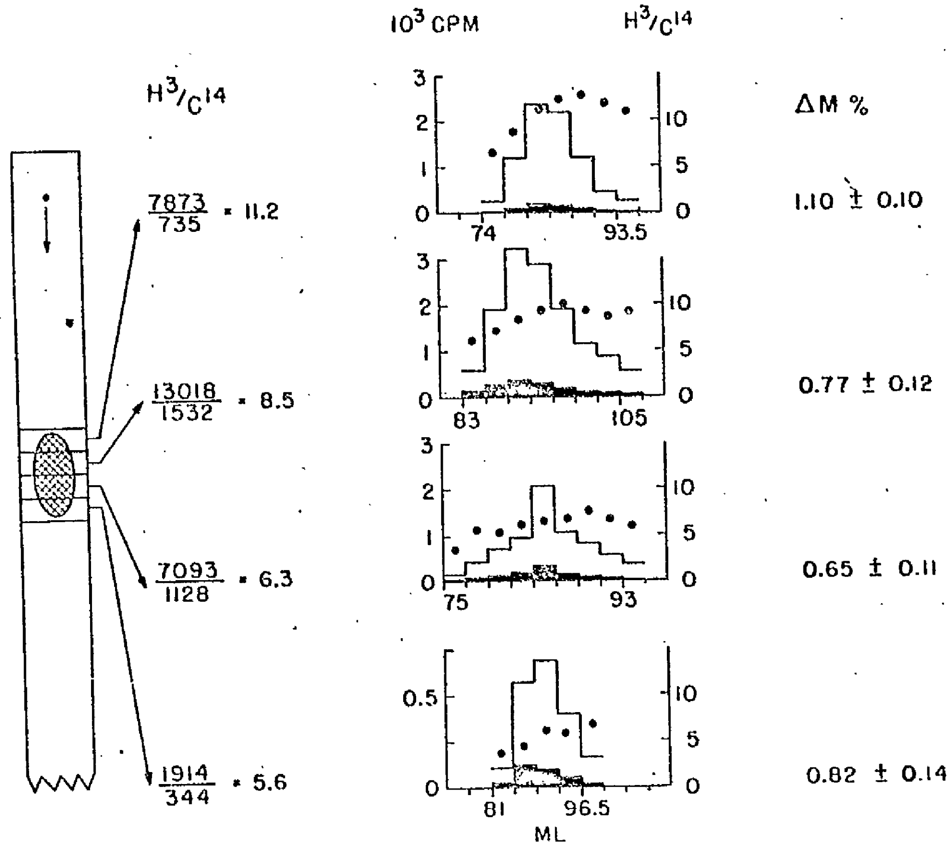
Other investigators have confirmed and extended our studies (115,116). A study by Cejka and Venneman (115) demonstrated that the presence of tritium in the molecule has the greatest influence on displacement. They found the greatest displacement (1.8%) between ^{3}H aldosterone and unlabelled aldosterone, and were unable to demonstrate any displacement of ^{14}C diacetate from ^{12}C diacetate. This data supports our own observations (10) that ^{3}H aldosterone diacetate was displaced more than ^{14}C diacetate from unlabelled diacetate.

The greater chromatographic displacement caused by introduction of ^{3}H into the steroid than by addition of ^{14}C to the acetate, may be because tritium is 200% heavier than hydrogen whereas ^{14}C is only 17% heavier than ^{12}C. The greater percent increase in size of the hydrogen molecule and its closer association with the steroid ring may change the conformation of the molecule and thus alter the relative running rates on chromatographic systems.

Analysis of the data suggests that peak enrichment should be avoided during all purification steps, and especially prior to acetylation. For this reason, wide bands should be cut in all paper chromatographic systems. Since ^{3}H aldosterone is

FIGURE 17

ISOTOPE FRACTIONATION DURING PAPER THEN COLUMN CHROMATOGRAPHY



Isotope fractionation during paper and column chromatography of ^{3}H aldosterone ^{14}C diacetate. A paper chromatogram of pure compound was run and the radioactive spot was located and cut into four segments. Each segment was eluted, counted, then individually chromatographed on a celite partition column. A progressive and significant increase in the $^{3}H/^{14}C$ ratio was apparent across the peak. ΔM% is the displacement of the two isotopes on each column.

more polar than the unlabelled compound, when the peak is identified by location of radioactivity (first chromatography), wider margins should be cut towards the solvent front. The reverse approach should be used for peaks in which the unlabelled compound is located (second and third chromatographies).

It was found that the most accurate method of calculating the correct $^{3}H/^{14}C$ ratio was to divide the area under the tritium peak by the area under the ^{14}C peak (10). The use of the ratio in the peak tube alone led to considerable error but ten percent truncation of the peak caused an error of less than 10%. Therefore, routinely, all tubes from the column which contain 20% of the counts in the peak tube are used to calculate the $^{3}H/^{14}C$ ratio. Each tube is counted separately and the sum of the counts are computed prior to the final calculation. Most accurate values would be achieved if the peak were divided into thirty fractions. However, the error introduced by using only 8-10 fractions is less than 8% as long as symmetrical cuts of the peak are made.

In summary, isotope fractionation is a problem in all chromatographic systems used in the measurement of aldosterone. However, knowledge of the characteristics of the effect can prevent errors throughout the purification steps. A constant $^{3}H/^{14}C$ ratio cannot be achieved in the final column but a consistent rise in the ratio across the peak is a good, although less reliable, indicator that radiochemical purity has been achieved. Column is preferable to paper chromatography for the last chromatographic system because the peak tubes can be chosen with precision.

5. Measurement of the secretion rate of aldosterone:

The method is identical to that described in Table 9 (page 74) except 1-2 μc of tritiated aldosterone are injected into the patient immediately preceding the 24 hour urine collection. No additional tritiated aldosterone is added in step #2. In the final calculation the counts added to the urine are replaced by the counts injected.

The measurement of the secretion rate of aldosterone has been replaced by excretion rate measurements for several reasons. Firstly it was demonstrated that, under most conditions, the fraction of aldosterone excreted as the acid-labile conjugate is quite constant at 10% of the secretion rate (9). Excretion measurements are preferable in studies requiring serial determinations since radioactivity is not injected into the patient. In addition quantitative injection of tritiated aldosterone is not always reliable and when all of the aldosterone is not injected directly into the vein the assay has to be discarded. Sometimes it takes several days before all of the radioactivity is excreted. This also occurs and is a real problem in patients with renal failure. Another disadvantage of secretory measurements is that larger volumes of urine may have to be extracted, unless a large amount of radioactivity has been injected, and this requires chromatography systems with greater capacity.

However, measurement of the excretion rate can be misleading in patients with renal or liver disease. Approximately 50% of the acid-labile conjugate is formed by the

kidney and the rest is made by the liver (117,118). In the presence of kidney disease there is both delay in excretion and a smaller fraction is converted to the acid-labile conjugate (119). In patients with liver disease, in whom the kidney becomes a major source of metabolism of aldosterone, as much as 50% of secreted aldosterone can be converted to the acid-labile conjugate (118).

6. Summary:

The measurement of either the secretion rate of aldosterone or the rate of urinary excretion of its acid-labile conjugate by double isotope derivative assay involves the use of multiple chromatography systems and the formation of a radioactive derivative of aldosterone. The two methods are complicated, expensive and time consuming but they have the advantage of accuracy because of the use of radioactive markers which account for losses during extraction and chromatographic procedures.

The problem of isotope fractionation during the chromatographic procedures can be overcome by taking wide cuts during preliminary purification steps and by accurately identifying the final peak on column chromatography.

B. Radioimmunoassay of Aldosterone.

The demonstration that antibodies to steroids could be induced in rabbits by injection of a conjugate of the steroid to bovine serum albumin (104), opened the door to measurement of steroids by radioimmunoassay. Recently, several radioimmunoassay methods have been developed for measurement of aldosterone in blood (12,105-109) and urine (11,108,110,111) utilizing antibodies induced in either sheep or rabbits. Conjugation of aldosterone to albumin was achieved by coupling through either a 3-oxime or 21-hemisuccinate derivative.

To date, antibodies to aldosterone lack the specificity needed for direct quantitation. Although cross-reactivity with other steroids is generally less than 1%, the high concentration of other steroids in blood and urine requires even greater antibody specificity.

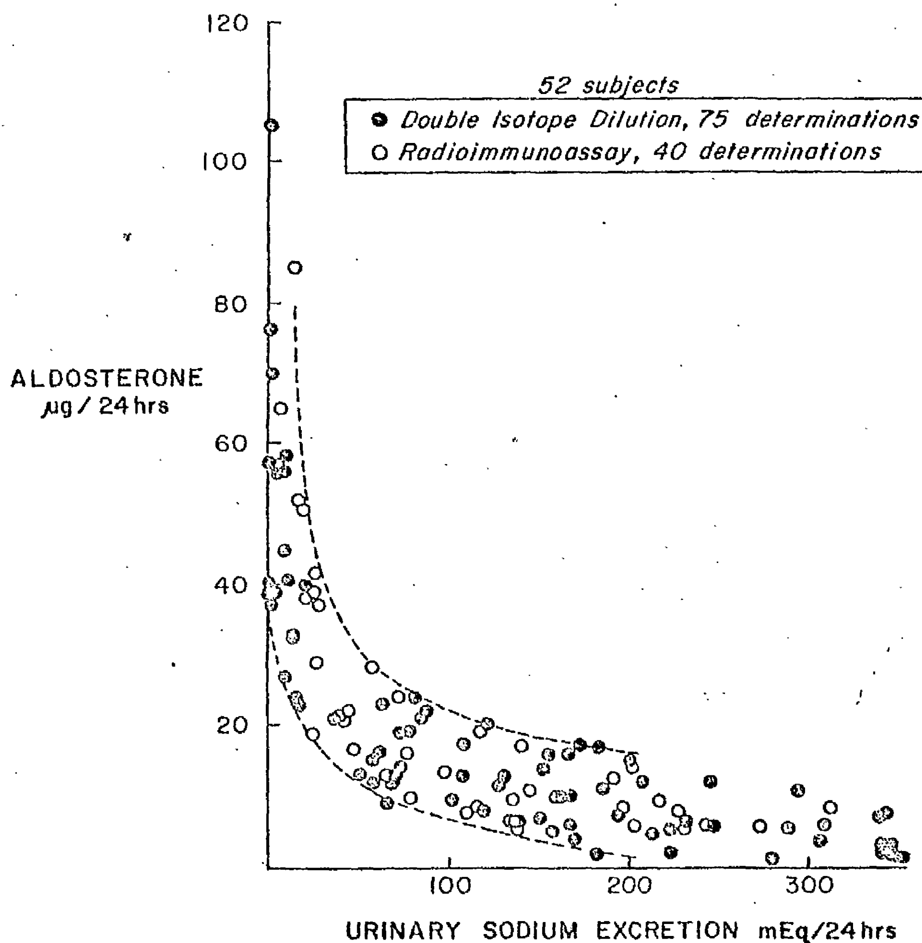
Radioimmunoassay of aldosterone is a much more sensitive method than double isotope dilution and is capable of detecting picogram amounts. However, the increased sensitivity resulted in, perhaps unforeseen, complications. The relatively large amounts of aldosterone measured by the double isotope derivative assay had caused considerable laboratory contamination. This contamination had not previously been a problem because it was below the limit of detection by double isotope dilution. Another problem is that steroids cannot be used as markers in chromatography systems prior to radioimmuno-

assay because the amounts needed for detection are very high and chances for contamination are great. Therefore, for measurement of aldosterone in plasma we adopted a chromatographic system which does not require the use of markers. In addition extraction, purification and radioimmunoassay steps are all carried out using disposable glassware.

Methods for measurement of aldosterone in both urine (11, 120) and plasma (12) have been developed in our laboratory. The two measurements fulfill different requirements. The 24 hour excretion of aldosterone is an integration of the 24 hour adrenal secretion rate whereas blood measurements reflect the level at only one point in time and are thus subject to wide fluctuations. Double isotope derivative assays of urine and plasma aldosterone are interchangeable with those measured by radioimmunoassay (Figure 18)(11,121). Urine aldosterone measurements are simpler and less time consuming than the plasma method but have the disadvantage that, in certain clinical situations such as malignant hypertension, renal and liver diseases or nephrectomy the excretion rate does not accurately reflect the rate of secretion because of alterations in metabolism or excretion. Blood measurements are especially applicable to the study of acute changes in aldosterone and also for analysis of those clinical situations in which urine measurement may be suspect.

When compared with the double isotope derivative assay, the technique of radioimmunoassay is more rapid, sensitive and

FIGURE 18



Hyperbolic relationship of urine aldosterone excretion to urine sodium excretion in normal subjects. There is complete overlap of values measured by double isotope derivative assay or radioimmunoassay.

has higher capacity. However, greater accuracy is only achieved with very strict quality control. Radioimmunoassay, although more sophisticated than in vivo bioassays, is still a biological assay and is fraught with many inexplicable variations. In general, once a successful radioimmunoassay technique has been developed, minimum changes should be attempted because, surprisingly frequently, one small change can affect the whole assay. In contrast, the double isotope derivative technique, while time consuming and relatively insensitive, has built-in safeguards which protect the reproducibility of the assay.

1. Principles of radioimmunoassay

Aldosterone is quantitated by utilizing (a) its affinity for highly specific antibodies and (b) competition between radioactive and unlabelled aldosterone for binding sites on the antibodies (63). The sample to be quantitated is added to a mixture of radioactive aldosterone and antibody in proportions such that half of the radioactive aldosterone is bound to antibody and the other half remains free. The extent to which radioactive aldosterone is displaced from the antibody by the unknown sample is a measure of the amount of aldosterone in the sample. The aldosterone content of the unknown sample is determined precisely by comparing it to the displacement caused by addition of unlabelled aldosterone standards.

In practice, a mixture of antibody and labelled aldoster-

one is added to a series of tubes containing known amounts of aldosterone or unknown sample. After 18 hours, the bound aldosterone is separated from the free by adsorption of the free aldosterone onto dextran coated charcoal. After centrifugation the bound aldosterone is decanted and counted in a scintillation spectrometer, while the free (charcoal plug) is discarded. From these counts the amount of aldosterone in the unknown sample can be calculated.

2. Urine aldosterone excretion (acid-labile conjugate): (11,120)

(i) Preliminary purification

Details of the method are illustrated in Table 10. Immunologically cross-reacting corticoids are pre-extracted into methylene chloride and discarded prior to acid hydrolysis. The methylene chloride insoluble acid-labile conjugate of aldosterone is then hydrolyzed with acid to liberate aldosterone which is subsequently extracted into methylene chloride. Thus, separation of aldosterone from immunologically cross-reacting compounds is accomplished simply by extraction, acid hydrolysis and then re-extraction. The methylene chloride extract is washed to remove non-specific cross-reacting substances and then aldosterone is quantitated by radioimmunoassay. Losses incurred during this preliminary purification step, are accounted for by addition of radioactive aldosterone to the hydrolyzed urine.

TABLE 10

PURIFICATION: HYDROLYSIS: EXTRACTION

(18 samples and 2 standard urines can be run as one batch)

Pre-extraction of impurities:

3 ml urine + 15 ml $MeCl_2$	Mix well

Hydrolysis at pH 1:

Pre-extracted urine + 1 drop each 6N & 3N HCL	Check pH
(+ additional drops 3N or 1N HCL)	Check pH (0.95 - 1.1)
Leave for 24 hours at room temperature	

Extraction:

1 ml hydrolysed urine + 2000 cpm ^3H aldosterone	Wait 30 minutes
+ 15 ml $MeCl_2$	Mix well
	Discard urine
15 ml $MeCl_2$ extract + 1.5 ml of 0.1N NaOH	Mix well; discard NaOH
+ 1.5 ml 0.1N NaOH	Mix well; discard NaOH
+ 1.5 ml 0.1N HAc	Mix well; discard HAc
+ 1.5 ml water	Mix well; discard water
	Evaporate to dryness

Dissolve extract in 2 ml ethanol

Transfer: 1 ml for recovery estimation (recovery vials)

2 x 0.4 ml aliquots for radioimmunoassay

* 2000 cpm are also pipetted into 3 counting vials for accurate estimation of the number of counts added to each urine sample (standard recovery vials).

The volume of urine extracted after hydrolysis is only 1 ml (150 ml was used in the double isotope derivative assay) and in the radioimmunoassay step 1/50th or less of this is assayed. The ^{3}H aldosterone which was added to detect losses during extraction, does not interfere with the radioimmunoassay step since the counts added to the assay tube account for less than 3% of the total counts.

(ii) Radioimmunoassay

The aldosterone content of the urine extract is dependent on both the excretion rate of aldosterone and the 24-hour urine volume. Prior to radioimmunoassay the extract is diluted if the aldosterone concentration is expected to be high.

Two different amounts of urine extract (10 and 20 ul) are assayed. The use of a maximum volume of 20 ul facilitates the evaporation of ethanol in the extract prior to radioimmunoassay. The rack of tubes is simply placed in a vacuum oven for 15 minutes.

Ten and 20 ul of three different aldosterone standards are added in duplicate to the standard curve (Table 11). Tubes are also included in the standard curve for measurement of the total counts (T), the binding of tritiated aldosterone in the absence of antibody (non-specific binding) and the binding of aldosterone to antibody in the absence of unlabeled aldosterone (B_o). In addition, two standard urine samples of known aldosterone content are assayed with each set.

TABLE 11

STANDARD CURVE:

TUBE #	ALDO STANDARD ng/ml	VOLUME µl	ALDO ng
1,2	0.5	10	.005
3,4	0.5	20	.010
5,6	2.0	10	.020
7,8	2.0	20	.040
9,10	8.0	10	.080
11,12	8.0	20	.160
13,14:	total counts; no charcoal (T)		
15,16:	non-specific binding; no antibody		
17,18:	no added aldosterone (B_o)		

A unique feature of this radioimmunoassay is the preparation of a mixture of antibody and radioactive aldosterone, prior to addition to the standard or unknown samples. This step reduces inaccuracies caused by pipetting separately buffer, tritiated aldosterone, and then antibody to each tube. Thus, only two pipettings are needed instead of four. This ^3H aldosterone/antibody solution can be stored for up to a month without loss of assay sensitivity.

After 18 hours at 4°C the aldosterone bound to antibody is separated from free aldosterone by adsorption of the free onto dextran-coated charcoal. This step causes the most problems. Charcoal quickly adsorbs free aldosterone but it also adsorbs very slowly antibody bound aldosterone. Thus, relatively more antibody bound aldosterone is adsorbed in those tubes to which charcoal is added first, and this causes the bound counts to appear to be higher in the last tubes. Because of this, the duplicates of the standard curve are divided and charcoal is added to one half at the beginning and to the other half at the end of the series. In this way the effect of charcoal to adsorb antibody bound aldosterone is averaged out.

Technically, addition of charcoal is the most variable step in the assay. The charcoal must be mixed vigorously, to maintain homogeneous suspension, added rapidly, and the tubes centrifuged immediately so that the charcoal is in suspension in the sample for a very short time. Different

Aldosterone: Urine RIA

TABLE 12

Sample #	Counts bound (B)	B/B_o	$\frac{B/B_o}{1-B/B_o}$ (Y)	Aldo pg
Standard Curve				
1	6387	.80	4.0	5
2	7035	.88	7.3	5
3	6186	.78	3.5	10
4	6189	.78	3.5	10
5	5090	.64	1.8	20
6	5232	.66	1.9	20
7	3972	.50	1.0	40
8	3901	.49	0.96	40
9	2687	.34	0.52	80
10	2753	.35	0.54	80
11	1909	.24	0.32	160
12	1868	.23	0.30	160
13	16095	total counts (T)		
14	15578			
15	545	4% non specific binding		
16	652			
17 (B_o)	7866	50% binding		
18	8073			

Standard Recovery Counts

23553
23464 mean = 23495
23467

Sample #	Counts bound (B)	B/B_o	$\frac{B/B_o}{1-B/B_o}$ (Y)	Aldo pg	Recovery counts	Chem* DF	Aldo (pg)	Error
Standard Urine Samples								
X(52)***	3650	.46	0.85	49	10147	1.16	57	+10%
X'(104)	2625	.31	0.45	102			121	+14%
Z(18)	5606	.70	2.3	15.1	10696	1.10	16.6	-8%
Z'(36)	4238	.53	1.13	34			37.4	+4%

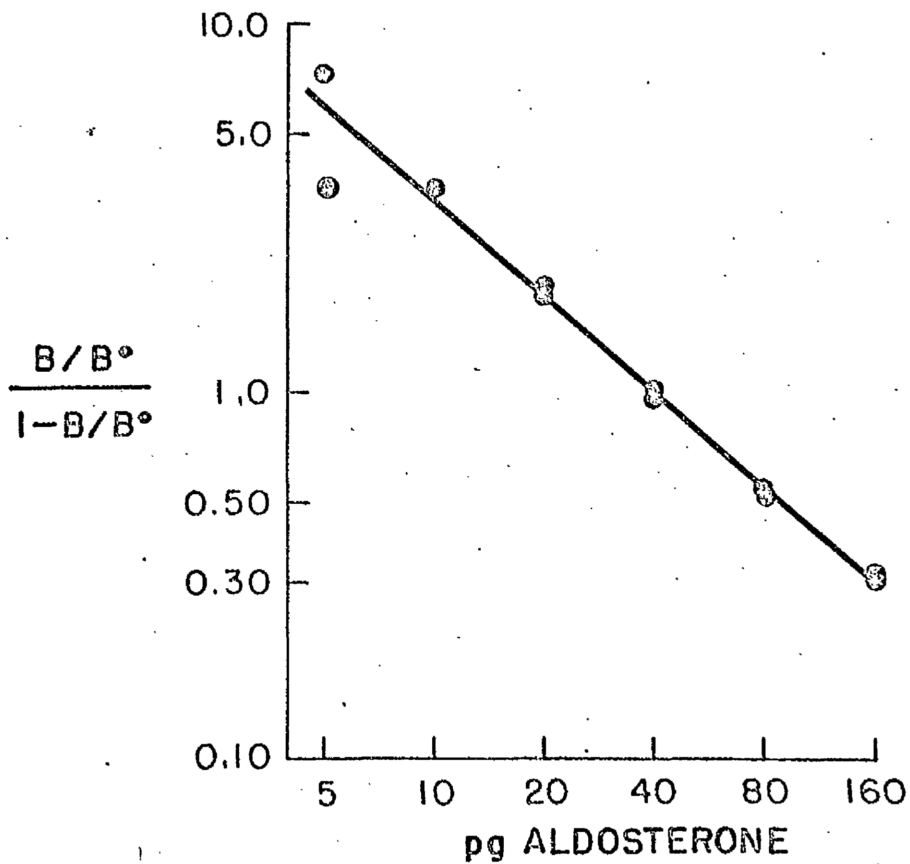
Sample #	Counts bound (B)	B/B_o	(Y)	Aldo pg	Recovery counts	Chem* DF	RIA** DF	Urine vol (ml)	Aldo ug/day	Mean value	error
Unknown Samples											
507	5148	.65	1.86	20	10147	1.14	500	1200	13.7	14.4	8%
507'	3832	.48	0.92	44			250		15.0		
508	5349	.67	2.0	18	10696	1.10	250	980	4.85	5.3	8%
508'	3919	.49	0.96	42			125		5.65		
515	3436	.43	0.74	56	10478	1.12	100	1320	8.28	8.1	2%
515'	2384	.30	0.43	108			50		7.98		

*Chem. DF = standard recovery counts(recovery counts x 2).

**RIA DF = dilution of sample prior to assay and volume of sample assayed (i.e., 10 or 20 ul).

***Numbers in parenthesis = pg aldo in standard urine sample.

FIGURE 19



Picograms of aldosterone added to each tube of the standard curve plotted on log/log paper against a function of the change in the counts bound caused by addition of unlabelled aldosterone.

batches of charcoal have widely different adsorptive capacity and each should be individually evaluated. It has been suggested that coating of charcoal with dextran is not essential (64) but we have been unable to confirm this.

After centrifugation the supernatant (bound) and the recovery vials are counted. The standard curve is calculated as illustrated in Table 12 (65) and plotted on log/log paper (Fig 19). Next, the two standard urine samples are calculated and the amount of aldosterone is derived from the standard curve. If the error in these samples (i.e. the percent difference from the true value) is less than ± 20%, the set is acceptable and the unknown samples are then calculated. Otherwise the whole set should be repeated. Duplicate values of unknown samples should not vary by more than ± 15%. If they do they should be repeated.

The modified logit plot (Fig. 19) has the advantage of forming a straight line. Routinely, the data is calculated using a computer (Table 13). The counts from the spectrometer are punched onto paper tape which is then fed into the computer. The standard curve is calculated automatically. The standard error (SE) of the curve is calculated and any point which differs from the line by more than 2 SE is discarded and the line recalculated. This procedure is repeated until all points fall within 2 SE.

Several parameters are useful for checking accuracy and for indicating the source of problems in the radioimmunoassay. The concentrations of the standard urine samples are chosen so that they span a wide portion of the standard curve. In

TABLE 13

RAAS CALCULATION RUN
MERGED DEC 09 73 SUN 12:24 BY JES FILE:UA27
RUN ON 1/ 1/74
*****************UA SYSTEM*******************

BACKGROUND	9 CPM	
TOTAL COUNTS	3226 CPM	ERROR=1%
NSB	2%	ERROR=6%
B(0)	1564 CPM	ERROR=1%
% BINDING	48%	

STANDARD CURVE CALCULATION

LOGY = -.932 *LOGX + 1.686
S.E.= 0.035 , COEF.COR= -.9973
LOGIT 1= 64.56

X	Y	Y(CALC)
5	9.23	10.84
5	12.71	10.84
10	5.30	5.68
10	6.07	5.68
20	2.79	2.98
20	3.11	2.98
40	1.54	1.56
40	1.63	1.56
80	0.81	0.82
80	0.86	0.82
160	0.40	0.43
160	0.45	0.43

RECOVERY STANDARD:2343

STANDARD URINE CALCULATIONS

PG ALDO	%ERROR	
47.5	-9	(52)
89.6	-14	(104)
21.1	5	(20)
43.7	9	(40)

AVERAGE ERROR= -1.91%

CALCULATION OF UNKNOWNS

POS	#	UG/DAY	PG	PG	ERROR	CHEM/DF	RIA/DF
33	1	2.5	22	40	4%	1.17	0.5
36	2	26.8	28	59	2%	1.16	10.0
39	3	6.8	6	16	7%	1.12	5.0
42	4	20.2	46	90	0%	1.17	5.0
45	5	13.4	18	37	1%	1.14	2.5

this way if either end of the standard curve is incorrect it will be detected.

A useful guide to changes in the radioimmunoassay is data derived from measurement of the non-specific binding (Table 12, tubes 15 and 16)(NSB, Table 13). If the duplicates of these tubes are variable, erratic addition of charcoal may have occurred. Data from these tubes also provide a simple check of the consistency of individual operators.

The aldosterone content at logit = 1.0 can be used to detect radical changes in the standard curve (Table 13). This parameter may vary even when the standard urine samples check perfectly and changes need not indicate error but only some variability in the assay. In the aldosterone radioimmunoassay, logit 1.0 should be quite constant since, unlike ^{125}I iodinated compounds, tritium labelled compounds are quite stable. Shifts in the logit 1.0 can indicate contamination, radioactive breakdown and inadequate addition of charcoal.

In summary, radioimmunoassay of urine aldosterone is a method of high capacity. It involves many steps, each of which is technically easy. Accuracy is dependent on the use of a highly specific antibody and rigid quality control, and is aided by the use of an automated device for calculation of the data.

3. Radioimmunoassay of plasma aldosterone (12,122)

Differences between plasma and urine aldosterone measurements are in the preliminary purification steps. The low con-

centration of aldosterone in plasma requires that a larger volume than urine be assayed. Thus, the extract from the equivalent of 1 to 40 ul of urine is added to the radioimmunoassay whereas the extract from the equivalent of 100 to 800 ul of plasma is assayed. These larger volumes of plasma require more stringent purification from other cross-reacting steroids present in much higher concentrations.

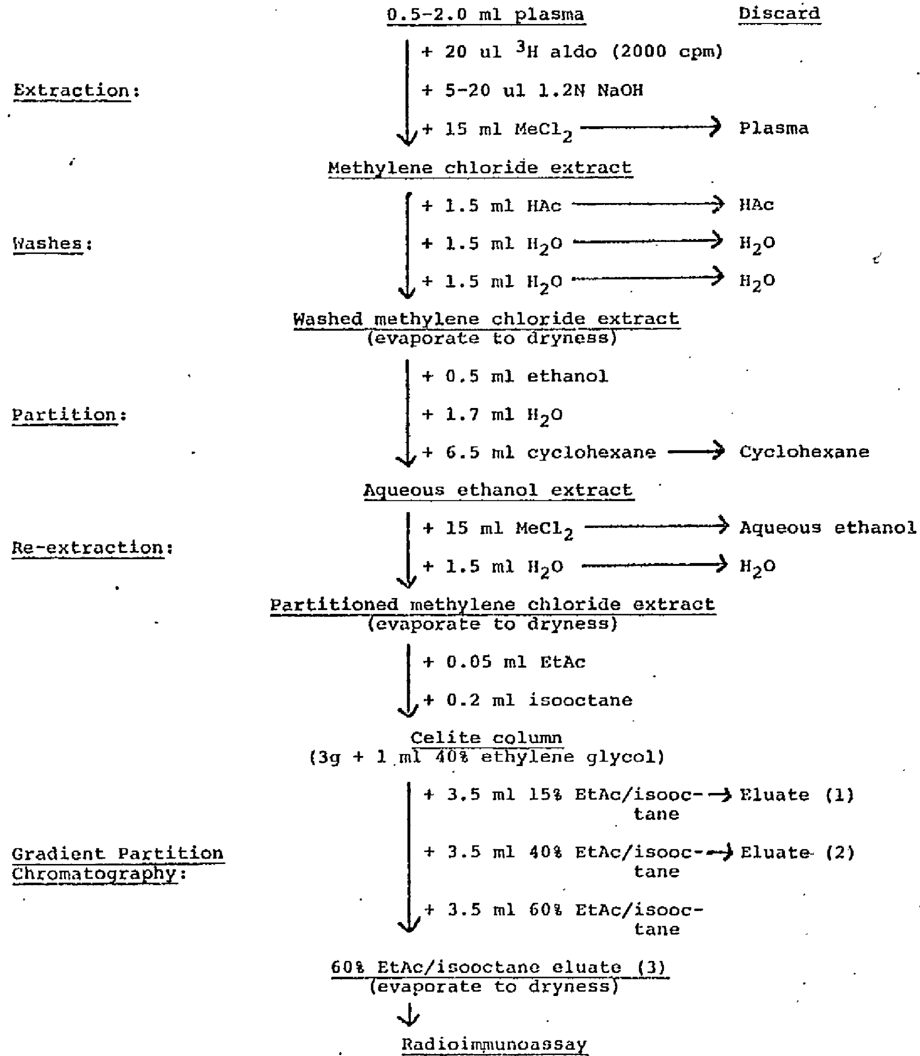
Purification of plasma by pre-extraction of cross-reacting steroids into methylene chloride is not possible. Instead, chromatography on a celite gradient partition column is used. To ensure consistent elution patterns of steroids from the column, lipids are first removed by partition between cyclohexane and aqueous alcohol.

For plasma, the number of counts and the amount of aldosterone in the tritiated aldosterone, added to detect losses in preliminary purification steps, must be taken into account in the calculations.

(i) Extraction and partition

Details of the method are illustrated in Table 14. Prior to extraction an educated guess is made of the aldosterone content of the unknown plasma. On the basis of this, either 0.5, 1, or 2 ml of plasma is extracted into methylene chloride. To minimize the formation of emulsions, the pH of plasma is increased prior to extraction by addition of sodium hydroxide and the methylene chloride is pre-chilled. The pH of the methylene chloride extract is returned to neutrality by acid and water washes and lipids are then separated by

TABLE 14



cyclohexane partition. Aldosterone is then re-extracted into methlene chloride which is evaporated to dryness prior to chromatography.

(ii) Column chromatography:

A single gradient partition column chromatography system is used which employs celite as an inert support. 40% ethylene glycol in water (stationary phase) is first mixed thoroughly with the celite. The mobile phase is ethylacetate/isooctane and the percent of ethylacetate is increased in three steps. Cross-reacting corticosteroids are washed from the column with 15% then 40% ethylacetate/isooctane and then aldosterone is eluted with 60% ethylacetate/isooctane.

The transfer of the extract onto the column is a crucial step affecting the overall recovery of the method. For optimum transfer it is necessary first to dissolve the extract in ethylacetate because aldosterone is poorly soluble in 15% ethylacetate/isooctane. The column is run under slight pressure to maintain an elution rate of about 10-15 drops per minute. Higher pressure causes the ethylene glycol to be washed off the column and this interferes with the radioimmunoassay step. The use of slight pressure on tightly packed columns helps to achieve uniformity of running rate which has the technical advantage that 20 columns can be run at one time without confusion.

The elution pattern of aldosterone relative to other steroids is consistently reproducible. Aldosterone is always eluted by 60% ethylacetate/isooctane and the other cross-

reacting steroids are consistently eluted by a lower percentage of ethylacetate. Alternate approaches to purification such as paper or thin layer chromatography require that a marker is run with each system. Since tiny amounts of steroids can be detected by radioimmunoassay, addition of steroids as markers inevitably leads to contamination and results in high blanks and inconsistent answers. Because of possible contamination, all glassware used in the assay, including the columns (5 ml disposable pipettes), is disposable. The reagents used in the purification steps do not interfere with the radioimmunoassay and thus the method is practically blank free.

An advantage of this particular chromatography system is that both corticosterone and hydrocortisone are eluted before aldosterone. In most other chromatography systems aldosterone is eluted between these two steroids and adequate purification from both in one system is difficult to achieve.

(iii) Radioimmunoassay

This step is similar to that used for urine except that 1/5 and 2/5 of the aldosterone eluted from the column are assayed. The number of counts in the plasma extract added to the assay are computed prior to calculation and are added to the total counts. Finally, the amount of aldosterone in the radioactive "tracer" added to detect losses is calculated and this value, approximately 20 pg, is subtracted from the final result.

A blank is run with each set, but the value is almost always below the lowest point of the standard curve and can be ignored.

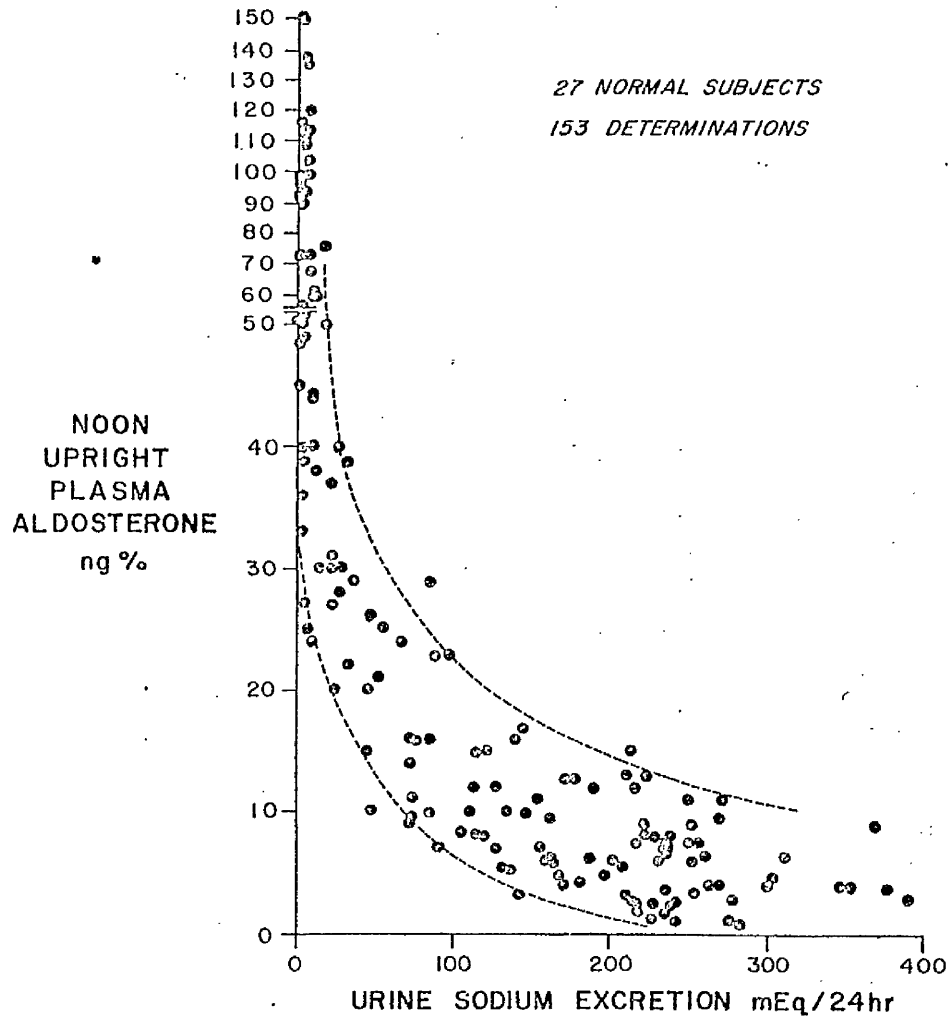
(iv) <u>Method sensitivity and relationship to urine aldosterone</u>

The use of column chromatography as a preliminary purification step combined with a highly specific antibody in the radioimmunoassay enables accurate detection of as little as 1 ng% aldosterone in plasma. No increase in the assay was found when other steroids such as hydrocortisone were added to plasma in concentrations as high as 100 µg%.

Plasma aldosterone concentration in normal subjects was found to change dynamically with the rate of urinary sodium excretion in a fashion similar to that observed for urine aldosterone excretion measurements (Figure 20). When the plasma aldosterone values from normal subjects were related to the concurrent 24-hour urine aldosterone excretion (Figure 21), a high degree of correlation was found between the two measurements (r=0.864). In general, when noon plasma aldosterone is 10 ng%, urine aldosterone excretion is approximately 10 µg/day and this increases proportionally so that when plasma aldosterone is 100 ng% urine aldosterone has increased to approximately 100 µg/day. Therefore, in normal subjects plasma aldosterone and urine aldosterone excretion change commensurately with changes in sodium balance. However, as indicated already, there are clinical situations in which urine aldosterone measurements do not accurately reflect the plasma level.

FIGURE 20

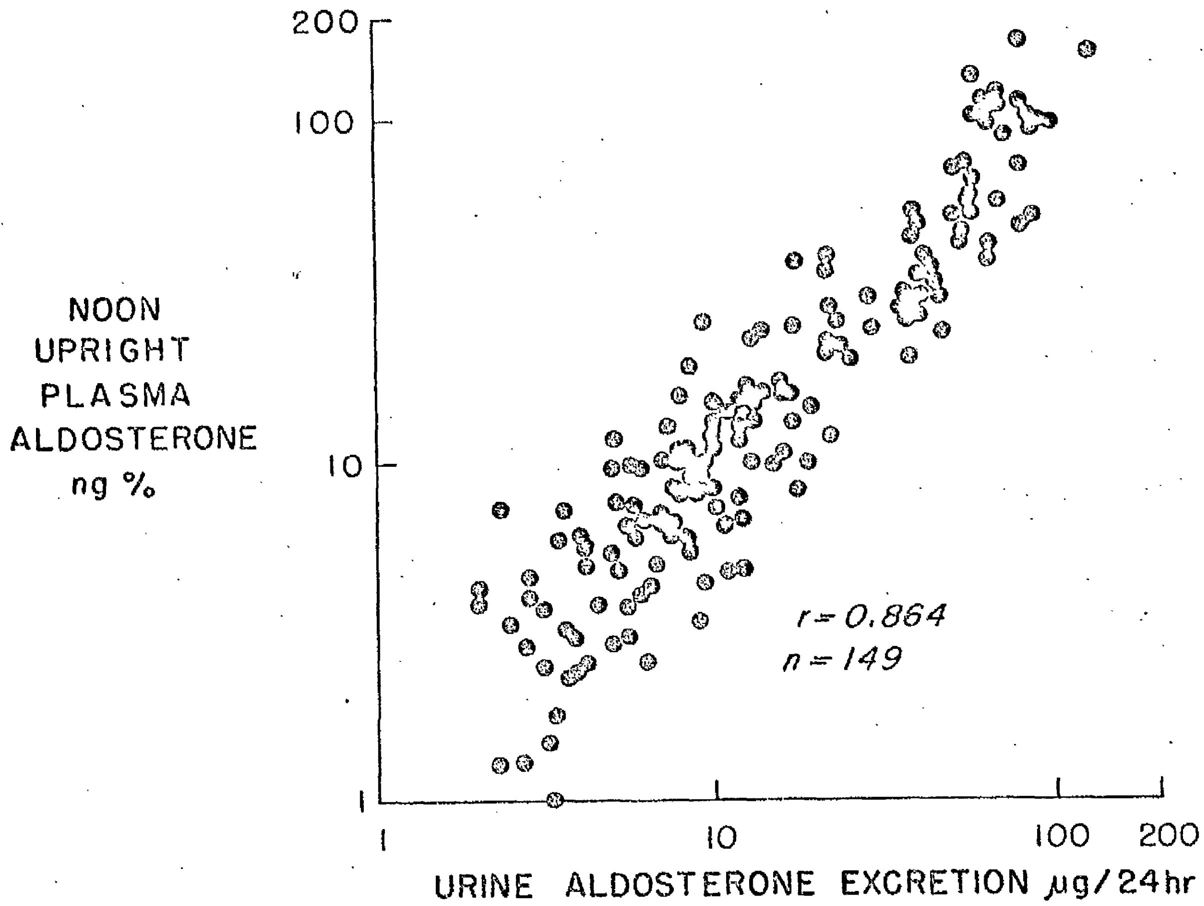
RELATIONSHIP OF PLASMA ALDOSTERONE TO URINE SODIUM EXCRETION IN NORMAL SUBJECTS



Hyperbolic relationship of plasma aldosterone to the concurrent 24 hour urine sodium excretion in 27 normal subjects. The relationship to sodium excretion is quite similar to that described by plasma renin activity and urine aldosterone excretion.

FIGURE 21

RELATIONSHIP OF PLASMA ALDOSTERONE TO URINE ALDOSTERONE IN NORMAL SUBJECTS



Linear relationship of urine aldosterone excretion to plasma aldosterone in normal subjects.

C. SUMMARY

A method has been presented for measurement of a urinary metabolite of aldosterone using a double isotope derivative assay. Tritiated aldosterone is added to urine and the specific activity of aldosterone in an isolated portion is used to calculate the urinary excretion rate. The amount of aldosterone in the isolated portion is quantitated by measuring the amount of ^{14}C incorporated into a diacetate derivative, which is prepared with ^{14}C acetic anhydride of known specific activity.

The method involves acid hydrolysis of the metabolite to form free aldosterone, then extraction and four chromatography steps in addition to preparation of aldosterone diacetate. The double isotope derivative assay is accurate but has the limitation of poor sensitivity, limited capacity, expense, as well as being time consuming.

During development of the method, isotope fractionation of ^{3}H from ^{14}C aldosterone diacetate was demonstrated in both paper and column chromatography systems. This is not a problem in the assay as long as wide cuts of the peaks are taken on paper chromatography and the peak is accurately identified on the final column system.

More recently, methods have been developed for quantitation of aldosterone in urine and plasma by radioimmunoassay. The methods are much simpler and more sensitive. However, preliminary purification from cross-reacting steroids is necessary prior to radioimmunoassay. For urine this is accomplished by extracting cross-reacting steroids into methylene chloride

prior to acid hydrolysis of the metabolite to liberate aldosterone. A celite partition column accomplishes the separation of plasma aldosterone from cross-reacting steroids.

Radioimmunoassay has made available simple and sensitive methods for detection of aldosterone in both urine and plasma. However, the potential for undetected errors is greater than in the double isotope derivative assay and strict quality control is essential.

III. Measurement of Natriuretic Hormone

These studies were designed to investigate whether or not there is a natriuretic substance involved in the regulation of sodium excretion (22-24). The possibility of such a hormone has been suspected since de Wardener and his associates (21, 123) showed that dogs could excrete a saline load under conditions involving no change in the parameters then known to be involved in the regulation of sodium excretion, i.e. glomerular filtration and aldosterone.

A meaningful assay for a natriuretic substance must involve changes in the rate of urine sodium excretion in an assay animal. Prior to our studies, other groups had suggested the existence of a natriuretic hormone by demonstrating activity assays which measured toad bladder or frog skin short-circuit current (124,125) and PAH uptake or sodium transport in kidney slices (126,127). However, in no instance was the suspected substance shown to be natriuretic in an assay animal. Hence our first approach was to develop a reliable assay in which changes in urine sodium excretion reflected changes in activity.

Concurrently, extraction concentration and purification procedures were investigated utilizing blood and urine from patients in whom a natriuretic hormone might be expected to be present in high concentration. Both urine and plasma were

studied in the hope that the substance, like aldosterone, might be excreted into the urine in large amounts. In fact this was found to occur and many of the physiological studies were carried out using urine extracts. The active material was separated from vasoactive peptides and the osmolarity of the injected extract was carefully monitored so as to rule out the possibility that natriuresis was induced by increases in arterial pressure or by osmotic diuresis.

Initially, urine and plasma from sodium loaded patients with primary aldosteronism were used as the source of potential activity. However, in later studies, sodium loaded normal subjects and sheep also provided active material.

Bioassay for Detection of Natriuretic Activity:

1. Type of Rats:

(a) Rats with congenital diabetes insipidus: (22).

Rats of the Brattleborough strain with diabetes insipidus were chosen since they cannot respond to injections of noxious extracts of urine or plasma with a sudden decrease in urine flow due to ADH release. In addition, in the basal state these animals have very high urine flows and adequate urine collections can be achieved using a bladder rather than ureteral catheter. Thus, surgery is minor and a stable and more physiological assay preparation is achieved. An additional advantage of the high urine flows was the ability to automate the urine collection system.

However, rats with fully developed diabetes insipidus were found to be sodium depleted and excreted dilute urine with low sodium concentration. Fortunately, heterozygous rats with diabetes insipidus were not as sodium depleted yet had high urine flows and rarely responded adversely to injection of the extracts. Therefore heterozygous rats with diabetes insipidus were routinely used as assay animals. In these animals urine flows approximating 200 ul/minute were achieved with rates of sodium excretion of from 0.5 to 4 uEq/minute.

(b) Normal rats: (23,24)

After extraction procedures had been developed, a few assays were performed in normal rats to demonstrate that the activity was not specific for a special type of rat or for rats in water diuresis. Two preparations were set up: (1) Normal hydropenic rats (ureteral catheters); sample injected intravenously. Urine flow = 3 ul/min., UNaV = 0.3 uEq/min. (2) Normal hydropenic rats; sample injected into the renal artery.

Although these assays were technically more difficult and less stable, natriuretic activity was easily demonstrable.

2. Assay Preparation: (22)

150 to 300 g rats were prepared by injecting 5 mg/100g body weight sodium pentobarbital and 0.13 mg/100 g atropine intraperitoneally. The bladder was catheterized through a small midline incision and one jugular vein was catheterized for injection of samples. A tracheostomy tube was inserted and carefully aspirated throughout the study since associated blood pressure changes caused by breathing disturbances could

markedly alter urine flow.

To induce a high rate of urine flow after surgery, the rat was given slowly by stomach tube up to 7 1/2% of its body weight of a dilute solution of sodium chloride (20 mEq/L). The rat was then placed on a balance located over a timed fraction collector. When urine flow reached approximately 100 µl/ min., 10 or 20 minute urine samples were collected whilst fluid lost was automatically replaced by oral administration of a 10 mEq/L solution chloride containing 18 mg/100 ml pentobarbital. With this system, in which the weight of the animal was kept constant, fluid balance and anesthesia were maintained constant throughout the day. The urine volume and sodium and potassium concentrations were measured for each 10 or 20 minute collection.

To prepare normal rats (23) 150-300 g female rats were anesthetized, tracheostomized and infused with isotonic saline (0.05 ml/min.) through a jugular vein catheter. The left kidney was ligated, but not removed, through a midline abdominal incision and a catheter was placed in the right ureter 2 cm below the renal pelvis so that ureteral peristalsis was not inhibited. The saline infusion was reduced to 0.02 ml/min. and 20 minute urine samples were collected under mineral oil. Extracts were injected into the jugular vein.

In a modification of this assay the aorta was occluded below the level of the right kidney and a catheter was placed in the aorta so that sample, when injected, passed in retro-

grade fashion directly into the kidney.

Ordinarily, from 2 to 4 hours elapsed before the first test sample was injected. This preparation time was for anesthesia, operating procedures, administration of the oral load, and establishment of a stable rate of urine sodium excretion.

A bolus of 0.5 ml of isotonic saline was routinely injected first. If an increase in sodium excretion occurred in response to this the animal was discarded. The unknown samples were given as either a bolus in 0.1 to 0.5 ml or occasionally as a constant infusion for 30 minutes at 0.02 ml/minute. Positive natriuretic responses were usually compared with the response to extracts from the same subject when not salt loaded.

The natriuretic response induced in the assay animal seemed related to the general health and preexisting state of salt balance of the assay animals. Insensitivity and an unsteady baseline could be caused by a fall in body temperature below 97°F, by too much anesthetic agent, or by occult bleeding.

Extraction and Purification Procedures

1. Preliminary Screening of Urine and Plasma for Natriuretic Activity: (22)

The objectives of the fractionation procedures were (a) to eliminate salts and small osmotically active or vaso-

active molecules, (b) to fractionate, according to molecular size, substances in plasma and urine and (c) to concentrate them as much as possible.

In initial studies, fractionation was carried out using a series of different gel filtration columns. 24-hour urines were lyophilized, reconstituted in 100 ml of 0.1 M acetic acid and centrifuged. The supernatant was desalted on a 5 x 30 cm column of polyacrylamide gel (Bio-Gel P2). 0.1 M acetic acid, was used as a buffer in all columns. Three fractions were collected; the first (about 400 ml), comprised substances of molecular weight greater than 200, except for those specifically retarded by the gel. The second fraction (about 800 ml) contained the salt peak which was discarded. A third fraction of about 1200 ml, collected after the salt peak, was lyophilized and prepared for assay.

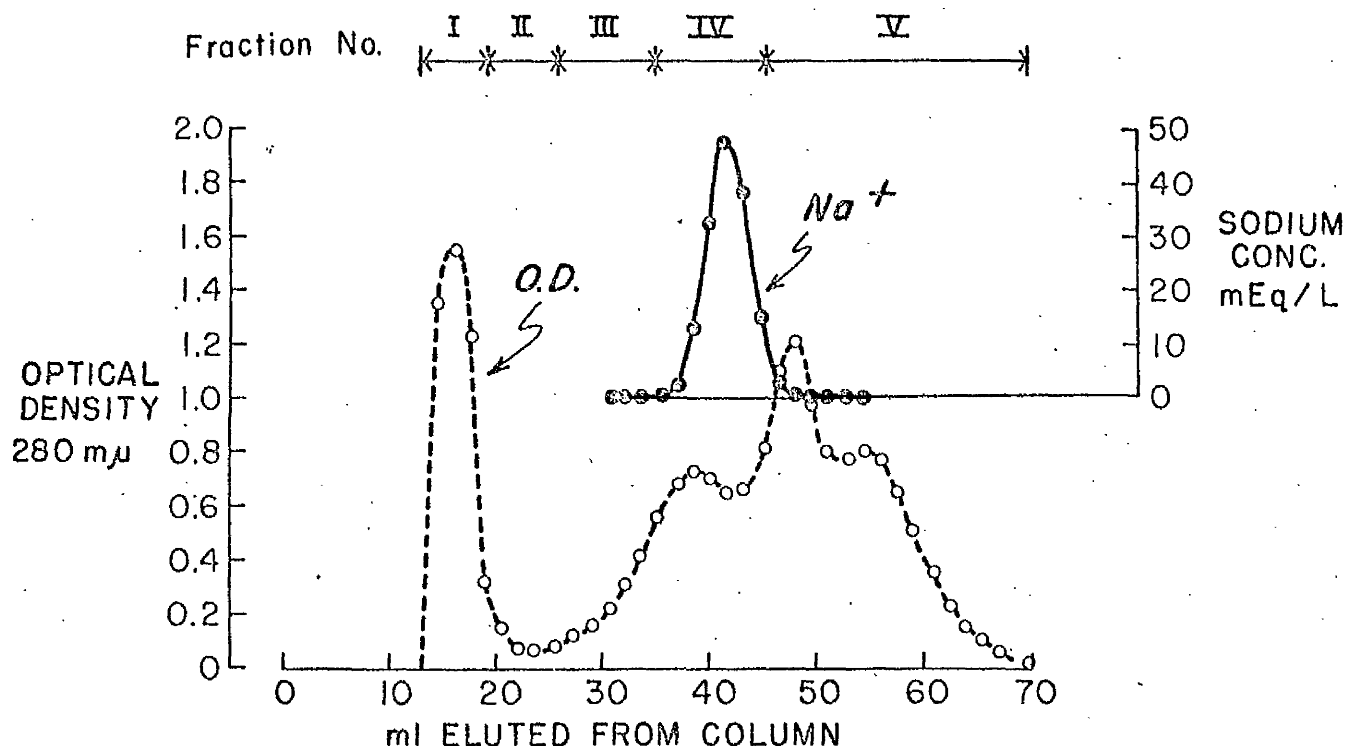
After lyophilization, the first fraction was dissolved in 24 ml buffer and 3 ml aliquots were applied to each of the following three 1.5 x 25 cm Sephadex columns: G25F, G75 and G100. The eluate from each of these columns was divided into five fractions. Fraction I contained substances completely excluded by the gel (Table 15). Fraction IV contained the salt peak and fraction V included substances eluted after the salt peak. Fractions II and III, collected between fractions I and IV, were arbitrarily divided as illustrated in Figure 22. 10 mg bovine serum albumin and 0.05 ml of 1 N mercaptoacetic acid were added to each frac-

TABLE 15

LOCATION OF ACTIVE FRACTIONS

Gel	Mol wt. fractionation range	Fraction No.				
		I, protein peak	II	III	IV, salt peak	V
P2	2,000-200	+?				-
G25F	5,000-1000	+++	-	-	-	-
G75	70,000-3000	++	++	-	-	-
G100	150,000-4000	-	+	++	-	-

FIGURE 22



Sheep urine, which had undergone preliminary purification on a Bio-Gel P2 column, was applied to a G75 Sephadex column (1.5 x 25 cm) using 0.1M acetic acid buffer. A plot is shown of the absorption at 280 mμ and the sodium concentration of fractions I to V.

tion prior to lyophilization and storage at -20°C. These two procedures reduced loss of activity during storage and they were routinely applied to all samples. Altogether 17 fractions were assayed (Table 15).

On the day of assay the samples were reconstituted in 0.3 ml of 0.45% saline. A 0.1 ml volume, representing the extract from 1 hr of urine, was injected into each of two assay animals. Positive assays were observed only in fraction I from G25F, in fractions I and II from G75, and in fraction III from G100 (Table 15).

Essentially the same fractionation procedures were employed for plasma samples. However, to remove plasma proteins the samples were first diluted, adjusted to pH 5.5, and placed in a boiling water bath for 20 minutes. This approach was used after it had been established that (a) boiled urine did not lose natriuretic activity and (b) the activity of positive plasma samples (deproteinized on Sephadex columns) remained after boiling.

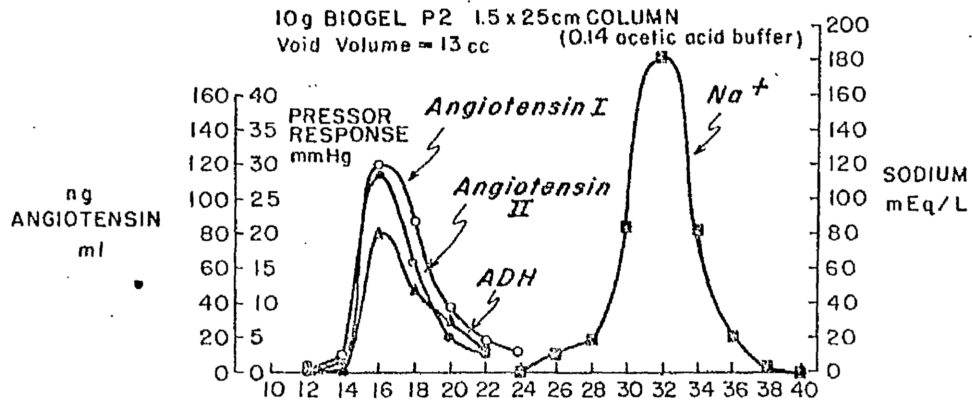
Those fractions were identified which contained the small pressor peptides angiotensin I, angiotensin II, and arginine vasopressin (Figure 23). They were found close to the salt peak on G25F and in the void volume on Bio-Gel P2. Clearly these peptides were not associated with any fraction which exhibited natriuretic activity.

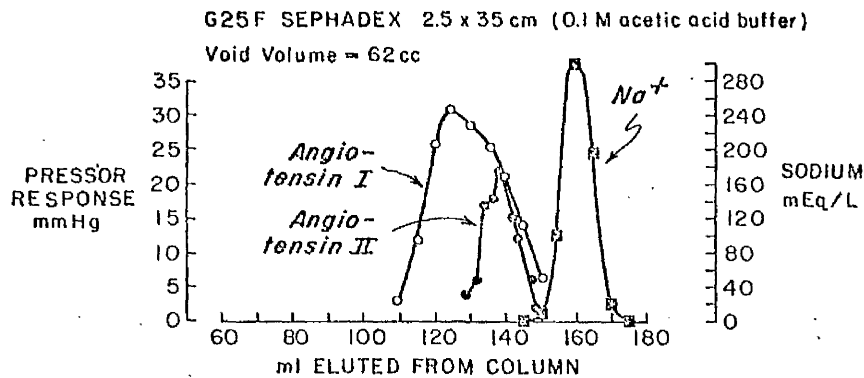
2. Simplified Procedure for Preparation of Urine and Plasma for Assay: (22)

24-hour urines were lyophilized, reconstituted in 100 ml

FIGURE 23

LOCATION OF VASOACTIVE PEPTIDES DURING FRACTIONATIONS





Location of vasoactive peptides on Bio-Gel P2 and on Sephadex G25F used for fractionation of urine and plasma samples. 750 ng angiotensin I and angiotensin II, dissolved in 2 ml of isotonic saline, were applied at different times to each column and their location was identified in a rat pressor bioassay. 10 pressor units of vasopressin were also applied to the Bio-Gel P2 column and subsequently located by pressor bioassay.

0.1 M acetic acid, and centrifuged. The supernatant was applied to a G 50M (medium mesh) column (2.5 x 60 cm). The fractions eluted before the salt peak were combined and lyophilized to a white fluffy powder. A quantity representing 2 hr of sample (assuming no losses) was reconstituted in 0.2 ml of 0.45% saline. A 0.1 ml volume of this was injected into the assay animal. The osmolarity of the final solution was repeatedly found to be in the region of 200 mOsm/liter.

3. Preparation of a Large Batch of Natriuretic Extract: (23)

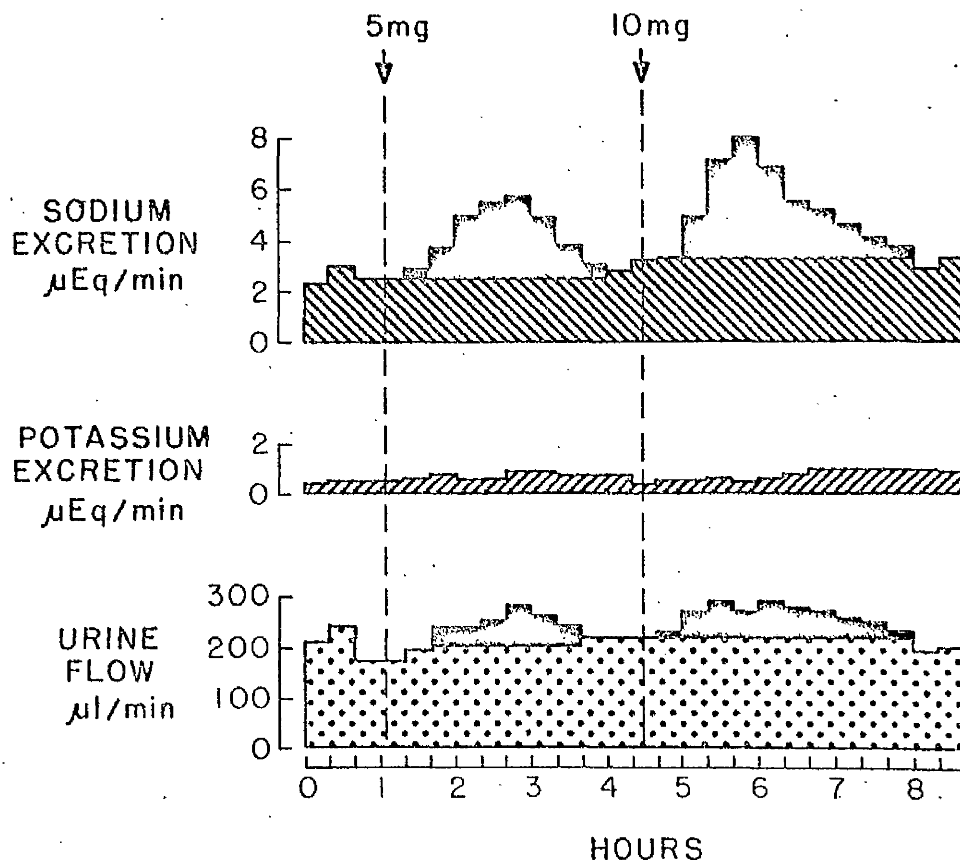
A large batch of natriuretic material was prepared using a preliminary ultrafiltration step. Fifty liters of urine were centrifuged for 20 minutes at 8000 rpm, and passed through 5-μ and then 0.45-μ Millipore filters. Subsequently, the urine was passed through an Amicon UM 10 filter. This latter filter theoretically holds back material of molecular weight greater than 10,000. The retentate was divided into 6 portions and applied to G-50 M and then G-100 Sephadex columns to accomplish the final desalting and purification.

Some Properties of the Natriuretic Extract: (17,22-24)

1. Typical Response in Assay Animal

The assays illustrated in Figure 24 were carried out in a heterozygous rat with diabetes insipidus. Sensitivity was greatest in animals excreting somewhat more than 0.5 μEq sodium per minute. While this is a high rate of sodium excretion for such a small animal, their endogenous natriuretic

FIGURE 24



5 and 10 mg of urine extract derived from a sodium loaded subject were dissolved in 0.1 ml 0.45% saline and injected into the same heterozygous rat with diabetes insipidus. Urine sodium excretion increased in response to both extracts. The higher dose gave the greater response. In these assays, urine flow increased slightly but potassium excretion was constant.

stimulus may not be high since volume expansion was achieved with water loading rather than sodium loading.

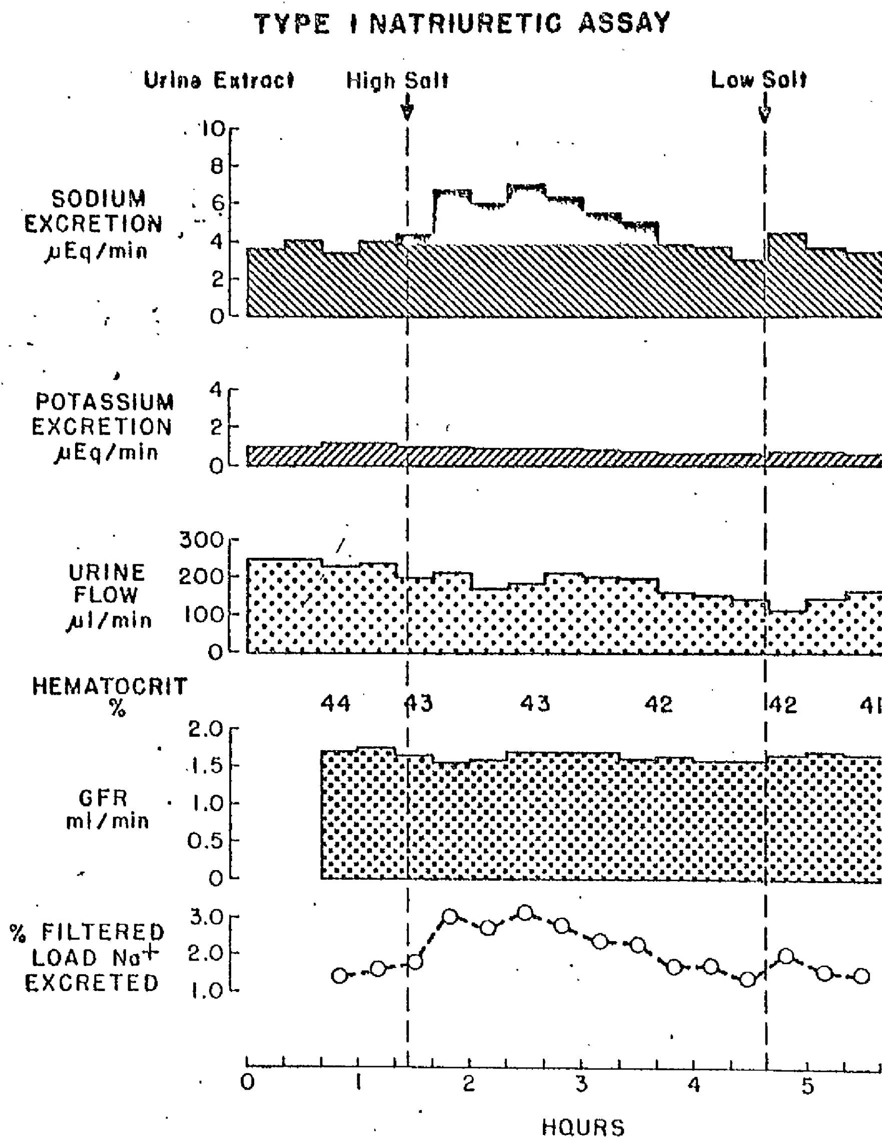
In these particular assays (Figure 24) sodium excretion increased from 2.5 to 5.5 μEq/minute in response to 5 mg of urine extract and from 3 to 7.5 μEq/minute in response to 10 mg of urine extract. Hence, there was a relationship between dose and response in the assay animal. Some of the positive responses involved the loss of more than 500 μEq i.e., more than 5% of the total body sodium content. No change in glomerular filtration rate occurred in response to the natriuretic extract (Figure 25) so that in some assays 5-10% of the filtered load was excreted.

All positive assays were associated with marked increases in urine sodium concentration and changes in urine flow were usually small or absent. Furthermore, there was no apparent association between the magnitude of the natriuresis and changes in urine flow.

In a few assays there were slight increases in potassium excretion, but most were similar to the ones illustrated in Figure 24 in which no change in potassium excretion was observed. The physiological implications of the lack of change in urine flow and potassium excretion are considered in a later section.

The occasionally observed delay in onset of natriuretic activity and its rather prolonged effect for up to 3 hours suggest that another active substance might be released

FIGURE 25



Extracts from 2 pools of urine derived from normal subjects on either high or low sodium intakes were injected into the jugular vein of a rat with mild diabetes insipidus. Natriuresis was induced only by the high salt extract and was observed to be independent of changes in potassium excretion, urine flow, hematocrit, or glomerular filtration rate (GFR).

from the large molecular weight substance we have detected. Alternatively, the hormonal agent itself, like aldosterone, may first enter cells and induce secondary changes.

Characteristics of the natriuretic response were quite similar, although less striking, in normal hydropenic rats. When samples were injected directly into the renal vein an immediate natriuretic response was always observed, followed by the more characteristic delayed natriuresis. However, the immediate response was always associated with an increase in blood pressure and was probably not due to an effect on tubular sodium transport mechanisms. The increase in blood pressure was only evident when the samples were injected directly into the kidney.

2. Specificity of the Natriuretic Response:

There was no response in the assay animal to injections of similar volumes of isotonic saline (0.1-0.5 ml) or to injections of extracts which were derived from subjects who were either not sodium loaded or who had been sodium deprived for 3 to 5 days. In addition, at the end of natriuresis, the rate of sodium excretion consistently returned to baseline.

3. Biochemical Characteristics:

On the basis of elution characteristics on several Sephadex columns and retention of the natriuretic activity by an Amicon UM 10 filter, the molecular weight of the substance appears to be above 10,000 and may be as high as 50,000. This large size is compatible with the observation

that the activity is non-dialysable.

The substance may be a protein since activity was destroyed by trypsin and by treatment with trichloracetic acid. However, it was not destroyed by boiling.

Further characterization of the biochemical properties is required but may have to be delayed until a simpler assay is developed which allows assay of several samples in the same animal. The limiting feature of the investigation is the fact that rarely can more than two samples be assayed accurately in the same rat and two assays can take more than 12 hours to complete.

Summary. These studies were designed to examine the question of whether or not there is a natriuretic humoral substance which participates in the regulation of urine sodium excretion.

Procedures for concentration and fractionation of plasma and urine were developed concurrent with a sensitive bioassay for detecting induced changes in renal sodium excretion. The assay utilized rats with mild diabetes insipidus which were maintained in sodium and water balance.

A natriuretic humoral substance was demonstrated in plasma and urine from salt-loaded normal man and sheep, and in patients with primary aldosteronism and essential hypertension who were maintained on a high sodium intake.

The substance appears to have physiological relevance

since it was not detectable in sodium-depleted subjects and it consistently appeared in sodium-loaded subjects.

The natriuretic activity was demonstrable in normal hydropenic rats as well as in rats with congenital diabetes insipidus. The induced natriuresis usually develops slowly and may last for several hours. The natriuresis is not usually associated with increases in water or potassium excretion. It occurs without measurable changes in glomerular filtration rate or arterial blood pressure. Altogether these results suggest that the substance acts at least in part to depress sodium reabsorption in the distal tubule at a site where sodium is normally reabsorbed without water.

Fractionation studies suggest that the natriuretic substance has a molecular weight of from 10,000 to 50,000. Further study will be necessary to determine whether or not this particular substance proves to be a physiologically important natriuretic hormone. However, of special relevance is its action to depress distal tubular sodium reabsorption because a large body of evidence indicates that depression of proximal tubular sodium reabsorption is not, by itself, sufficient to cause a sodium diuresis (128-130) [See page 183].

PART II

PHYSIOLOGY OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

In the preceding sections, methods have been presented for measurement of the components of the renin-angiotensin-aldosterone hormonal system and for detection of natriuretic factors in urine and plasma. The following analysis will be concerned with the role of this hormonal system in regulation of sodium and potassium balance and blood pressure homeostasis.

In general, the hormonal system appears to act as two, almost independent negative feed back control systems which regulate (1) sodium balance and blood pressure and (2) sodium and potassium homeostasis. The first system responds to reductions in arterial pressure, renal perfusion pressure or blood volume. When any of these events occur, renin secretion is increased and arterial pressure is restored both by the vasoconstrictor action of angiotensin II and by aldosterone induced sodium retention which increases tissue perfusion and turns off the signal for renin release. The second control system regulates potassium balance. An increase in plasma potassium directly stimulates adrenal aldosterone secretion. Increased circulating aldosterone promotes renal potassium loss which returns potassium balance to normal and thus turns off the stimulus for increased aldosterone biosynthesis. However, since changes in sodium balance affect both of these arbitrarily separated systems, in the following memoir the hormonal system will be analysed with respect to sodium balance and blood pressure homeostasis on the one hand

and sodium balance and potassium balance on the other hand.

Before turning to the operation of the system and its function in regulating electrolyte balance and blood pressure, the responses and interactions of the hormonal components will be examined in more detail.

A. Renin Secretion and Its Relation to Plasma Renin Activity

I. Determinants of Plasma Renin Activity

Renin occupies a key role in the hormonal system which regulates blood pressure and electrolyte homeostasis (Figure 1) (page 7). When, as a result of such events as sodium depletion, hemorrhage, transudation or alimentary loss, effective blood volume contracts and arterial pressure falls, the kidney's perfusion is threatened and it reacts by secreting renin into the blood stream. This initiates the process whereby the other hormonal components of the system are activated and work to restore equilibrium.

The kidney is the major source of plasma renin for both man and animals (131-133). Renin has also been found in other tissues such as the submaxillary gland, placenta and uterus (134-136). However, there is no evidence that plasma renin is ordinarily derived from any of these other sources.

The nature of the renal sensor device which transmits the signal to the renin containing juxtaglomerular cells for the release of renin is the subject of some controversy (5). Postulated mediators include [1] a baroreceptor or stretch receptor in the afferent arteriole which responds to changes in arterial pressure (137) and [2] changes in the amount of sodium passing the macula densa area of the loop of Henle (138). Both the macula densa and the afferent arterioles are

in physical contact with the juxtaglomerular cells and both could transmit messages to these cells to release renin. (3) Circulating catecholamines and neural stimulation have also been shown to affect renin secretion (139,140) and it is likely that each of these mechanisms is important. In fact it is possible that they all act through a final common pathway.

Renin, released into the blood, acts on plasma renin substrate to generate angiotensin I which is converted to angiotensin II during passage through the lungs (Figure 1, page 7) Since plasma renin substrate concentration is normally rate limiting (15,59,141), the rate of angiotensin generation in response to secreted renin could vary depending on the concentration of circulating substrate.

Plasma Renin Substrate in Normal and Hypertensive Man:

To study whether or not differences in plasma renin substrate can be primary determinants of plasma renin activity, plasma renin substrate was related to the concurrent measurement of plasma renin activity in 28 samples collected from 19 normal subjects in whom urine sodium excretion was above 40 mEq per day (6). Mean plasma renin activity in 14 samples with substrate concentration less than an arbitrary cut off point of 1450 ng/ml (range 919-1447) was 2.6 ng/ml/hr. $\pm$ 1.32 (SD). In 14 samples in which plasma renin substrate concentration was greater than 1450 ng/ml (range 1463-2228)

mean plasma renin activity was almost identical, 2.7 ng/ml/ hr. ± 1.52 (SD). Thus differences in renin substrate do not appear to be physiological determinants of plasma renin activity. Renin appears to be the dynamic component of the renin system which reacts to changes in renin substrate to maintain an appropriate rate of angiotensin generation.

In another study of patients who were taking oral contraceptive medication and in whom renin substrate rose progressively during the first few weeks of medication, the initial increase in renin substrate was at first accompanied by a rise in plasma renin activity (62). However, this increase was not sustained in most patients, and plasma renin activity gradually returned to normal levels (Figure 11, p. 48).

Both of these studies support the view that when substrate concentration varies, renin secretion feeds back to maintain the rate of angiotensin generation.

Renin substrate concentrations in normal and hypertensive patients, measured by radioimmunoassay, are presented in Table 16 (6,16). There was no significant difference in renin substrate from any of the sources. The slightly higher mean substrate concentrations in patients with essential hypertension is in agreement with the observations by Gould and Green (142) that males with essential hypertension have higher substrate levels than normotensive males. The absence of statistical significance in the present study may be explained by the failure to discriminate between males and

TABLE 16

PLASMA RENIN SUBSTRATE IN NORMAL AND HYPERTENSIVE PATIENTS

	RENIN SUBSTRATE ng/ml	(SD)
NORMAL SUBJECTS (N=19)	1517	346
ESSENTIAL HYPERTENSION		
High Renin (N=9)	1670	318
Normal Renin (N=23)	1644	364
Low Renin (N=18)	1855	348
RENOVASCULAR HYPERTENSION (N=5)		
Artery	1690	194
Vena Cava*	1739	252
Right Renal Vein	1688	239
Left Renal Vein	1668	318

* Blood collected from vena cava below level of kidneys

females in the analysis.

In a study of normal subjects on different sodium intakes, there was no consistent relationship between sodium intake and plasma renin substrate concentration (6). This is in contrast with the report of Rosset and Veyrat (143) in which substrate concentration was inversely related to plasma renin activity. In addition, in our studies there was no difference in the concentration of renin substrate in patients with essential hypertension who had high, normal or low plasma renin activity (Table 16). In animal studies it has been found that infusion of renin or angiotensin II at low doses causes an increase in substrate concentration but at higher doses of renin, substrate concentration falls in plasma, probably because of excess utilization (144,145).

When renin substrate was measured in plasma collected from the renal veins, aorta and vena cava of five patients with renovascular hypertension, no differences in mean values were found (Table 16)(16). Hence, in this study there is no evidence for clearance of renin substrate by the kidneys.

Altogether, the data indicate that although in normal subjects and patients with essential hypertension, plasma renin substrate concentrations in different individuals can span a two-fold range, mean values are quite similar in the different groups. Physiologically, changes in renin substrate are not normally determinants of plasma renin activity. However, since measurements of plasma renin activity depend on a first order

reaction, acute changes in substrate concentration could markedly affect the rate of angiotensin generation and thus could transiently affect plasma renin activity until renin secretion adjusts (Figure 11).

Studies of the Metabolic Clearance Rate of Renin

Peripheral plasma renin levels are determined by both the secretion rate of renin and the metabolic clearance rate. Thus, under steady state conditions, the metabolic clearance rate of renin is equal to renal renin secretion. It has been shown by Haecox and associates that there is little, if any, clearance of renin by the kidneys (146). Most available data point to the liver as the prime route of metabolism of renin (146-150). Schneider and co-workers and others have shown in the dog that, in general, clearance of renin by the liver is proportional to blood flow through the liver (148-150). Thus, changes in liver blood flow exert an important modulating effect on plasma renin levels. However, there is no evidence that alterations in liver blood flow ordinarily exert a rate limiting influence on plasma renin levels since increases in plasma renin activity caused by reductions in the metabolic clearance rate are normally compensated for by suppression of renal renin secretion (150).

An unanswered question regarding the metabolic clearance rate of renin is whether or not a constant fraction of plasma is cleared over a wide range of plasma renin activity.

Analysis of studies of metabolic clearance rate in dogs (150) suggests this possibility. However in these studies renin secretion and liver blood flow both changed at the same time and it was not possible to determine from this data whether changes in renin secretion per se had an effect on the metabolic clearance rate.

Under steady state conditions the clearance rate of renin must equal the secretion rate. If the liver always clears a constant fraction of the plasma renin, then plasma renin would be a direct reflection of renal renin secretion and under steady state conditions peripheral plasma renin would have a constant direct relationship to renal renin secretion. However, if the fraction of renin cleared by the liver were shown to fluctuate with changes in renin secretion then the relationship of renin secretion to peripheral renin would not be constant.

Renin secretion can be calculated from the product of renal plasma flow and the increment in renin added to the renal vein (V-A) (where V = renal vein renin and A = arterial renin). Thus, under conditions of constant renal plasma flow, (V-A) is directly proportional to renin secretion (151). If a constant fraction of plasma is cleared of renin then, under steady state conditions, (V-A) in relation to peripheral renin (A) should be constant. Hence measurement of renin in simultaneously collected arterial and renal venous blood could provide information about the

metabolic clearance rate of renin.

Patients with essential hypertension who had no evidence of liver or renal disease were chosen for the study (16) since these patients as a group normally exhibit a wide range in plasma renin activity (88). Thus a wide spectrum of peripheral plasma renin values were obtained under conditions in which both liver and renal blood flows would be expected to be normal. Many patients with essential hypertension come to arteriography because of suspicion of renovascular hypertension. All patients with hypertension who underwent arteriography were studied under controlled conditions and those who had no evidence of renovascular disease and who were classified as having essential hypertension form the basis for this study.

Renin activity and renin substrate were measured in four sources of plasma from 43 patients with essential hypertension. Blood was collected from the right and left renal veins, the vena cava (below the level of the kidneys) and the aorta. The study was carried out under steady state conditions, after the patients had been on a constant diet for a minimum of three days and after they had been in the supine position for at least two hours.

The patients were subdivided into three groups on the basis of their peripheral plasma renin activity, considered in relation to the concurrent 24-hour urine sodium excretion. Eleven patients were classified as low, 28 as normal and four

had high plasma renin activity. This spectrum of plasma renin activity is fairly typical of the population of patients with essential hypertension(88).

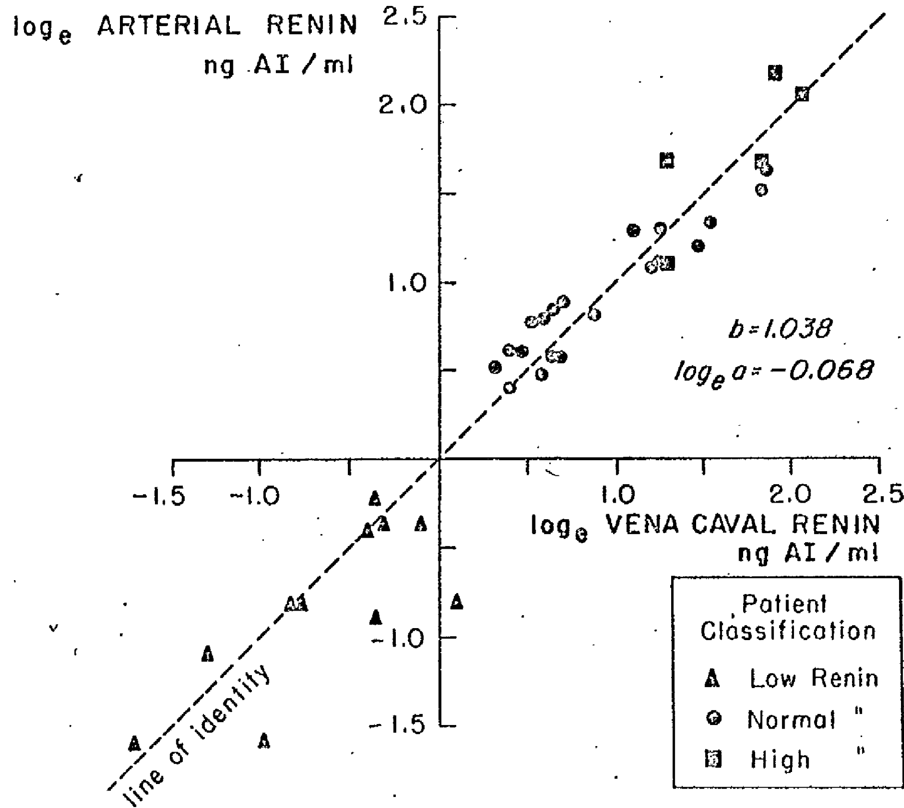
Renin substrate was normal and similar in the four sources of plasma at all levels of peripheral plasma renin activity. Hence it can be assumed that differences in plasma renin activity reflect differences in plasma renin concentration.

Plasma renin activity in blood collected from the aorta was similar to that found in blood collected from the vena cava (Figure 26). The slope and intercept of the line which best fit the relationship of arterial to caval plasma renin activity did not differ from the line of identity. This supports the view that plasma renin is derived exclusively from the kidney and removed exclusively by the liver. Pragmatically, it means that venous and arterial plasma renin values can be used interchangeably and they will be designated as (A) in the following analysis. (V) will represent renal vein renin values.

Plasma renin activity was similar in blood collected from the right or left renal vein (Figure 27) which suggests that there is no consistent physiological difference in the secretory capacity of the right and left kidneys. However, as expected, renal vein renin was almost invariably higher than arterial renin (Figure 28).

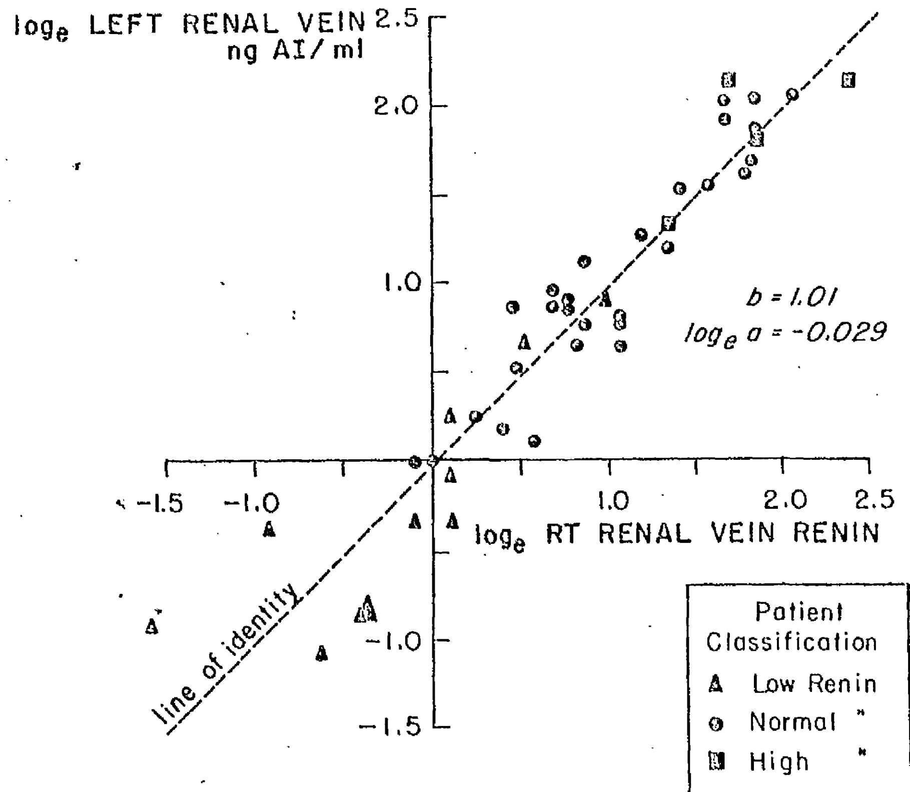
The key observation of the study was that the increment

FIGURE 26



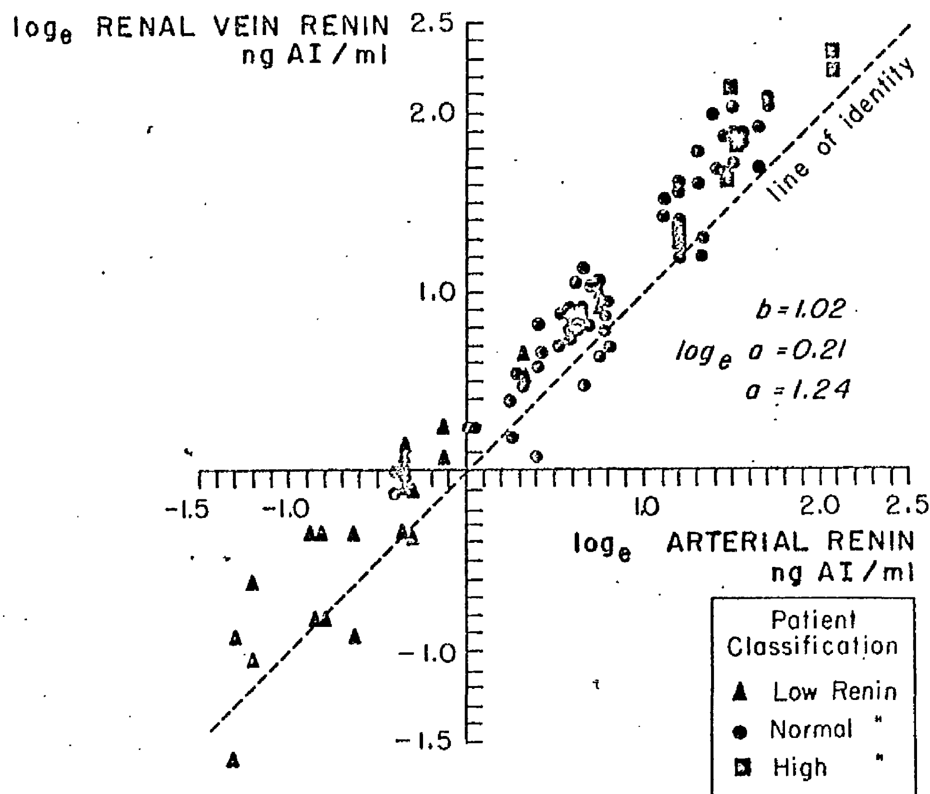
Relationship of renin activity in blood collected from the aorta to that found in blood collected from the vena cava, below the kidneys, of patients with essential hypertension. The line which best fits the points is not significantly different from the line of identity. This supports the view that venous and arterial plasma renin activity are identical.

FIGURE 27



Relationship of renin activity in blood from the right renal vein to that from the left renal vein of 43 patients with essential hypertension. The line expressing the relationship is not different from the line of identity and the intercept is not significantly different from zero. Therefore, renal vein renin in the right renal vein is similar to that found in the left renal vein of patients with essential hypertension.

FIGURE 28



Relationship of renal vein renin to arterial (or venous) renin in patients with essential hypertension. The slope of the line is not different from the line of identity, indicating that the relationship of renal vein renin to arterial renin is constant at all levels of plasma renin found in essential hypertension. The intercept of 1.24 indicates that the renal vein renin in patients with essential hypertension is normally 124% of arterial renin

of renin added to the renal vein (V-A) is a constant proportion of the arterial renin at all levels of plasma renin activity found in essential hypertension. The slope of the line which best fit the relationship of renal vein renin to arterial renin was a line parallel to the line of identity, with a significantly positive intercept. In this group of patients, renal vein renin activity was 124% of peripheral plasma renin over a wide range of plasma renin activity (Figure 28). This value is similar to that reported by Brown and co-workers (125%) for samples with plasma renin concentration that were not abnormally high (152).

The constancy of this relationship suggests that under steady state conditions the metabolic clearance rate of renin is always proportional to the plasma concentration of renin. In other words, the fraction of blood that is cleared of renin by the liver is constant irrespective of the blood level of renin. Thus, in essential hypertension, peripheral plasma renin is directly proportional to renal renin secretion, i.e., renin secretion = (arterial renin) x constant.

II. Practical Applications of Differential Renin Measurements:

(i) Renin Secretion Rate: In patients with presumably normal renal blood flows the renal vein renin increment (V-A) was shown to be consistently 24% of the peripheral level (A). Thus, renal vein renin is equal to A + 0.24A.

Renin secretion can be calculated from the product of renal plasma flow and the renal vein renin increment (V-A). (V-A) is equal to 0.24A (Table 17) and renal plasma flow in patients with essential hypertension can be presumed to be normal and equal to roughly 300 ml/minute from each kidney. Therefore, as illustrated in Table 17, renin secretion = 144A.

Thus, under steady state conditions similar to those of the present study, renal renin secretion per minute is approximately 144 times peripheral plasma renin.

Confirmation of this formula can be found in a study of renin secretion by Kaneko and co-workers (153) in which renal plasma flow was measured in patients with essential hypertension. From their data renin secretion per minute was calculated to be 136 times peripheral plasma renin. Thus, calculated and measured values are quite similar.

(ii) Renal Plasma Flow: Variations in renal plasma flow must influence the renal vein renin increment (V-A) since reductions in flow would result in less dilution of secreted renin by renal vein blood and (V-A) would increase (16,151). Since,

$$\text{Renin secretion} = \text{renal plasma flow} \times (V-A)$$

$$\text{Renal plasma flow} = \text{renin secretion}/(V-A) = \frac{144A}{(V-A)} = \frac{144}{(V-A)/A}$$

Thus renal plasma flow is inversely related to (V-A)/A.

However peripheral renin is normally derived from two kidneys.

TABLE 17

RELATIONSHIP OF RENIN SECRETION TO PERIPHERAL PLASMA RENIN

V-A = Renal vein renin increment

V-A/A = V-A expressed as fraction of peripheral renin

V-A/A = 0.24 when renal and liver blood flows are normal

V-A = 0.24A

Renin secretion = renal plasma flow x (V-A)

= 600 x 0.24A when renal plasma flow is normal

= 144A

Renin secretion per minute = 144 times peripheral plasma renin

i.e. Renin secretion = $RPF_1(V_1-A) + RPF_2(V_2-A)$

But, renin secretion = 144A

Therefore $144A = RPF_1(V_1-A) + RPF_2(V_2-A)$

Rearranging: $RPF_1 = \frac{144}{(V_1-A)/A} - RPF_2\frac{(V_2-A)}{(V_1-A)}$

This equation provides a basis for estimating renal plasma flow from each kidney. However, since there are two unknowns in the equation (RPF_1 and RPF_2) absolute determination of renal plasma flow from each kidney can only be calculated from two different sets of data, preferably collected from two different physiological settings (e.g., sodium depletion and sodium loading). However, since one of the components of the right hand side of the equation has negative value, a maximum value for renal plasma flow from each side can be estimated from one set of measurements.

When $(V_1-A)/A$ is greater than 0.48, the value for $144/(V_1-A)/A$ must be less than 300 and renal plasma flow to that kidney must be less than 300 ml/minute. Therefore, irrespective of the renin secretion and renal plasma flow from the contralateral kidney, a value of (V-A)/A greater than 0.48 from either kidney is always associated with reduced plasma flow.

This formula has an important application in the study of patients with renovascular hypertension. This will be discussed in detail in a later section (page). However, it is relevant to point out that in classic Goldblatt hypertension, reduction in blood flow to one kidney is associated

with hyper-secretion of renin from that kidney and complete suppression of renin secretion from the contralateral kidney. Under these circumstances, $(V_2\text{-}A) = 0$) and the right hand side of the equation for calculation of renal plasma flow is reduced to zero, then, renal plasma flow from the sole secreting kidney can be calculated with precision. It follows that the closer $(V_2\text{-}A)$ is to zero from the uninvolved kidney, i.e., the lower the secretion rate of renin, the more accurate the estimation of renal plasma flow from the suspect kidney.

In a companion study of 28 patients with suspected unilateral renovascular hypertension who came to surgery, the combination of the following three indicators (28) consistently predicted curability: (1) Reduction in renal plasma flow to the suspected kidney ($V\text{-}A/A > 0.48$); (2) suppression of renin from the unaffected kidney ($V\text{-}A/A < 0.38$) and (3) elevated peripheral plasma renin activity (i.e., increased secretion of renin). Thus increased understanding of the determinants of plasma renin concentrations in various blood vessels has proved useful in evaluating the presence or absence of curable renovascular hypertension.

III. Summary

Plasma renin activity appears to be regulated to sustain an appropriate rate of angiotensin production. Although changes in both renin substrate and the metabolic clearance rate of renin can affect plasma renin activity, available

evidence suggests that renal renin secretion is the dynamic component of the system which responds to physiological stimuli. Thus renal renin secretion appears to be that component of the system which responds to stimuli which reflect a need for changes in angiotensin production. In addition, we have shown that in patients with essential hypertension plasma renin activity directly reflects renal renin secretion.

The constant relationship of plasma renin activity to renal renin secretion has been used to evaluate changes in renal plasma flow. In patients with renovascular hypertension who have unilateral secretion of renin, increases in renal vein renin in relation to arterial renin are inversely related to reductions in renal blood flow. When bilateral renin secretion occurs, a renal vein renin increment greater than 148% arterial renin is always associated with renal plasma flows of less than the normal value of 300 ml/minute.

B. Renin and Aldosterone and Their Interrelationship in Response to Changes in Sodium and Potassium Balance:

In the previous section data were presented to support the view that plasma renin activity is directly related to renal renin secretion so that changes in plasma renin activity can be used as an indicator of changes in the secretion rate of renin. Since angiotensin II is the effector hormone of the renin limb of the hormonal system, its blood level would be expected to be the most meaningful indicator of changes in renin secretory activity. However, many methods for measurement of angiotensin II are not sufficiently sensitive to detect low levels of the hormone with confidence. Plasma renin activity measurements can be used instead since all evidence suggests that angiotensin I generation, i.e. plasma renin activity, reflects angiotensin II levels (154), there being no evidence that lung converting enzyme activity is ever rate limiting.

In this section, the responses of renin and aldosterone to extreme changes in sodium and potassium balance will be evaluated in normal man and in rats. Although it has been generally accepted that renin and aldosterone are important for regulation of sodium excretion at low and normal sodium intakes, the role of the hormonal system in regulating salt balance at high levels of sodium intake has not been clarified. In addition, some investigators have recently

postulated that angiotensin II may not be the sole mediator of the increased aldosterone biosynthesis which occurs in response to sodium deprivation (155-157). In the following presentation new data will be analysed to determine if there is a need to postulate another trophic stimulus for aldosterone, in addition to plasma potassium and angiotensin II.

I. Effect of Variations in Sodium Intake on Renin and Aldosterone

The well known relationship of plasma renin activity to variations in sodium intake is illustrated in Figure 10 (page) (2,6,158,159). These data are derived from a study of 52 normal subjects, most of whom were maintained on constant dietary regimen. The 24-hour urinary sodium excretion, plotted on the abscissa, is taken as an index of the state of sodium balance. Measurements were usually made after the subjects had been on a constant diet for five days. In some subjects, however, daily analyses were made, beginning on the first day of the constant regimen and continuing for up to eleven days.

The plot confirms that plasma renin activity moves dynamically in relation to the rate of urinary sodium excretion. Thus plasma renin activity increases with sodium deprivation and, conversely, falls to a minimum when salt is present in excess.

As illustrated in Figures 18 (page 88) and 20 (page 105),

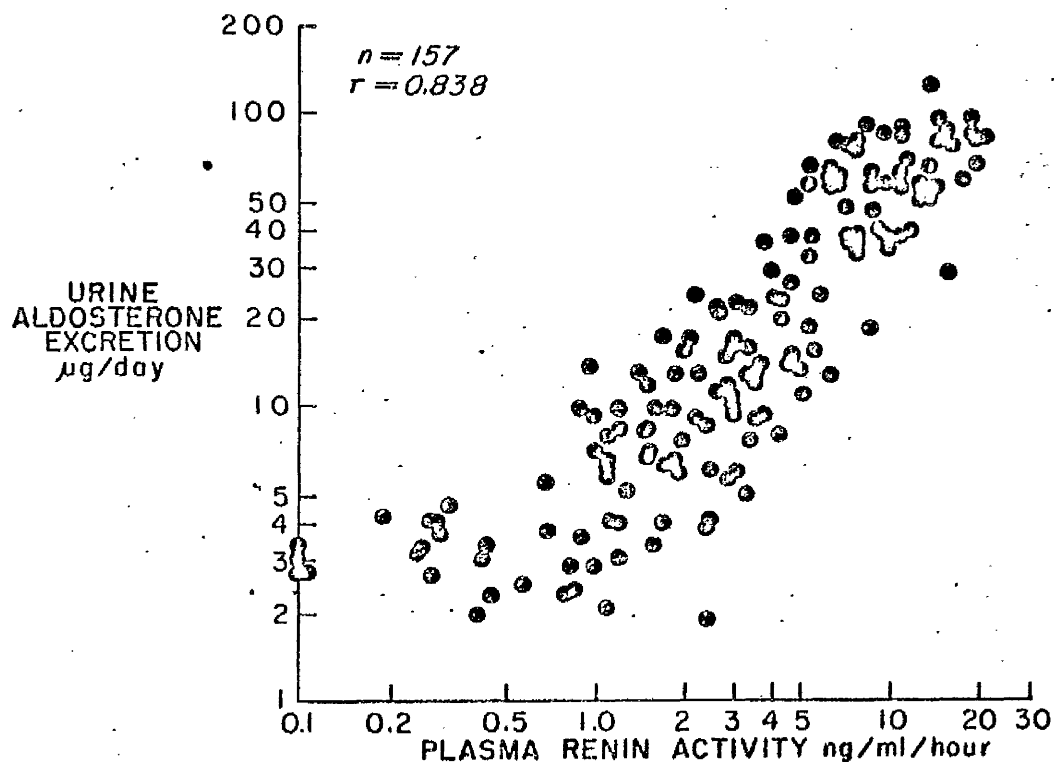
urine aldosterone and plasma aldosterone exhibit an entirely similar relationship to changes in sodium balance (2,9,11). This provides strong circumstantial evidence that the aldosterone secretory response to changes in sodium balance is mediated via changes in renin activity. The greater spread in plasma renin values compared to aldosterone measurements may be explained by the more rapid response of renin secretion to various stimuli. Large amounts of renin are stored in the kidney (30) and can be released rapidly (160), whereas angiotensin II stimulates aldosterone biosynthesis (161) and this slower response may result in less marked fluctuations in hormonal levels.

The close association between renin and aldosterone secretions is illustrated in Figure 29 which summarizes data from 157 studies of 61 normal subjects in whom the daily aldosterone excretion was related to the concurrent plasma renin activity (18). Included are data from subjects maintained on various constant dietary regimens and from subjects eating random diets. The plot reveals a highly significant direct relationship between the activity of these two hormones. The observed correlation coefficient of 0.84 is impressively high, but does not exclude the operation of other factors in this close hormonal interaction.

As might be expected, a similar direct relationship was found between plasma aldosterone levels and plasma renin activity in normal subjects in response to changes in sodium intake (Figure 30)(122).

FIGURE 29

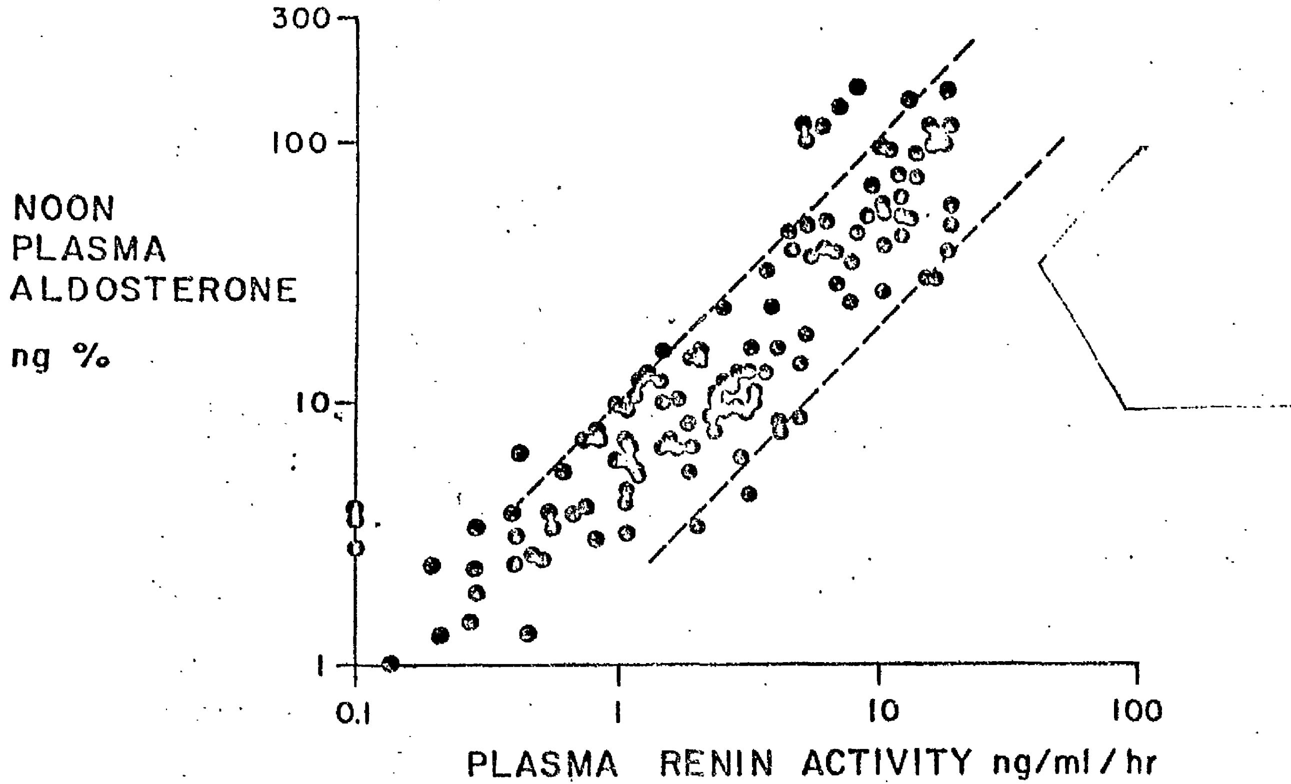
RELATIONSHIP OF URINE ALDOSTERONE EXCRETION TO PLASMA RENIN ACTIVITY IN NORMAL SUBJECTS



Relationship of plasma renin activity to the concurrent level of urine aldosterone excretion in 61 normal subjects. Both parameters are plotted on logarithmic scales. The correlation coefficient of 0.84 is strikingly high, especially when one considers the considerable interindividual variation in the renin-aldosterone relationship which occurs even in normal subjects.

FIGURE 30

RELATIONSHIP OF PLASMA ALDOSTERONE TO PLASMA RENIN ACTIVITY IN NORMAL SUBJECTS



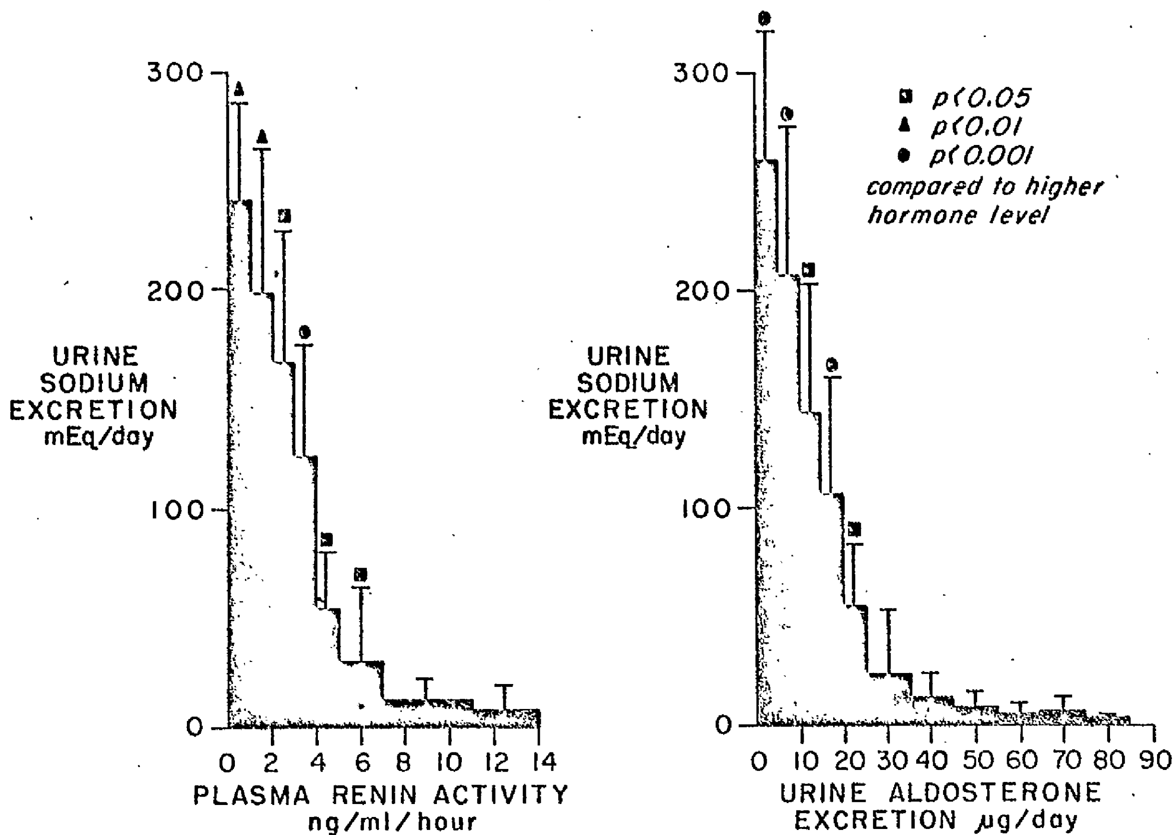
Plasma aldosterone is linearly related to plasma renin activity in 27 normal subjects.

II. Renin and Aldosterone During Sodium Loading:

The hyperbolic relationships inscribed by changes in renin and aldosterone activity in relation to urine sodium excretion strongly suggest that these two hormones play an important interrelated role in sodium conservation since their activity increases sharply during sodium depletion. However, a question which remains unsettled is whether or not the two hormones play any role when diatary sodium intakes are relatively high. To investigate this question the data in Figures 10 (page 46) and 18 (page 88) were replotted (17). The mean urine sodium excretion associated with a given range of renin or aldosterone activity was calculated and the mean value for each increment of hormone activity and its standard deviation were plotted.

As illustrated in Figure 31, under circumstances of sodium loading, when renin and aldosterone activity are low, these two hormones continue to be serially inversely related to sodium excretion and continue to change in concert. Thus when normal subjects have a plasma renin activity of from 1 to 2 ng/ml/hr., mean sodium excretion was found to be about 200 mEq/day. But at plasma renin activities of less than 1 ng/ml/hr., mean sodium excretion was still higher -- in this study approximately 250 mEq/day. Statistical treatment of these differences indicates that they are highly significant at all of the higher rates of sodium excretion. Entirely similar results were obtained from an analysis of the behavior of aldosterone excretion.

FIGURE 31



Mean urine sodium excretion is plotted for eight different ranges of plasma renin activity and eleven ranges of urine aldosterone excretion. The data are derived from 62 normal subjects. The vertical lines represent the standard deviation of urine sodium excretion for a given hormone range. Urine sodium excretion continues to increase significantly as both hormones fall, suggesting a causal relationship, even at low levels of the hormones.

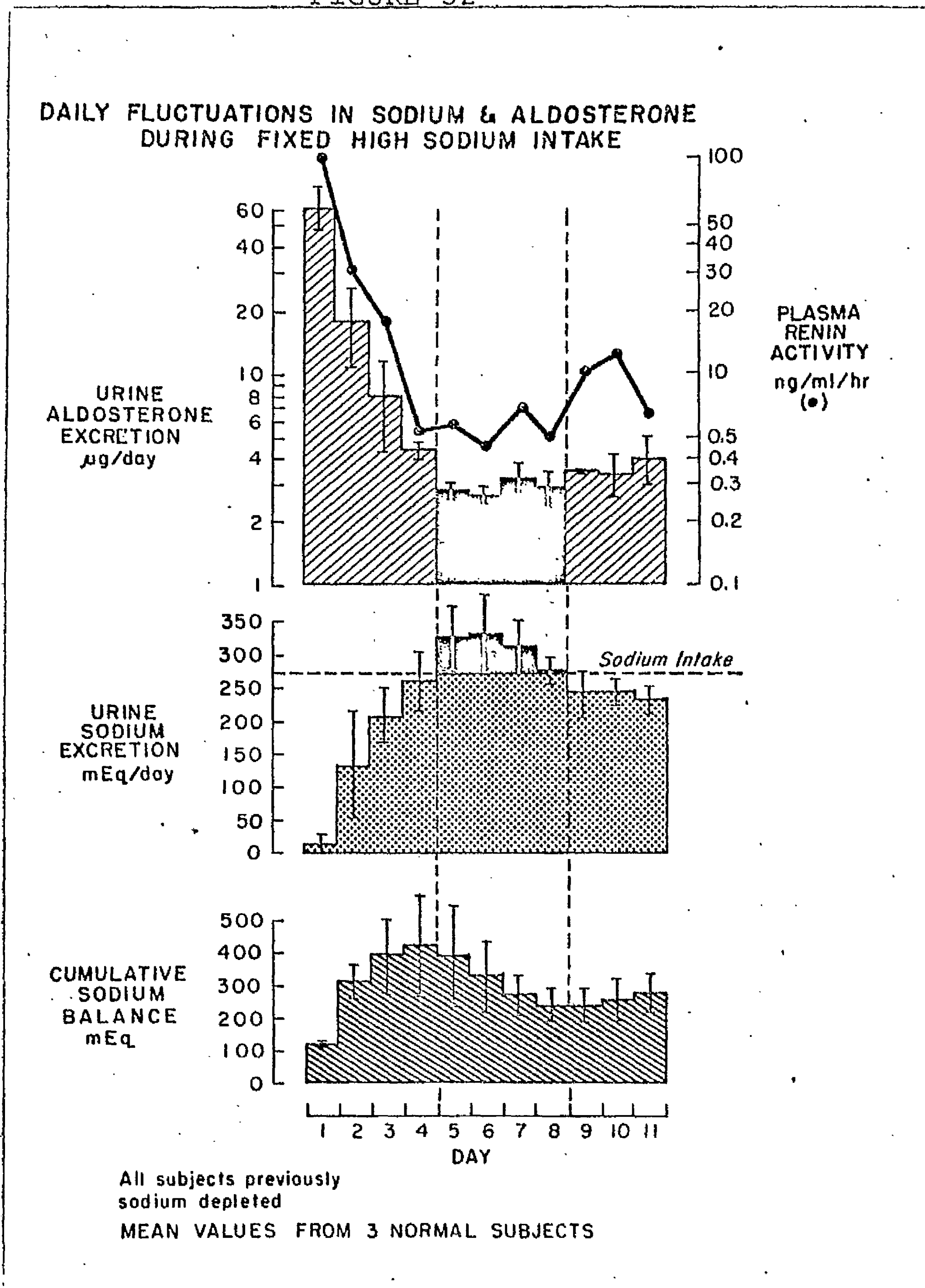
These data suggest that changes in both renin and aldosterone secretory activity are physiologically meaningful and interrelated even at very low levels of secretory activity. Thus renin and aldosterone appear to affect sodium balance at both high and low levels of sodium intake and excretion.

Oscillations in the System During Sodium Loading:

The responses of renin and aldosterone during sodium loading are illustrated in another study in which three normal subjects received a constant high sodium intake of 270 mEq/day for 11 consecutive days (11). The diet was instituted after 9 days of sodium depletion. The results of this study are presented in Figure 32.

In all three subjects there was an initial period of sodium retention, lasting for 4 days, in which the intake exceeded the output. This was followed by a second phase in which the rate of sodium excretion actually exceeded the intake. This phase lasted for 4 days and was followed by another period of mild positive sodium balance lasting for at least the next 3 days when the study terminated. Thus the high sodium intake caused marked sodium retention. The diet of 270 mEq/day resulted in mean net positive sodium balance of some 270 mEq at the end of the 11 day period. However, this equilibrium point was only achieved after a period during which the subjects had retained approximately 400 mEq of sodium.

FIGURE 32



Fluctuations in sodium excretion, sodium balance, plasma renin activity and aldosterone excretion during a maintained 270 mEq sodium diet. Mean values ± SD from three normal subjects are presented. Each subject exhibited similar and corresponding fluctuations in urine sodium, plasma renin and aldosterone. The highest levels of sodium excretion were associated with the lowest rates of aldosterone excretion.

Both aldosterone excretion and plasma renin activity, measured daily, fell serially in each subject as urinary sodium excretion increased so that the highest rates of urine sodium excretion were associated with the lowest levels of renin and aldosterone. Note that in the third phase, when urinary sodium excretion began to fall, renin and aldosterone both exhibited a slight but consistent rise. These data strongly suggest that the phasic, albeit relatively slight, changes in sodium excretion which occur during circumstances of prolonged sodium loading are mediated by aldosterone in response to changes in renin secretion.

These data also indicate that renin and aldosterone secretion are more closely related to the rate of sodium excretion than to the state of overall sodium "balance." Thus on day 3 when sodium balance was almost at its peak aldosterone and renin levels were double those of the lowest values. Moreover, during the natriuretic phase, cumulative sodium balance declined while aldosterone and renin remained constant, but low. This is not to say that aldosterone and renin do not in the first place respond to changes in "balance." Some function of a change in balance obviously is the stimulus which induces a change in hormonal activity. However, the hormonal response appears sluggish so that large overshoots in balance occur, both positively and negatively, before equilibrium is achieved by continuing

adjustment of the hormonal secretions.

These consistent daily patterns of renin and aldosterone activity in relation to urine sodium excretion suggest that in normal subjects daily fluctuations in aldosterone do in fact have a modulating effect on urine sodium excretion tion, even at high levels of sodium intake.

In summary, there is a close relationship between renin and aldosterone and sodium excretion in normal subjects which points to a close causal relationship in the three parameters. The relationship obtains over a wide range of intakes, suggesting that renin and aldosterone play a physiological role in regulating salt balance during sodium loading as well as during sodium depletion. However these studies do not rule out the possibility that other factors are involved in the regulation of any or all of the three parameters.

III. Effect of Changes in Potassium Balance on Plasma Renin Activity:

Changes in sodium balance have been shown to exert a marked affect on renal renin secretion. Sodium depletion increases renin secretion and sodium administration decreases it (158). The direct effect of potassium on aldosterone secretion is also well documented (162-165). However, until recently, the direct effect on potassium on renin secretion was less clear. Potassium adminstration had been shown to suppress renin secretion (166-168)

but the effect of potassium deprivation had not been studied.

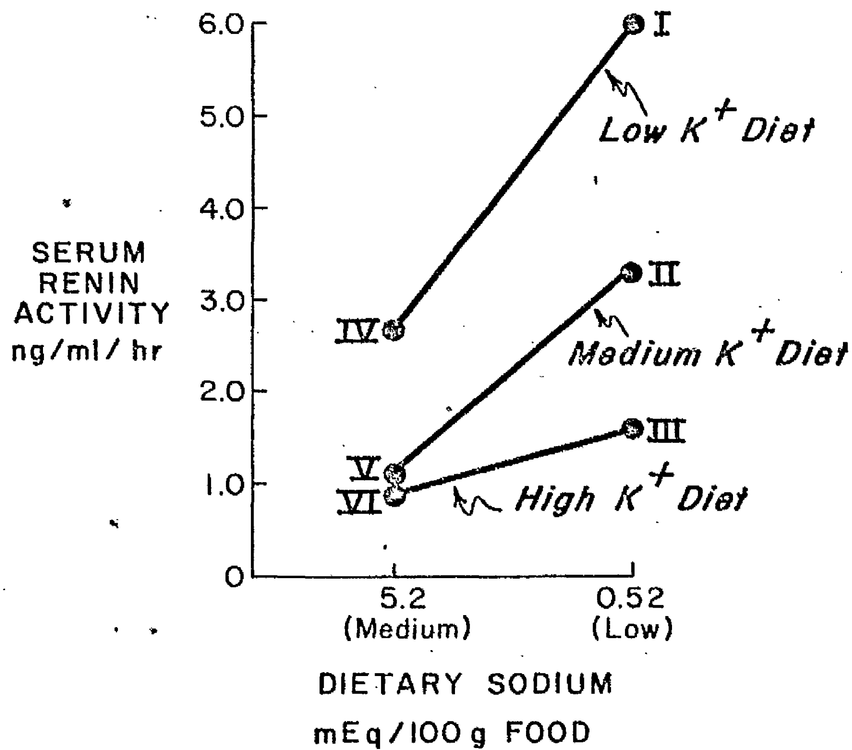
1. Experimental studies in rats.

Studies were designed to investigate the effect of potassium on renin secretion by employing an animal model in which extreme and rigidly controlled changes in both potassium and sodium balance could be imposed (19). Rats, weighing between 275 and 285 g were divided into nine groups of ten animals each. The nine groups encompassed all possible combinations of three levels of dietary intake (low, medium, or high) for both sodium and potassium. A 12 day balance study was carried out in which sodium and potassium intake and excretion were carefully monitored and then the animals were maintained on the same regimen for an additional 35 days (total 53 days) at which time they were sacrificed by decapitation.

Among all nine groups no consistent or impressive changes were observed in the concentrations of renin substrate. Accordingly, changes in serum renin activity were most likely a reflection of changes in serum renin concentrations.

As expected, sodium deprivation produced significant increases in serum renin levels. Thus on the medium K^+ intake, sodium deprivation caused an increase in renin activity from 1.1 to 3.3 ng/ml/hr. However, changes in the potassium intake exerted a highly significant modulating influence on this characteristic renin response (Figure 33).

FIGURE 33



Effect of three different levels of potassium intake on serum renin activity in normal rats. Potassium deprivation resulted in significant increases in renin and augmented the response of renin to sodium depletion. Increasing dietary potassium progressively blunted renin secretion.

Thus, on the high potassium intake the typical response to sodium depletion was greatly attenuated. The control value for serum renin was lower (0.9 ng/ml/hr.) and it increased to only 1.6 ng/ml/hr. despite an even greater degree of sodium depletion associated with the high K^+ administration. Conversely, the basal renin level, as well as its response to sodium depletion, was greatly augmented by potassium depletion. In this latter situation sodium depletion caused the values to rise from 2.7 to 6.0 ng/ml/hr. The highest of all renin values occurred in the low K^+, low Na^+ dietary group.

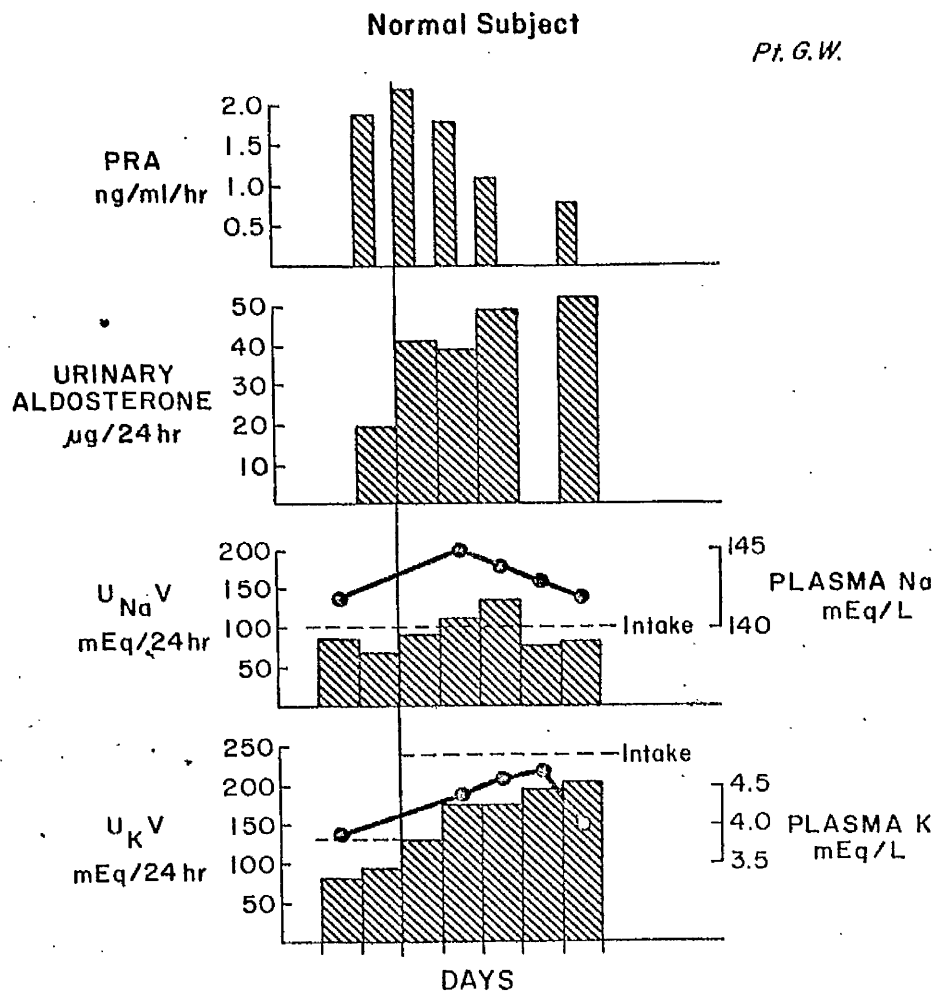
2. Companion studies in man

The response of plasma renin activity to less extreme changes in potassium balance was investigated in man (20). Potassium administration reduced plasma renin activity in 18 of 28 normal or hypertensive subjects. Suppression of renin often occurred despite the slight sodium diuresis induced by potassium administration. The renin suppression was directly related to induced changes in plasma potassium concentration and urinary potassium excretion.

In six studies potassium deprivation invariably increased plasma renin activity even though a tendency for sodium retention often accompanied this procedure.

Figure 34 presents the details of a representative study in a normal subject who was studied under metabolic balance ward conditions. Potassium administration produced

FIGURE 34



Study of the effect of increased potassium administration for five days in a normal subject maintained on constant dietary regimen. Plasma renin activity was serially suppressed. As expected, urinary aldosterone, sodium and potassium excretion all increased.

a serial and progressive fall in plasma renin activity. It also induced a slight sodium diuresis during the first three days of the experimental period. The serial suppression of plasma renin activity thus occurred in the face of an induced natriuresis, a change which would be expected to stimulate plasma renin activity.

It is interesting to note the aldosterone excretion increased in response to potassium administration, despite the fall in plasma renin activity.

Analysis

These long-term balance studies in rats and in man define an impressive inverse relationship between, on the one hand, potassium administration, plasma potassium concentration, the rate of urinary potassium excretion and potassium balance and on the other hand, the concurrent level of plasma renin activity.

The influence of the changes in potassium metabolism on circulating renin levels could not be explained by induced changes in sodium balance. Indeed, the suppressing effect of potassium administration on renin activity was often observed despite the fact that the potassium feeding occasionally induced natriuresis and hyponatremia which would ordinarily induce increased renin secretion. Furthermore, the striking increases in serum renin activity associated with K^+ depletion were never associated with sodium diuresis and in some situations may in fact have occurred despite

induced sodium retention.

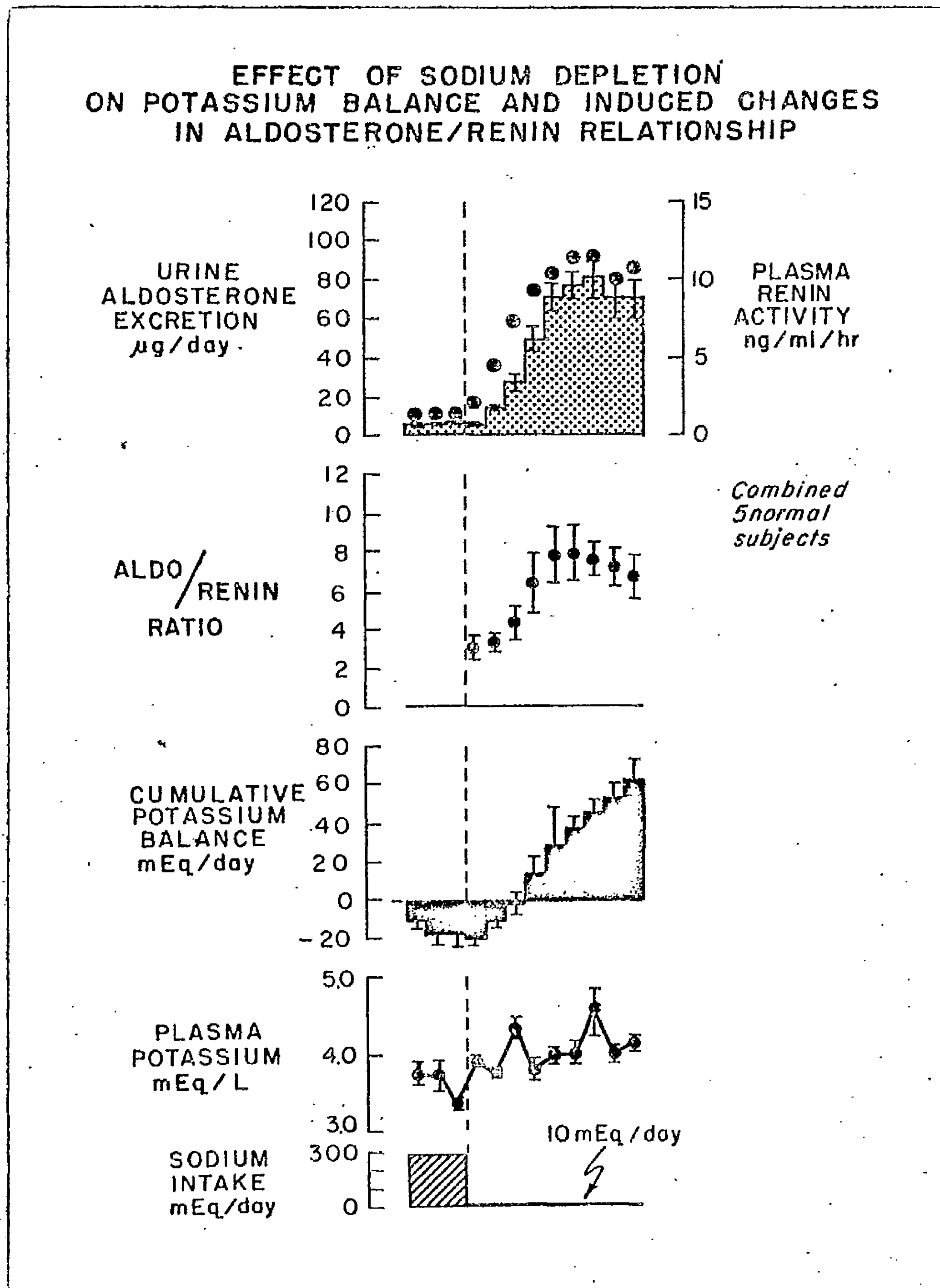
Thus, potassium administration affects the renin-angiotensin-aldosterone system in two ways. It both suppresses renin secretion and increases aldosterone production. Potassium deprivation has entirely opposite effects. Accordingly, potassium might exert a much greater stimulatory effect on aldosterone secretion if plasma renin levels were not concurrently suppressed. The reason for the suppressive effect of potassium on renin secretion is not clear, but it may be related to the simultaneous actions of aldosterone to maintain both sodium and potassium balance. (See section C, page 172).

IV. Sodium Depletion: Effect of Concurrent Changes in Sodium and Potassium Balance on Renin and Aldosterone Secretions

Since both sodium and potassium balance have now been shown to affect both renin and aldosterone secretion, either directly or indirectly, it was of interest to examine the effect of concurrent changes in balance of both of these cations on renin and aldosterone secretions.

In a study of five normal subjects who were sodium deprived for nine days, sodium and potassium balance were carefully monitored (18). It was noted that as sodium depletion was induced, potassium balance became progressively positive so that a mean accumulation of some 60 mEq occurred (Figure 35). In keeping with these changes, a general trend

FIGURE 35



Positive potassium balance induced by sodium depletion. Mean data and standard errors are presented from five normal subjects during nine days of sodium deprivation. In the upper plot, renin is represented by dots and aldosterone by bars. Potassium retention induced a change in the ratio of aldosterone to renin secretion such that relatively less renin was neede to maintain adequate aldosterone levels during continued sodium depletion.

was observed for plasma potassium levels to increase. However, the range of variation in plasma potassium was considerable so that plasma levels were not reliable indicators of the serial changes in cumulative balance.

As expected, during the first five days, sodium deprivation produced serial increases in both aldosterone excretion and plasma renin activity. In fact, at first glance, they appeared to rise in concert. However, when the aldosterone/renin ratio was calculated for each day it was found that the ratio rose progressively during sodium deprivation rather than remaining constant. The observed changes in cumulative potassium balance may well explain the change in the ratio. As described in the previous section, the positive potassium balance would be expected to increase aldosterone secretion (162) while retarding the increases in renin secretion (19,20) in response to continued sodium depletion and this would cause an increase in the aldosterone/renin ratio.

These findings suggest that the summation of changes in plasma renin activity and potassium balance provides an adequate explanation for changes observed in aldosterone secretion during sodium deprivation. They also serve to illustrate that changes in balance of sodium and potassium are intimately related so that a change in intake of only one of the two cations does not preclude changes in balance of the other and these changes are not necessarily reflected in differences in plasma electrolyte concentration. Thus

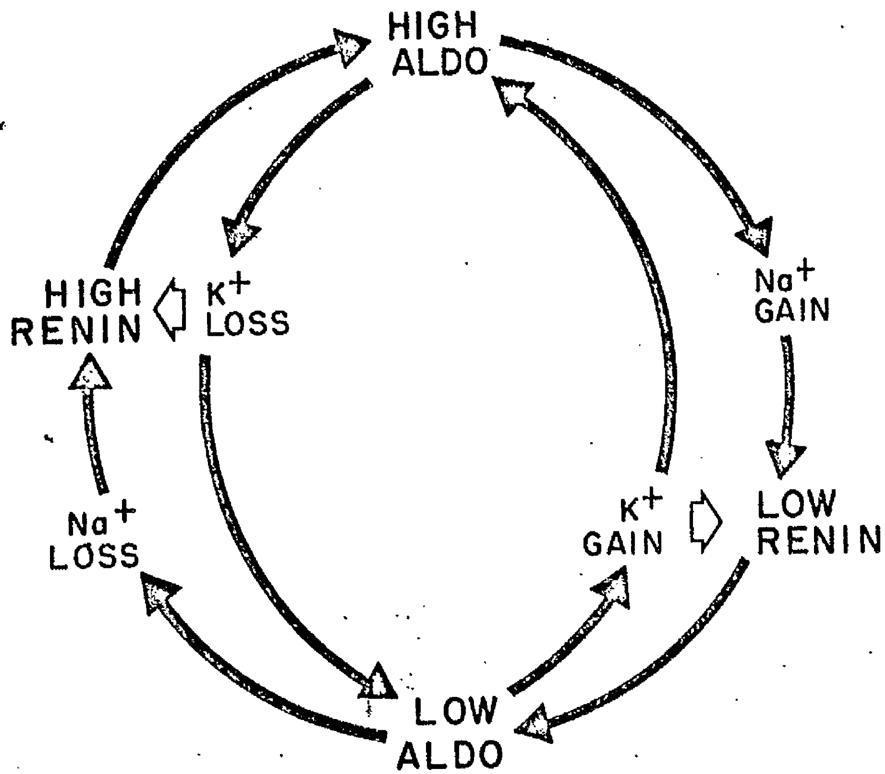
in studies of sodium deprivation in which evidence for the existence of unknown aldosterone stimulatory factors are sought (155,156), the effect of changes in both sodium and potassium balance must be taken into account before the existence of other factors is postulated.

V. Summary: The Coordinated Hormonal Control of Sodium and Potassium Homeostasis

The data just presented can provide the basis for a cybernetic scheme for sodium and potassium homeostasis (18). Figure 36 describes this "double-cycle feedback" cybernetic system involving renin, angiotensin and aldosterone which simultaneously controls sodium and potassium homeostasis. For simplicity, angiotensin has been omitted since a large body of evidence indicates that the plasma level of this hormone is determined by changes in plasma renin activity.

The Sodium Cycle. The outer cycle of Figure 36 describes the system for regulation of sodium balance via changes in renin and aldosterone secretion. In this cycle, any stimulus producing sodium or volume depletion activates renal renin secretion and this in turn stimulates aldosterone secretion. Aldosterone then causes sodium retention with attendant hydremia, and this effect, by restoring renal perfusion, operates to turn off the original signal for renin secretion and bring the aldosterone secretory rate back to the null point.

FIGURE 36



The Potassium Cycle. The inner cycle describes the system which maintains potassium balance. Ingested potassium ions, by raising plasma potassium, stimulate aldoster-secretion. Aldosterone in turn, by acting on the renal tubules, restores plasma potassium to normal by promoting renal potassium excretion. As potassium levels in the blood fall, aldosterone secretion is again turned off and restored to the null point. Simultaneously, changes in plasma potassium also produce direct effects on renal renin secretion so that a rising plasma potassium level suppresses renin secretion and vice versa. The changes in renin secretion induced by changes in plasma potassium may operate to amplify or retard the capacity for renal excretion of potassium. It should be noted that the concurrently induced changes in plasma renin activity tend to modulate the effects of potassium on the aldosterone secretory mechanism so that although potassium stimulates aldosterone secretion, this action is dampened by a concurrently induced decrease in plasma renin activity. Accordingly, potassium might exert a much greater stimulatory effect on aldosterone secretion if plasma renin levels remained constant.

C. Aldosterone: Integration of Its Effect with Changes in Intrarenal Physical Factors.

There is an apparent conflict in the simultaneous regulation of sodium and potassium balance by one hormone, aldosterone. Changes in sodium and potassium balance act in opposite directions to modify aldosterone secretion (Figure 36). Positive potassium balance (K^+ gain) increases aldosterone secretion but positive sodium balance (Na^+ gain) decreases it. Sodium depletion (Na^+ loss) increases aldosterone but potassium depletion (K^+ loss) depresses secretion. At the same time aldosterone has been shown to act to retain sodium but promote potassium excretion. Hence, one would expect that sodium depletion-induced increases in aldosterone would cause potassium loss and potassium-induced increases to cause sodium retention. This does not happen. In fact, the tendency is for the converse situation to occur (18,162,168-172).

I. Effect of Changes in Sodium or Potassium Intake on the Overall Balance of the Other Cation

The influence of sodium depletion on potassium balance is illustrated in Figure 35 (page 167)(17,18). During nine days of sustained sodium deprivation in five normal subjects, potassium balance became slowly but progressively positive so that a mean accumulation of some 60 mEq occurred. This positive balance is to be contrasted with the neutral or

slightly negative balance during the three day control period. As expected, sodium deprivation induced a serial rise in both aldosterone excretion and plasma renin activity. Thus, sodium depletion and associated hyperaldosteronism did not induce hypokalemia (Figure 36) but actually caused potassium retention and a tendency towards hyperkalemia.

In another study (19) of the effect of various sodium and potassium intakes in rats, potassium loading caused significant natriuresis in both sodium depleted rats and in rats on normal sodium intake. There was also some tendency for increasing amounts of sodium in the diet to induce kaliuresis. Thus changes in sodium or potassium intake seem to affect the other cation in the opposite direction to that expected on the basis of induced changes in aldosterone secretion.

II. Effect of Changes in Distal Sodium Supply on Urine Sodium and Potassium Excretion:

To reconcile this seeming paradox, other factors which respond to changes in sodium and potassium intake and which are involved in the regulation of urine sodium and potassium excretion must be considered (173).

The amount of sodium excreted in the urine is dependent on the rate of glomerular filtration (GFR) minus the net sum of sodium reabsorbed from the proximal tubule, loop of Henle, distal convoluted tubule and collecting duct. Recently, active sodium secretion has been demonstrated but only in response to injection of uremic serum (174). Under physio-

logical conditions, maintenance of sodium balance depends on adequate reabsorption of sodium from the nephron.

Reabsorption of sodium from the loop of Henle appears to be load dependent and there is no evidence to suggest that changes in either sodium or potassium balance alter the fraction of sodium that is reabsorbed in this portion of the tubule (175). However, changes in both sodium and potassium balance have been shown to affect both GFR (176-181) and proximal sodium reabsorption (180,182-188). Increases in both sodium and potassium balance act to increase GFR and depress proximal sodium reabsorption. Both of these two changes increase the amount of sodium passing out of the proximal tubule. Since reabsorption from the loop of Henle is load dependent (175), increases in net sodium leaving the proximal tubule must result in an increase in the amount of sodium presented to the distal tubule. Conversely a fall in either sodium or potassium balance, by reducing GFR and increasing proximal tubule sodium reabsorption, results in depression of distal tubule sodium supply. A summary of the effects of changes in sodium and potassium balance on distal sodium supply are presented in Table 18A.

It can be appreciated that if sodium reabsorption in the distal tubule and collecting duct, like the loop of Henle, were load dependent, then changes in distal sodium supply would inevitably result in changes in urine sodium excretion (Table 18B).

Filtered potassium is almost completely reabsorbed

TABLE 18

A. EFFECTS OF CHANGES IN SODIUM AND POTASSIUM BALANCE ON DISTAL TUBULE SODIUM SUPPLY

Increased sodium balance ⟶ Increased distal Na^+ supply
Decreased sodium balance ⟶ Decreased distal Na^+ supply

Increased potassium balance ⟶ Increased distal Na^+ supply
Decreased potassium balance ⟶ Decreased distal Na^+ supply

B. EFFECTS OF CHANGES IN DISTAL SODIUM SUPPLY ON URINE SODIUM EXCRETION

Increased distal Na^+ supply ⟶ Increased urine Na^+ excretion
Decreased distal Na^+ supply ⟶ Decreased urine Na^+ excretion

C. EFFECTS OF CHANGES IN DISTAL SODIUM SUPPLY ON URINE POTASSIUM EXCRETION

Increased distal Na^+ supply ⟶ Increased urine K^+ excretion
Decreased distal Na^+ supply ⟶ Decreased urine K^+ excretion

prior to the distal convoluted tubule (189). Urine potassium excretion appears to be dependent almost entirely on secretion in the distal tubule (189-191). Thus maintenance of potassium balance depends on adequate secretion of potassium into the nephron. Evidence suggests that changes in distal sodium supply also affect potassium excretion (175,190). Since sodium reabsorption in the distal tubule is not accompanied by commensurate chloride reabsorption, a negative electrochemical gradient develops across the distal tubule and potassium passes from the cells into the tubule in an attempt to restore neutrality (192). Hence, increases in net sodium reabsorption would result in increased electronegativity and thus an increase in net potassium secretion. It can be appreciated that, even if fractional sodium reabsorption in the distal tubule remained unchanged, increases in the sodium load to the distal tubule would result in a net increase in the amount of sodium reabsorbed and thus increased potassium secretion would occur. Therefore, changes in distal sodium supply can affect potassium excretion (Table 18C). Increases in distal sodium supply cause more potassium elimination and decreases result in a fall in urine potassium excretion.

When changes in intrarenal physical factors are considered alone, sodium induced increases in distal sodium supply ought to cause potassium elimination and potassium induced increases ought to cause sodium diuresis. Conversely, sodium depletion should be accompanied by potassium retention and

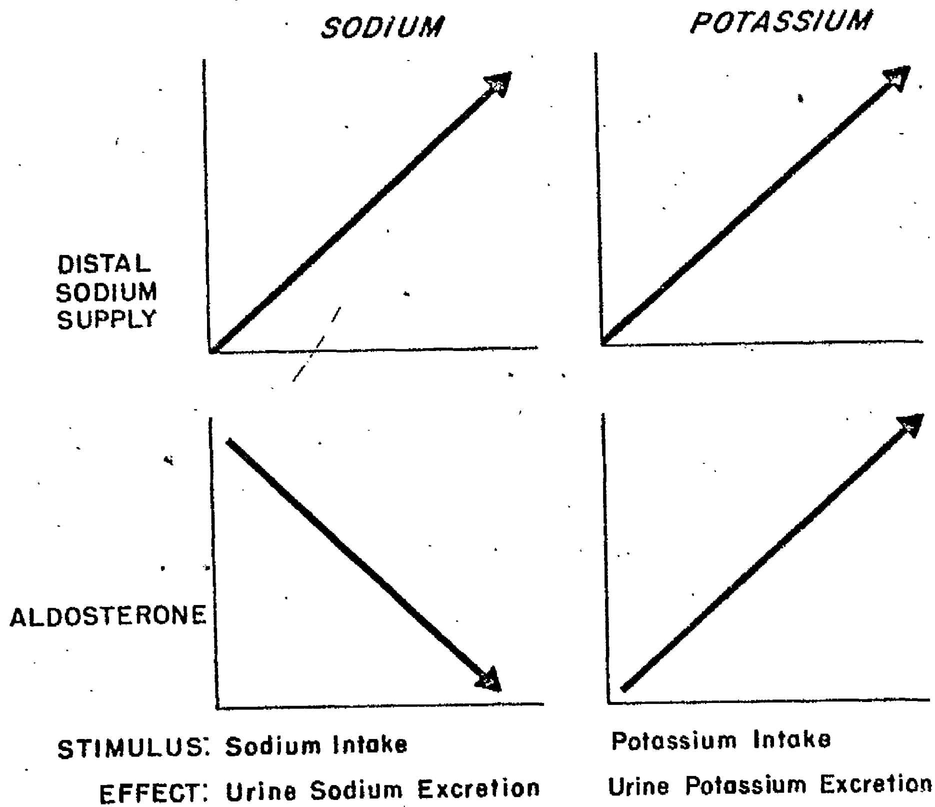
potassium deprivation should be associated with a marked increase in sodium balance. In fact, the tendency is for these changes to occur, but the magnitude of the effect is quite small.

III. Coordination of Changes in Distal Sodium Supply with Changes in Aldosterone.

Reabsorption of sodium in the distal tubule is not exclusively load dependent. Aldosterone markedly affects distal sodium reabsorption (193,194) and thus affects potassium secretion at least in part via resultant increases in the electrochemical gradient. Therefore, in analysing the renal response to changes in sodium and potassium balance, changes in distal sodium supply and aldosterone must be considered together.

The effect of variations in sodium and potassium balance on aldosterone and distal sodium supply and the effect of the induced changes in aldosterone and distal sodium supply on urine sodium and potassium excretion are illustrated diagramatically in Figure 37. Sodium loading results in an increase in distal sodium supply and reduction in aldosterone secretion. Both of these changes augment urine sodium excretion. However, although the increase in distal sodium supply promotes urine potassium excretion, the concurrent fall in aldosterone acts to reduce net distal sodium reabsorption and thus retard potassium excretion. Altogether, these two changes protect potassium balance and result in no net change

FIGURE 37



The upper panels represent diagramatically the increase in distal sodium supply which occurs in response to increasing sodium and potassium intake and the resultant increase in urinary excretion of both ions, assuming no change in aldosterone. The lower panels represent the opposite effects of dietary sodium and potassium on aldosterone and the opposing effects of aldosterone on sodium and potassium excretions, assuming no change in distal sodium supply.

in potassium excretion.

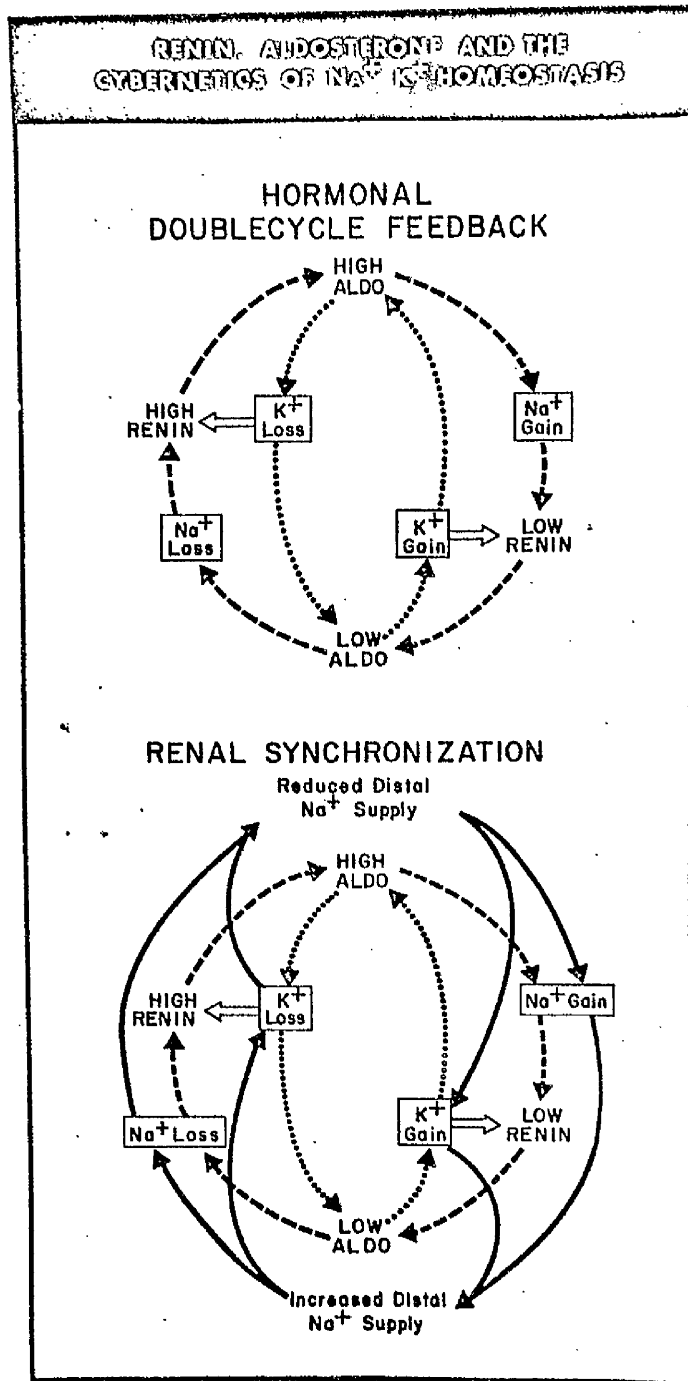
Alternatively, increasing potassium intake results in augmentation of both distal sodium supply and aldosterone excretion, both of which promote potassium elimination. However, although increased distal sodium supply should result in sodium loss, the concurrent elevation of aldosterone secretion increases net distal sodium reabsorption and the net effect is no change in sodium excretion.

IV. A Cybernetic System for Sodium and Potassium Homeostasis.

The coordination of changes in distal sodium supply with changes in aldosterone secretion is illustrated in Figure 38 (18). In the lower panel, factors which alter distal sodium supply and the effect of these changes are superimposed on the hormonal variations discussed in the previous section.

This diagram illustrates the effect of changes in intake of one cation on its own excretion and on the excretion of the other cation. For example, sodium loss, via increased aldosterone production and decreased distal sodium supply results in renal sodium retention which feeds back to turn off the initial signal. However, increased aldosterone promotes potassium excretion but decreased distal sodium supply retards potassium excretion. Thus, while the system compensates to prevent further sodium loss, potassium balance is maintained neutral.

FIGURE 38



Upper panel: Hormonal double cycle feedback system for simultaneous sodium and potassium homeostasis. Lower panel: Complementary changes in renal tubular sodium transoprt consequent to changes in sodium and potassium balance.

Aldosterone: Renal physical factors

The effect of changes in one or both cations on aldosterone and distal sodium supply and the result of the compensation by hormonal and intrarenal physical factors is presented in Table 19. Under all conditions the system is able to maintain sodium and potassium balance. Thus, even when both sodium and potassium intake change together the system is almost perfectly balanced. Concurrent increases or decreases in both cations together result in increases or decreases in distal sodium supply but have no effect on aldosterone secretion. Hence excretion or retention of both ions is concurrently achieved. However, opposite changes in sodium and potassium intake cause marked compensation of the aldosterone component, with little change in intrarenal physical factors. Since aldosterone affects each cation in the opposite direction, balance is achieved.

The effect of the collecting duct to modulate urine sodium excretion and the hormonal response to extreme conditions of sodium loading will be considered in the next section.

TABLE 19

COORDINATION OF THE EFFECTS OF CHANGES IN DISTAL SODIUM SUPPLY AND ALDOSTERONE ON URINE SODIUM AND POTASSIUM EXCRETION

Increased Sodium Intake	Effect on Na+ excretion	Effect on K+ excretion
Increased Distal Na+ Supply	increased	increased
Decreased Aldosterone	increased	decreased
Net Change:	increased	no change
Increased Potassium Intake		
Increased Distal Na+ Supply	increased	increased
Increased Aldosterone	decreased	increased
Net Change:	no change	increased
Decreased Sodium Intake		
Decreased Distal Na+ Supply	decreased	decreased
Increased Aldosterone	decreased	increased
Net Change:	decreased	no change
Decreased Potassium Intake		
Decreased Distal Na+ Supply	decreased	decreased
Decreased Aldosterone	increased	decreased
Net Change:	no change	decreased
Increased Na+ and K+ Balance		
Increased Distal Na+ Supply	increased	increased
No Change Aldosterone	no change	no change
Net Change:	increased	increased
Decreased Na+ and K+ Balance		
Decreased Distal Na+ Supply	decreased	decreased
No Change Aldosterone	no change	no change
Net Change:	decreased	decreased
Increased Na+: Decreased K+ Balance		
No Change Distal Na+ Supply	no change	no change
Decreased Aldosterone	increased	decreased
Net Change:	increased	decreased
Decreased Na+: Increased K+ Balance		
No Change Distal Na+ Supply	no change	no change
Increased Aldosterone	decreased	increased
Net Change:	decreased	increased

D. Natriuretic Hormone: Its Role in Sodium Homeostasis:

Considerable interest and debate centers around whether or not there is convincing evidence for the existence of a natriuretic hormone. The possibility of such a hormone has been suspected since de Wardener and his associates (21,123) showed that dogs could excrete a saline load under conditions in which no change occurred in the parameters then known to be involved in the regulation of sodium excretion; i.e., glomerular filtration and aldosterone.

Studies from several groups, utilizing cross-circulation techniques demonstrated evidence for a natriuretic hormone (21,123,195-198), but others reported negative findings (199, 200). Other groups, utilizing such assays as toad bladder and frog skin short-circuit current (124,125), and PAH uptake or sodium transport in kidney slices (126,127) had positive assays in samples collected under conditions in which natriuretic activity might be present. However, the active material was not shown to increase urine sodium excretion.

Concurrent with the search for a natriuretic hormone was the development of evidence that changes in intrarenal physical factors can alter the rate of sodium reabsorption in the proximal tubule (185,188). The natriuretic hormone had been thought by some investigators to affect proximal tubular sodium transport, since during saline diuresis the amount of sodium reabsorbed from the proximal tubule is reduced (183). The means by which changes in proximal tubular sodium reab-

sorption occur are still not completely understood. However, evidence suggests that the rate of removal of resorbate from the peritubular environment of the proximal tubule is the determinant (185,188,201) and there is only scanty evidence for hormonally induced changes in active sodium transport. Fractional sodium reabsorption in the proximal tubule appears to fluctuate with changes in hydrostatic pressure in the post-glomerular circulation (202-204). In addition, changes in viscosity and oncotic pressure in the peritubular capillaries have also been shown to affect the rate of sodium reabsorption (202,205). Therefore, although a hormonal effect on sodium reabsorption in the proximal tubule has not been ruled out, it may not be necessary to postulate one.

However, reduction in proximal tubule sodium reabsorption by itself is inadequate to account for sodium diuresis since proximal sodium reabsorption can be reduced when urinary sodium excretion is not increased (128,129). These results, and other more recent data (130,206) suggest that differential changes in sodium reabsorption, which are not the result of changes in aldosterone, can occur in a more distal portion of the nephron. Thus saline or hyperoncotic albumin infusions equally depress aldosterone excretion and proximal sodium re-absorption, but only saline infusion results in large increases in urinary sodium excretion (128-130,207). Therefore the site of action of a natriuretic hormone which would be important for regulation of urine sodium excretion, should be in a distal portion of the nephron. In fact, recent evidence

suggests that the collecting duct may be the site for final regulation of sodium excretion (130,206).

The bioassay, extraction and purification procedures, described on pages 109-125 , were used to demonstrate that there is a substance which appears in the urine and blood of salt-loaded man or sheep which can induce natriuresis when injected into an assay animal (17,22-24). The response is characterized by increased sodium excretion which is accompanied by inconsistent and usually small changes in potassium excretion and urine flow. Because of the nature of the response we suggested that this substance could be a natriuretic hormone which acts in the distal tubule to promote sodium excretion. The natriuretic material appears to be a protein-like substance with a molecular weight in the region of 50,000.

Physiology of the Natriuretic Substance:

(1) Response to changes in sodium intake in normal subjects:

Table 20 presents data from assays of urine and plasma extracts from normal subjects on two different sodium intakes. Positive assays were found only in extracts from salt-loaded subjects. The mean increase in sodium excretion in the assay animal was 25 times greater than the response to extracts prepared from sodium depleted normal subjects. Similar directional changes were observed with two plasma extracts, although the magnitude of the response was less.

TABLE 20

ASSAY RESULTS: CUMULATIVE Na^+ LOSS IN ASSAY ANIMAL

1. Effect of dietary sodium intake in normal subjects

(a) Urine

	Low Na^+ diet	High Na^+ diet
		289*
		369
		196
		178
	2*	297
	15	111
	27	404
	19	98
	0	529
	29	346
	-15	876
	18	339
	0	221
	10	39
	27	224
Mean	12	301

(b) Plasma

	Low Na^+ diet	High Na^+ diet
	4*	39*
	4	77
Mean	4	58

2. Effect of saline infusion in normal subjects and hypertensive patients

(a) Urine

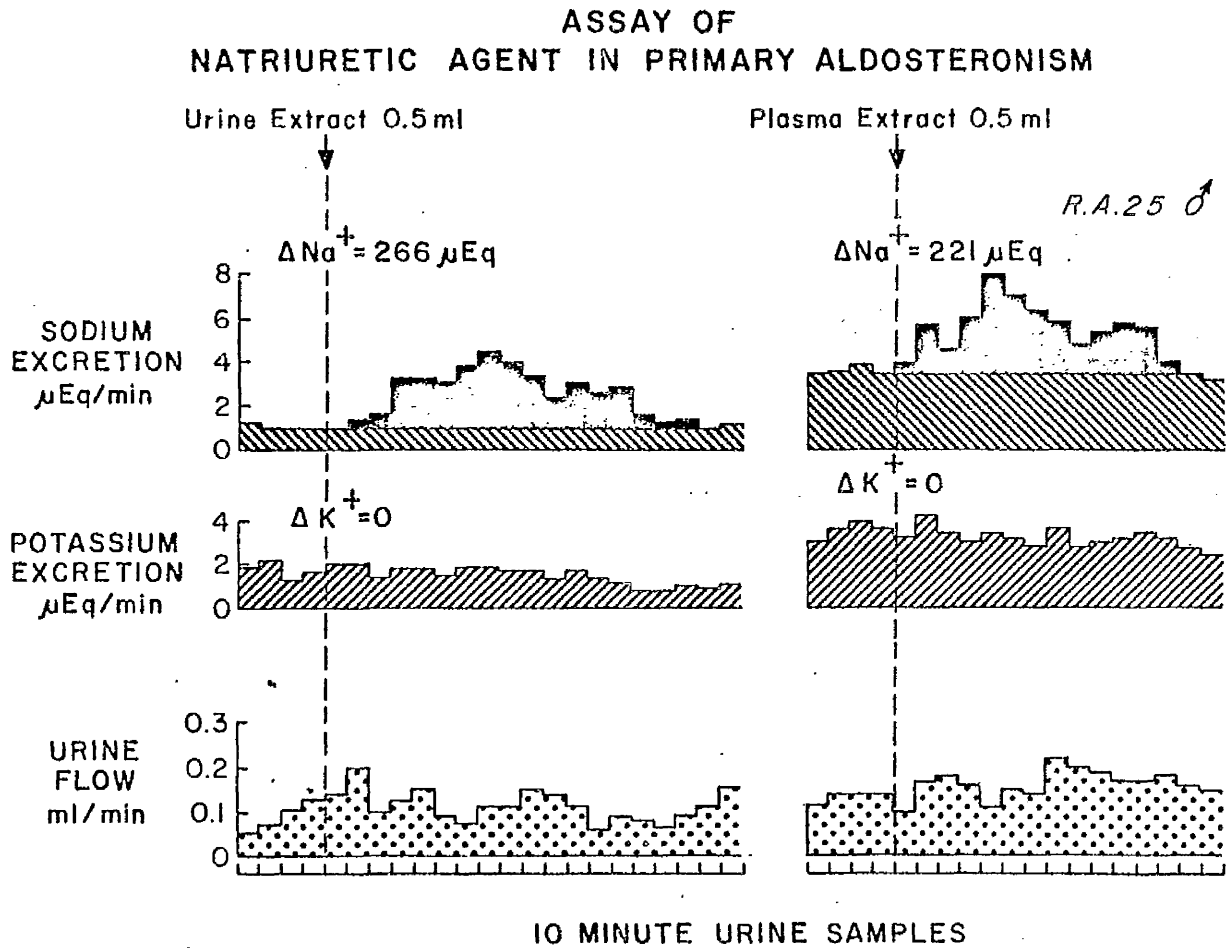
	Normal	Hypertensive
		356*
		209
		435
	30*	77
	10	355
	0	219
Mean	13	292

(b) Plasma

	Normal	Hypertensive
		36*
		69
		306
		96
		77
		363
		76
	2*	120
	-5	276
	0	415
	15	61
	7	323
	-7	161
	16	242
	17	15
	5	33
	0	252
	2	147
	2	92
Mean	4	166

* = Change in cumulative Na^+ balance

FIGURE 39



Positive assays for natriuretic activity in extracts of urine and plasma taken from a patient with primary aldosteronism. Assay animals were rats with diabetes insipidus in maintained water diuresis.

(2) Response to changes in sodium intake in hypertensive patients:

Urine and plasma extracts from salt-loaded patients with hypertension also induced natriuresis in the assay animal. Figure 39 presents assays of extracts from a patient with primary aldosteronism who was maintained on a high sodium intake. Both urine and plasma extracts induced impressive increases in sodium excretion in the test animal. Sodium excretion increased from 1 to 4.5 μEq/minute in one assay and from 3 to 8 μEq/minute in the other. Cumulative losses were 266 and 221 μEq. Extracts from all hypertensive patients studied had a similar effect.

(3) Response to saline infusion:

On the right side of Table 20 data are presented from assays of plasma and urine extracts derived from normal subjects and hypertensive patients who received saline infusions. Prior to infusion, a 2 hour control urine specimen was collected and 60 ml of blood was drawn. Two liters of saline were then infused during the next hour and a second blood specimen was drawn. The second urine collection included the hour of infusion and 3 hours after the infusion was completed.

There was little response to control extracts, suggesting that sodium loading is needed to expose the presence of the hormone. Samples collected during saline infusion were

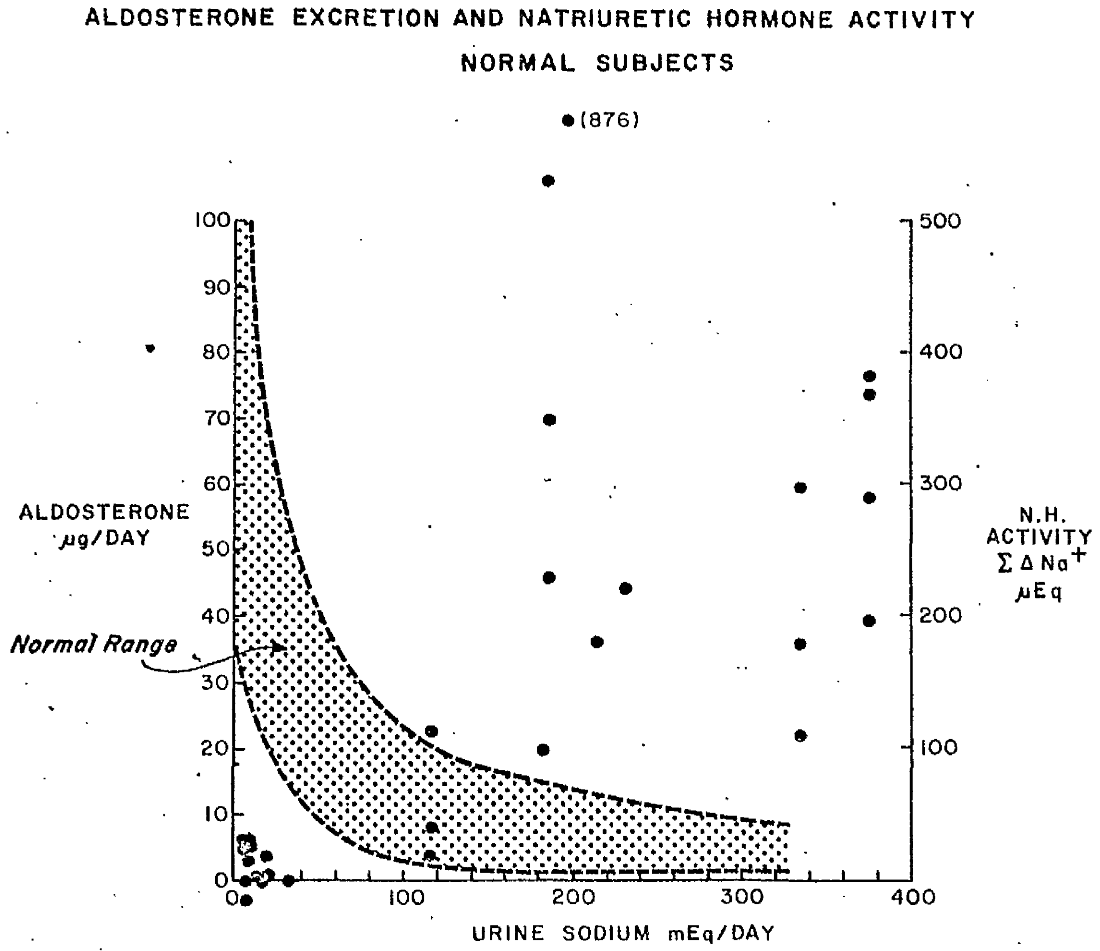
consistently natriuretic and the natriuretic response in the assay animal seemed to be greater with urine than with plasma extracts.

Taken altogether, these studies suggest that there is a natriuretic substance which circulates in blood and is excreted into urine in response to increases in sodium balance. Natriuretic activity appears when urine aldosterone excretion is falling (Figure 40) suggesting that when aldosterone is excreted in increased amounts, natriuretic activity is very low. However, when aldosterone exerts the least action, i.e., during positive sodium balance, the natriuretic substance is present in blood and urine in its highest concentrations.

(4) Effect of natriuretic extract on glomerular filtration rate (GFR):

No changes in GFR were detected in response to injection of natriuretic extract. One example of the studies in which GFR was measured (23) is illustrated in Figure 25, (page 122). The natriuretic response was not accompanied by any change in urine flow, potassium excretion, hematocrit or glomerular filtration rate. The percent of the filtered load of sodium excreted increased from 1.3% to 3%. Thus, the hormone appears to act on tubular sodium reabsorption and not via changes in filtration rate.

FIGURE 40



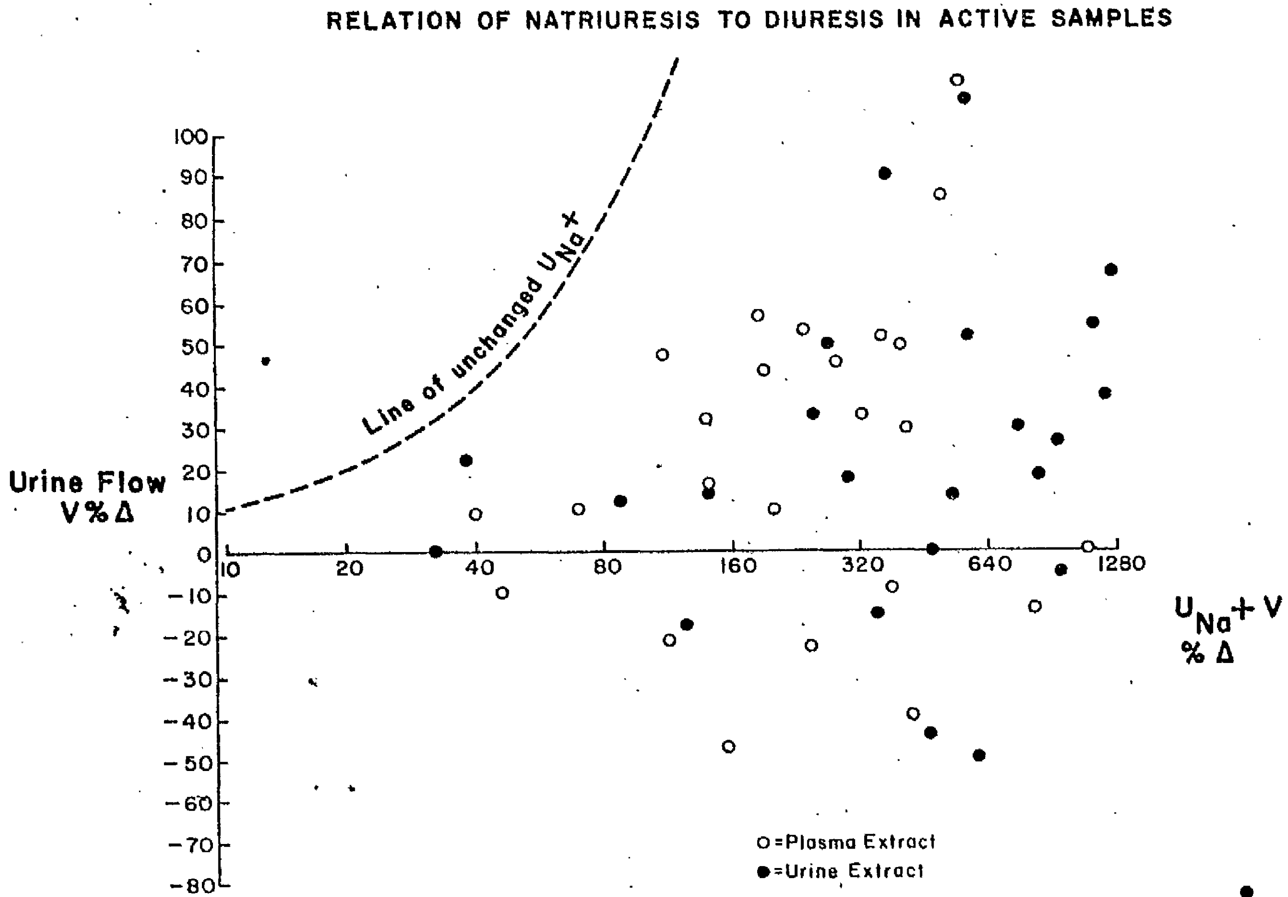
Reciprocal relationship of natriuretic activity to urine aldosterone excretion in normal subjects.

(5) Site of action of natriuretic activity:

Figure 41 presents an analysis of the relationship between induced changes in urine flow and sodium excretion. The percent change in sodium excretion is plotted logarithmically on the abscissa. There is no relationship between the magnitude of the natriuretic response and changes in urine flow. The curved dotted line represents the change in urine flow relative to sodium excretion which would be expected if increases in sodium excretion were accompanied by commensurate increases in urine flow. Such a situation might occur during water diuresis if the hormone acted to depress sodium reabsorption in the proximal tubule. In all assays (the assay rat was always in water diuresis) the response fell to the right of this line, indicating that the natriuresis was associated with increased urine sodium concentration. Thus, the natriuretic substance must act, at least in part, in a more distal portion of the renal tubule where dilution normally takes place.

No consistent changes in potassium excretion were observed during the natriuretic response. This observation is also compatible with the absence of a proximal action of the hormone since decreased proximal sodium reabsorption would cause increased sodium delivery to the sodium-potassium exchange site and this would be expected to result in increased urine potassium excretion . The absence of any change in potassium excretion in many assays (Figures 25,(page 122),39) suggests that the site of action might be beyond the distal

FIGURE 41



Relationship between the percent change in urine flow and the percent change in the rate of sodium excretion plotted on a logarithmic scale. Data are presented for all positive natriuretic assays. The curved line represents those increases in UNaV in which the only component which increased is the urine flow. Any points in which there was an increase in urine sodium concentration fall to the right of this line. Since all points fall to the right of this line, the results suggest that the natriuretic agent depresses distal tubular sodium reabsorption at the diluting segment.

convoluted tubule, where potassium is secreted, perhaps in the collecting duct. This would be in accord with some recent studies which have demonstrated that, in response to saline or hyperoncotic albumin infusions, no differences in fractional reabsorption of sodium can be measured in fluid collected from the late distal tubule (130) but urine sodium excretion is much greater in animals receiving the saline infusion. These and other investigators (175,206) suggest that the collecting duct may be the site at which the final amount of sodium in the urine is determined.

(6) Organ source:

In a preliminary investigation of the organ source we extracted the pituitary, brain, spleen, kidneys, liver, thyroid, parathyroid, pancreas, heart and lungs of a sheep that had been salt-loaded for several months. Fractionation procedures similar to those used for blood and urine were used. The organ source which exhibited the most impressive natriuresis was the liver. The characteristics of the natriuretic response were quite similar to those induced by plasma and urine extracts. However, since this was by weight the largest organ extracted and probably contained large amounts of blood, it is possible that the effect was not due to storage or synthesis by the liver. Also, lesser degrees of natriuretic activity were found in other organs (for example kidneys, heart, lungs and spleen) so that the data do not permit us at this time to positively

identify the organ source of the natriuretic substance.

Summary:

We have demonstrated that there is a natriuretic substance in urine and plasma of salt-loaded normal and hypertensive man and sheep. This substance was undetectable in subjects on a normal or low sodium diet. Thus the substance may have physiological relevance.

These observations have been confirmed by other investigators using assays which detected changes in sodium excretion in the rat (188,208) or changes in sodium and potassium transport in fragments of renal tubules (209). These other investigators were also able to distinguish between extracts from sodium depleted or sodium loaded subjects.

The molecular weight of the natriuretic material appears to be around 50,000 but this is only a gross estimate since it is derived from elution characteristics on gel filtration columns and ultrafiltration. The active substance may be a polymer of a smaller molecule, or perhaps a fragment of the large molecule. Other investigators, using different assays and different fractionation procedures, have suggested that the molecular weight of their active agent is around 1000. We investigated lower molecular weight fractions from gel filtration columns but were unable to completely separate them from vasoactive substances or fractions with very high osmotic activity, both of which could induce natri-

uresis even in the absence of a substance affecting tubular sodium transport.

The site of action of the hormone appears to be in the late distal tubule or collecting duct since the natriuresis was not accompanied by any consistent changes in urine flow, potassium excretion or glomerular filtration rate.

In the previous section it appeared that aldosterone and distal sodium supply could adapt and compensate for all possible combinations of changes in sodium and potassium balance. However, as discussed earlier in this section, there is evidence during massive saline loading in the rat that final adjustment of urine sodium excretion can occur in the collecting duct under conditions of constant aldosterone secretion.

Available evidence suggests that aldosterone can affect sodium reabsorption in both the distal convoluted tubule and the collecting duct (210). However, secretion of potassium appears to be largely confined to the earlier site, i.e., the distal convoluted tubule. Since distal potassium secretion is associated with approximately a ten to fifty fold greater reabsorption of sodium (191) final regulation of sodium excretion at the same site seems unlikely. Aldosterone has been shown to affect both sodium and chloride excretion. Aldosterone has also been shown to act in the collecting duct, where potassium secretion is minimal or absent. Thus the final regulation of urine sodium excretion could be under aldosterone control, but at a site distal to the potassium

secretory site.

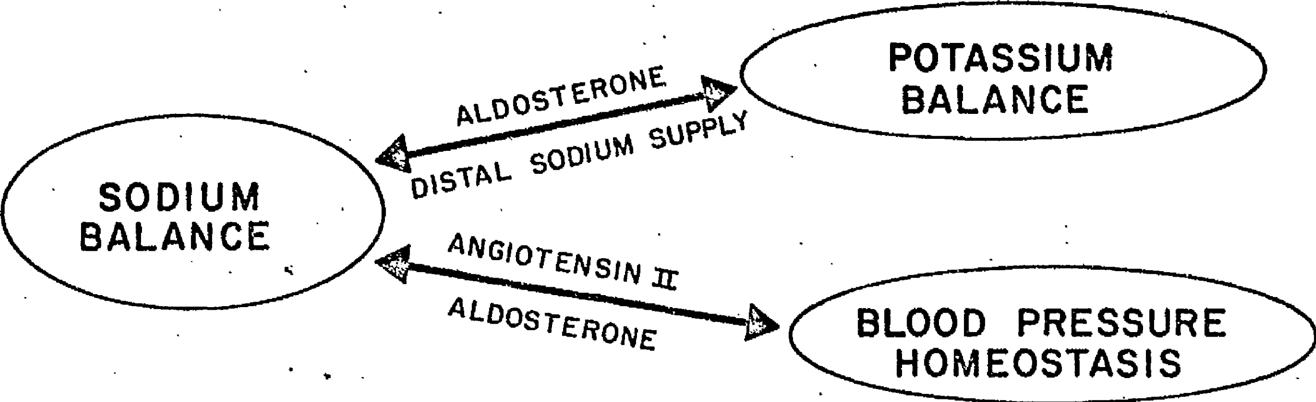
The natriuretic hormone could act to modify the sodium chloride retaining action of aldosterone. Alternatively, it could directly affect the sodium pump, alter the permeability to sodium ions or perhaps enhance the back diffusion of sodium into the tubule. Thus, the natriuretic hormone might be part of an emergency mechanism which copes with extreme changes in salt balance such as are found during saline loading or in end-stage kidney disease. A reactive natriuretic mechanism could operate to complement the more sluggishly responding control effected by the renin-aldosterone system.

E. Relationship of Sodium Balance and the Renin-Angiotensin-Aldosterone Hormonal System to Blood Pressure Homeostasis:

In the previous sections the hormonal system has been analysed with respect to its involvement in maintenance of sodium balance and potassium balance. This aspect of the hormonal system appears to function as an almost independent negative feed-back control system. From the analysis in Section C (pages 172-182) it can be appreciated that sodium balance and potassium balance can be maintained almost independently of each other, despite being under the control of one hormone, aldosterone, and despite the fact that potassium secretion is dependent on the net amount of sodium delivered to the distal tubule. Therefore it is not unreasonable to consider the second control system -- for sodium balance and blood pressure homeostasis -- separately since changes in sodium balance which affect blood pressure are unlikely to alter the equilibrium of potassium (Figure 42).

As illustrated in Figure 1 (page 7), the hormonal system can directly affect blood pressure via the vasoconstrictor action of angiotensin II. In addition, it can be appreciated that changes in sodium balance, by affecting blood volume, can also influence blood pressure via alteration in filling of the arterial tree (211-213). Thus the second limb of the hormonal system, aldosterone induced sodium retention, can also directly support blood pressure. The

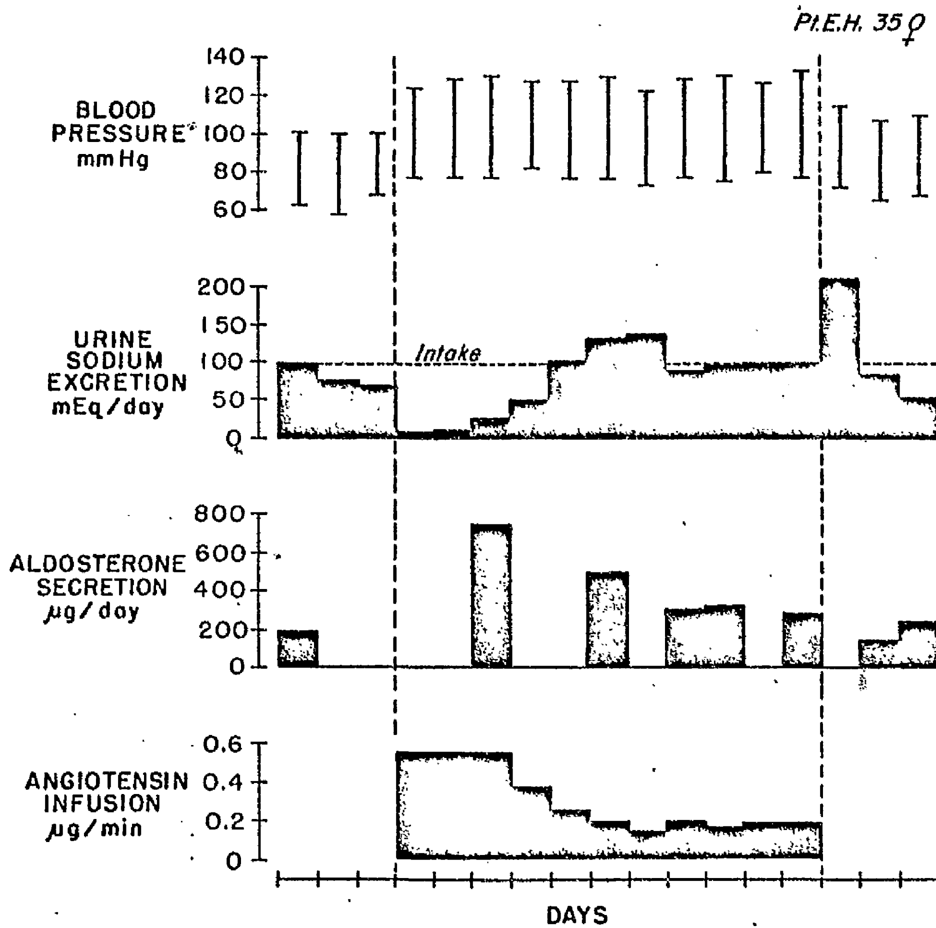
FIGURE 42



interrelationship of these two components of blood pressure, vasoconstriction and arterial filling, is illustrated by a study by Ames and coworkers (214) in which prolonged infusions of angiotensin II were administered to man (Figure 43). Angiotensin II infusion caused an immediate and sustained increase in blood pressure. However, after three days it was found that diminishing amounts of angiotensin II were needed to sustain the increase in blood pressure. This gradual increase in potency of the peptide appeared related to the accompanying sodium retention, secondary to angiotensin II mediated increases in aldosterone production. Thus the vasoconstrictor action of angiotensin was greatly potentiated by concurrently induced volume expansion(215).

The question arises as to the mechanism of this increased sensitivity to angiotensin II during positive sodium balance. One explanation is that the accompanying increased filling of the arterial tree directly increased blood pressure so that less and less angiotensin induced vasoconstriction was needed to sustain the blood pressure elevation. An alternate or perhaps additional explanation is that, under conditions of positive sodium balance the vascular tree is more sensitive to the vasoconstrictor action of angiotensin. Such a possibility is supported by studies which have demonstrated that the pressor response to exogenous angiotensin II is greater in sodium depleted than in sodium loaded man and animals (3,216-218).

FIGURE 43



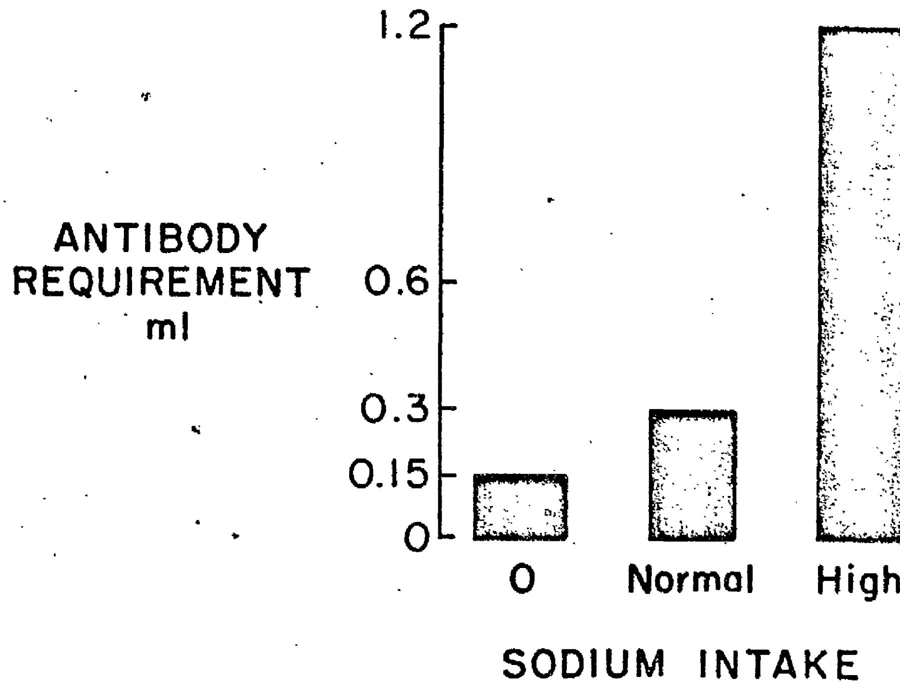
Angiotensin infusion caused an increase in blood pressure which was accompanied by increased urine aldosterone excretion and marked sodium retention. After three days progressively less angiotensin was required to sustain the blood pressure increase. The increased pressor sensitivity appeared to be consequent to the sodium retention.

Studies of Angiotensin II Vascular Receptors:

To study the mechanism whereby changes in the pressor action of angiotensin II occur, antibodies specific for angiotensin II were injected into rats maintained on different sodium intakes (25). The minimum volume of antibody-rich serum required to eliminate the blood pressure response to injection of 50 ng of angiotensin II was determined. As illustrated in Figure 44, wide differences were observed in the volume of antibody required depending on the sodium intake of the rats. However, at each level of sodium intake entirely similar amounts of antibody were needed. Thus, rats on normal sodium balance required 0.3 ml of antibody to eliminate the pressor responsiveness to angiotensin II, whereas only 0.15 ml were needed in sodium depleted rats but 1.2 ml were required for sodium loaded rats.

Our interpretation of these studies was that the amount of antibody required to block the pressor response to angiotensin II is a measure of the affinity of vascular receptors for angiotensin II. Vascular receptors are visualized as competing with the antibody for binding of exogenous angiotensin II. Thus the more avid the vascular receptors, the more antibody required to block them. Hence, under circumstances of sodium loading, vascular receptors appear to have greater binding affinity for angiotensin II than during sodium deprivation. An alter-

FIGURE 44



The amount of angiotensin required to block the blood pressure response to exogenous angiotensin was directly related to sodium intake. This appeared to be consequent to increased vascular affinity for angiotensin. Vascular receptors are visualized as competing with the antibody for binding of exogenous angiotensin.

native interpretation is that during sodium administration a larger number of receptors are unoccupied because of the lower level of circulating angiotensin (219).

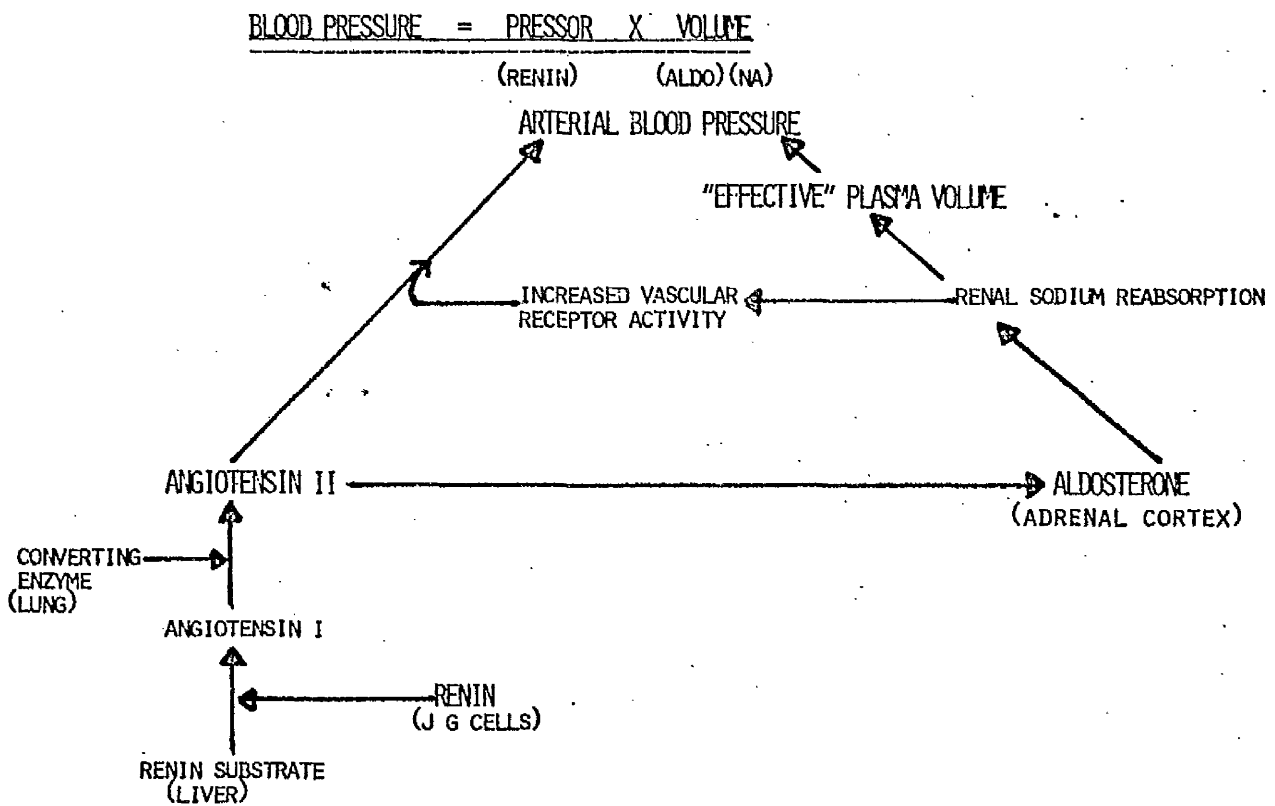
Whatever the true interpretation, increased vascular affinity for angiotensin II could explain in part the increased pressor responsiveness to angiotensin II which was found during prolonged infusions, after aldosterone-induced sodium retention has occurred.

The Role of the Renin-Angiotensin-Aldosterone System in Normal Blood Pressure Homeostasis:

It is quite apparent that the renin-angiotensin-aldosterone system can affect blood pressure both by altering the capacity of the blood vessel via angiotensin II induced vasoconstriction and by affecting the filling of the arterial tree by aldosterone induced sodium retention and volume expansion (Figure 45)(211,212). Of interest is the question of whether or not the renin-angiotensin-aldosterone hormonal system is normally involved in the minute to minute maintenance of blood pressure. Recent studies utilizing angiotensin II inhibitors have demonstrated that angiotensin blockade of sodium deprived animals causes a fall in blood pressure (27,220). However almost no effect was seen in animals maintained on a normal sodium intake (27,221). These studies suggest that in animals the vasoconstrictor action of angiotensin only participates in maintenance of blood pressure when blood

FIGURE 45

DUAL SUPPORT OF BLOOD PRESSURE BY THE RENIN-ANGIOTENSIN-ALDOSTERONE HORMONAL SYSTEM



volume is compromised. However, in man, angiotensin II seems to be more important since marked depression of blood pressure has been noted in subjects who were studied on a tilt table during angiotensin II blockade (222). This confirms that the increases in renin seen during change in posture (223) are important for support of blood pressure.

Altogether it appears that the vasoconstrictor action of angiotensin is important for maintenance of blood pressure during adjustment to the upright position, during sodium deprivation and under any circumstance in which blood volume and perfusion pressure are compromised. In this construct, the system is viewed as being part of a contingency mechanism which responds to situations in which blood pressure is compromised.

The system appears to function as follows:

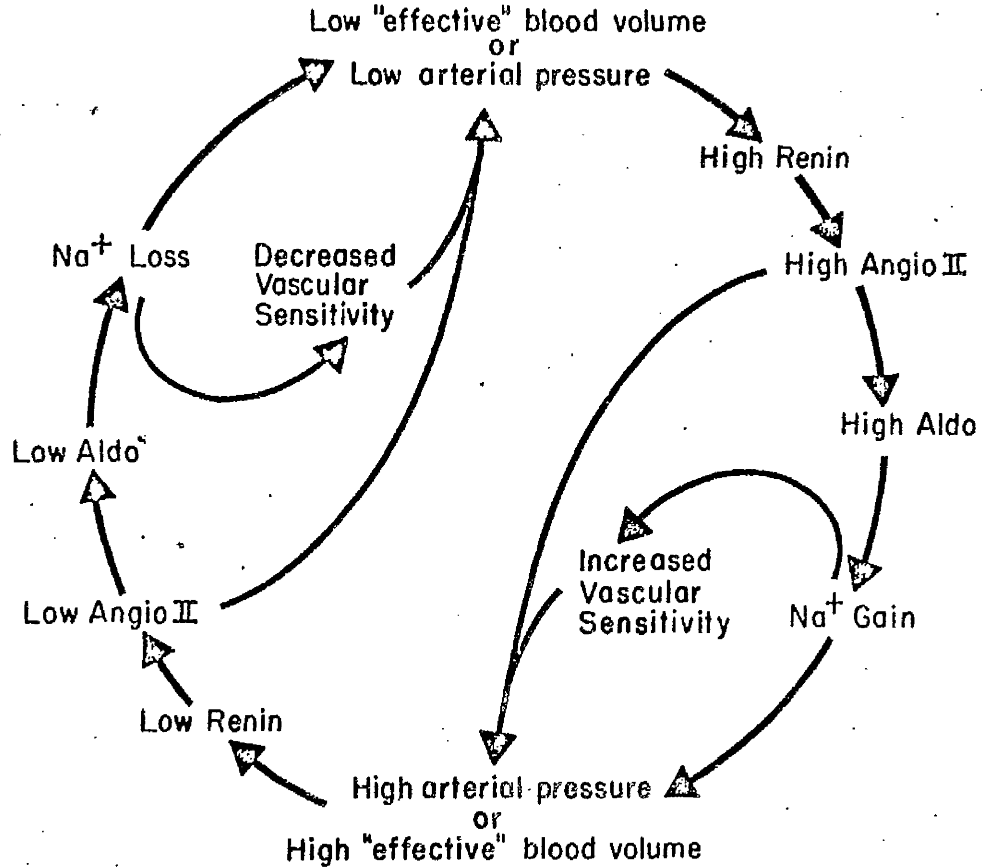
Renin is released rapidly from the kidney in response to reductions in blood pressure or in "effective" blood volume. Renin then reacts with renin substrate, present in blood in high concentration, to liberate angiotensin I (Figure 45). Angiotensin I is converted rapidly to angiotensin II during passage through the lungs and blood pressure is quickly restored by the vasoconstrictor action of angiotensin II. Meanwhile, angiotensin II acts more slowly to stimulate aldosterone production. Only small amounts of aldosterone are stored in the adrenal and aldosterone takes up to an hour to act, probably because it first increases

protein synthesis in the target organ, kidney (194). Thus, the secondary sodium retaining response of the system slowly restores sodium balance and improves "effective" blood volume which turns off the signal for renin release. Moreover, the sodium retention gradually potentiates the vascular reactivity or vasoconstrictor action of angiotensin II so that less and less is needed to sustain the blood pressure.

Therefore, as illustrated in Figure 46, arterial pressure is protected by the renin-angiotensin-aldosterone system in three ways. (1) The primary release of renin results in increased plasma angiotensin II and direct vasoconstrictor support of blood pressure. (2) Secondary increases in aldosterone induce sodium retention and thus help to increase blood volume and pressure and tissue perfusion and (3) sodium-induced increases in vascular receptor affinity potentiate the responsiveness of angiotensin II as the induced sodium retention and volume expansion act to shut off the signal for renin release.

In this context, the hormonal system is seen as acting as an emergency mechanism which responds to reductions in arterial pressure. Obviously, other factors which include the neural system respond more rapidly to sustain perfusion pressure (224) and these are followed and gradually superseded by a somewhat slower angiotensin II vasoconstrictor effect. The net effect of vasoconstriction is to maintain perfusion pressure at the expense of reduced blood flow.

FIGURE 46



The other limb of the renin-aldosterone system restores blood flow and tissue perfusion by aldosterone induced sodium retention and consequent volume expansion. Therefore the two components of the hormonal response act in sequence first to maintain perfusion pressure and then to restore the compromised blood volume. The first response is supportive, the second restorative.

The Causal Role of the Renin System in Renovascular Hypertension.

Since the vasoconstrictor action of angiotensin II is accompanied by sodium retention (mediated by aldosterone), angiotensin II excess can maintain chronic hypertension. Other vasoconstrictor substances cannot sustain chronic hypertension since increased blood pressure normally results in pressure natriuresis and this tends to restore blood pressure to the null point so that more and more hormone is needed to sustain blood pressure elevations (214). However, the dual actions of angiotensin II to cause both sodium retention (via aldosterone) and vasoconstriction can result in sustained hypertension because the pressure natriuresis can be offset by the sodium retaining action of aldosterone. Thus the renin-angiotensin-aldosterone system is admirably constructed to sustain chronic increases in blood pressure.

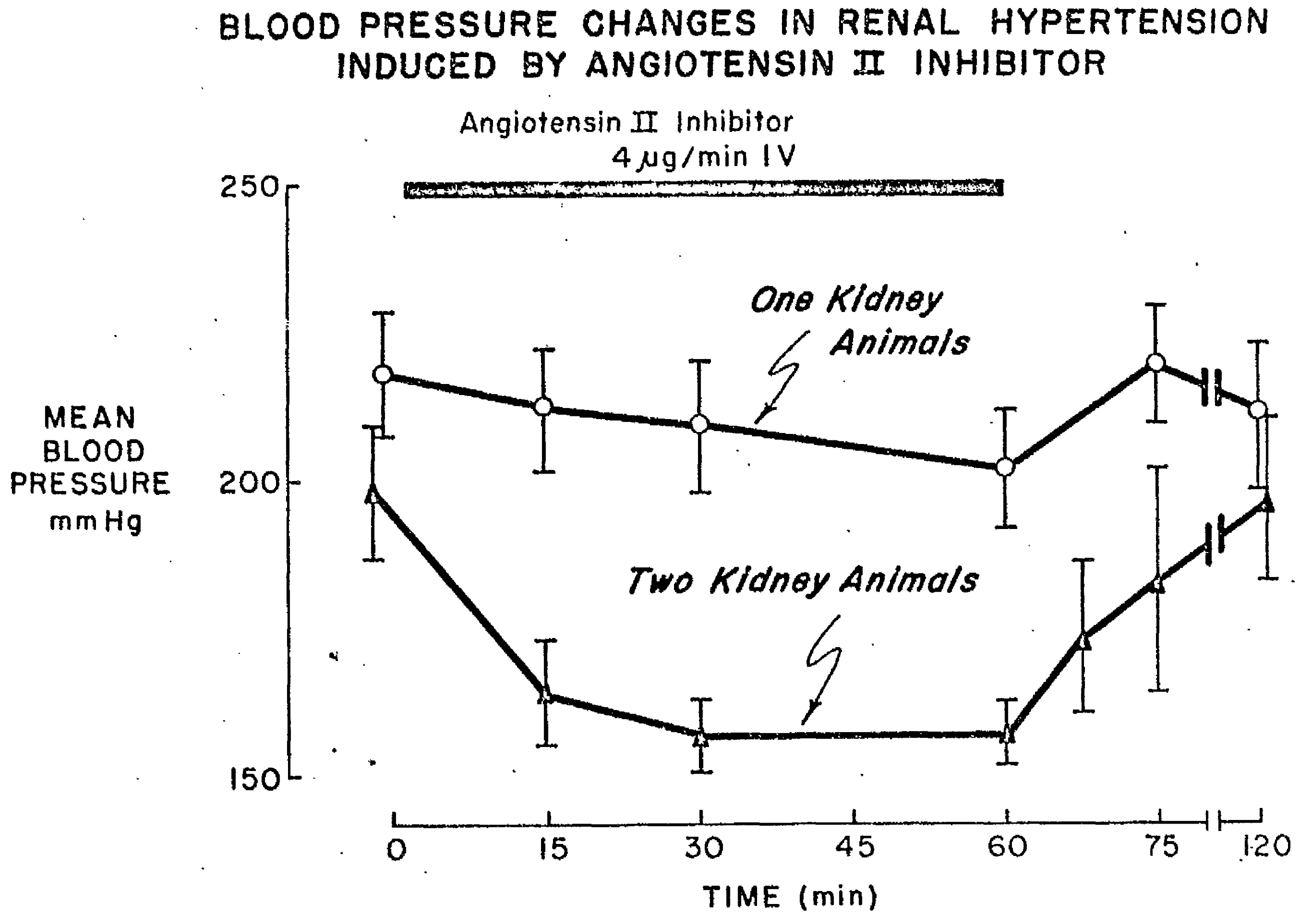
A causal role for renin has long been suspected in renovascular hypertension -- both experimental and natur-

ally occurring (2,212,225). However, the participation of renin in maintenance of this form of hypertension has been questioned since renin is not always elevated in renovascular hypertension (226-229) and not all patients who have evidence of the disease are cured by renal surgery (230,231).

To investigate the role of renin in experimental renal hypertension two different rat models were employed. In one model, one renal artery was clamped to less than 50% of its diameter and the contralateral kidney was left in place. This model is associated with increased peripheral plasma renin due to hypersecretion of renin from the clipped kidney, but renin release is suppressed from the contralateral kidney (232,233). The second model is similar, but the contralateral kidney is removed. Renin secretion is not increased in this form of experimental hypertension (232,233). In two sets of studies, either angiotensin II antibody was injected into these two rat models or a specific angiotensin II inhibitor (sar^1-ala^8-angiotensin II) was infused (26). The elevated blood pressure was reduced by both procedures for angiotensin blockade only in the animals with two kidneys (Figure 47). An insignificant fall in blood pressure was observed in the rats with only one kidney.

These data suggest that angiotensin II is critically involved in the pathogenesis of the two kidney form of renal hypertension but appears to play no role in the main-

FIGURE 47



Infusion of a specific angiotensin II inhibitor, sar-1-ala-8-angiotensin II, to hypertensive rats lowered blood pressure only in the model in which one renal artery was clamped, the other kidney left in place. Blood pressure was not lowered in the uninephrectomized model with the remaining renal artery clamped.

tenance of hypertension in the one kidney model. Thus when renin is not elevated, it does not appear to participate in the maintenance of renovascular hypertension. This study points to the key role that renin can play in hypertension, but it also serves to illustrate that the kidney has an alternate means for sustaining hypertension.

The role of sodium in maintaining the elevated blood pressure in the one kidney model was revealed in a subsequent study by Gavras and his co-workers (27) who demonstrated that blood pressure could be reduced in these hypertensive animals by angiotensin II inhibitor after a period of sodium deprivation. Thus, sodium depletion converted the hypertension from being caused by another factor (presumably excessive volume)(213,234) to one which was dependent on abnormal renin secretion.

The physiological basis for the differences in renin secretion and in the hypertensive process in these two experimental forms of renal hypertension has application to the study of human renovascular hypertension. In the two-kidney animals, the decrease in renal perfusion caused by the clip around the renal artery has been shown to result in increased renin secretion from that kidney (151,235) and in the studies just described this excess renin has been shown to be the cause of the increased blood pressure. In addition, reduction in perfusion pressure to the clipped kidney resulted in its becoming a sodium-retaining kidney

(234) but at the same time the elevation in systemic arterial pressure has been shown to result in pressure natriuresis in the contralateral normal kidney (236,237). This sodium loss may have provoked further increases in renin secretion from the ischemic kidney. In contrast, in the one kidney model, contralateral pressure natriuresis cannot occur. The resultant chronic sodium retention turns off renin release and it also sustains a volume dependent hypertension.

Renovascular Hypertension in Man.

In the following analysis, information gleaned from the studies of experimental renal hypertension have been applied to the naturally occurring forms of renovascular hypertension. There has been some controversy as to whether or not renin is involved in maintaining the blood pressure elevation in human renovascular hypertension (2,212,225). The use of peripheral and renal vein renin measurements to predict curability by unilateral repair or nephrectomy has not been consistently successful. Since mortality from associated surgery is as high as 10% in some studies (230,231,238) it is important to be able to identify accurately patients potentially curable by surgery.

Since most patients with renovascular hypertension have two kidneys, theoretically in man one might expect to find the analogue of the two-kidney experimental hyperten-

sion, i.e., increased peripheral plasma renin caused by unilateral hypersecretion of renin in association with suppression of renin from the contralateral kidney. However, bilateral renal ischemia is not uncommon. Bilateral renovascular hypertension might be similar to the one-kidney form of experimental hypertension since contralateral pressure natriuresis might not ensue if the contralateral kidney is also underperfused and cannot excrete salt normally. Cure of hypertension in these patients by uninephrectomy might not be expected since reduction in renal mass, while removing some excess renin, might result in more sodium retention because of reduction in the number of nephrons and cause elevation of blood pressure by volume expansion. Thus, the hypertension might be converted from a renin-dependent form into a volume-dependent form (211).

Hence, it is important to identify accurately those patients who might benefit from uninephrectomy from those with bilateral disease in whom repair of the renal artery might be the process of choice in order to increase functioning renal mass and thus perhaps increase sodium excretion.

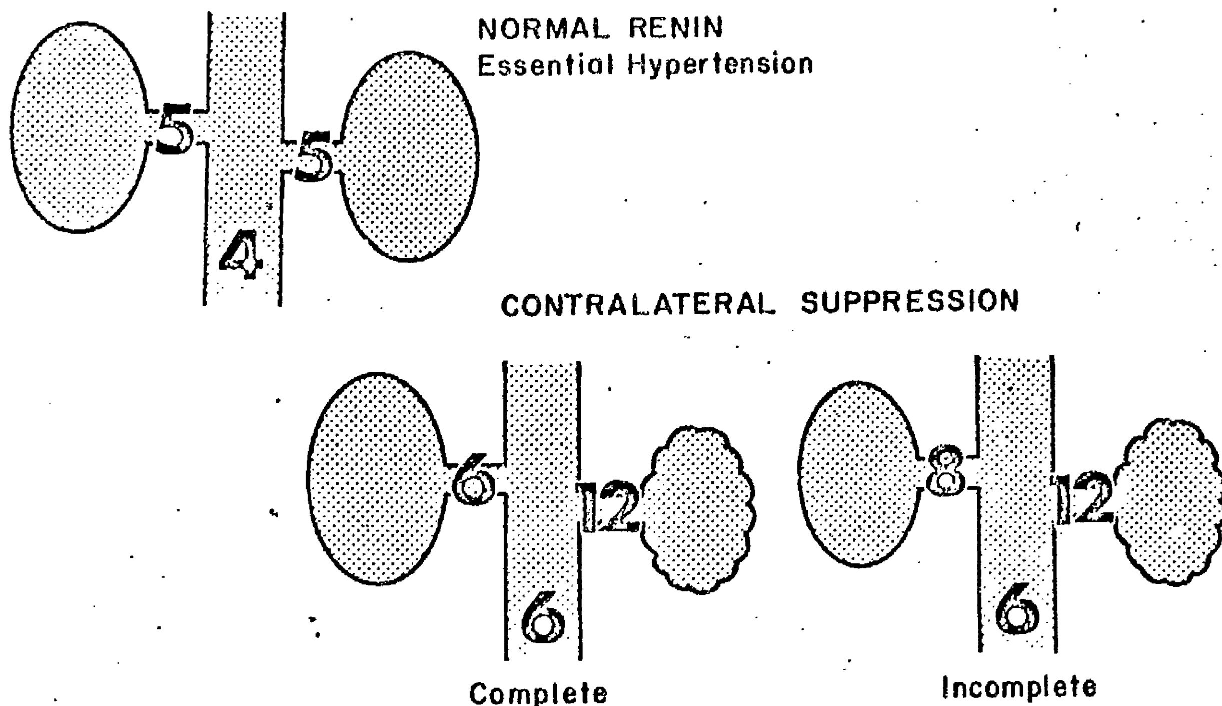
Theoretically, to identify two-kidney renovascular hypertension in man, the following parameters should be identifiable: (1) elevation of peripheral plasma renin activity; (2) suppression of renin secretion from the contralateral kidney -- an indication that that kidney is normal and (3) renal ischemia of the suspect kidney.

In a study of 28 patients with suspected renovascular hypertension who came to surgery (28) we were able to analyse, retrospectively, the value of these three parameters in predicting curability. All patients who had elevated peripheral plasma renin were cured by surgery with the exception of one who had post-operative graft closure. However, of 10 patients who had normal plasma renin, 2 were cured, 3 improved, and in 5 hypertension was not reduced by surgery. Hence, elevated peripheral plasma renin is a good indicator for cure, but its absence does not preclude curability.

Suppression of renin secretion from the contralateral kidney is a good indication that the kidney is functioning normally and could sustain the patient in the event of unilateral nephrectomy. Contralateral suppression is defined in Figure 48. Since renin is not cleared to any great extent by the kidney, when a kidney is secreting no renin, renal vein renin should equal arterial renin and all of the renin which contributes to the peripheral level should be derived from the other kidney. However, in the absence of contralateral suppression of renin secretion V (renal vein renin) minus A (arterial renin) would have positive value.

Theoretically, V-A = 0 is a good indicator for cure, but pragmatically it is difficult to predict definitively that the two values are idential. This problem is compounded by the fact that in patients with essential hypertension V-A is

FIGURE 48



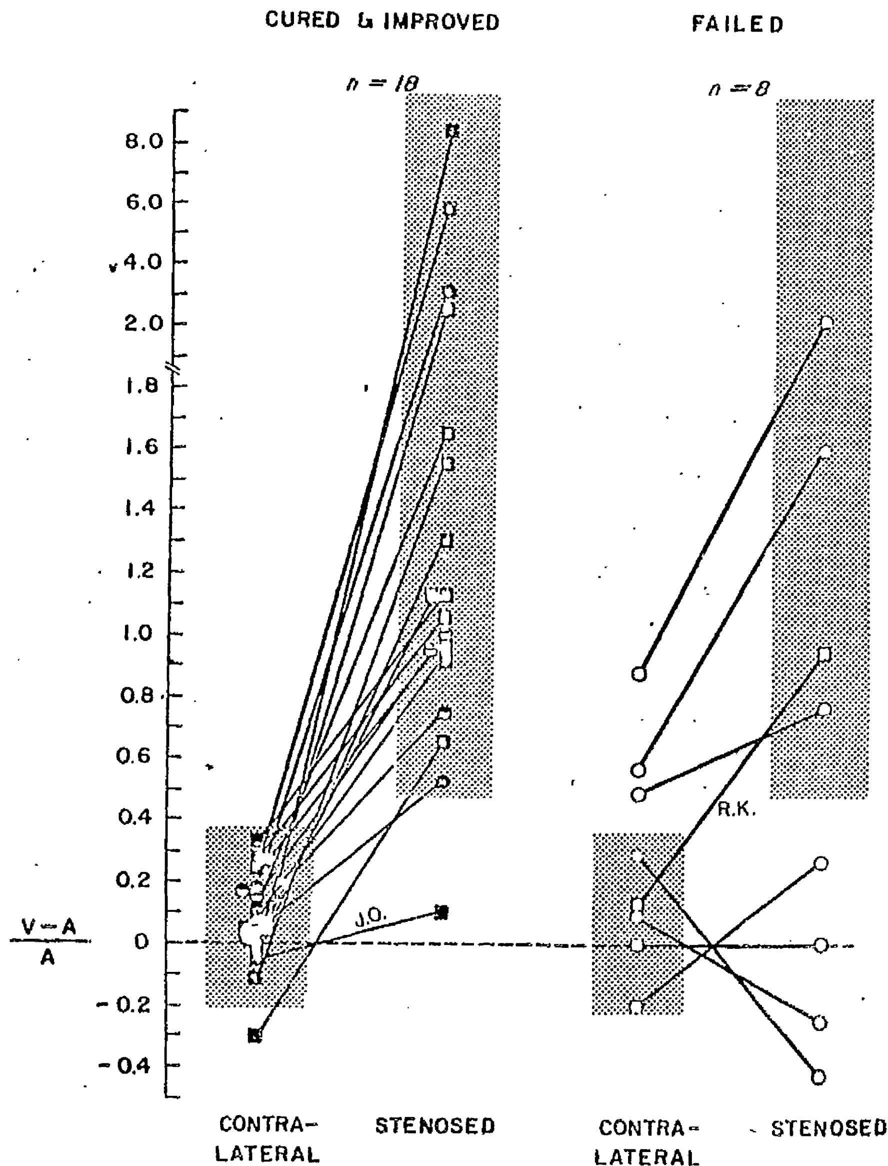
Since renin is not cleared to any great extent by the kidney, renal vein renin is equal to arterial renin when renin secretion is suppressed. Incomplete suppression of renin secretion is associated with renal vein renin greater than arterial renin. Contralateral suppression of renin secretion is difficult to identify accurately since, normally renal vein renin is only 24% higher than arterial renin (upper figure).

normally only 24% higher than arterial renin (see page 146). Therefore although contralateral suppression is a good theoretical indicator, it is difficult to identify accurately.

For this reason, the third parameter -- identification of renal ischemia -- becomes more important. Hosie and coworkers (151) pointed out that renal vein renin concentration can be increased both by increasing renin secretion and by reducing renal blood flow. In a previous section, it was demonstrated that renal plasma flow is inversely related to renal vein renin when considered in relationship to arterial renin [(V-A)/A] and renal blood flow can be calculated from renal vein renin measurements when renin is secreted from only one kidney (16). Under these conditions, $(V-A)/A > 0.48$ is always associated with reduced renal blood flow. (V-A)/A was estimated for both kidneys of the 28 patients included in the study (Figure 49). All but one of the cured or improved patients had an elevated (V-A)/A from the suspect kidney and all exhibited contralateral suppression $(V-A)/A < 0.38$ (i.e. mean + 2 SD of cured patients). The one exception was patient J.O. in whom the combined renal vein renins were only 10% higher than the arterial level. This low value suggests that renal venous blood was not collected since this value is not high enough to sustain the peripheral level (see page 145).

Seven of 8 patients in whom surgery was not successful either failed to demonstrate elevated (V-A)/A from the sus-

FIGURE 49



Preoperative stenotic and contralateral renal vein renin relative to arterial renin in 28 patients. All cured or improved patients (18) had contralateral renin suppression (shaded area = 0.08 $\pm$ 2 SD) and all but JO, in whom there was a technical error, had abnormally elevated (V-A)/A from the stenotic kidney [shaded area= (V-A)/A < 0.48] indicating renal plasma flow reduction. In contrast, no patient who failed to respond to successful surgery (7) met both of these criteria. RK had postoperative graft occlusion.

pect kidney (N=4) or secreted renin from the presumably normal kidney (N=3). The one patient in whom we would have predicted cure had post-operative graft occlusion.

A scoring system has been developed which mathematically evaluates the weight of each of the three parameters (1) elevated peripheral plasma renin, (2) contralateral suppression, i.e. $(V-A)/A < 0.38$, and (3) renal ischemia, i,e, $(V-A)/A > 0.48$. Using this system we have been able to predict consistently the curability of patients suspected of having curable renovascular hypertension.

Summary

The renin-angiotensin-aldosterone hormonal system appears to participate in normal blood pressure regulation and in causation of some forms of renovascular hypertension. The system restores reductions in perfusion pressure by the direct vasoconstrictor action of angiotensin II followed by restoration of blood volume via aldosterone mediated sodium retention which turns off the signal for renin release, while potentiating the vasoconstrictor effect of angiotensin II.

The hormonal system can sustain chronic hypertension because the increased arterial pressure associated with angiotensin II excess is not associated with sustained pressure natriuresis since it is accompanied by aldosterone mediated sodium retention.

An understanding of the mechanisms which can sustain

experimental renovascular hypertension, together with an appreciation of the normal relationships of renal vein renin to arterial renin has enabled differentiation of those patients with renovascular hypertension who can be cured by surgery. The success of this approach lends credence to the idea of the dual nature of blood pressure support -- i.e., volume and vasoconstriction -- and supports the view that the renin-angiotensin-aldosterone hormonal system can be importantly involved in maintenance of hypertension.

SUMMARY

This memoir has considered the regulation of sodium and potassium balance and blood pressure homeostasis by the renin-angiotensin-aldosterone hormonal system.

First, improved methods for measurement of renin and aldosterone have been described. Plasma renin activity is measured by a radioimmunoassay in which angiotensin I, generated by the reaction of plasma renin with its substrate during 3 or 18 hours incubation, is quantitated. Blank subtraction is eliminated and accuracy enhanced by complete inhibition of angiotensinases and converting enzyme. Improved assay sensitivity has been achieved which is important for detecting about 30% of patients with essential hypertension who exhibit subnormal renin levels. However, using three other methods recommended by commercial kits, most low renin samples were undetectable and indistinguishable from many normal renin samples.

A double isotope derivative method was described for measurement of the urinary acid-labile conjugate of aldosterone. Though time consuming, the method provides accuracy and reproducibility. Evidence is presented for isotope fractionation of ^{3}H from ^{14}C aldosterone diacetate during column and paper chromatography. Data from this method are interchangeable with those derived from a more recent radioimmunoassay method which provides greater sensitivity and capacity. Procedures were developed for radioimmunoassay to

separate aldosterone from cross-reacting steroids. For urine samples, the acid-labile conjugate of aldosterone is separated merely by extracting cross-reacting steroids into methylene chloride. For plasma aldosterone, a celite partition column accomplishes separation.

To investigate the existence of a natriuretic hormone, extraction, concentration and purification procedures were developed in conjunction with a semi-automated rat bioassay for detecting changes in urine sodium excretion. Urine and plasma samples were fractionated and a natriuretic substance separated which could have physiological relevance since it was found only in extracts from sodium-loaded patients. Evidence suggests that this substance affects sodium reabsorption in the distal tubule at a site beyond that for potassium secretion.

Studies of plasma renin "activity" revealed that, normally, renin substrate concentration is rate limiting. However, under steady state conditions, no relationship was found between substrate concentration and plasma renin activity. Therefore from a physiologic standpoint, renin secretion appears to compensate for changes in substrate concentration to maintain an appropriate rate of angiotensin generation. Heparin was shown to be a specific competitive inhibitor of the reaction between renin and renin substrate.

In patients with essential hypertension, a constant fractional increment in renal venous renin, relative to arterial renin was observed over a wide range of peripheral

renin levels. These data indicate that the clearance of renin is a constant fraction of peripheral renin and that plasma renin activity reflects renin secretion.

Studies in normal subjects revealed that renin and aldosterone respond to, and appear to affect, urine sodium excretion, even at high sodium intakes. Also, during 11 days of sodium deprivation, as expected, renin and aldosterone secretions increased. But aldosterone increased proportionally more than renin. The discrepancy could be explained by accompanying potassium retention. This study highlights the importance of both trophic stimuli for aldosterone,—angiotensin II and potassium.

The importance of another coordinate in the renin-aldosterone system was revealed when potassium administration to man and rats suppressed renin secretion and potassium deprivation increased it.

Notwithstanding these interrelationships, sodium and potassium homeostasis cannot be explained solely in terms of the two renal actions of aldosterone to promote sodium reabsorption and potassium secretion since the simultaneous effect on both ions would lead to inappropriate variations in one when intake of the other changed. A balanced control system can be proposed if the concurrent effect of both sodium and potassium on glomerular filtration rate and proximal tubular sodium reabsorption, and consequently distal sodium supply, are taken into account. Changes in distal sodium supply

can affect urine sodium excretion, but when aldosterone is high, more sodium is reabsorbed distally. This results in increased potassium secretion and reduction in sodium excretion. Thus, urine sodium excretion is inversely related to aldosterone activity and directly related to distal sodium supply, whereas potassium excretion is directly related to both aldosterone and distal sodium supply. Hence, changes in distal sodium supply counterbalance the dual and opposing actions of aldosterone, allowing one ion to be held constant in the face of large excess or paucity of the other.

Control of blood pressure by the renin-angiotensin-aldosterone system proceeds simultaneously. Studies indicate that a positive sodium balance increases pressor responsiveness to angiotensin II both hydraulically and by increasing vascular receptor affinity for it.

Studies in rats of the role of renin and angiotensin in renovascular hypertension, revealed that hypertension is maintained in one model (two kidneys, one renal artery clamped) by vasoconstriction due to increased angiotensin II. However, in another model (uninephrectomized, remaining renal artery clamped) blood pressure is not sustained by excess angiotensin. Improved understanding of the mechanisms of hypertension in these models led to a new analysis of the human counterpart. Three criteria were developed which, in combination, have been successful in identifying curable

unilateral renovascular hypertension: (1) elevated peripheral plasma renin in relation to sodium excretion, i.e., demonstration that excess renin secretion sustains the hypertension, (2) complete contralateral suppression of renin secretion, i.e. an indicator that the contralateral kidney is unaffected and (3) renal ischemia of the suspect kidney. This last parameter can be quantitated simply by comparing the relationship of renal renin to peripheral renin with data derived from patients with essential hypertension.

It is proposed that the renin-angiotensin-aldosterone system comprises two interacting negative feed-back loops. In one, sodium and potassium balance are maintained by aldosterone coordinated with changes in distal tubular sodium supply. In the other, blood pressure is supported by aldosterone coordinated with angiotensin. When the effective blood volume is compromised, angiotensin II vasoconstriction acts to support blood pressure until aldosterone induced sodium retention restores blood volume and turns off renin release.

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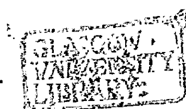
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