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As it has always been, my parents and sister continue to give love and support which is so important to me.

To 112 Huntington St., a tradition and joy which I will carry with me always.

And to Abby, who has my unending love. She survived the preparation of this thesis; thanks to her, so did I.

Introduction

Prorenin is a polypeptide with a molecular weight of approximately 56,000 daltons (3,73,75,78) having no inherent enzymatic activity. It does, however, have the potential for renin-like properties which occur when prorenin is exposed to the correct environment and the proper structural changes take place. Sine 1971 when Lumbers (45) discovered prorenin, activation of the molecule has been demonstrated by a number of methods including cryoactivation, acid dialysis and exposure to proteolytic enzymes. Much of the research aimed at understanding the physiologic role of prorenin, if any, has been done by analyzing the possible mechanisms for this activation (see Appendix I).

Evaluation of the prorenin system has proven to be a complex problem. Plasma is an enormously complicated medium and assigning a unique role to any one component is foolhardy. Conversely, experimental data obtained from more limited systems is not easily applied to physiological conditions. Interspecies differences have also complicated the understanding of prorenin. Trypsin activation of prorenin has never been well documented in the rat (57), a commonly used animal model for hypertension. An understanding of the prorenin system of the rat is important in interpreting past experiments and in elucidating the possible significance of prorenin.

The objective of this research is to demonstrate the presence of inducible plasma renin activity in rat plasma and to show that this

increased activity is secondary to renin formation from prorenin. The first step is proof that inducible renin activity exists in rat plasma, both from normal and nephrectomized rats. This will be accomplished by use of trypsin as an activator of angiotensin I production. Increased generation in normal rat plasma would show presumed activation of prorenin as has been done in other species. Since there is no circulating active renin 24 hours after nephrectomy in rats and other animals (70), demonstration of increased activity in the plasma of nephrectomized animals would eliminate the possibility of any alteration in endogenous active renin as a source for increased angiotensin I production. It would also support an extra-renal source for at least a portion of circulating prorenin.

While renin's sole function appears to be the production of angiotensin I, there are other sources of angiotensin I generation under the right conditions. Thus, angiotensin I measurement and renin activity cannot be assumed to be synonymous. Therefore, the second aspect of investigation is an attempt to show renin to be the source of elevated angiotensin I levels. This will be done in two ways. The profile of angiotensin I generation with variation of pH will be examined in an attempt to show the "fingerprints" of renin activity. Secondly, the hemodynamic properties of activated plasma will be evaluated by injection of this plasma into animals prepared for blood pressure monitoring (bioassay animals). The effects of specific pharmacological blockade will help to characterize the reasons for any blood pressure alterations.

Materials and Methods

Plasma samples - Plasma was collected from donor animals by cannulation of the carotid artery of male and female Sprague-Dawley rats weighing 200-300 grams; all plasma was pooled. Ketalar (ketamine HCl, Parke-Davis) 50mg/kg intraperitoneally was used for anesthesia.

Bioassay animals - Animals were male and female Sprague-Dawley rats weighing 200-300 grams. Surgical preparations are described below.

Plasma renin activity - Activity was determined at pH 6.0 by the Renak renin RIA kit (Roche). EDTA, PMSF and 8-hydroxyquinoline were used as angiotensinase inhibitors. All plasma was stored at -40°C and thawed in an ice bath just prior to assay. There was no multiple thawing and refreezing.

Trypsin activation - Trypsin solution was prepared by addition of lypholized enzyme (Sigma) to 0.001N HCl. Fresh trypsin solution was prepared each day of use. Trypsin activation was done with 1 mg enzyme/ml plasma unless otherwise noted. Activation was done at 4°C for 48 hours. Plasma was dialyzed against 0.1M Tris buffer in normal saline (pH 7.4) throughout the activation, as was control plasma.

Renin activity pH profile - Plasma pH profile was obtained by the method of Gallagher (25). Plasma, following 48 hours of dialysis, was adjusted to the desired pH by addition of 0.01N malaeic acid or 2.0N KOH. Plasma renin activity was then determined by RIA as described above with the exception that addition of MES-HCl buffer was omitted.

Nephrectomy and adrenalectomy - Both procudures were performed under ether anesthesia. Nephrectomy procedures were identical for donor

and bioassay animals. Bilateral incisions were made just below the costal margins and the organs were isolated. Ureters and renal vessels were clamped and ligated prior to removal of kidneys. Excision of adrenal glands (in the case of bioassay animals) without ligation gave no significant hemorrhage. Following surgery, food was withheld and water (or normal saline for adrenalectomized rats) was provided ad lib. Adrenalectomized rats also received 0.5mg deoxycorticosterone SC at the time of surgery. Both operations were done 24 hours before plasma was collected or blood pressure recording was done.

Blood pressure monitoring - Bioassay animals were anesthetized with Ketalar (50mg/kg IP) and a tracheotomy was performed. Blood pressure monitoring was done by cannulation of the common carotid artery with PE50 Intramedic polyethylene tubing filled with heparinized saline. Recording was done using a Narco Physiograph and a Slather P23dB transducer. The vagus nerve was not severed. For IV administration, one or both internal juglar veins were cannulated using tubing of known dead space.

Hemodynamic experiments - Each in vivo experiment began with injection of 200ng of angiotensin I to insure proper operation of equipment and normal responsiveness of the animal (brief, rapid increase in blood pressure). The ganglionic blocker pentolinium (Ansolysen, Wyeth) was used, 10mg/kg IP, except under conditions denoted as "baseline." Saralasin (1-sar-8-ala-angiotensin II, 120ug/ml), when used, was administered at 0.044ml/min via a constant infusion pump beginning ten minutes prior to other studies and was continued throughout. Effective blockade was always demonstrated by lack of a pressor response to 200ng of angiotensin I. For alpha-adrenergic blockade, phentolamine (Regetine, CIBA)

was slowly administered IV (1 mg/kg). Blockade was shown by absence of a pressor response to a 600ng infusion of eqinephrine and norepinephrine.

Results

Trypsin activation - Exposure of plasma to trypsin yielded approximately a doubling of angiotensin I production at pH 6.0. This increase occurred in the plasma of normal and nephrectomized animals. Further analysis showed a linear increase in plasma renin activity with increasing trypsin concentration up to 2mg trypsin/ml plasma. At 0.1 mg/ml, however, an unexpectedly sharp rise is present (graph 1). This is seen clearly in normal plasma and not in plasma from nephrectomized rats.

pH optima - Plots of plasma renin activity with varying pH did not yield the classic smooth curve of renin (graph 2). The low pH range showed surprisingly high levels of angiotensin I generation with increasing production as pH decreased. The mid pH range (5.0 to 7.0) was composed of a gradual increase in activity with lowering of pH and a peak at about 5.5. In trypsin treated samples, there is also a peak of activity at pH 7.5. With the exception of pH 4.0, activity of untreated plasma was always greater than that of trypsin treated plasma from nephrectomized animals and activity of normal plasma which was trypsin activated was greater than either of the other sample groups at corresponding pH.

Hemodynamic studies - Introduction of 0.1 ml of dialyzed, untreated plasma into a bioassay animal had no effect on blood pressure. Injection of trypsin treated plasma from nephrectomized or normal rats yielded a characteristic biphasic response composed of a rapid drop in pressure (phase A) followed by a slow rise to baseline. After a brief delay (1-2 minutes), a mild pressor effect was noted (phase B), after which blood

pressure returned to baseline. Quantitative summarization of results can be found in tables 1 and 2. Graphic representation is located in graphs 3, 4 and 5. No differences were noted between injection of treated plasma from normal versus nephrectomized donor animals. Plasma of nephrectomized rats was generally used since it contained no inherent renin activity prior to activation.

The phase B rise in blood pressure above baseline was equal in normal and nephrectomized bioassay animals. A greater sensitivity to angiotensin I would not have been surprising in nephrectomized animals since their plasma should not contain any active renin. In an attempt to show renin dependence of this pressure peak, saralasin was administered prior to plasma injection. In spite of the elimination of a response to angiotensin I, no change could be detected in the phase B phenomenon (table 6). The pressor response was totally eliminated, however, by preliminary treatment with phentolamine. Prior adrenalectomy of bioassay animals also obliterated the rise in pressure; administration of phentolamine to adrenalectomized animals had no additional effect.

While an initial drop in blood pressure following plasma injection (phase A) was a fairly consistent finding, different experimental conditions were noted to modulate this response. A comparison of adrenalectomized rats with and without pentolinium shows that those with pentolinium had a significantly smaller drop in pressure. Another inconsistency noted was that rats treated with pentolinium and phentolamine did not always respond to treated plasma with a statistically significant drop in pressure. Animals treated with only pentolinium had significant phase A periods.

Discussion

These experiments clearly document an increase in angiotensin I generation following trypsin treatment. In both types of plasma (that from normal and nephrectomized donor animals) production of angiotensin I is doubled when measured at pH 6.0. Thus, inducible angiotensin I generation exists in rat plasma. Because this was the case in plasma from nephrectomized animals, alteration of endogenous active renin cannot account for this increase in activity. Although active renin is no longer present in animals 24 hours after nephrectomy, prorenin appears to be present in near normal levels (65,66). Anephric patients also continue to have circulating plasma prorenin (9,76). If the increase in angiotensin I activity demonstrated in this work proves to be renin, this would be strong support for a non-renal source of prorenin in the rat.

The degree of increased production is generally linearly correlated to the concentration of trypsin used during incubation. This linear relationship, however, did not hold at 0.1mg trypsin/ml of plasma, where a disproportionate increase in activity was noted. We cannot explain this increase.

Having demonstrated that increased angiotensin I levels could be induced by trypsin, the next step was an attempt to prove it came from renin. Profiles of renin activity with variation of pH revealed a group of curves not typical of plasma renin activity (peak at pH 5.5-6.0 with a smooth fall off on either side (25,46,69)). Angiotensin I production at low and mid pH ranges were similar among different groups tested, independent of whether trypsin activation was carried out. In the pH range of 5.0-6.5, activity was somewhat like that of renin in

that a production peak was present at pH 5.5-6.0 with a decrease in activity moving away from that point. Another peak of angiotensin I generation was present at pH 7.5 and was dependent on the plasma sample having been treated with trypsin.

The results of pH profiling do not support the assumption that angiotensin I production is the result of renin or that trypsin specifically increases active renin content. The facts that part of the curves are similar to that of renin and that the newly created activity might have resulted from the cleavage of prorenin where no circulating active renin was originally present, however, justified an attempt to look at the hemodynamic effects of the activated plasma to see if these were characteristic of renin. Activated plasma from nephrectomized donor animals was generally used to overcome any potential difficulty in separating the effects of endogenous renin from newly activated renin.

Hemodynamic experimentation with injection of activated plasma into bioassay animals yielded complicated and rather unexpected results. If renin is injected, one would expect a rapid rise in pressure (5) but such was not the case; the initial cardiovascular event was a fall in blood pressure (phase A). While a rise in pressure above baseline did occur consistently (phase B), it was after a significant delay. Additional and more persuasive evidence against this pressor response being renin mediated is the demonstration that saralasin, an extremely effective blocker of the vasoconstrictive and pressor actions of angiotensin II (29), did not prevent this response. Adequate blockade was always demonstrated prior to each experiment. Therefore, specific blocking studies also failed to support the presence of active renin in trypsin activated plasma.

What can be inferred from the results of the blocking experiments? Once the phase B pressor effect was shown not to be due to renin, the contribution of other possible mediators was investigated. The rise in blood pressure was consistently eliminated by pretreatment with phentolamine. Phentolamine is a moderately effective alpha-adrenergic blocker (20). While angiotensin II may in some way potentiate the pressor effect of catecholamines (8), phentolamine does not seem to alter the interaction of angiotensin analogues with its receptor (79). Thus, the effect of phentolamine can be interpreted as evidence that phase B may be due to catecholamines. The observation that adrenalectomy is as effective as phentolamine in preventing a rise in blood pressure would also implicate catecholamines, particularly those from the adrenal.

Bradykinin, too, can stimulate adrenal catecholamine release (22,23,59). The initial fall in blood pressure followed by a catecholamine mediated pressure rise would be consistent with activation of the kinin system. Kallikrein may have a role in prorenin activation (see Appendix I). It should be noted that angiotensin II receptros controlling epinephrine and norepinephrine release from the adrenal medula may be less specifically blocked by saralasin than those receptors governing vascular tone (26). Angiotensin II also has the capability to stimulate adrenal catecholamine release (22,23,59), so lack of phase B modulation by saralasin does not totally rule out some contribution of renin in the production of the hypertensive response.

The etiology of the phase A hypotensive period following plasma injection is uncertain. The involvement of the kinin system seems like a reasonable possibility, although the large changes in blood

pressure with the rapid time course being discussed would call for vast kinin activation. There is evidence that trypsin can activate kallikrein (78). No evidence was generated in these experiments to lend support to this model.

In rats treated with pentolinium and phentolamine, the typical hypotensive episode did not always reach statistical significance. There is no obvious explanation for this. Most likely, the lowering of baseline pressure brought about by phentolamine attenuated further vasodilation. Phentolamine has its own vasodilatory action independent of alpha blockade (20). The reason why normal bioassay animals had significant pressure drops during phase A when pretreated with phentolamine, in spite of having baseline pressure lowered similarly to nephrectomized and adrenalectomized bioassay rats, is unknown.

Pentolinium was given routinely to animals in an effort to achieve a clearer baseline pressure. Its use gave only a small decrease in baseline pressure. While ganglionic blockers have variable effects on blood pressure based on the extent of autonomic discharge at the time of use, they generally produce only mild reductions in pressure; postural hypotension is a more significant and consistent effect (47). Ganglionic blockers also reduce reflex accommadation to exogenously administered substances. Use of these blockers is a standard technique in bioassay systems (6). In only one situation did results from baseline (untreated) and pentolinium treated animals differ. The fall in blood pressure following injection of trypsin treated plasma to adrenalectomized rats was greater in controls than with pentolinium.

One difference between past studies using trypsin activation and these experiments is that activation was carried out over a longer

period of time in the present work. Instead of minutes to a couple of hours, trypsin was incubated with the plasma for 48 hours. Most evidence seems to support activation being a very rapid process, perhaps even terminating within a few minutes (9,69). The effect of prolonged incubation should only result in a guarantee of full dialysis. The sudden peak in the trypsin titration curve (graph 1) at low trypsin concentration does raise the possibility that higher concentrations with long incubation could be creating angiotensin I by some other route (see below and figure 1).

Was renin generated by the action of trypsin in these experiments? Based on these results, the answer is no. The possibility of prorenin activation, however, has not been absolutely eliminated. Relatively small renin pressor effects could have been masked by the sharp phase A fall in pressure. Injection of activated plasma of normal rats, where there is pre-existing active renin, did not give a noticable increase in blood pressure.

The lack of support for prorenin activation is surprising in view of ample evidence in the literature that, in some species, prorenin is converted to active renin. Plasma activation has been shown to correlate with the loss of a chromatographic protein peak felt to represent prorenin (3,7,50). Likewise, the peak known to correlate to renin undergoes an associated increase (3,7,50,71,75,78). Column fractions containing the prorenin peak have no innate renin activity (75).

Looking at the kinetics of activated prorenin, several studies have shown that its K_m is the same as that for plasma renin, regardless of the method of activation chosen (2,19,69,71). V_{max} has been found to increase following trypsin treatment (69), showing an increase in

enzyme content without a change in allosteric activator or inhibitor concentration or a change in substrate affinity.

Another fact in support of conversion of prorenin to renin is that activated prorenin appears to be immunologically identical to renin. Antibodies raised against renin inhibit the activity of both substances equally as effectively (71,72).

Given the lack of evidence for prorenin activation being responsible for increased angiotensin I production, other possible sources must be considered. There are enzymes other than renin which can form angiotensin I under the proper conditions. Pseudorenin, for example, is a normal plasma constituent of both animals and man. It has the potential to directly hydrolyze human substrate to form angiotensin I, although this is normally totally inhibited in plasma (74). There is substantial evidence that pseudorenin is actually the protease cathepsin D. This enzyme has a pH optimum of about 4.5, significantly below that of renin. Dependence of apparent plasma renin activity on cathepsin D, or any other enzyme with a pH profile different from that of renin, would have been demonstrated by typical pH curves. The curve found was not typical of cathepsin D.

Another pathway for production of angiotensin I independent of renin is unique to the use of trypsin as an activator. Tetradecapeptide (TDP) is a cleavage product of renin substrate. It has the potential to simulate angiotensin I in an RIA as well as to be quickly hydrolyzed to antiotensin I by both renin and cathepsin D. While trypsin does not appear to cleave TDP from human substrate (9,54,69), it does appear to have this capability in dog plasma (25). TDP is a dialyzable peptide, so it should have been eliminated prior to assay in these experiments.

The formation of a non-dialyzable cleavage product with characteristics similar to that of TDP cannot be ruled out.

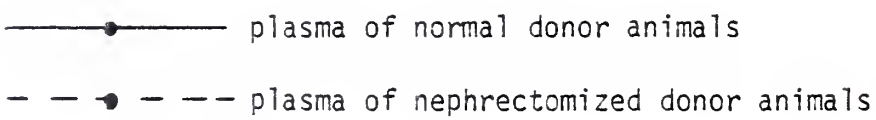
The results of pH profiling have already been reviewed. The increased activity in the area of pH 7.5 is present only in trypsin treated plasma and corresponds to the pH optimum of trypsin. Gallagher (25), doing similar experiments using dog plasma, also noted increased activity at this pH, although this also occasionally appeared in acid activated plasma. The data presented here would suggest some kind of direct participation of trypsin (i.e.-direct conversion of prorenin to renin, formation of a cleavage product similar to TDP). Another possibility is the involvement of another enzyme which has optimal activity at pH 7.5. Trypsin might unmask the effect of this enzyme; proteolytic activation of other enzymes has been recognized in the prorenin literature (52).

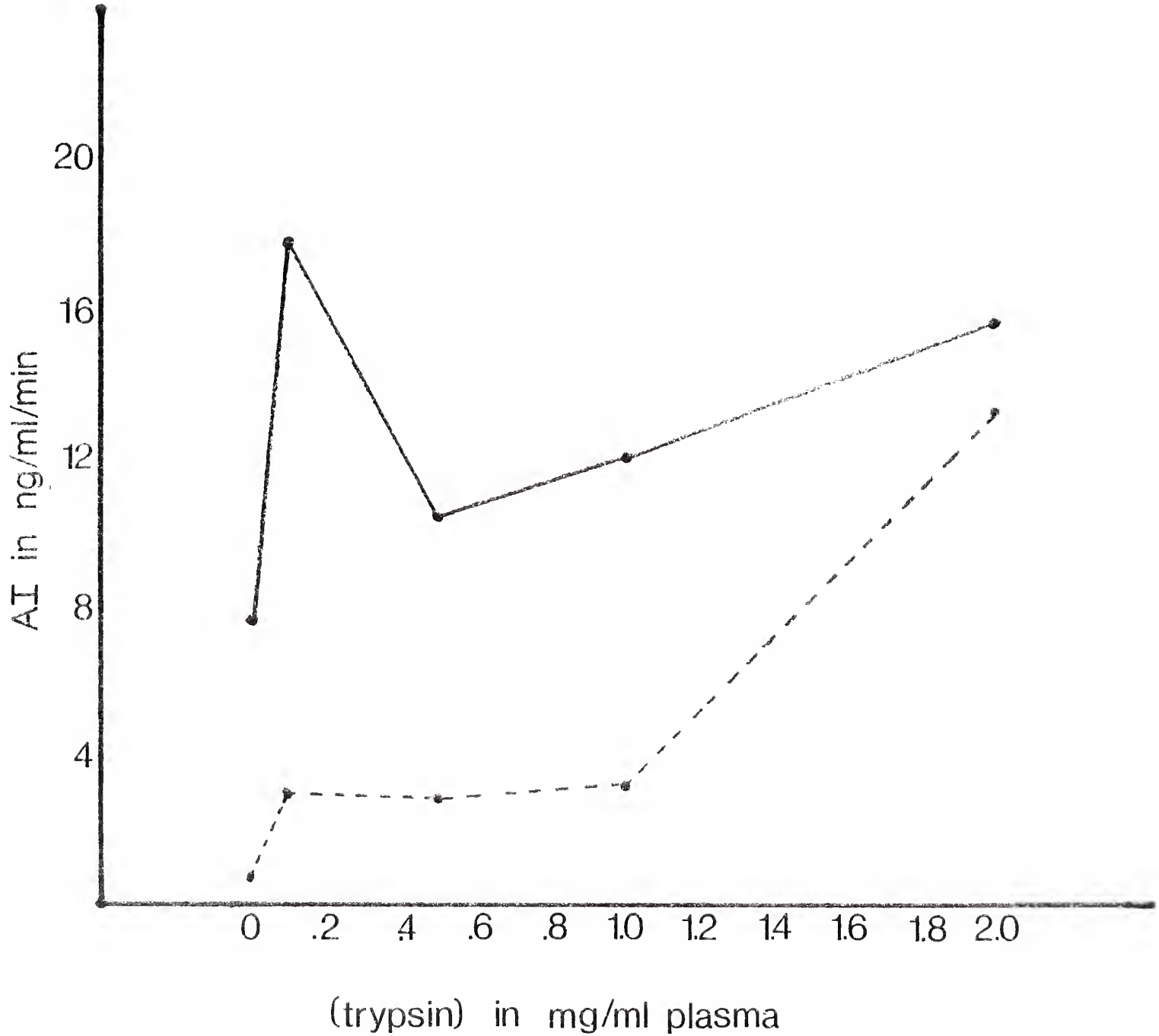
A shift in renin activity at low pH also appears in Gallagher's work. He attributes the increase in angiotensin I production to cathepsin D. Results from the present study do not show a peak at pH 4.5 as was found by Gallagher. Activity continued to rise through pH 4.0. Matoba (46) showed that a small synthetic molecule used as rat renin substrate gave a pH optimum of about 4.0 and a fall below that point. Conceivably, trypsin could form a cleavage product that would give pH profile results similar to this synthetic substrate. There is no data in this study to substantiate this postulate and the increase in low pH renin activity could be secondary to cathepsin D or some other acid protease.

Trypsin activation of plasma consistently increases the production of angiotensin I. This is true not only for plasma of normal rats but also in plasma of nephrectomized donor animals which contained no active renin prior to trypsin treatment. Profiles with variation of pH and

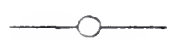
in vivo hemodynamic studies do not support a renin source for this increase generation. At the same time, the conversion of prorenin to renin has not been eliminated. Further investigation of trypsin activation poses a dilemma. More isolated systems would be easier to understand but would also be further removed from physiologic conditions. Ultimately, chromatological isolation of the components of angiotensin I production will be necessary to fully characterize prorenin activation. Inconsistencies such as high levels of activation with low trypsin concentration must be analyzed. Once this work is accomplished, it can be augmented by *in vivo* experimentation as well as by clinical investigations dealing with interactions between prorenin and renin under varying normal and pathological conditions. It is only through this combination of efforts that the role of prorenin may be properly understood.

Graph 1: EFFECT OF VARIABLE TRYPSIN CONCENTRATION ON PLASMA ANGIOTENSIN I PRODUCTION



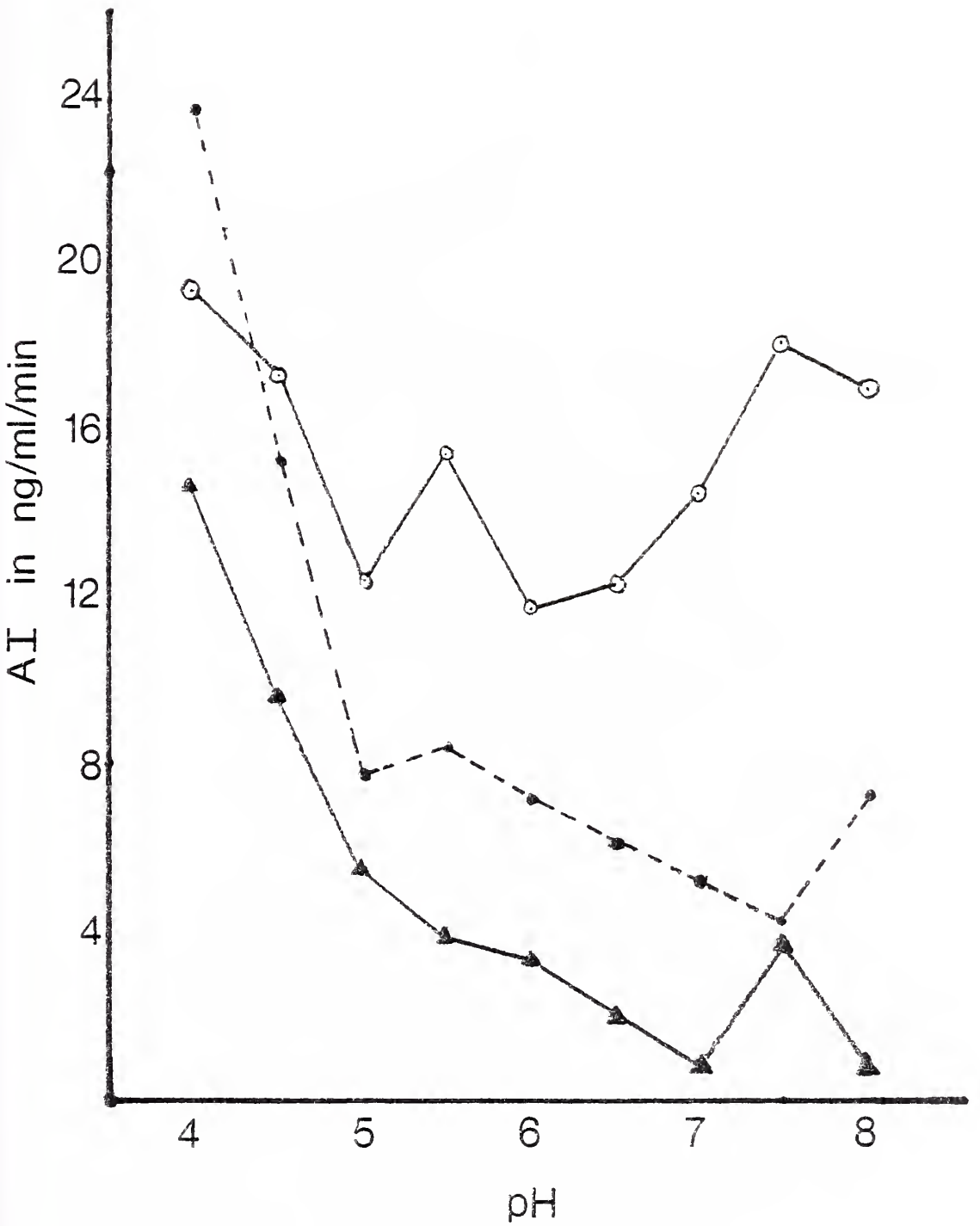

20
16
12
8
4
AI in ng/ml/min
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
(trypsin) in mg/ml plasma

Graph 2: EFFECT OF pH ON PLASMA ANGIOTENSIN I PRODUCTION

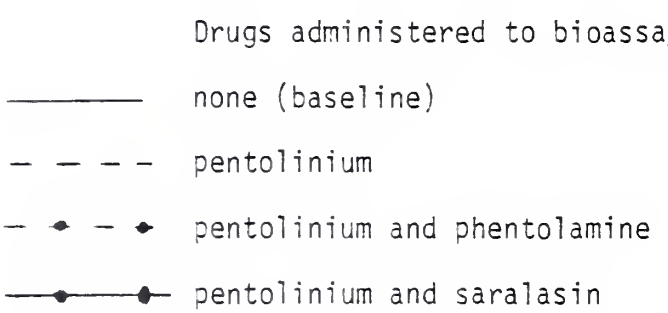

plasma of normal donor animals + trypsin (1 mg/ml)

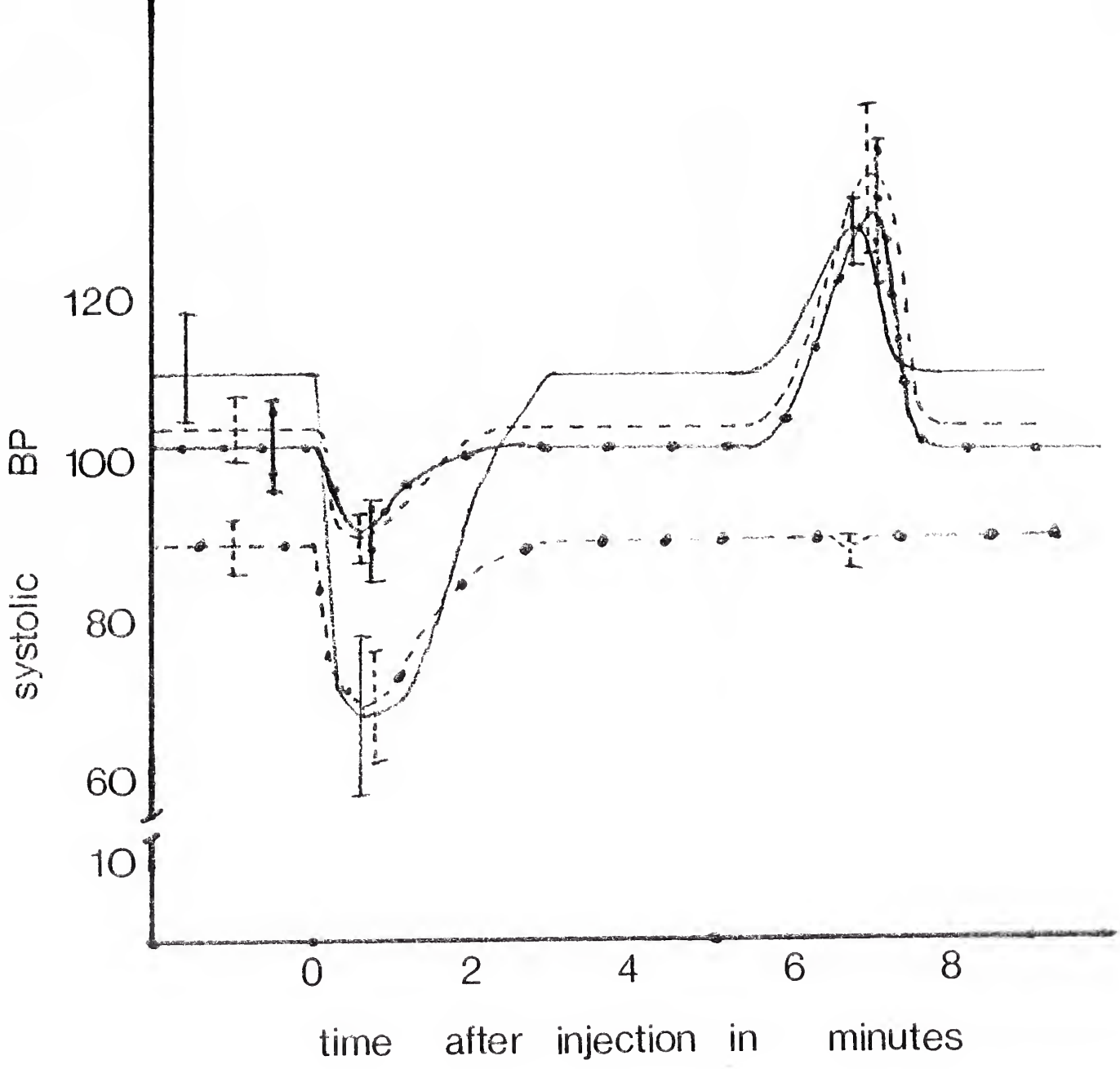

plasma of normal donor animals without trypsin

plasma of nephrectomized donor animals + trypsin (1 mg/ml)

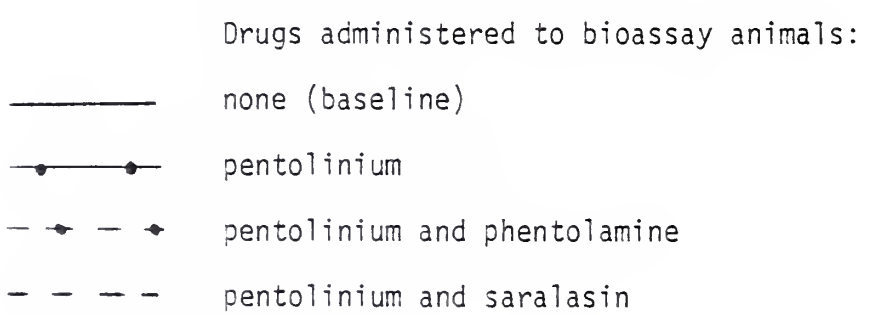

24
20
16
12
8
4
AI in ng/ml/min
4
5
6
7
8
pH

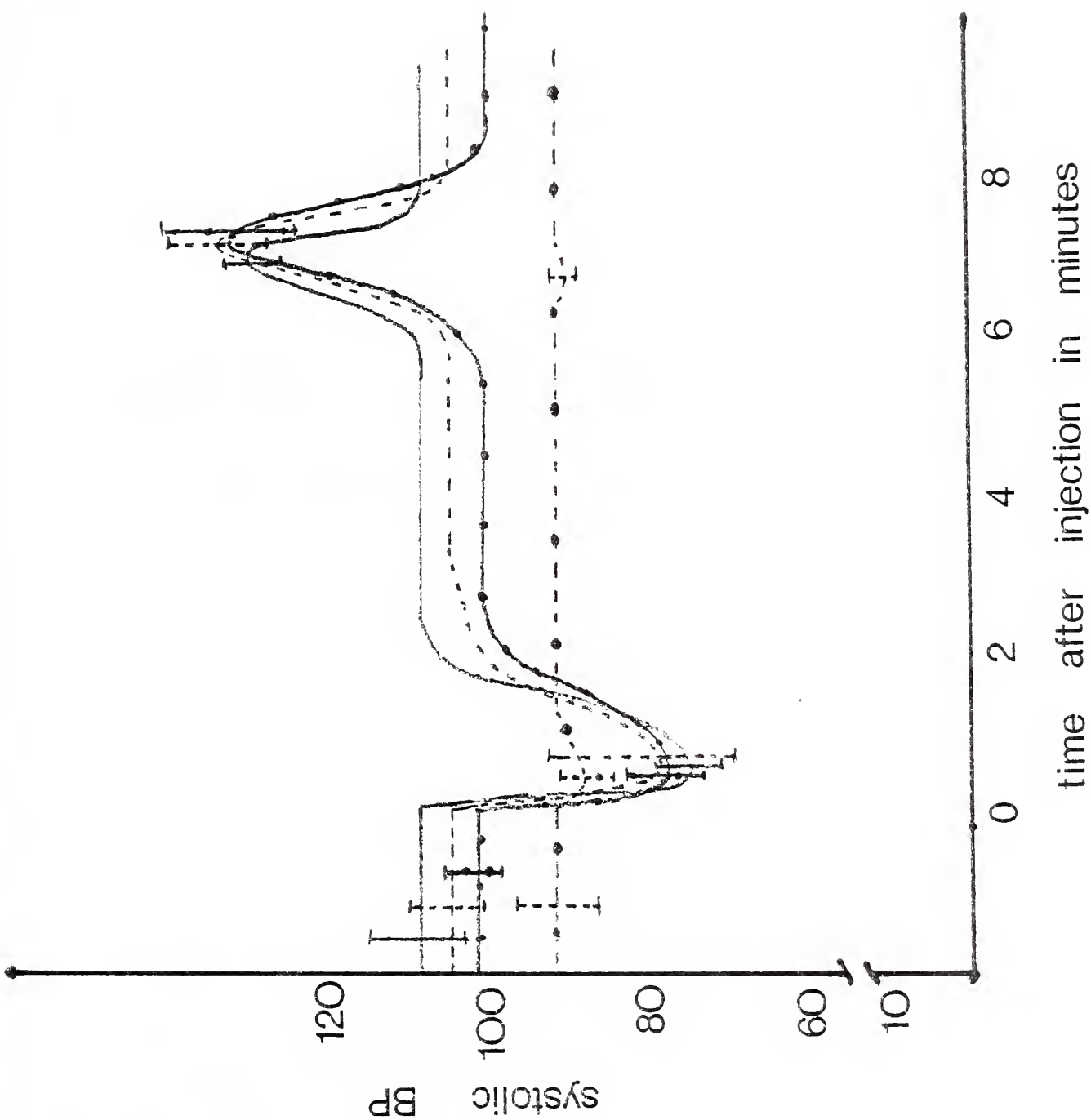
Graph 3: SYSTOLIC BLOOD PRESSURE FOLLOWING INJECTION OF 0.1ml OF TRYPSIN TREATED PLASMA OF NEPHRECTOMIZED DONOR RATS INTO NORMAL BIOASSAY ANIMALS




systolic BP
120
100
80
60
10
0
2
4
6
8
time after injection in minutes

Graph 4: SYSTOLIC BLOOD PRESSURE FOLLOWING INJECTION OF 0.1ml OF TRYPSIN TREATED PLASMA OF NEPHRECTOMIZED DONOR RATS INTO NEPHRECTOMIZED BIOASSAY ANIMALS




systolic BP
120
100
80
60
10
0
2
4
6
8
time after injection in minutes

Graph 5: SYSTOLIC BLOOD PRESSURE FOLLOWING INJECTION OF 0.1ml OF TRYPSIN TREATED PLASMA OF NEPHRECTOMIZED DONOR RATS INTO ADRENALECTOMIZED BIOASSAY ANIMALS

Drugs administered to bioassay animals:

———— none (baseline)

— — — pentolinium

—•——•— pentolinium and phentolamine

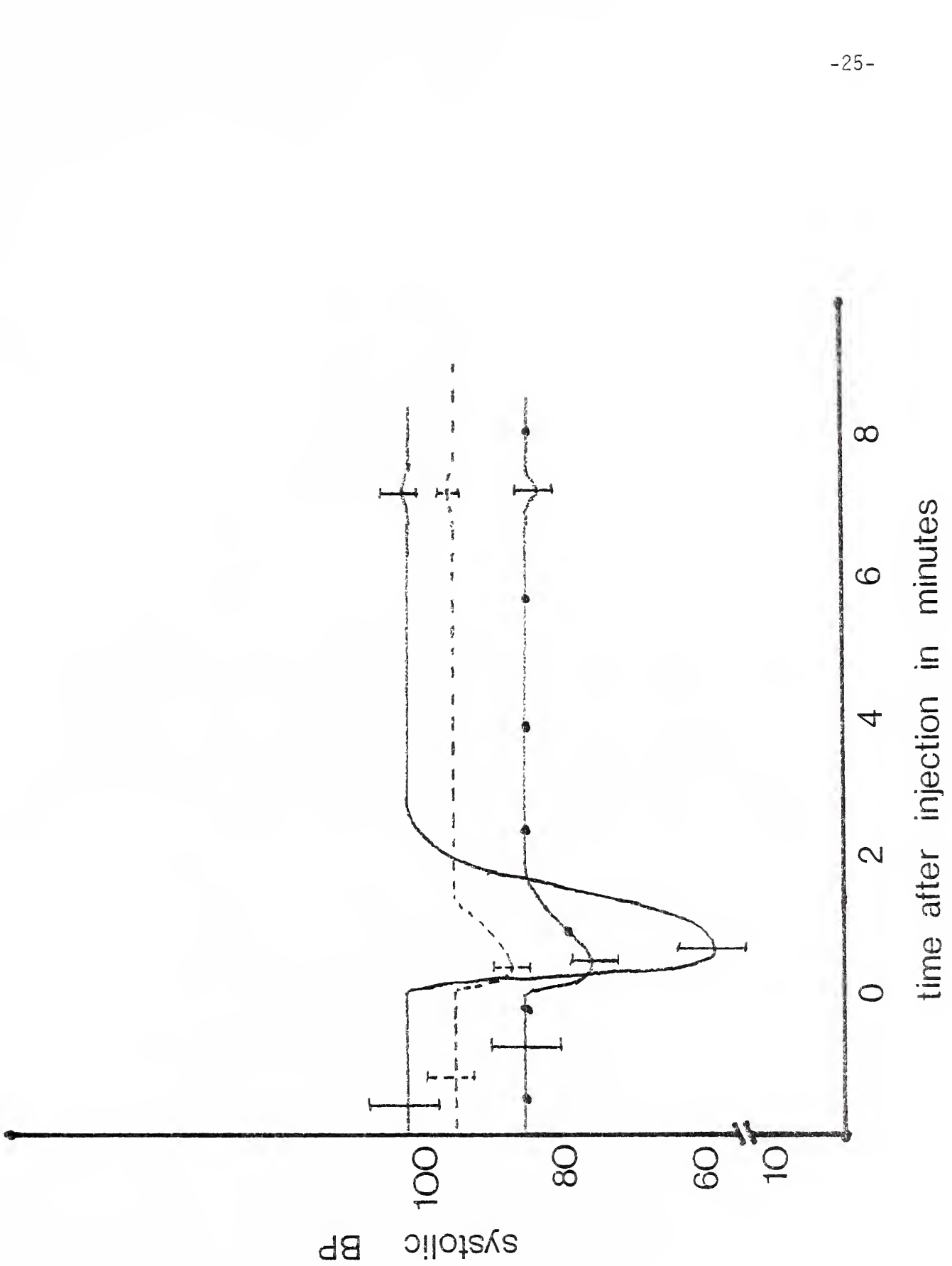

systolic BP
100
80
60
10
0
2
4
6
8
time after injection in minutes

Table I: MEAN PHASE A FALL IN SYSTOLIC BLOOD PRESSURE INDUCED BY INJECTION OF 0.1ml DIALYZED TRYPSIN TREATED PLASMA OF NEPHRECTOMIZED ANIMALS

Values given are mean±S.E. in mm of Hg. First number in parenthesies is the number of rats used to determine pressure values. Following is statistical significance of the change in pressure from baseline based on Student's t-test and Mann-Whitney Rank order test. These test agree unless noted.
*significant by Mann-Whitney test, $p < .05$.

Table I

	normal rats	nephrectomized rats	adrenalectomized rats
baseline	42.1±10.0 (7,p<.01)	34.0±4.6 (10,p<.001)	45.8±4.7 (6,p<.001)
pentolinium	12,9±2.9 (7,p<.005)	24.2±5.2 (6,p<.01)	8.3±1.7 (6,p<.025)
pentolinium & phentolamine	23.0±5.1 (5,p<.025)	4.0±2.9 (5,n.s.)	10.0±2.8 (3,n.s.)
pentolinium & saralasin	11.0±1.9 (5,p<.01)	37.5±13.3 (4,n.s.*)	

Table II: MEAN PHASE B RISE IN SYSTOLIC BLOOD PRESSURE INDUCED BY INJECTION OF 0.1ml DIALYZED TRYPSIN TREATED PLASMA OF NEPHRECTOMIZED ANIMALS

Values given are mean±S.E. in mm of Hg. First number in parenthesies is the number of rats used to determine pressure values. Following is statistical significance of the change of pressure from baseline based on Student's t-test and Mann-Whitney Rank Order test. These tests agree unless noted.

Table II

	normal rats	nephrectomized rats	adrenalectomized rats
baseline	17.1±4.0 (7,$p<.01$)	21.0±3.5 (10,$p<.001$)	0.8±1.5 (6,n.s.)
pentolinium	37.9±8.9 (7,$p<.01$)	31.7±7.5 (6,$p<.01$)	0.3±1.1 (6, n.s.)
pentolinium & phentolamine	1.0±1.0 (5,n.s.)	-1.0±1.0 (5,n.s.)	-1.3±1.7 (3,n.s.)
pentolinium & saralasin	28.0±8.6 (5,$p<.05$)	27.5±5.9 (4,$p<.025$)	

Table III: COMPARISONS OF HYPOTENSIVE (PHASE A) EPISODES IN NORMAL, NEPHRECTOMIZED AND ADRENALECTOMIZED RATS WITH VARIATION OF DRUG MODULATORS

Statistical significance based on Student's t-test and Mann-Whitney Rank Order test. Tests agree unless noted.
*n.s. by Mann-Whitney test

Table III

	normal vs. nephrectomized	nephrectomized vs. adrenalectomized	normal vs. adrenalectomized
baseline	n.s.	n.s.	n.s.
pentolinium	n.s.	$p < .025$*	n.s.
pentolinium & phentolamine	$p < .025$	n.s.	n.s.

Table IV: COMPARISONS OF HYPERTENSIVE (PHASE B) EPISODES IN NORMAL, NEPHRECTOMIZED AND ADRENALECTOMIZED RATS WITH VARIATION OF DRUG MODULATORS

Statistical significance based on Student's t-test and Mann-Whitney Rank order test. Tests agree unless noted.

Table IV

	normal vs. nephrectomized	nephrectomized vs. adrenalectomized	normal vs. adrenalectomized
baseline	n.s.	$p<.001$	$p<.005$
pentolinium	n.s.	$p<.005$	$p<.005$
pentolinium & phentolamine	n.s.	n.s.	n.s.

Table V: COMPARISON OF PHARMACOLOGICALLY MEDIATED DIFFERENCES OF BLOOD PRESSURE CHANGES ASSOCIATED WITH HYPOTENSIVE (PHASE A) EPISODES IN NORMAL, NEPHRECTOMIZED AND ADRENALECTOMIZED RATS

Statistical significance based on Student's t-test and Mann-Whitney Rank order test. Tests agree unless noted.

+n.s. by Mann-Whitney test

**p<.002 by Mann-Whitney test

*pentolinium was always used when phentolamine or saralasin were administered

Table V

	normal	nephrectomized	adrenalectomized
baseline vs. pentolinium	$p<.025^{+}$	n.s.	$p<.001$
baseline vs. phentolamine*	n.s.	n.s.**	$p<.005$
pentolinium vs. phentolamine*	n.s.	$p<.025$	n.s.
pentolinium vs. saralasin*	n.s.	n.s.	

Table VI: COMPARISON OF PHARMACOLOGICALLY MEDIATED DIFFERENCES OF BLOOD PRESSURE CHANGES ASSOCIATED WITH HYPERTENSIVE (PHASE B) EPISODES IN NORMAL, NEPHRECTOMIZED AND ADRENALECTOMIZED RATS

Statistical significance based on Student's t-test and Mann-Whitney Rank order test. Tests agree unless noted.
*pentolinium was always used when phentolamine or saralasin were administered

Table VI

	normal	nephrectomized	adrenalectomized
baseline vs. pentolinium	n.s.	n.s.	n.s.
baseline vs. phentolamine*	$p<.01$	$p<.001$	n.s.
pentolinium vs. phentolamine*	$p<.01$	$p<.005$	n.s.
pentolinium vs. saralasin*	n.s.	n.s.	

Figure 1: MULTIPLE PATHWAYS FOR ANGIOTENSIN I PRODUCTION - THE ROLE OF TRYPSIN

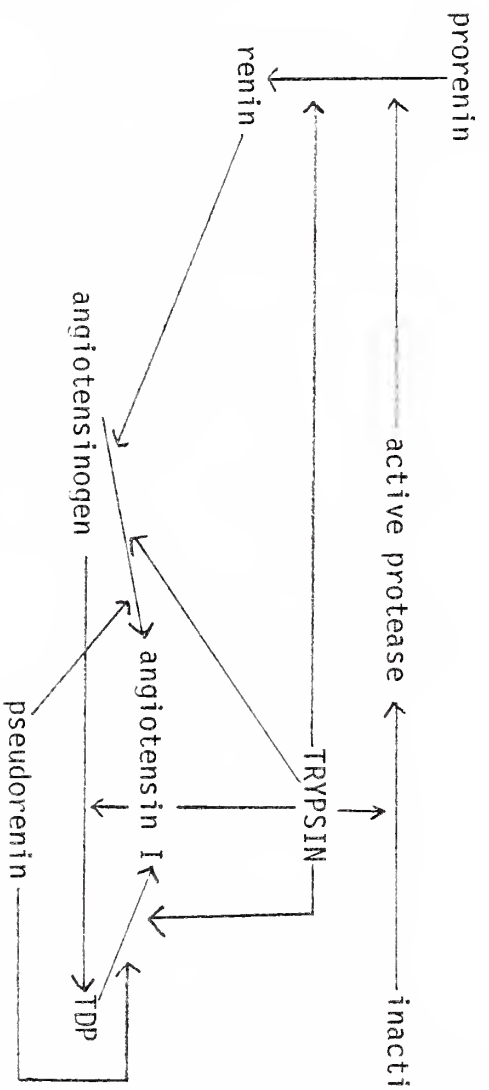

prorenin
renin
active protease
inactive protease
angiotensinogen
TRYPSIN
angiotensin I
pseudorenin
TDP

Figure 1

Appendix I

Possible Mechanisms of Activation

Many models for activation of prorenin have been put forward. It is far from certain which, if any, is correct, nor is it clear if different methods of induction are based on different molecular mechanisms. Most models center on one of two concepts - direct action on the prorenin moleculare or removal of an inhibitory substance, thereby allowing activation to occur. All methods of induction seem to affect the same pool of prorenin. Acid treatment following cryoactivation (2) and pepsin following acid induction (71) do not add any further increase in renin activity.

During acid activation, plasma is dialyzed to a pH of between 3.0 and 3.5; activation does not occur if the final pH is greater than 4.5 or less than 3.0 (71). Maximal activation depends on sufficient exposure to both acidic and relatively more alkaline environments (2). The combined process takes somewhat less than 24 hours for full effect (40).

Acid activation is blocked by exposure to Trasylol (non-specific serine protease inhibitor) and soy bean trypsin inhibitor (19,40). It is also prevented by unacidified plasma (67). Treatment with N-ethylamide, a thiol protease inhibitor, has no effect (67). Thus, acid activation requires the action of a serine protease.

It appears to be appropriate to divide the process of acid activation into two phases - acid and alkaline. Only about one-third of prorenin is activated during dialysis to pH 3.3 (68). It has been postulated that this part of activation is mediated by an acid protease. Cathepsin D has been thought of as a likely condidate because of its low optimal pH. It is also relatively localized to the kidney, a more likely site for

physiologically significant activation than plasma. It is known that the enzyme is capable of activating renin (51). One problem with this theory is that no chromatographically isolatable protease seems to be involved; column isolation of prorenin does not consistently eliminate its capacity for acid activation (70).

The remaining two-thirds of activation occur during the alkaline phase. It is this phase which is affected by serine protease inhibitors. Plasma can also block this phase; its inhibitory capacity is eliminated by prior acidification (70). Exposure to low pH appears to irreversibly destroy a protease inhibitor. Kotchen (37) collected plasma fractions containing neither renin nor renin substrate. Addition of certain fractions to an in vitro renin-renin substrate system gave significant reduction of angiotensin I generation. Prior acidification greatly reduces this inhibitory potential. Long chain, unsaturated fatty acids were already known to inhibit renin. Low pH was found to eliminate the capacity of arachidonic and linoleic acids to retard renin activity. Cold exposure did not prevent inhibition. The current model of cryoactivation is basically equivalent to an isolated alkaline phase (2); the serine protease inhibitor that blocks the alkaline phase also blocks cryoactivation. Low temperature has been shown to decrease the effectiveness of enzyme inhibitors (9). Thus, the proof of a fatty acid in the role of inhibitor is far from conclusive. Nor is it clear that disinhibition is the only mechanism responsible for acid activation. Hseuh (30) has noted that the acid phase of activation is not effected by dilution while the alkaline phase is modified. She has interpreted this to show a direct effect of acid on the prorenin molecule (such as changing hydrogen bonding). Hseuh also found that the extent of kallikrein activation of prorenin in a mixture

of normal and acid-exposed plasma was equal to the percentage of acid exposed plasma in the mixture. If activation were limited by inhibitors, then large percentages of untreated plasma should have prevented all activation and, conversely, excess kallikrein activity should have lead to total activation. These findings are consistent with a structural change in prorenin at low pH, making the molecule susceptible to modification.

Trypsin activation is carried out at physiological pH; an optimal trypsin concentration of 0.5mg trypsin/ml plasma has been found in human plasma (53). Trypsin activation is also dependent on temperature.

Trypsin is a very non-specific protease and its actions are far from limited to activation of prorenin. High doses of trypsin can yield a net decrease in renin activity secondary to destrction of renin substrate, other modifiers or even renin itself (25,53,57). Activation also appears to begin extremely quickly as lima bean trypsin inhibitor added prior to trypsinization does not eliminate all activation (57).

The action of trypsin may be more direct than that of acidification. Both trypsin and acid activation were equally inhibited by renin antibodies, substrate analouges and pepstatin (acid protease inhibitor); when prorenin was isolated by gel filtration, however, trypsin retained full activating potential while acid dialysis did not. Noth (53) added partially purified renin to plasma and then activated this plasma with trypsin. Activation resulted in a linear rise in angiotensin I production just like that of activated plasma without exogenous renin. Since there is no difference created by addition of exogenous renin, it can be inferred that trypsin does not increase activity by altering active renin or renin substrate.

The body of data does not argue for direct action of trypsin on prorenin. Although trypsin can activate isolated prorenin, it is difficult to relate this to the in vivo process. It has also been shown that a protease can activate renin via an intermediary step (activation of an andogenous protease or degradation of inhibitor) (52). Other observations lend support for such a process occuring with trypsin activation. Trypsin is normally about seventeen times more active at 37°C than at -4°C, yet it activates renin far better at -4°C than at the higher temperature (67). The fact that cold has been shown to decrease the potency of plasma protease inhibitors (1) makes this an attractive explanation, especially in view of the phenomenon of cryoactivation.

Over the past few years, evidence has surfaced which suggests that kallikrein may be the activated (or disinhibited) protease of acid, and possibly trypsin, activation. Both urinary and pancreatic kallikrein have the capacity to activate semipurified prorenin (16), although activation in plasma is less predictable (16,56). Acid dialyzed plasma is much more susceptible to kallikrein activation, presumable secondary to destruction of an endogenous inhibitor (16,30,78). Kallikrein is not unique as a normal blood protease with this capacity, however; plasmin and other constituents can also activate prorenin (28). Kallikrein is a very potent activator, being fifty times more active than trypsin, even though trypsin is normally the more powerful protease (67). Combining this fact with the observation that renal kallikrein is distributed in roughly the same pattern as renin makes a model linking the two attractive. Sealey (67,69) found that, with patients on a random sodium intake and having normal plasma renin activities, there is a direct relationship between daily rate of urinary kallikrein excretion and the percentage of

the total plasma renin present as active renin. The authors hypothesize that this seems reasonable teleologically, bradykinin production maintaining local perfusion while prorenin conversion ultimately increases systemic angiotensin I levels.

Genetic deficiences of various components of the kinin pathway allow further analysis of the system's role in renin activation. Sealey (68) showed that plasma deficient in either Hageman factor or prekallikrein almost totally lacks the alkaline phase during acid activation. Acid phase was unaltered. A one to one mixture of these two deficient plasmas gave near normal activation, suggesting dependence on both substances. Lack of a cofactor for contact activation of Hageman factor and prekallikrein gave a diminished but present alkaline phase. Direct addition of purified plasma kallikrein to plasma deficient in any component resulted in full correction of the activation abnormality. Thus, kallikrein may be the essential compound missing in these different samples. Nothin g can be concluded about the role of kallikrein in acid activation beyond that it appears to be obligatory. Other substances may also be necessary and the action of kallikrein may be direct or indirect. Sealey has hypothesized that a kallikrein inhibitor is removed. The work of Hseuh (30) discussed earlier suggests that acid activation may structurally change prorenin, thereby making it more susceptible to kallikrein's proteolytic activity.

The discussion of activation has centered on the prorenin found in circulating plasma. Renal extracts contain large molecular weight renin differing from prorenin in molecular weight and in posessing some innate activity (6,7,11,24). Body (6) showed that a crude kidney extract separated on a column had two different pressure patterns on bioassay -

one the same as renin and one slower and more prolonged. Both produced angiotensin I. Acidification of the high molecular weight form changed its pressure tracing to that of normal renin. Incubation with 3M NaCl, 1M NaI, 0.005% SDS and other denaturants had the same effect. The change in pressure tracing corresponded to a change in molecular weight from about 68,000 to that of normal renin. A substance eluted off the column was a protein capable of transforming normal renin back to the large form (24). Antibodies raised to this binding protein blocked this modification. These results were confirmed by Leckie and McConnell (39) who also showed that this inhibitor had no innate vasoactive properties. There is some evidnece to suggest that binding is dependent on blockage of thio groups (34). The fact that this high molecular weight renin appears to be located in cellular granules in renal tissue suggests a storage form of renin. Its interactions with plasma prorenin are unknown.

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