HUMAN PRORENIN
HUMAAN PRORENINE

PROEFSCHRIFT

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"Twee keer twee is vier: da's waarheid.
Jammer genoeg dat het zo licht en leeg is.
Liever had ik heldre klaarheid.
Over dat wat vol en zwaar is."

W. Busch (1909): Schein und Sein.
Geciteerd door Karl R. Popper (De groei van kennis, Hoofdstukken uit Conjectures and Refutations: The growth of scientific knowledge, vertaling van Zeno Swijtink)

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1. ACTIVE RENIN, INACTIVE RENIN AND PRORENIN

1.1. INTRODUCTION

1.1.1. HISTORY

Robert Tigerstedt (1853-1923), professor of physiology at the Karolinska Institute in Stockholm, discovered renin at the turn of the century[1]. The scientific achievements of Richard Bright (1789-1858), Karl Ludwig (1815-1895) and Charles Brown-Séquard (1817-1894) had set the stage for this discovery[2]. It was Bright who observed that many patients dying with small contracted kidneys had cardiac hypertrophy and a 'hard, full, pulse.' Ludwig introduced the mercury manometer for recording intra-arterial pressure on a revolving kymograph. Of equal importance was the study of the process of internal secretion by Brown-Séquard. In a series of classic experiments published in 1898, Tigerstedt and Bergman[3] were able to demonstrate that a saline extract of rabbit kidneys, when injected intravenously into rabbits, raised blood pressure up to fifty percent within two minutes. The substance concerned was present in the renal cortex and in the renal venous effluent. It was absent in renal medulla and urine. The substance was soluble in water, non-dialysable, stable at 56 C but not at 100 C and extremely potent. Tigerstedt and Bergman called this unknown substance, renin. Their main conclusion was that renin was a blood pressure-raising substance released by the kidney into the circulating blood. They considered this hormone to be the connection explaining the association between renal and cardiac disease described by Bright.

This discovery laid dormant for more than 30 years before its significance was recognized by Goldblatt and coworkers, who produced hypertension in the dog by clamping the renal artery[4,5]. These experiments indicated that the pressor substance, renin, was released from the kidney with the constricted artery. Goldblatt's group later defined the 'Goldblatt Unit' of renin, which is the quantity of renin that, after intravenous injection, raises the directly measured mean arterial pressure with 30 mm Hg in an unanaesthetized, trained, dog[6]. At present renin is defined in relation to a standard preparation supplied by the World Health Organization. This standard has been accepted as the international reference preparation[7]. It is to be expected that, as a result of the recent purification of renin, a less arbitrary definition of the Goldblatt Unit will be established.

Ruyter[8] first described the juxtaglomerular epitheloid granular cells present in the wall of the afferent arteriole of the glomerulus of the mouse kidney. These cells exhibit features of endocrine and smooth muscle cells and may be

considered as 'myoendocrine' cells. It was Goormaghtigh[9] who postulated that they were the source of renin. Nairn[10] and Edelman[11] demonstrated the localization of renin in the granules of the juxtaglomerular cells in the afferent arteriole by the fluorescent antibody technique. However, because of the unspecific nature of the renin antisera, these observations were be questioned. With the introduction of highly specific antibodies and by using immunohistochemical techniques, renin could be unequivocally demonstrated in the secretory granules of the epithelioid cells of the juxtaglomerular apparatus[12–14]. By microtechniques Cook et al.[15] were able to isolate the content of the granulated juxtaglomerular cells and demonstrated that the isolated material had vasopressor activity.

There is now general agreement that renin is stored in membrane-bound cytoplasmic granules of modified cells of the afferent glomerular arteriole, just before it enters the glomerular capillary tuft. Both the afferent and efferent glomerular arterioles are anatomically and functionally closely associated with a group of cells of the distal tubule forming the macula densa. These cells, together with the modified cells of the afferent arteriole, form the juxtaglomerular apparatus. The anatomy of the juxtaglomerular apparatus has recently been reviewed[16].

The proteolytic activity of renin was discovered just before the second world war, independently by Brown-Ménendez[17,18] and his group in Argentina and by the group of Page[19,20] in the United States. Renin catalyses the hydrolysis

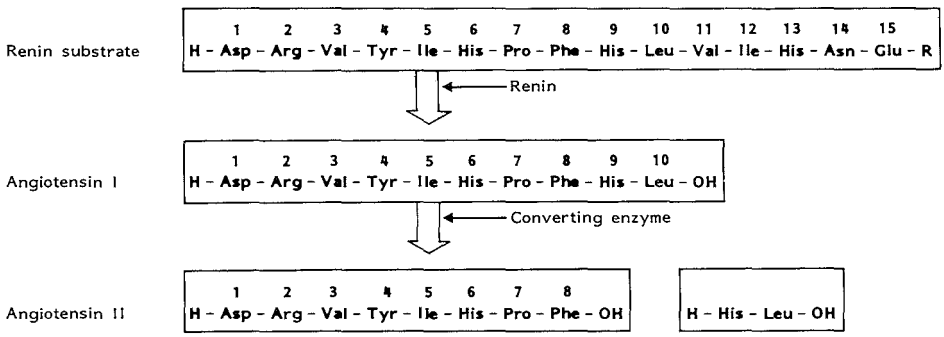

Fig. 1.1. Outline of the biochemistry of the human renin-angiotensin system. The aspartic protease renin cleaves its substrate (angiotensinogen) between leucine 10 and valine 11 to form the decapeptide angiotensin 1. Converting enzyme removes residues histidine 9 and leucine 10 to form the octapeptide angiotensin II, the biologically active endproduct of the system.

of the Leu-10-Val-11 bond of human angiotensinogen[21–23] (Fig.1). Because this reaction is the rate-limiting step in a series of reactions leading to the formation of angiotensin-II, a highly potent vasoconstrictor and aldosterone-releasing hormone, renin is considered to have a key role in blood pressure regulation and in sodium and water homeostasis[24–27].

1.1.2. BIOCHEMISTRY OF RENIN

The family of protein-splitting enzymes or proteases are classified according to the structural features of their active site i.e. serine, cysteine, metallo- and aspartic (aspartyl) proteases, also called acid proteases, because of their low pH optimum of activity[29,30]. The name aspartyl protease has been adopted by the Committee of Enzyme Nomenclature of the International Union of Biochemistry[31] because all enzymes in this group have two aspartyl residues in their active site[29–30,32]. The systematic number of the aspartyl proteases is EC 3.4.23.[31]. Renin (EC 3.4.23.15) belongs to the group of aspartyl proteases, which also includes proteases such as pepsin A (commonly known as pepsin), pepsin C or gastricsin, chymosin or rennin, penicillopepsin and cathepsin D[29–36]. The aspartyl proteases are all specific for peptide bonds with large hydrophobic residues on either side of the scissile bond[32,36]. However, the nearly neutral pH optimum and the highly restricted substrate specificity of renin are properties not shared by the other aspartyl proteases.

Investigations on the tertiary structure of the aspartyl proteases have shown that these enzymes have a bilobal structure[32–39]. The two domains are separated by a deep and extended cleft perpendicular to the largest diameter of the molecule. The two aspartyl residues of the active site are located in this cleft and are in intimate contact. The two catalytically active residues in the aspartyl proteases are Asp-32 and Asp-215, in the amino-acid sequence of porcine pepsin A[32,36]. Renin is completely inactivated by the active-site specific irreversible inhibitors of acid proteases, i.e diazo compounds and epoxides[40–43]. Diazo compounds, such as N-diazoacetyl-L-phenylalanine methyl ester, react with un-ionized carboxyl groups, specifically with the side chain carboxyl of Asp-215[32,36,44–48]. Epoxides, such as 1.2-epoxy-3-(p-nitrophenoxy)propanol, which react specifically with ionized carboxyls, modify Asp-32[32,36,49,50]. From measurements of the pH dependence of the inhibition, the side chain carboxyl group of Asp-32 and Asp-215 of porcine pepsin A have been found to have pKa values of 1.2 and 4.5 respectively[32]. This implies that, within the pH range of the optimum catalytic activity of pepsin A, Asp-32 is in the ionized form, whereas Asp-215 is not ionized[32].

The hexapeptide, pepstatin A (isovaleryl-L-valyl-L-valyl-statyl-L-alanyl-statine) which contains the unusual amino acid statine (4-amino-3-hydroxy-6-methylheptanoic acid) and which has been isolated from various species of actinomyces, is a potent inhibitor of pepsin and other acid proteases[51,52]. Pepstatin binds as an extended chain in the center of the apparent substrate binding site. Statine is the major structural component responsible for the inhibition by pepstatin and is an analog of the transition state for catalysis by pepsin and other aspartyl proteases[53]. Pepstatin A also inhibits renin *in vitro* as well as *in vivo*[52,54–56]. The pepstatin concentration required to inhibit human renin by 50 percent (IC 50) was 10^{-6} M, which is much higher than the IC 50 for pepsin A and cathepsin D, which have an IC 50 of about 10^{-9} M to 10^{-10} M[51–53]. Recently

, some novel inhibitors containing the amino acid statine incorporated into analoques of porcine angiotensinogen, have been developed. These are highly potent inhibitors of human renin; their IC 50 is 10^{-9} M to 10^{-10} M[57]. Szelke et al.[58] obtained highly selective potent inhibitors of human renin by reduction of the Leu-10-Val-11 scissile peptide bond, i.e. replacement of the peptide bond (-CO-NH-) by a reduced bond (-CH2-NH-), in the 5-12 amino acid sequence of human angiotensinogen.

A 56,000-fold purification of hog renal renin, with a specific activity of 780 Goldblatt Units per mg of protein was reported by Haas and coworkers already in 1953[59]. An important improvement in the purification of renin has been the use of chromatography on pepstatin coupled to agarose[54,60]. The purification of renin from human kidneys is more difficult because its concentration is 20 times lower than in hog kidney. Recently, the purification of human renal renin obtained from cadaver kidneys, with a specific activity of about 1,000 Goldblatt Unit per mg of protein has been accomplished[60–63]. Galen and coworkers[64] reported on a fully purified human renin isolated from a renin-secreting tumor. Juxtaglomerular cells constitute the main source of renin but the concentration is too low to purify it in quantities high enough to allow determination of its primary structure. However , as much as five percent of the total protein content of the submandibular (often, but less correctly, called submaxillary) salivary glands of an inbred strain of male mice is an isoenzyme of renin. This isoenzyme of renin has physicochemical, enzymatic and immunochemical properties closely resembling those of mouse renal renin[68–72].

Using Edman degradation techniques, Misono and coworkers[73,74] demonstrated that renin from mouse submandibulary gland consists of two polypeptide chains, connected by one sulfide bond. The heavy chain consists of 208 amino acids, molecular weight (Mr) 31,306, and the light chain contains 48 amino acids, Mr 5,458. The complete amino acid sequence of mouse submandibular gland renin precursor has been reported by Panthier and Corvol[75,76]. They were able to present the analysis of bacterial DNA recombinant clones harbouring an essentially complete transcript of renin mRNA from the mouse submandibular gland. The decuded amino acid sequence showed that renin was synthesized as a Mr 44,209 precursor, preprorenin.

Murakami[77,78], Hobart[79] and Morris[80,81] reported on the primary structure of human renin. They also used the technique of nucleotide sequencing of the complementary DNA (c-DNA) coding for the human renin precursor. The predicted amino acid sequence consists of 406 amino acid residues with a N-terminal pre- and prosegment consisting of 20 and 46 residues respectively (Fig.2). There is a close similarity between the primary structure of human renin and mouse renin. A high degree of sequence homology, particularly in the catalytically important region, was found upon comparison of human and mouse renins with other aspartyl proteases[73–81]. This suggest that the tertiary structure of renin is similar to that of other acid protesases. Blundell[82,83] has presented a model for the three dimensional molecular structure of mouse submandibular renin.

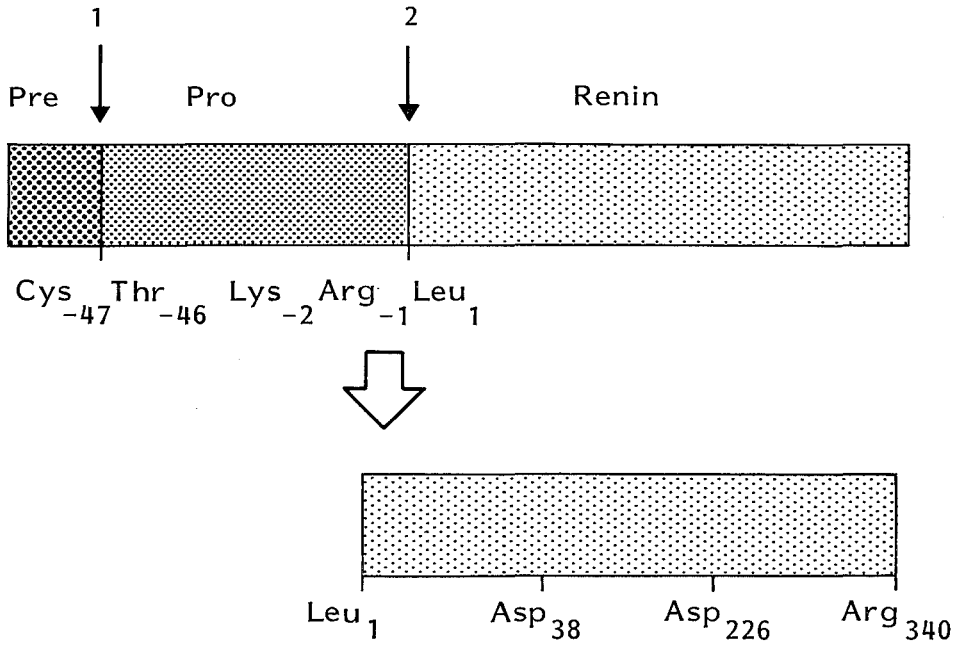

Fig. 1.2. Schematic representation of structure of human kidney renin and renin precursor. Arrow 1 depicts the cleavage of the signal peptide (pre-part) from the parent molecule, pre-pro-renin. Arrow 2 depicts the processing of prorenin (inactive renin) into renin.

The tertiary structure of human renin was reported by Morris[81]. It consists of two lobes forming a cleft where the active-site is located, which is comparable to the structure of other acid proteases.

Aspartyl proteases, such as pepsin A and cathepsin D, are processed from inactive precursors, with the release of a prosegment to give the active enzyme. These prosegments or proparts are located on the N-terminus of the parent molecule. Their is a striking similarity between the prosegment of porcine pepsinogen A (44 amino acids), bovine pepsinogen (45 amino acids) and the propart of mouse and human prorenin (45 and 46 amino acids respectively)[75–81,84–88]. The enzymatic activity of pepsin A is inhibited by fragments of the prosegment of pepsinogens[86–88]. Peptides related to the 12-19 amino acid sequence of the prosegment of mouse submandibulary prorenin have been synthesized and these peptides were found to inhibit mouse submandibulary renin[84] as well as human renin[85].

1.1.3. RENIN AS A POLYPEPTIDE HORMONE

After the classic work of Steiner[89] on the precursor of insulin and Chrétien[90]

on lipoprotein it has become clear that most, if not all, protein hormones and other secretory proteins begin their life as part of a larger precursor molecule. These precursor molecules are subsequently processed by limited proteolysis to the biologically active proteins[91,92]. According to Steiner[91] the precursor proteins can be classified in two major groups depending on the localization of the maturation processes. The first group of proteins are processed within their cell of origin, before exocytosis. The polypeptide hormones, insulin and parathyroid hormone, are examples of this group. The second group consists of proteins that are processed only after secretion, either in a single step or in a series of sequential cleavages. The cleavage of the proenzymes of the clotting system is a typical example of the latter group of proteins. The proenzymes are enzymatically inactive and become activated during proenzyme-enzyme conversion.

The precursor proteins contain a short hydrophobic N-terminal extension of 15 to 30 amino acids, the signal peptide or prepeptide, which is cleaved shortly after attachment and penetration of the nascent protein through the lipid membrane of the endoplasmic reticulum. The proprotein, which is located intracisternally, is transported through the endoplasmic reticulum, Golgi apparatus and condensing vacuoles and finally stored in the secretory granules. The polypeptide chain of the propart can vary markedly in size as well in location within the full length polypeptide chain of the proprotein.

Polypeptide hormones are usually converted into the mature hormone during this intracellular transportation process or within the secretory granules. The cleavage occurs in most cases after a pair of basic amino acids (lysine,arginine). The inactive precursors of enzymes, proenzymes or zymogens, are activated outside the cell of origin. The mechanism of renin maturation is similar to that observed in other cases of preproprotein processing[93–103]. Protein synthesis *in vitro* is studied in fractionated cell extracts, also known as cell-free systems. The complete machinery of protein synthesis is present in the rabbit reticulocyte lysate and other systems. If m-RNA isolated from mouse salivary gland or mouse and human kidney is added to such extracts, the added m-RNA will be translated to the proteins coded for. Total poly(A)$^+$RNA or mRNA isolated from mouse salivary gland and mouse and human kidney were translated in the presence of 35-S-methionine using the cell-free rabbit reticulocyte system and other systems. The translated products were subsequently subjected to immunoprecipitation using specific renin antibodies. The cell free translation of m-RNA yielded a 45,000 Mr preprorenin.

In the presence of microsomal membranes, mouse preprorenin was rapidly converted to prorenin, Mr 43,000 and also, rapidly further converted to a single chain form of renin, Mr 38,000. Mouse renin was then slowly hydrolyzed to give two chain renin, the heavy chain of Mr 33,000 and the light chain of Mr 5,000 held together by one disulfide bond[93–100]. Both single- and two chain mouse renin have an exposed active site. The specific activity of the purified one-chain renin has been reported to be five-fold higher than of two-chain renin. This

difference in specific activity of the one- and two-chain renin is surprising because they differ by only two amino acids. Purified heavy chain, obtained by dithiotreitol treatment of the two-chain form, had less then 4% of the activity of the native two-chain renin[99]. This indicate that the light chain is essential for enzymatic activity. The one-chain renin is immediately secreted into the medium after synthesis, whereas the two-chain renin is secreted later. This might indicate that there are two separate pathways for renin secretion.

Recently two groups of investigators reported on renin biosynthesis by a human juxtaglomerular cell tumor[102–103]. Studies with tissue slices from this tumor showed that renin was first synthesized as an inactive 55,000 Mr species followed by conversion to the active molecule with an molecular weight of 44,000. Prorenin is probably packed and converted to active renin within the secretion granules. Renin producing cells isolated from the tissue and grown in culture secreted the inactive 55,000 molecular weight form. No conversion to renin was observed. Therefore the existence of two pathways for the processing, packaging, and secretion of renin in the juxtaglomerular cell were proposed[102]. Hirose[103] and coworkers isolated poly(A)$^+$RNA from a juxtaglomerular cell tumor and translated it in rabbit reticulocyte lysate in the absence or presence of microsomal membranes. The primary translation product, preprorenin, had a molecular weight of 45,000 that was further processed to glycosylated prorenin (Mr 47,000). Thus post-translational modification leads to an increase in Mr despite the removal of the signal peptide.

1.1.4. RENIN AS A PROTEOLYTIC ENZYME

The discussion so far considers renin being a polypeptide hormone secreted by the kidney. This consideration, however, does not take into account that renin , in contrast to the polypeptide hormones, does not appear to act via target cell receptors. Renin acts by virtue of its enzymatic activity. In this respect renin is comparable to other proteolytic enzymes in plasma. These enzymes have specific functions in important physiological systems, such as coagulation, fibrinolysis and the complement cascade[104–109]. One important mechanism for regulating the proteolytic activity of such systems rests on the fact that the individual enzymes participating in the cascade circulate in plasma as inactive precursors or proenzymes, which are converted into the active enzyme through limited proteolysis by other enzymes of the cascade. The activation step is irreversible since proteolysis is an exergonic reaction and since, under normal physiological conditions, there is no simple biological mechanism to repair a broken peptide bond. Potent protease inhibitors, which are present in plasma in abundant quantities, serve to terminate enzymatic action and constitute a second mechanism for controlling proteolytic activity[110].

It is generally accepted that the precursors of polypeptide hormones are processed to the active hormone before or at the time of their release into the

circulation. The concentration of precursor in plasma, as compared to the active hormone, is usually low. In contrast, the proteolytic enzymes in plasma are largely present as inactive precursor molecules that are activated outside their site of production. In the case of the coagulation system, for instance, 99 percent or more of the total quantity of the individual enzymes circulates in plasma as inactive precursor[104–109]. It was therefore of great interest that Lumbers and Morris[111,112] and Skinner[113] and later others[114–122] reported on the presence of an inactive form of plasma renin that can be converted into active renin by limited proteolysis.

The literature on the subject of inactive renin has been reviewed by several groups of investigators[123–131]. It now appears that more than 90 percent of the renin in plasma is present in this inactive form. It is therefore tempting to speculate that the renin angiotensin system has a dual function. It acts as a circulating hormonal system but it may also act as a proteolytic system comparable to, and perhaps interconnected with, the coagulation, fibrinolytic and complement systems. It is the second possibility that is the subject of this thesis.

1.1.5. AIM OF THE THESIS

Our interest in inactive renin originates from the observation that plasma renin can be activated by several apparently unrelated modes of treatment. Dialysis of plasma against a pH 3.3 buffer at 0 C for 24 hours followed by another 24 hours of dialysis at neutral pH causes a five-fold increase in renin activity[113,132–137]. Renin activity of plasma is also increased, albeit to a lesser extent, when plasma is stored at 0 C without prior acidification[134–141]. Finally, the addition of trypsin and some other proteases, such as plasma kallikrein and plasmin, to plasma also increases renin activity[137–155].

Theoretically, the increase in the enzymatic activity of renin could occur by several mechanisms:

1) changes of the fluid milieu of the enzyme, for instance changes in pH, certain metal ions, chaotropic agents,
2) reversible binding of an activator to the active site or to an allosteric site of the enzyme molecule,
3) destruction of an inhibitor of renin or dissociation of the inhibitor moiety from a renin-inhibitor complex,
4) alteration of the primary structure of inactive renin by limited proteolysis.

The last possibility of activation by limited proteolysis is in agreement with the concept of a proenzyme-enzyme conversion. The existence of inactive forms of renin with a molecular weight greater than that of active renin has been demonstrated in plasma but the Mr-figures that have been reported are widely different[126,127,130,131]. Such a concept is also in agreement with the presence of an additional nucleotide sequence in mouse submandibulary renin and human

kidney renin c-DNA, which may correspond to N-terminal amino acid sequences that belong to the so-called prepropart of renin[75–81]. Furthermore, there is recent evidence that the propart amino acid sequence of human kidney prorenin is also present in inactive renin of human plasma[103,156]. Prorenin-renin conversion, within or outside the juxtaglomerular cells where prorenin is synthesized, could be an important regulatory step in the renin-angiotensin system. With this in mind we have studied some biochemical aspects of the activation of inactive renin and addressed the the possible physiological and clinical significance by measurements of inactive renin in plasma and other body fluids. This thesis describes our contributions to this subject in the past five years

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2. PRORENIN-RENIN CONVERSION; ROLE OF ACTIVATORS

2.1. ACTIVATION OF INACTIVE PLASMA RENIN BY TISSUE KALLIKREINS

2.1.1. ABSTRACT

It has been reported that inactive (acid-activatable) human renin could be converted into the active form by adding urinary kallikrein to acid-pretreated plasma. Without prior acidification, however, only a small portion of the total amount of inactive renin present in plasma was converted (activated) by kallikrein, probably because native plasma contains protease (kallikrein) inhibitors that are destroyed by acid. We have separated inactive renin and active renin by DEAE-Sepharose column chromatography of normal human plasma at pH 7.5 and a linearly increasing sodium gradient. Inactive renin isolated in this way could be activated at pH 7.5 by highly purified pancreas and urinary kallikreins. With the semipurified preparation of inactive renin, prior acidification was not required for obtaining virtually complete activation by kallikrein. The kallikreins were effective at concentrations as low as 10 nM. It is therefore possible that one or more tissue kallikreins act as physiological activators of inactive renin.

2.1.2. INTRODUCTION

Renin activity of normal human plasma increases by a factor of 3-7 after treatment at pH 3.0-4.0; this is caused by the conversion of enzymatically inactive renin or prorenin into the active form[1–7]. Acid activation of inactive renin is a two-stage process; renin activity increases slightly during the acidification step, but most of the rise in activity occurs after pH has been restored to neutral, probably through the action of one or more serine proteases[6,8–10]. It seems unlikely that this pathway is operative in circulating plasma because of the inhibitory effect of various plasma proteins. But *in vivo* activation of inactive renin at the tissue level through a serine protease is a possibility, and there is now some evidence to support this. Preliminary experiments by Morris and Day[11] showed increased renin activity of amniotic fluid after the addition of large quantities of a crude preparation of pancreas kallikrein. Sealey et al.[12] reported that the rate of renin activation in acid-treated plasma was increased after the addition of urinary kallikrein, which is known to originate from the kidney[13,14]; the renin activity ultimately attained was not higher than that attained with acid treatment alone.

The possibility that kallikrein acts as an activator of inactive renin is interesting, since kallikrein is produced close to the site where renin is synthesized[15–18] so that an effect of kallikrein on renin biosynthesis could influence renal circulation and sodium handling. Here we describe additional data on the activation of inactive renin by tissue kallikreins. We have separated inactive plasma renin from active renin by ion exchange chromatography and found that the inactive fraction could be activated at physiological pH by highly purified pancreas and urinary kallikreins without prior treatment with acid.

2.1.3. MATERIALS AND METHODS

Ion exchange chromatography. Blood from healthy male subjects was collected in plastic tubes containing disodium-EDTA (5 mM). Within 5 min, the blood was centrifuged at 8,000xg for 10 min. The plasma was immediately frozen at -20 C. For ion exchange chromatography, EDTA-plasma was thawed and dialyzed for 24 h against 0.024 M Tris/acetate buffer, pH 7.5, which contained 0.02 M NaCl. The dialyzed plasma (30 ml) was applied to 40 x 2.6-cm columns of DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden), which had been equilibrated with the same buffer. Elution was performed with a linear gradient of NaCl up to 0.2 M. Flow rate was 20-22 ml/h, and the eluate was collected in 5.0 – to 5.5-ml fractions. All procedures were carried out at 4 C.

Activation of inactive renin by acid. Samples (2 ml) of EDTA-plasma or fractions isolated from plasma by ion exchange chromatography were dialyzed for 24 h at 4 C against a glycine/ HCL buffer, pH 3.3, which contained 5mM EDTA[19]. After pH had rapidly been restored to 7.5 with 1 M NaOH the samples were dialyzed for various periods, as indicated, at 4 C against a phosphate buffer, pH 7.5, containing 1 mM EDTA[19].

Binding of kallikrein to Sepharose. Highly purified hog pancreas kallikrein (Kallikrein, Bayer KZ 45/32, Leverkusen, West Germany) was a gift from professor GL Haberland and Dr E Wischhöfer. This preparation had a specific activity of 1180 biological kallikreins units (Frey Units)/mg protein, which corresponds with 9.4 enzymatic units. One enzymatic unit is defined here as the amount of enzyme hydrolyzing 1 μM of the synthetic chromogenic substrate H-D-valyl-L-leucyl-L-arginine-p-nitroanilide (S-2266, KABI, Stockholm, Sweden)/min under the conditions of the spectrophotometric assay, as described below. Urinary kallikrein was isolated from human urine and purified according to Geiger et al.[20] using affinity chromatography on aprotinin-Sepharose: its specific activity was 6.3 enzymatic units/mg protein. Both kallikrein preparations were homogeneous at electrophoresis on 7.5 % polyacrylamide gel at pH 8.6[21]. The enzymes were covalently bound to CNBr-activated Sepharose-4B (Pharmacia) at a ratio of 10 mg protein/g dry Sepharose[22]. The advantage of binding the enzymes to an insoluble carrier is that they can be removed so that they cannot interfere with the assay of renin. The activities of the Sepharose-bound enzymes

were measured through their amidolytic actions on the synthetic chromogenic substrate S-2266 (KABI) (23) and compared with standard solutions of unbound pure hog pancreas kallikrein (Kallikrein KZ 45/32). The enzyme suspensions (50 μl) in 0.2 M Tris buffer, pH 8.2, were added to 0.15 mM substrate and the volume was adjusted to 1.1 ml with the same buffer. The mixtures were slowly shaken in a water bath for 30 min at 37 C. The reaction was then stopped with 50% acetic acid, and absorbance at 450 nm was measured in a 1-cm semimicrocuvette. Identical mixtures with aprotinin (Trasylol, Bayer) added (100 kallikrein-inhibiting units/ml incubate) served as blanks.

Activation of inactive renin by Sepharose-bound kallikreins. The immobilized kallikreins were suspended in 0.1 M phosphate buffer, pH 7.5, containing 0.075 M NaCl and 0.1 ml of the suspension was added to EDTA-plasma (1 ml) or to fractions (1 or 2 ml) isolated from plasma by ion exchange chromatography. Pepsin (3000 U/mg protein:Sigma Chemical Co., St.Louis, MO, USA), which was bound to Sepharose in the same way as the kallikreins but which is known to be inactive at neutral pH, was used as a control. The suspensions were slowly shaken at 4 C for various periods up to 48 h, as indicated. The enzymes were then removed by centrifugation at 8,000 x g for 10 min. Experiments in which the immobilized enzymes were added to 0.15 M NaCl did not result in detectable renin activity.

Assays of naturally occurring active renin and in vitro activated renin. The method for measuring naturally occurring active renin, which has previously been described[3,24], was slightly modified. Briefly, aliquots (0.1 or 0.2 ml) of EDTA-plasma or fractions isolated from plasma by ion exchange chromatography were mixed with purified sheep renin substrate (final concentration, 0.7 μM angiotensin I equivalents/liter), and the total volume was adjusted to 1.0 ml with 0.1 M phosphate buffer, pH 7.5, which contained 75 mM NaCL. EDTA (1 mM) was present in both substrate and buffer solutions. After the addition of 10 μl 8-hydroxyquinoline sulfate (0.34 M), 5 μl phenylmethylsulfonylfluoride (0.3 M) in ethanol, and 10 μl aprotinin (Trasylol, Bayer; 10,000 kallikrein-inhibiting units/ml), the mixtures were incubated for 3-12 h at 37 C. At the end of the incubation period, no more than 10% of the renin substrate had been consumed, and generation of angiotensin I was linear for the whole period. For measuring *in vitro* activated renin, the acid- or kallikrein-pretreated samples were similarly incubated with sheep renin substrate. The quantity of angiotensin I that was generated during incubation with renin substrate, was measured by RIA and compared with the quantity generated by standard human kidney renin (MRC standard 68/356, WHO International Laboratory for Biological Standards, Holly Hill, Hampstead, London, United Kingdom). With the protease (angiotensinase) inhibitors we have used, the recovery of angiotensin I, which was added to plasma after treatment with acid or kallikreins, was 98.8 $\pm$ 4.9% (mean $\pm$ sem; n = 15). The recovery of standard renin, which was added to the samples before treatment with acid or kallikreins, was 97.0 $\pm$ 4.0 % (n = 15). Renin concentration is expressed as microunits of the renin standard (MRC standard 68/356) per ml.

2.1.4. RESULTS

In contrast with acid treatment, pancreas and urinary kallikreins had no effect on inactive renin in whole plasma (Fig. 1). The elution profile of DEAE-Sepharose chromatography showed two peaks of renin activity: peak A at a sodium concentration of 0.060 M, and peak B at 0.110 M (Fig.2). The renin content of peak B was about 80% of the total quantity of naturally occurring active renin present in plasma. Acid treatment of this peak did not alter its renin activity. Acid treatment doubled the renin activity of peak A, indicating that this peak contained inactive renin. However, acidification of this fraction did not activate more than 5% of the total quantity of inactive (acid- activatable) renin present in the plasma. In contrast, the addition of Sepharose-bound pancreas kallikrein to peak A (final concentration 16 nM), led to the activation of more than 80% of the inactive (acid-activatable) renin present in the plasma. Similar results were obtained with urinary kallikrein (Fig.1). Both pancreas and urinary kallikreins were effective at concentrations as low as 10 nM. The kallikreins had no effect on the renin activity in peak B, probably because this peak did not contain inactive renin. The inability of added kallikreins to activate inactive renin in whole plasma can be explained by the presence of protease inhibitors[25]. Thus, ion exchange chromatography has resulted in the separation of inactive renin from active renin as well as from inhibitors that interfere with the proteolytic activation of inactive renin.

2.1.5. DISCUSSION

The results demonstrate that pancreas and urinary kallikreins in concentrations as low as 10 nM are capable of activating inactive plasma renin at neutral pH without prior acidification. The finding with urinary kallikrein confirm and extend the results of Sealey et al.[12]. The precise chemical relationship between active and inactive plasma renin is still unknown. While there is agreement that the molecular weights of both naturally occurring active renin and in vitro activated renin are about 44,000, there is no consensus on whether inactive (acid-activatable) renin in plasma represents a higher molecular weight form[4,7,26-29]. Recent findings seem to indicate that the difference in molecular weight with active renin is small or even absent[4,7]. Our findings confirm the results of Shulkes et al.[7], who have shown that the active and inactive forms of renin can be readily separated on the basis of their difference in net electrical charge.

The present study was restricted to tissue kallikreins. They differ in substrate specificity from plasma kallikrein[30]. Nevertheless, we have recently found that prekallikrein (Fletcher factor)-deficient plasma had much lower renin activity after acid treatment than after trypsin treatment[31], whereas in normal plasma, identical results were obtained with the two procedures[32]. This is strong evidence that plasma kallikrein is involved in the acid activation of the inactive renin. Under these artificial conditions, plasma kallikrein can unfold its action on renin,

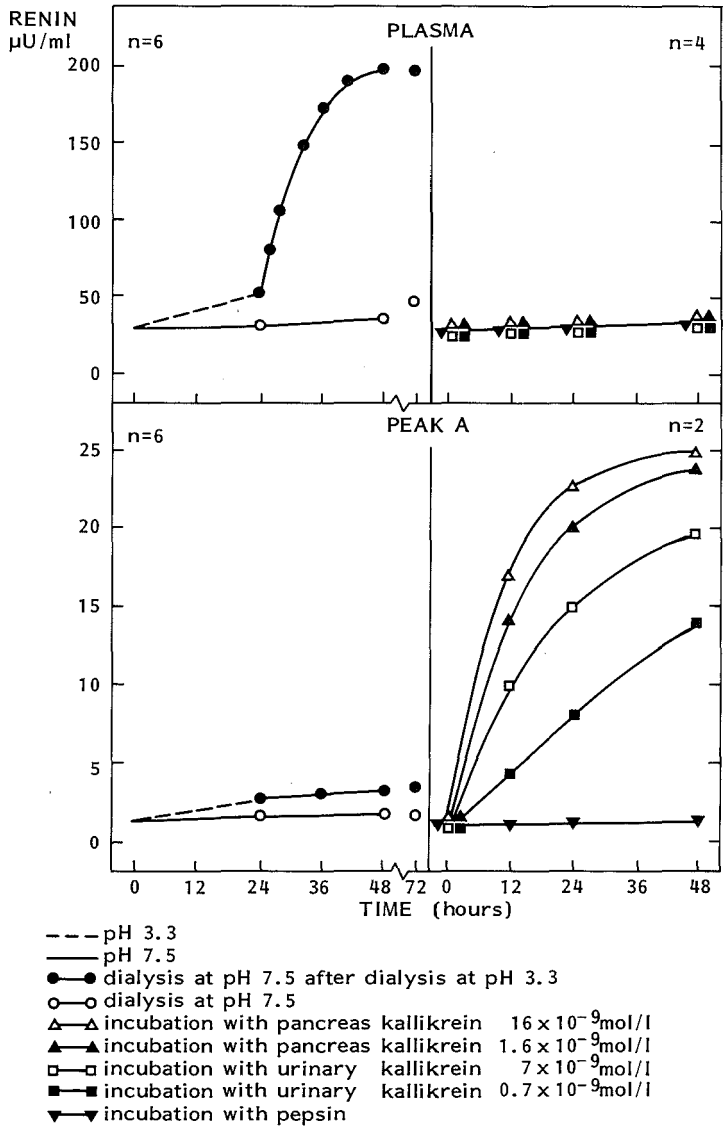

Fig. 2.1.1. Activation of inactive renin as a function of time in whole plasma and in a fraction isolated by DEAE-Sepharose column chromatography with a linear sodium gradient. Six tubes (5 ml each) with the highest renin content from the renin peak which was eluated at a sodium concentration of about 0.06 mol/l (peak A, see Fig. 2.1.2.) were pooled. Samples were treated as follows: 1) dialysis at pH 7.5; 2) dialysis at pH 7.5 with Sepharose-bound pancreas kallikrein, urinary kallikrein and pepsin. After these procedures, which were carried out at 4 C, the samples were incubated (3-12 h) with an excess of sheep renin substrate at pH 7.5 and 37 C for measuring renin.

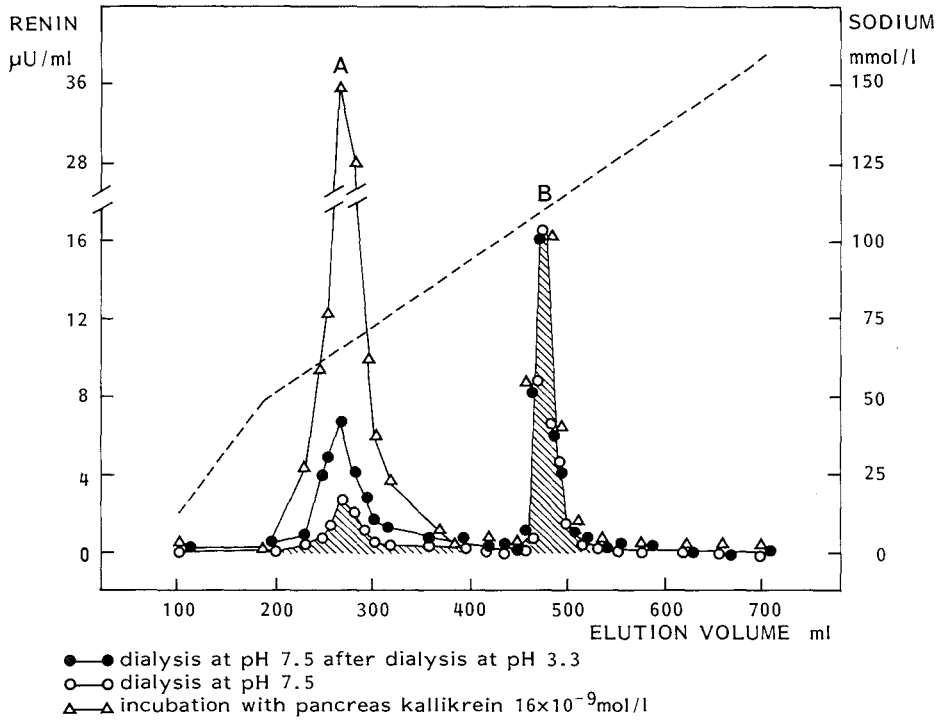

Fig. 2.1.2 Separation of inactive renin (peak A) and active renin (peak B) by DEAE-Sepharose column chromatography. Elution was carried out with a linear sodium gradient (--). Eluates were treated as follows: 1) dialysis at pH 7.5 (48h); 2) dialysis at pH 3.3 (24h), followed by dialysis at pH 7.5 (24h); or 3) incubation at pH 7.5 with Sepharose-bound pancreas kallikrein (24 h). After these procedures which were carried out at 4 C, the samples were incubated (3-12 h) with an excess of sheep renin substrate at pH 7.5 and 37 C for measuring renin.

probably because the protease (kallikrein) inhibitors have been destroyed by acid. Under physiological conditions however, any newly formed kallikrein will be rapidly inactivated in the circulating plasma.

The ability of tissue kallikreins to act as activators of inactive renin may have physiological implications. Evidence is accumulating from studies in animals that both hormones are produced in close anatomical association with a strategically important part of the nephron, the juxtaglomerular apparatus[17,18]. Intrarenal activation of inactive renin by kallikrein might therefore affect some aspects of renal function, particularly sodium handling. Recent observations in patients with renovascular hypertension have provided some evidence that inactive plasma renin can be activated as it passes through the kidney[33]. Furthermore, several studies have revealed some striking quantitative correlations among plasma renin activity, plasma bradykinin, and urinary kallikrein. These hormones increased in parallel after both standing and sodium depletion[34–37]. Plasma renin

and bradykinin were suppressed by excessive mineralocorticoid activity, whereas urinary kallikrein was increased[37]. Another link is formed by the enzyme that converts angiotensin I into angiotensin II. This enzyme also inactivates bradykinin[30]. Thus, there is growing evidence from both *in vitro* and *in vivo* work favouring the existence of intricate relationships between two important vasoactive systems, i.e. the renin-angiotensin system and the kallikrein-kinin system.

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2.2. ACTIVATION OF INACTIVE PLASMA RENIN BY PLASMA AND TISSUE KALLIKREINS

2.2.1. ABSTRACT

Normal human plasma contains a pro-activator of inactive renin. The pro-activator is activated at physiological pH in plasma that has been pretreated with acid. This activation *in vitro* leads to the conversion of inactive renin into the active form with simultaneous generation of kallikrein activity. The endogenous activator of inactive renin has the same pH profile and inhibitor spectrum as plasma kallikrein. Inactive renin can also be activated by exposure of plasma to exogenous trypsin, and in normal plasma the quantities of inactive renin that are activated after acidification and with trypsin are identical. Prekallikrein (Fletcher factor)-deficient plasma, however, has much lower renin activity after acidification than with trypsin. Thus acid activation of inactive renin depends on plasma prekallikrein, whereas the action of trypsin is independent of prekallikrein. Highly purified tissue (pancreatic) kallikrein, in a concentration of less than 20 nM, activates inactive renin that has been isolated from plasma by ion-exchange chromatography. In this respect it is at least 100 times more potent than trypsin. It is therefore possible that plasma and/or tissue (renal) kallikreins are also involved in the activation of inactive renin *in vivo*.

2.2.2. INTRODUCTION

Plasma and tissue kallikreins act on kininogens in plasma to form bradykinin and kallidin respectively. The preferred substrate for plasma kallikrein is high-molecular-weight kininogen, whereas the tissue kallikreins react more readily with low-molecular-weight kininogen[1]. Furthermore, the formation of kallikrein is an early step in the cascade-like activation of proteolytic plasma factors involved in coagulation, fibrinolysis and complement-mediated reactions[2]. Here we report evidence that kallikreins are also capable of activating inactive renin.

2.2.3. METHODS

Collection of blood. Blood from 20 healthy male subjects was collected in plastic tubes, which contained 5 mmol of edetic acid (EDTA)/l of blood. Within 5 min the blood was centrifuged at 8,000 g for 10 min. The plasma was pooled and immediately frozen at -20 C. Plasma was also obtained from a patient with prekallikrein (Fletcher factor)-deficiency (less than 1% of normal).
Ion-exchange chromatography. EDTA-treated plasma (30 ml) was dialyzed for 24 h against 24 mM Tris/acetate buffer, pH 7.5, which contained 20 mM NaCl. The dialyzed plasma was applied to a 2.6cm x 40cm column of DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden), which had been equilibrated against the same buffer. Elution was performed with a linear gradient of NaCl up to 200

mM. Flow rate was kept constant at 22 ml/h, and the eluate was collected in 5.5 ml fractions. All procedures were carried out at 4 C.

Activation and assay of renin.

Activation of inactive renin by acid. Samples (2ml) of EDTA-treated plasma, or fractions, isolated from plasma by ion-exchange chromatography, were dialyzed for 24 h at 4 C against a glycine/HCl buffer, pH 3.3, which contained 5 mM EDTA[3,4]. After the pH had rapidly been restored to 7.5 with 1 M NaOH, the samples were dialyzed for another 24 h at 4 C against sodium phosphate buffer, pH 7.5, which contained 1 mM EDTA[3]. The pH-dependency of renin activation was assessed by adjusting the pH after the first dialysis step to values ranging from 4.0 to 9.5, again with 1 M NaOH, followed by dialysis against citric acid/phosphate buffers of the same pH. In experiments where the rate of activation was followed, the second dialysis step was omitted, and the samples were kept in plastic tubes for various times at 4 C as indicated, after pH had been adjusted to 7.5.

Activation of inactive renin by trypsin and kallikrein. Trypsin, 12,000 α-N-benzoyl-L-arginine ethyl ester (BAEE) units/mg of protein, was obtained from Sigma Chemical Co. Ltd, St Louis, U.S.A. Highly purified porcine pancreatic kallikrein was kindly supplied by Professor GL Haberland and Dr E Wischhofer (Kallikrein KZC 45/32, Bayer, Leverkussen, West Germany). The specific activity of this preparation was 1180 kallikrein units/mg of protein. The enzymes were covalently bound to CNBr-activated Sepharose-4B (Pharmacia) in a ratio of 10-30 mg of protein/g of dry Sepharose (Pharmacia). The immobilized enzymes were added to EDTA-treated plasma or to fractions isolated from plasma by ion exchange chromatography. The suspensions were slowly shaken at 4 C, for various times as indicated, and the enzymes were then removed by centrifugation at 8000 g for 10 min.

*Activation of inactive renin by low temperature.*Samples (2ml) of EDTA-treated plasma were kept in plastic tubes for various times at 4 C as indicated.

Inhibition of activation in vitro of inactive renin. Samples (2ml) of EDTA-treated plasma were dialyzed for 24 h at 4 C against glycine/HCL buffer, pH 3.3. The pH was then restored to 7.5 with NaOH 1 M, and the samples were redialyzed at pH 7.5 in the presence of various protease inhibitors. The following inhibitors were studied: 1) aprotinin (Trasylol, Bayer); 2) soybean trypsin inhibitor (Sigma); 3) lima bean trypsin inhibitor (Sigma); 4) trypsin inhibitor from chicken egg-white (ovomucoid, Sigma); 5) benzamidine/HCL (Sigma).

Assay of naturally occurring active renin. The method, which has previously been described[4], was slightly modified. Briefly, samples (2ml) of EDTA-treated plasma or fractions, isolated from plasma by ion exchange chromatography, were dialyzed for 2x24h at 4 C against a phosphate buffer, pH 7.5, which contained 1 mM EDTA. Aliquots (0.1 or 0.2 ml) of the dialyzed samples were mixed with sheep renin substrate (concentration 6.1 Km), and the total volume was adjusted to 1.0 ml with phosphate buffer, pH 7.5. After addition of 10 μl of 8-hydroxy-quinoline (0,34 M), 5 μl of phenylmethylsulfonylfluoride (0,3 M) in ethanol and

10 μl of aprotinin (10,000 kallikrein-inhibiting units/ml) the mixture was incubated for 3 h at 37 C. The angiotensin I that was generated during incubation with renin substrate was measured by RIA, and compared with that generated by standard human kidney renin (MRC standard 68/356). With the protease (angiotensinase) inhibitors we have used, recovery of angiotensin I, which was added to plasma after dialysis, was 98.8 $\pm$ 4.9% (n = 15). The recovery of standard renin, which was added to plasma before dialysis, was 97.0 $\pm$ 4.8% (n = 15).

Assay of renin activated in vitro. After the samples had been treated with acid, trypsin, kallikrein or low temperature, their renin content was measured as described before.

Assay of kallikrein. The method is based on the amidolytic action of plasma kallikrein on the synthetic chromogenic substrate H-D-propyl-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride (PPAN), as described by Claesson et al.[5]. Kallikrein was measured in EDTA-treated plasma which had been treated with acid, trypsin or low temperature. Aliquots (10 or 100 μl) of the treated plasma were mixed with the chromogenic substrate (PPAN, Kabi, Stockholm, Sweden; concentration 3.7 Km), and the total volume was adjusted to 1.2 ml with pre-warmed (37 C) 50 mM Trisbuffer, pH 7.5. The increase in absorbance at 405 nm was followed for 2 min in a 1 cm semi-microcuvette at 37 C. In experiments where the pH-dependency of kallikrein activity was assessed, the Tris buffer, pH 7.5, was replaced by citric acid/phosphate buffers with pH values ranging from 4.0 to 9.5. As mentioned before, these buffers were also used for studies on the pH-dependency of renin activation. The results of both sets of experiments could therefore be compared.

Units for expression of results. Renin concentration is expressed as micro-units of the renin standard (MRC standard 68/356)/ml of plasma (μU/ml). The concentration of inactive renin is the concentration of renin measured in plasma after dialysis for 24 h at pH 3.3 and subsequent dialysis for 24 h at pH 7.5 (total renin), minus the concentration of renin measured after dialysis for 2x24h at pH 7.5 (active renin). Kallikrein concentration is expressed as units/ml of plasma (U/ml), 1 unit being the quantity of enzyme that hydrolyzes 1 μmol of the synthetic substrate PPAN in 1 min at pH 7.5 and 37 C.

2.2.4 RESULTS

Simultaneous activation of inactive renin and prekallikrein in plasma. In plasma treated at pH 3.3, the activation of inactive renin occurred after pH had been restored to a value above 5.0. The pH-dependency of this process followed a bell-shaped curve, which was identical with the pH/activity curve of plasma kallikrein (Fig.1).

Results of various activation procedures, carried out on whole plasma at 4 C, are shown in Fig. 2. In normal plasma (Fig. 2a) both inactive renin and prekallikrein were simultaneously activated by either acid or trypsin treatment. Activation of inactive renin after acidification with subsequent restoration of pH to 7.5 was maximal after 16-24 h. The same maximum was reached by

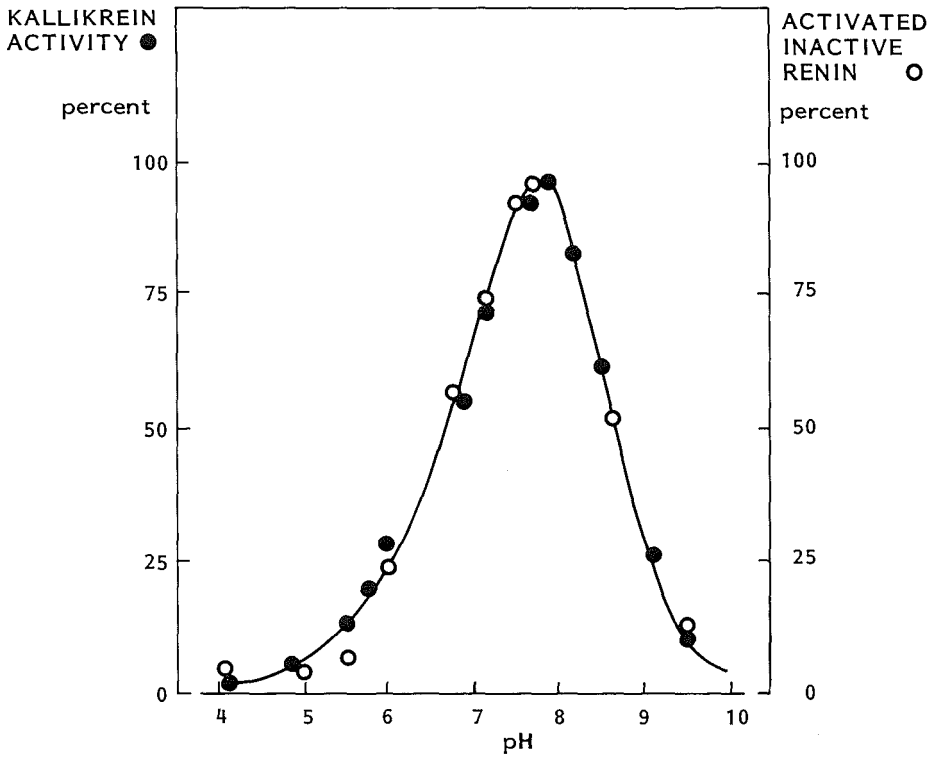

Fig. 2.2.1. Identical pH profiles for the amidolytic action of plasma kallikrein on the substrate PPAN (●) and for the activation of inactive renin (O). In this experiment prekallikrein was activated by exposure of normal plasma to pH 3.3 followed by restoration of pH; the procedure was the same as that for acid activation of renin (see text). Identical results were obtained with trypsin- and cold-activated prekallikrein. No detectable amidolytic was generated in prekallikrein (Fletcher factor)-deficient plasma (see Fig. 2.2.2.), which indicates that PPAN had adequate specificity.

treatment with trypsin at pH 7.5 for 24 h. Treatment for longer periods up to 72 h did not result in any further increase of renin activity.

In contrast with normal plasma the amount of inactive renin that was activated in prekallikrein-deficient plasma after acidification was much smaller than with trypsin (Fig.2b, lower part). There was no detectable amidolytic action on the chromogenic substrate, PPAN, after acid or trypsin treatment of the deficient plasma (Fig.2b, upper part), which demonstrates that, for the purpose of our experiments, PPAN has adequate specificity for kallikrein.

When normal plasma was kept at 4 C, without any other form of treatment, the activation of both inactive renin and prekallikrein proceeded more slowly (Fig.2a). Renin activity was 31 $\pm$ 6% above control after 48 h, and rose to 45 $\pm$ 3% above control after 72 h. The differences from control were significant $P < 0.01$, paired t-test). In prekallikrein-deficient plasma no activation occured under these circumstances.

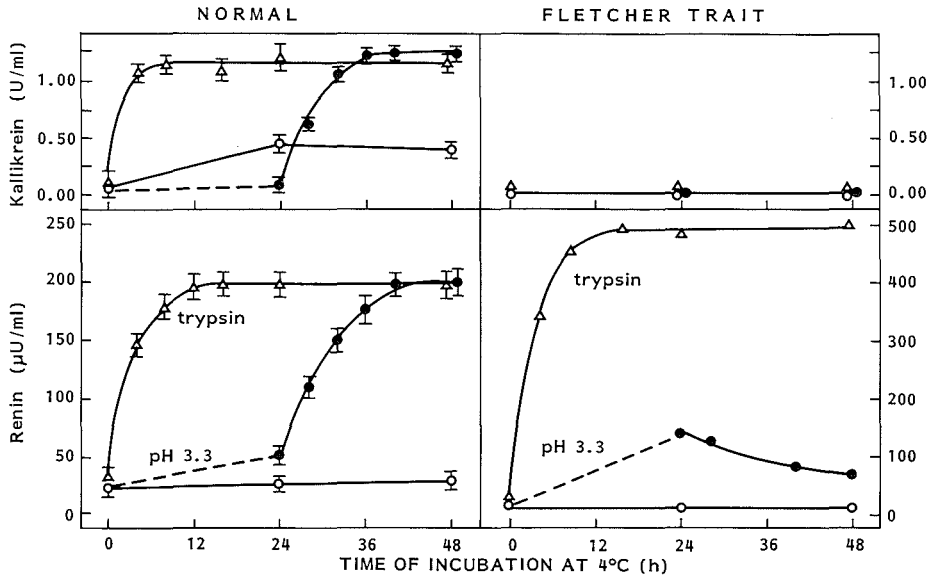

Fig.2.2.2. Effects of various procedures on the generation of active renin and kallikrein in normal plasma (M ± SEM,n = 10) and in prekallikrein (Fletcher factor)-deficient plasma. The plasma samples were treated as follows: O, storage at 4 C (cold activation); ●, dialysis at pH 3.3 , followed by dialysis at pH 7.5; and Δ, incubation with trypsin (final concentration 1.2 mM. in normal plasma inactive renin and prekallikrein are simultaneously activated with each procedure. No active renin is formed in deficient plasma after storage at 4 C or at pH 7.5 after dialysis at pH 3.3

Effects of protease inhibitors on the activation of inactive renin in plasma. Table 1 shows the effects of protease inhibitors, which were added to acid-treated plasma after the pH had been restored to neutral. The inhibitor/plasma mixtures were kept at 4 C for 24 h. Among the substances tested, aprotinin (Trasylol), soybean trypsin inhibitor and benzamidine/HCl had the greatest effect on the activation of inactive renin. The inhibitor spectrum for renin activation was similar to that reported for plasma kallikrein[6,7]. None of the protease inhibitors interfered with the reaction between active renin and its substrate. This was demonstrated by adding the inhibitors to known amounts of human renin (MRC standard 68/356) before incubation with excess of sheep renin substrate.

Activation of inactive plasma renin by tissue kallikrein. Pancreas kallikrein, added to whole plasma and left there for 24 h at 4 C, did not activate inactive renin (Table 1). This contrasts with the findings when a semipurified preparation of inactive plasma renin was used instead of whole plasma. As illustrated in Fig. 3, the elution pattern, obtained after DEAE- Sepharose column chromatopgraphy of plasma, showed two peaks of renin activity at sodium concentrations of 60 mM (peak A) and 100 mM (peak B). The renin content of peak B comprised 80-90% of the total quantity of active renin present in whole plasma. Acid

Table 2.2.1. Activation of inactive renin by proteases and effects of protease inhibitors.

The same plasma pool was used in all experiments. The quantity of renin that was activated in whole plasma after 24 h dialysis at pH 3.3 with subsequent 24 h dialysis at pH 7.5 without serine protease inhibitors was taken as 100%. Acid-activatable renin in the plasma pool was 170 μU/ml. As stated in the text, 90-95% of the acid-activatable renin in whole plasma was calculated to be recovered as kallikrein-activatable renin after chromatography (concentration of added kallikrein in eluate approximately 120 nM). The activation of semipurified inactive renin was therefore considered to be complete under these circumstances.

Protease (1) and protease inhibitors (2)	Prior treatment of plasma	Percentage of renin activated (M±SEM)	No
(1)			
Kallikrein	None		
approx. 120 nM		<5	6
approx. 12 nM		<5	6
Kallikrein	Ion-exchange		
approx. 120 nM	chromatography	100±5	6
approx. 12 nM	(peak A)*	72±4	6
Trypsin	None		
approx. 1.2 μM		102±6	6
approx. 0.6 μM		75±6	6
Trypsin	Ion-exchange		
approx. 1.2 μM	chromatography	56±6	6
approx. 0.6 μM	(peak A)	35±4	6
(2)			
Aprotinin	Dialysis at pH 3.3 with		
1250 I.U./ml**	subsequent restoration	11±4	16
125 I.U./ml	of neutral pH	70±4	16
Soybean trypsin inhibitor	Idem.		
1250 I.U./ml		17±9	16
125 I.U./ml		26±7	16
Lima bean trypsin inhibitor	Idem.		
1250 I.U./ml		87±4	4
125 I.U./ml		95±2	4
'Ovomucoid'	Idem.		
1250 I.U./ml		81±4	4
125 I.U./ml		100±1	4
Benzamidine/HCl	Idem.		
(10 mM)		20±1	4

* This plasma fraction was eluted at a sodium concentration of 60 mM. Four 5.5 ml fractions of peak A (fig. 3) with the highest renin content were pooled.

** One inhibitor unit inhibits a quantity of trypsin that will produce an absorbance at 253 nm of 0.001/min at pH 7.6 and 25 C, with α-N-benzoyl-L-argenine ethyl ester (BAEE) as substrate; reaction volume equals 3.2 ml (1 cm light-path).

treatment doubled the renin content of peak A but had no effect on peak B, indicating that peak A contained inactive renin. After acid treatment, however, only a small proportion of the total quantity of inactive renin present in peak A was activated, whereas addition of pancreatic kallikrein, in a concentration of 120 nM of eluate, had a much larger effect. The quantity of inactive renin

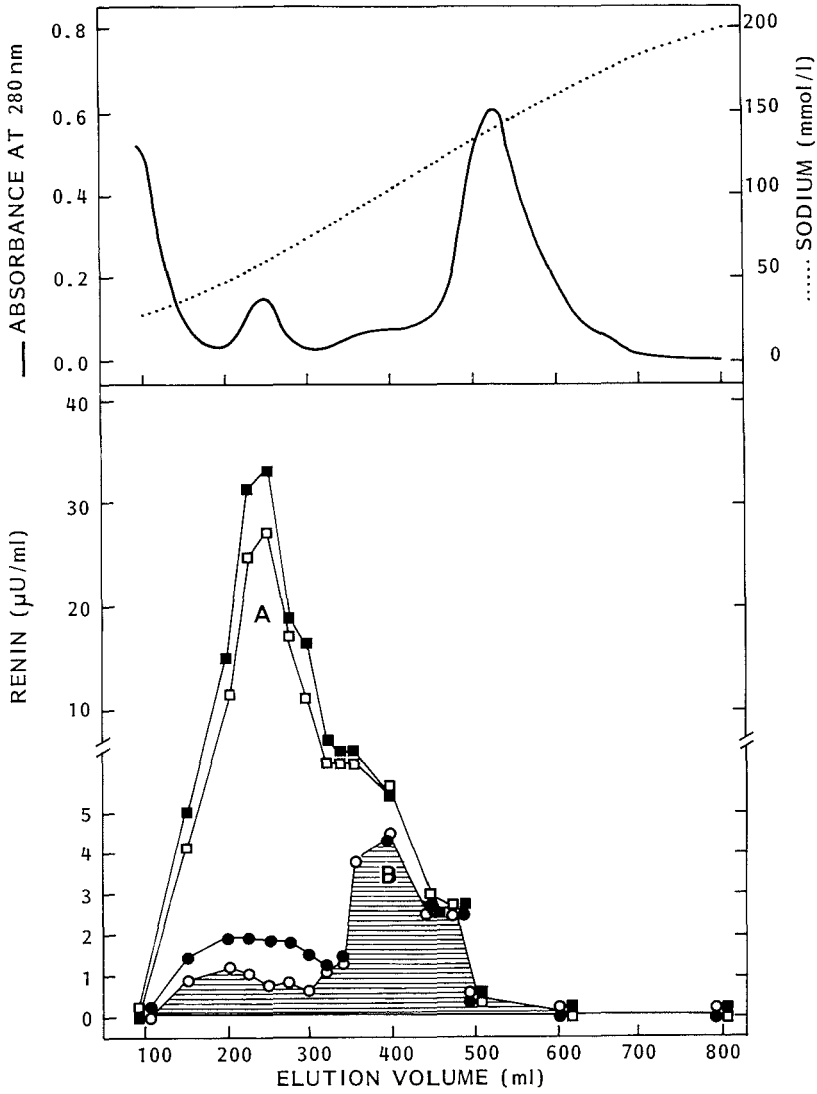

Fig. 2.2.3. Separation of inactive renin from active renin by ion-exchange chromatography of normal plasma and its activation with pancreatic kallikrein (final concentration approximately 120 nM or acid. The absorbance by plasma proteins and the sodium gradient are shown in the upper part of the Figure. Peaks A and B in the lower part of the Figure correspond to inactive renin and active renin respectively (for explanation, see the text). Fractions of the column were treated as follows: O, dialysis at pH 7.5 for 2 × 24 h; ●, dialysis at pH 3.3 for 24 h, followed by dialysis at pH 7.5 for 24 h; □, dialysis at pH 7.5 for 24 h with subsequent incubation with kallikrein for 24 h; ■, dialysis at pH 3.3 for 24 h with subsequent restoration of pH to 7.5 and incubation with kallikrein for 24 h. These procedures were carried out at 4° C.

that could be activated by kallikrein after ion-exchange chromatography was calculated to be 90-95% of the quantity that could be activated by acid in plasma before chromatography. This indicates that 90-95% of the acid- activatable renin in whole plasma is recovered as kallikrein- activatable renin after chromatopgraphy. Kallikrein had no effect on peak B. In Table I the quantities of kallikrein and trypsin that were needed for the activation of inactive renin are compared. It can be seen that kallikrein is at least 100 times more potent.

2.2.5 DISCUSSION

Kallikrein-mediated activation of inactive renin. Most of the kallikrein and renin circulating in plasma is inactive. The activation of the kallikrein-kinin system may be linked with the activation of the renin-angiotensin system. The evidence for this is as follows: 1) prekallikrein and inactive renin in normal plasma are simultaneously activated after exposure of plasma to acid and subsequent restoration of pH, 2) the pH-dependency of the endogenous plasma enzyme that activates inactive renin closely parallels the pH/activity profile of kallikrein, 3) after acidification there is little activation of inactive renin in prekallikrein-deficient plasma, 4) substances that are known to inhibit plasma kallikrein, such as aprotinin, soybean trypsin inhibitor and benzamidine[6,7] interfere with the activation of inactive renin, 5) other protease inhibitors that do not inhibit plasma kallikrein significantly, such as lima bean trypsin inhibitor and 'ovomucoid' [6] , have little effect, and 6) highly purified tissue(pancreatic) kallikrein is a potent activator of inactive renin, which has been isolated from plasma by ion-exchange chromatography. Exogenous kallikrein has no effect when added to untreated whole plasma, probably because of the inhibitory action of some plasma proteins[2].

Admittedly, the procedures that active renin, also activate other plasma factors besides prekallikrein, and the pH-optimum curve and inhibitor spectrum of kallikrein are shared by several serine proteases involved in the processes of clotting, fibrinolysis and complement activation. Furthermore although both plasma kallikrein and tissue kallikrein are involved in kinin formation, their substrate specificity is somewhat different[1]. Thus we have no proof that inactive renin is directly activated by kallikreins. On the other hand, generation of kallikrein is an early step in the activation of other serine proteases in plasma[2,7]. Furthermore, in prekallikrein-deficient plasma, renin activity after acidification was much smaller than with trypsin, whereas, in normal plasma, the results with acid and trypsin were identical. This is strong evidence that acid activation of inactive renin depends on plasma prekallikrein. Our data therefore suggest that the activation of inactive renin is at least in some way linked with the kallikrein-kinin system. During the course of this work some preliminary data, which are keeping with this view, have been published[8–11]. Recently Sealey and coworkers[11] reported that renin activity increased more rapidly when urinary kallikrein, which originates from the kidney , was added to acid-treated plasma than with acid treatment alone. Our work extends these observations in that

it demonstrates that inactive renin can be fully activated by tissue kallikrein without prior acid treatment.

Physiological implications. The physiological importance of these data remains to be established, but some observations might be relevant. Active kallikrein is secreted by kidneys and salivary glands, and these organs also produce active renin[12,13]. Furthermore, there is some evidence for intrarenal activation of inactive renin[14]. Plasma bradykinin and urinary kallikrein increase when the release of active renin is stimulated by standing up or sodium depletion[15–17]. Furthermore, some stimuli for plasminogen activation, such as exercise and isoprenaline [18], increase the release of active renin[19–22] and may activate prekallikrein[2]. Possibly related to this is the frequent association of malignant hypertension with low-grade disseminated intravascular coagulation and high concentrations of circulating active renin[23]. In the intact organism, kallikrein-mediated activation of proteolytic enzymes involved in clotting and fibrinolysis is thought to occur in close contact with the blood-vessel wall, and after their release into the circulation they are rapidly inactivated or removed[2]. This may also be true for components of the renin-angiotensin system, so that circulating levels of these hormones correlate poorly with their physiological effects. Finally, the biologically active endproducts of the kallikrein-kinin and renin-angiotensin systems act on vascular smooth muscle. Kinins are potent vasodilators, and angiotensin II is a potent vasoconstrictor. A direct interaction of kallikrein and renin, possibly in close association with the blood-vessel wall, might therefore provide a mechanism for blood-pressure regulation.

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2.3. AN INTRINSIC FACTOR XII-PREKALLIKREIN DEPENDENT PATHWAY ACTIVATES THE HUMAN PLASMA RENIN ANGIOTENSIN SYSTEM

2.3.1. RESULTS AND DISCUSSION

Exposure of normal human plasma to an activating (negatively charged) surface leads to the activation of the intrinsic coagulation and fibrinolytic system and the generation of vasoactive peptides (kinins)[1,2]. This contact activation mechanism is known to be initiated by factor XII (Hageman factor). Surface-activated factor XII converts prekallikrein (Fletcher factor) into kallikrein, which leads to further activation of factor XII. Prekallikrein circulates in plasma as a complex with the kallikrein substrate, high molecular weight kininogen, and the latter acts as a cofactor in the reciprocal activation of factor XII by kallikrein. The experiments described here demonstrate that the renin-angiotensin system is also activated by kallikrein through the factor XII-dependent pathway.

Normally, about 80% of the renin present in plasma is in an enzymatically inactive form[3–5]. It is called 'inactive renin' or 'prorenin', as opposed to naturally occurring 'active renin'. Both forms can be separated by ion exchange chromatography[6]. Inactive renin is converted into the active form by limited proteolysis at neutral pH, either in acid-pretreated plasma by one or more endogenous serine proteases[7–9] and by urinary (renal) kallikrein[10], or in untreated plasma with exogenous trypsin[5–9]. Active renin is also formed during prolonged exposure of plasma to low temperature (cryoactivation)[11].

Recent reports have suggested that factor XII and prekallikrein might be involved in the generation of active renin, but the results were contradictory[12–13]. To investigate the possible involvement of the factor XII-dependent pathway in the activation of prorenin, we treated normal plasma (n = 10), factor XII-deficient plasma (n = 2) and prekallikrein-deficient plasma (n = 1) by 1) dialysis at pH 3.3 and 4 C for 24 h with subsequent restoration of pH to 7.5[14], or 2) incubation with Sepharose-bound trypsin at pH 7.5 and 4 C . Renin was then measured by radioimmunoassay of angiotensin I formed after incubation of the plasma samples with an excess of purified sheep renin substrate at pH 7.5 and 37 C for 3 h[4]. Results in normal plasma showed identical amounts of activated renin after acid or trypsin treatment (Fig. 1, left-hand scale). Smaller increments of active renin were obtained during cryoactivation. In plasma from patients with Hageman trait, levels of naturally occurring active renin were 17 and 21 μU/ml, and those of inactive (trypsin-activatable) renin were 180 and 210 μU/ml. These values were within the normal range found in our laboratory, which is 12-45 μU/ml for active renin and 70-230 μU/ml for inactive renin (n = 25). In samples from the patient with Fletcher trait, active renin was low 8.4 μU/ml, but inactive renin was markedly elevated, 510 μU/ml. In contrast to normal plasma, both factor XII- and prekallikrein-deficient plasma generated small amounts of active renin after acidification, whereas trypsin generated normal

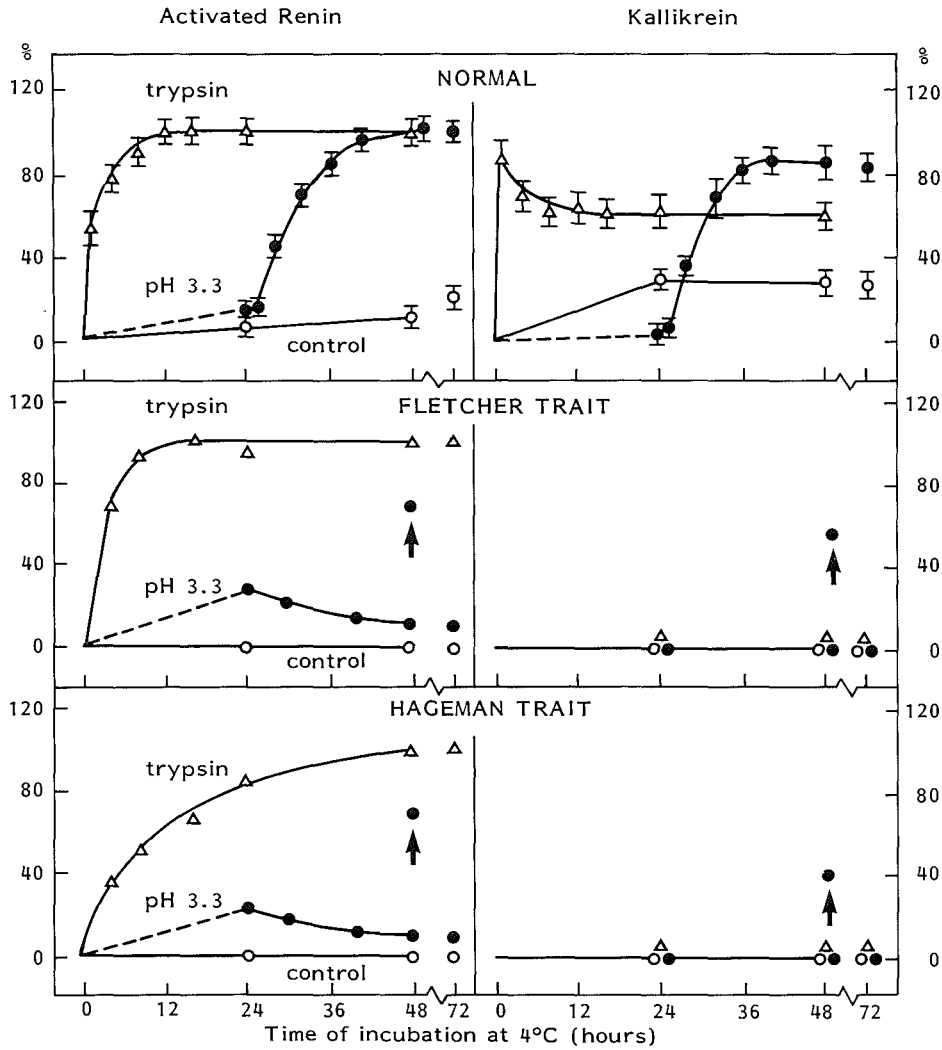

Fig. 2.3.1. Generation of active renin and kallikrein in normal plasma (mean ± sem, n = 10), prekallikrein (Fletcher factor)-deficient plasma and factor XII (Hageman factor)-deficient plasma. Two procedures were used: acidification and addition of trypsin.Plasma, which contained 5 mM EDTA, was acidified (------) by dialysis against 0.15 M glycine/HCl buffer of pH 3.3 for 24 h[14]. The pH was then restored to pH 47.5 with 1 M NaOH, and the generation of active renin and kallikrein was followed for 1-72 h (●—●). Trypsin (14,000 BAEE units per mg protein (Sigma) was covalently bound to CNBr-activated Sepharose (Pharmacia) in a ratio of 10 mg protein per g dry Sepharose. The immobilized enzyme was added to the plasma (Δ—Δ) at a final concentration of 3,000 BAEE units per ml. After 1-48 h it was removed by centrifugation.All these procedures were carried out at 4 C. Plasma that was kept at 4 C without any other form of treatment served as a control (O O). Pretreated and control plasma samples were then assayed for renin and kallikrein

activity. For renin measurements the samples were incubated with purified sheep renin substrate (final concentration 6.7 x Km)[4] at pH 7.5 and 37 C for 3 h in the presence of the following protease inhibitors: 5 mM EDTA,3 mM phenylmethylsulphonylfluoride, 3.4 mM 8-hydroxyquinoline and aprotonin (Bayer) at a concentration of 100 kallikrein-inhibiting units per ml of incubate. The increment of renin after 48 h exposure to trypsin was taken as 100 % activation. Kallikrein was measured as amidolytic activity using the chromogenic substrate ester H-D-Pro-Phe-Arg-p-nitroanilide (PPAN, Kabi) as a substrate[16]. Plasma (15 μl) was mixed at 37 C with 815 μl Tris buffer of pH 7.5 and 200 ul 4.1 mM PPAN. The linear release of p-nitroanilide was followed at 405 nm for 1-2 min in a 1-cm semi-microcuvette at 37 C. Complete activation of prekallikrein was obtained by mixing 100 μl of the untreated plasma with 100 μl dextran sulphate 25 mg/l [18].After 7 min incubation at 4 C, 30 μl of the mixture was added to 800 μl Tris buffer and 200 μl PPAN, and kallikrein was measured as before. The values obtained after dialysis at pH 3.3 and restoration of pH to 7.5 and with trypsin were expressed as a percentage of total activatable prekallikrein as obtained with dextran sulphate. Lineweaver-Burk plots for the reactions of acid-activated renin, trypsin-activated renin and naturally occurring active renin with sheep renin substrate (1 h incubation) gave identical Km values (0.06 μM). Thus, the increase of renin activity after acidification and with trypsin is caused by a parallel change in the number of molecules of active renin rather than by changes in the enzymatic activity per molecule of active renin. When untreated normal plasma was kept at 4 C, small increments in renin and kallikrein activity were observed ($P < 0.001$ for difference of values at 72 h from those at 0 h). Prekallikrein and factor XII were isolated from normal human plasma and purified[19]. They were added to, respectivily, prekallikrein-deficient plasma (final concentration of prekallikrein 50 μg/ml) and factor XII-deficient plasma (final concentration of factor XII 28 μg/ml). Deficient plasmas to which Tris buffer of pH 7.5 was added, instead of prekallikrein or factor XII, served as controls. The reconstituted plasmas and controls were treated by dialysis at pH 3.3 for 24 h, and the renin and kallikrein activities were measured 24 h after the pH had been stored to 7.5 (results indicated by arrows).

or increased amounts. There was no detectable cryoactivation of renin in the deficient plasmas. This suggested that acid activation and cryoactivation of plasma renin depend on the presence of both factor XII and prekallikrein.

Further experiments substantiated this hypothesis. Reconstitution of factor XII-deficient plasma and prekallikrein-deficient plasma with, respectively, purified factor XII and prekallikrein resulted in a correction of the generation of active renin after acidification. Prekallikrein in normal plasma is converted into active kallikrein after exposure of the plasma to low pH, trypsin or low temperature (cryoactivation)[15]. Simultaneous determinations of renin and kallikrein measured as amidolytic activity[16] showed parallel increments of both activities (Fig.1, right-hand scale).

The acid-activation of prekallikrein was confirmed by the parallel cleavage of ^{125} I-labeled prekallikrein added to normal plasma before acidification (Fig.2). Previous experiments in which the activation of prekallikrein by activated factor XII was studied had shown that the generation of kallikrein activity was directly correlated with the extent of proteolytic cleavage[17]. Analysis of the reaction mixture by SDS-polyacrylamide gel electrophoresis in the presence of reducing agents demonstrated that the disappearance of prekallikrein of apparent molecular weight 85,000 was associated with the simultaneous generation of cleavage fragments; the major radiolabeled fragment had an apparent molecular weight of 52,000.

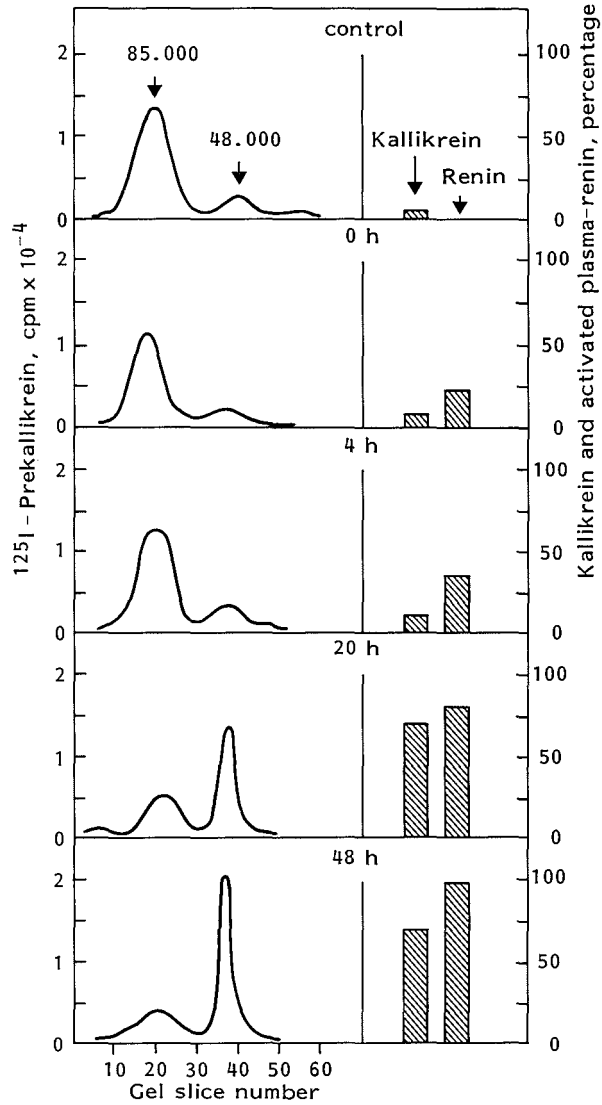

Fig. 2.3.2. Cleavage of ^{125} I-prekallikrein in acid-pretreated normal plasma. Prekallikrein was isolated from normal plasma[19] and labelled by the method of Bolton and Hunter[20]. ^{125} I- prekallikrein (2.7 μCi in 15 μl) was added to 600 μl normal plasma. The mixture was dialyzed at pH 3.3 for 24 h. Immediately (0 h) after restoration of pH to 7.5 with 1 M NaOH, and 4-48 h later, the reaction mixtures were analysed for the presence of cleavage products of ^{125} I-prekallikrein (left) and for the kallikrein and renin activity (right). After reduction by β-mercaptoethanol the reaction mixture was subjected to SDS-polyacrylamide gel (7.5 %) electrophoresis. The gels were sliced and the radioactivity in each slice (1.2 mm) was counted. The appearance of cleavage products with an apparent molecular weight of 52,000 indicates activation of prekallikrein[17].

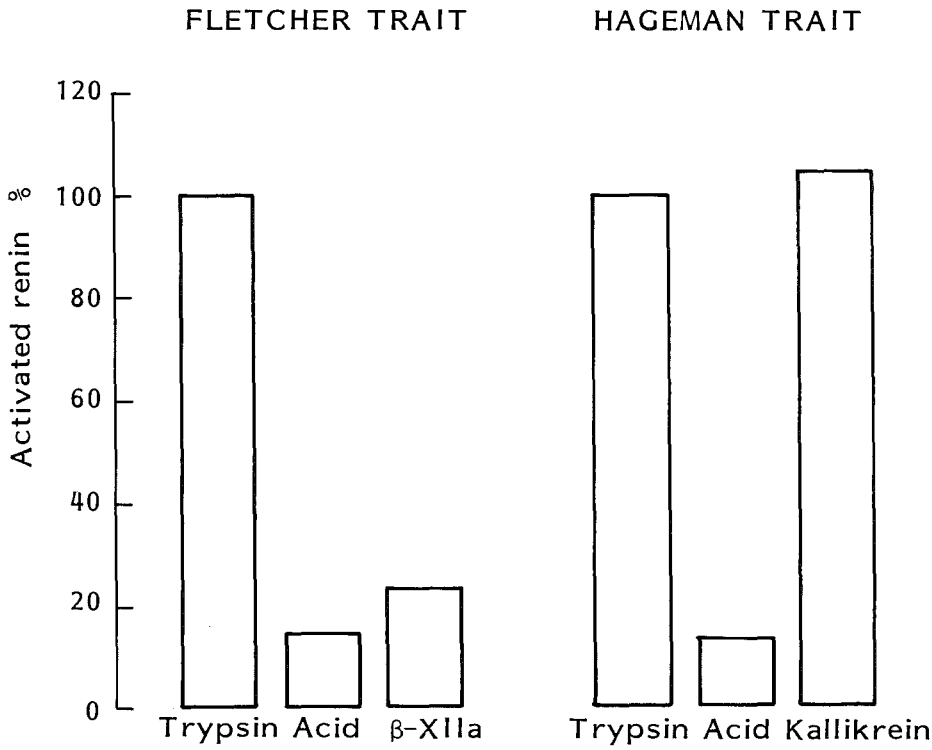

Fig. 2.3.3. Generation of active renin in prekallikrein (Fletcher factor)-deficient plasma and factor XII (Hageman factor)-deficient plasma. Plasma was treated with Sepharose-bound trypsin at pH 7.5 and 4 C for 48 h or was dialyzed at pH 3.3 for 24 h followed by dialysis at pH 7.5 and, 4 C for 24 h. Factor β-XIIa (final concentration 10 μg/ml) was added to prekallikrein-deficient plasma after 24 hdialysis of the plasma at pH 3.3 and restoration of pH to 7.5. Plasma kallikrein (final concentration 32 μg/ml) was added to factor XII-deficient plasma again after 24 h dialysis of the plasma at pH 3.3 and restoration of pH to 7.5. The mixtures were incubated at 4 C for 24 h. Factor β-XIIa (600 μg/ml) (from Dr John Griffin) gave a single band on SDS- polyacrylamide gel electrophoresis with an apparent Mr of 28,000. Kallikrein (200 μg/ml) was prepared by activation of plasma prekallikrein by factor β-XIIa and was separated from factor β-XIIa by DEAE-Sephadex chromatography at pH 8.25.[19]

Addition of a physiological amount of purified plasma kallikrein to factor XII-deficient plasma after the acidification step, caused complete activation of inactive renin (Fig.3). Active factor XII fragment (β-XIIa) in a physiological concentration, however, was not capable of generating active renin in prekallikrein-deficient plasma. This indicates that in the factor XII-dependent pathway, plasma kallikrein is the major activator of inactive renin. The factor XII dependency can be explained by the capacity of this factor to convert plasma prekallikrein into kallikrein.

We conclude that factor XII and prekallikrein are part of an endogenous pathway that activates renin *in vitro*. It is of interest that inactive (trypsin-activatable) plasma renin was grossly elevated in the patient with Fletcher trait, whereas his plasma level of naturally occurring active renin was abnormally low. This may indicate that the the factor XII-prekallikrein pathway is also important for the activation of renin *in vivo*. Possibly, inactive renin that is adsorbed or bound to the blood vessel wall, at sites that are inaccessible to circulating protease inhibitors, is activated through this pathway. The reported activation of prorenin after the addition of renal kallikrein to acid-pretreated plasma[10], suggest a further analogy with the clotting and fibrinolytic systems: plasma kallikrein is part of an intrinsic pathway for activating the renin-angiotensin system, whereas tissue (renal) kallikrein might be involved in extrinsic activation.

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2.4. PLASMA KALLIKREIN AND PLASMIN AS ACTIVATORS OF PRORENIN: LINKS BETWEEN THE RENIN-ANGIOTENSIN SYSTEM AND OTHER PROTEOLYTIC SYSTEMS IN PLASMA

2.4.1. RENIN AND PRORENIN

Renin is a circulating proteolytic enzyme acting at the physiological pH of blood; a circulating inhibitor of this enzyme has not been identified. Therefore, most studies on the biological significance of renin are based on the classical model of a circulating hormone. Renin, however, does not act on cellular receptors; it acts by virtue of its enzymatic activity.

Evidence has accumulated in recent years that a proportion of the renin in human plasma has little or no enzymatic activity. About 80 % of the total potential renin activity of normal plasma is in this inactive form[1–4]. Possibly we are dealing here with a proenzyme comparable with the proenzymes that are activated by limited proteolysis in important physiological processes, such as coagulation, fibrinolysis, inflammatory responses and complement-mediated reactions. Usually, these processes are initiated in close contact with the blood vessel wall and not in circulating plasma. This could also apply to the renin-angiotensin system, so that the circulating renin activity is not always a good index for the biological activity of the system. The traditional endocrinologist's view on renin is perhaps too limited.

Inactive renin can be separated from active renin by ion exchange chromatography[5,6] and by affinity chromatography with pepstatin and certain dye ligands[7]. As will be discussed inactive renin is converted (activated) *in vitro* into an active form of renin by various proteinases. Plasma renin that is activated *in vitro*, naturally occurring active plasma renin and renin isolated from human kidneys as described by Haas, Goldblatt and Gibson[8] have similar properties, in terms of pH optimum, Km and inhibitory effects of pepstatin[9–12]. It is not certain, however, that inactive renin in plasma is a precursor of active renin in the course of its biosynthesis. Despite this uncertainty we will use here the term prorenin, because this form of renin has potential renin activity; its reacts, after activation, with natural renin-substrate to form angiotensin I.

Our knowledge of the biochemistry of prorenin is still preliminary. It may or may not represent a single molecular entity. A completely inactive form of renin has been isolated from plasma[7] but other forms of prorenin with some enzymatic activity before full activation may exist[13]. There is much debate on differences between prorenin, naturally occurring active renin and active renin generated *in vitro*, in terms of molecular weight and chromatographic behaviour[4–16]. Here we shall not discuss these questions but rather focus on the proteolytic enzymes , particularly the enzymes present in plasma, that are involved in the various pathways of prorenin activation *in vitro*. We shall consider the possible significance of these pathways *in vivo*.

2.4.2. INTRINSIC, EXTRINSIC AND EXOGENOUS PRORENIN ACTIVATORS

The term activator is used here to designate proteolytic enzymes that are capable of activating prorenin in crude plasma or in partly purified fractions prepared from plasma. The use of this term therefore does not imply that such an activator directly acts on prorenin.

2.4.2.1. INTRINSIC ACTIVATORS

Factor XII kallikrein pathway. The renin activity of human plasma is increased after 24 h-dialysis at pH 3.3 with subsequent restoration of pH[2]. This also applies to human amniotic fluid. Lumbers[1] and Morris and Lumbers[17] were the first to show that this process of 'acid-activation', which is now known to be caused by conversion of prorenin into an active form of renin, is dependent on one or more proteolytic enzymes. The renin activity of plasma also increases during exposure of plasma to -4 C. Tatemichi and Osmond[18] have provided some evidence that this process of 'cryo-activation' depends on clotting factor XII (Hageman factor). Thus renin activity did not rise in Hageman-trait plasma. Experiments, in which serine proteinase inhibitors were added to plasma in the neutral phase after pH 3.3-dialysis, have subsequently shown that one or more serine proteinases are responsible for the increase in renin activity[19–21]. Studies carried out independently by Derkx, Bouma, Schalekamp & Schalekamp[22] and by Sealey, Atlas, Laragh, Silverberg & Kaplan[23] have demonstrated that both factor XII and prekallikrein (Fletcher factor) are involved here; the renin activity of Hageman-trait plasma and Fletcher-trait plasma did not rise in the neutral phase after pH 3.3-dialysis, whereas it rose after addition of trypsin, which seems to act on prorenin independently of factor XII and plasma kallikrein (Fig. 1). By adding active factor XII fragment (factor β-XIIa, Mr 28,000) and highly purified plasma kallikrein (obtained by factor β-XIIa-activation of prekallikrein) to acid-pretreated Fletcher-trait plasma and acid-pretreated Hageman-trait plasma respectively, it could be demonstrated that kallikrein was the more direct activator of prorenin in this pathway. The factor XII-dependency of prorenin activation can be readily explained by the capability of this factor to convert plasma prekallikrein into kallikrein (Fig. 2).

C_1-Esterase inhibitor and α2-macroglobulin are the most important inhibitors of kallikrein in plasma. The former is activated at pH 4.5-5.0. After treatment of plasma at this pH, however, little activation of prorenin is observed. α2-Macroglobulin is inactivated at pH 3.0-3.5 and this coincides with the generation of uninhibited kallikrein and the formation of active renin after pH has been restored[24]. It is possible that acid treatment is not only required for the generation of enough uninhibited kallikrein, and possibly other proteinases, but also for rendering the prorenin more susceptible to activation by these proteinases[25].

Plasmin. This also is an intrinsic activator of prorenin[26]. α2-Antiplasmin is the most important inhibitor of plasmin in human plasma. It is inactivated at pH 5.5-6.0[24]. After dialysis of plasma at pH 4.0 for 24h and subsequent restoration

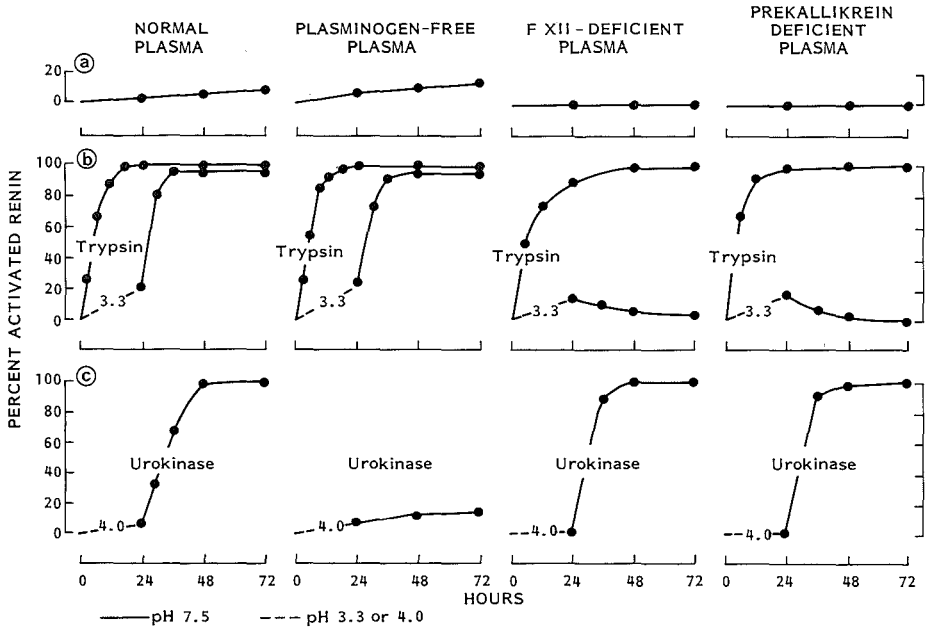

Fig. 2.4.1. Activation of prorenin (mean values) at 4°C in normal plasma (n=5), normal plasma from which plasminogen was removed by affinity chromatography on lysine-Sepharose as described by Deutsch & Mertz [29] (n= 4), factor XII-deficient plasma (Hageman-trait, n=2) and pre kallikrein-deficient plasma (Fletcher-trait, n=2). Plasmas contained EDTA in a total concentration of 5 mmol/l. (a) Plasmas were dialyzed against phosphate buffer of pH 7.5, made up 0.15 mol/l with NaCl, and stored at 4°C for various time periods as indicated. (b) Plasmas were treated with trypsin and with acid. Trypsin treatment: plasmas were dialyzed against phosphate buffer of pH 7.5, made up 0.15 mol/l with NaaCl, and incubated at 4°C with Sepharose-bound trypsin in a final concentration of 3000 α-N-benzoyl-L-arginine ethyl ester units/ml [22, 27] for various time periods as indicated. Acid treatment: plasmas were dialyzed at 4°C for 24 h against glycine/HCl buffer of pH 3.3, made up 0.15 mol/l with NaCl. The pH was then restored to 7.5 with NaOH solution (1.0 mol/l) and the plasmas were stored at 4°C for various time periods as indicated. (c) Plasmas were dialyzed at 4°C for 24 h against glycine/HCl buffer of pH 4.0, made up 0.15 mol/l with NaCl. At this pH α_2-antiplasmin is destroyed. The pH was then restored to 7.5 with NaOH solution (1.0 mol/l) and the plasmas were incubated at 4°C with urokinase in a final concentration of 1000 Ploug units/ml [27] for various time periods as indicated.

of pH α2-antiplasmin is not detectable any more, but there is little increase in renin activity. In contrast, large quantities of active renin are generated in pH 4.0-pretreated plasma after the addition of plasmin or the plasminogen activators, streptokinase and urokinase[27] (Fig. 1). By affinity chromatography on Cibacron-blue Sepharose columns, using a 0.02 M phosphate buffer of pH 7.1, containing 0.2 M NaCl, for elution, we have isolated from plasma a fraction

which contained practically all the prorenin and plasminogen present in crude plasma. This fraction was virtually free of active renin and plasmin inhibitors. Addition of streptokinase, urokinase and highly purified plasminogen activator isolated from human uterine tissue[28] all led to the activation of prorenin. The plasminogen activators did indeed act via generation of plasmin; after removal of plasminogen by lysine-Sepharose affinity chromatography[29] the plasminogen activators had no effect on renin activity (Fig. 1).

Interactions of plasmin and the factor XII-kallikrein pathway. Experiments with purified factors have demonstrated that plasmin is capable of activating factor XII and that kallikrein can activate plasminogen. The factor XII-kallikrein pathway for prorenin activation, however, can proceed in the absence of plasmin[23]. Moreover, plasmin-mediated prorenin activation can proceed in the absence of factor XII and kallikrein[27] (Fig.1). Kallikrein, via activation of prekallikrein by factor XII, and plasmin are the only intrinsic prorenin activators identified as yet, but there is no proof that these activators act directly on prorenin. Possibly, intermediate steps will be discovered.

Fig. 2 gives a schematic representation of the intrinsic pathways of prorenin activation and their possible connections with other proteolytic processes. The discovery that prorenin is activated via the factor XII-kallikrein pathway links the renin-angiotensin system with the surface-mediated reactions of coagulation, fibrinolysis and kinin formation[30]. Both prekallikrein and factor XI circulate in plasma as complexes with the kallikrein-substrate, high molecular weight

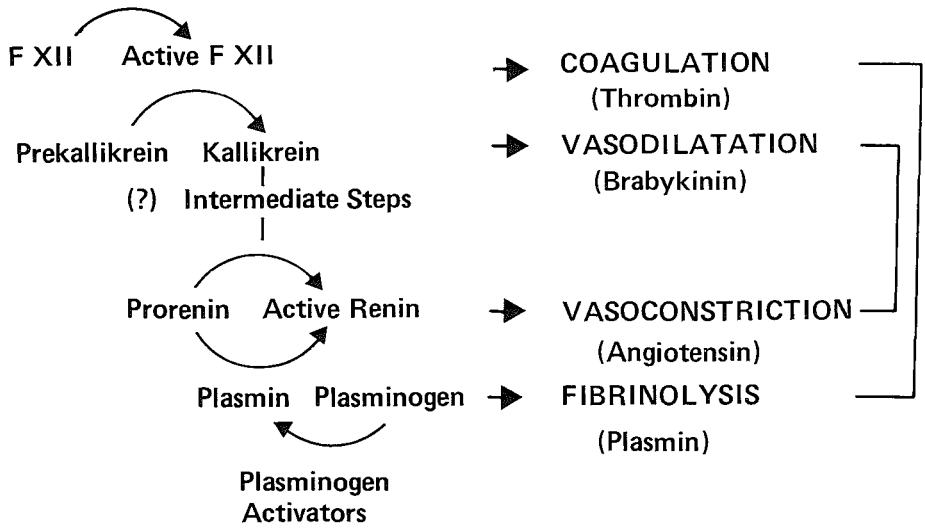

Fig. 2.4.2. Factor XII-kallikrein and plasmin pathways of prorenin activation; connections with coagulation, fibrinolysis and kinin formation.

kininogen. Factor XII and kallikrein reciprocally activate one another in a positive feedback mechanism that also requires high molecular weight kininogen. Initial activation of factor XII may occur on exposure to negatively charged surfaces such as vascular basement membrane. Activated factor XII then triggers the cascades of coagulation and fibrinolysis. The biochemistry of these surface-mediated reactions ha been reviewed[31].

2.4.2.2. EXTRINSIC ACTIVATORS

Glandular kallikrein. Two types of kallikrein are to be distinguished, plasma kallikrein and glandular kallikrein. High molecular weight kininogen is the preferred substrate for plasma kallikrein, and the product is bradykinin. Glandular kallikrein is found in exocrine glands and in the kidney. It is about equally active on low- and high molecular weight kininogen of plasma and the product is lysylbradykinin.

Sealey, Atlas, Laragh, Oza & Ryan[32] have demonstrated that glandular kallikrein, isolated from urine, is capable of activating prorenin at neutral pH in pH 3.3-pretreated human plasma. It has subsequently been shown that activation of prorenin by glandular kallikrein does not require prior acidification of prorenin[33].

Renal kallikrein has been localized to the distal tubule, possibly including the macula densa. This would favour an interaction with renin. Furthermore some kallikrein is released in the perfusate of isolated kidneys and small quantities of immunoreactive glandular kallikrein have been detected in plasma. However, immunochemical staining of kallikrein in the kidney was most intense on the luminal surface of tubular cells, whereas renin is localized in the afferent arteriole. This localization would indicate that these enzymes are released into urine and blood respectively, so that both proteins should never meet. For a more detailed discussion on renal kallikrein the reader is referred to Carretero & Scicli[34] and Keiser[35].

Vascular plasminogen activator. We have already mentioned that tissue plasminogen activator, which is probably similar or identical with the vascular activator (see the review by Collen[36]), is capable of initiating plasmin-mediated prorenin activation. This is an interesting observation since the release of vascular activator is increased by stimuli that are also known to increase the renin activity of plasma. We will come back to this in a later section of this review.

2.4.2.3. EXOGENOUS ACTIVATORS

Trypsin is now often used for the conversion of prorenin into active renin in crude plasma *in vitro* as a first step in measuring prorenin[37–39]. The acid proteinases, pepsin and cathepsin D, also are capable of activating prorenin[40]. The effect of these exogenous proteinases on the renin activity of plasma does not seem to be mediated by the intrinsic prorenin activators, kallikrein and plasmin. We have already mentioned streptokinase and urokinase as exogenous initiators of plasmin-mediated prorenin activation.

2.4.3. MEASUREMENT OF PLASMA PRORENIN

A direct assay specific for prorenin is not available. As yet, prorenin can only be measured after it has been converted into active renin and the conversion has to be complete to obtain a valid estimate. Kinetic studies on the activation of prorenin in crude plasma by trypsin, plasma kallikrein (after pH 3.3-dialysis, which causes inactivation of C_1-esterase inhibitor and α2-macroglobulin and plasmin (after pH 4.0-dialysis, which causes inactivation of α2-antiplasmin) have demonstrated that under optimal conditions the same plateau of renin activity is reached (Fig. 1). These data indicate that all the prorenin that can be converted by serine proteinase is indeed converted under these circumstances.

It is generally held that equimolar amounts of naturally occurring active renin and prorenin that is activated *in vitro* are equally active, but this has still to be proven. With this caveat, the difference between the concentrations of 'total' renin and naturally occurring active renin is considered an estimate of the prorenin concentration. A difficulty arises when the difference is either small or very large. For instance, the plasma concentration of renin in a normal individual in the recumbent position may be 20 μU/ml (with human kidney renin as a standard for expressing activity as described by Bangham et al.[41] before activation *in vitro*, and 120 μU/ml after activation. The prorenin concentration is then calculated to be 100 μU/ml. After this individual has assumed the upright posture, his naturally occurring renin may rise by 10 μU/ml, a 50% change. If we assume that this increase is caused by prorenin into active renin conversion, and if we also assume that equimolar quantities of naturally occurring active renin and prorenin activated *in vitro* are equally active, then the calculated concentration of prorenin will have decreased from 100 to 90 μU/ml, a 10% change, which is hard to detect.

2.4.4. ACTIVATION OF PRORENIN IN VIVO*: DOES IT OCCUR?*

Parallel measurements of 'total' renin and naturally occurring active renin in plasma suggested a fall in prorenin in hypertensive subjects after heap-up tilting and after intravenous injection of the potent vasodilator, diazoxide[3]; these stimuli caused a rapid rise of active renin in plasma. Similar responses to upright posture and intravenous injection of the potent diuretic, frusemide, have been observed by Weinberger, Aoi & Grim[42] and Munday, Noble & Richards[43] respectively. However other investigators using the competitive angiotensin II antagonist, saralasin, or the angiotensin I-converting enzyme inhibitor, captopril, did not find that an increase of active renin was associated with decreased prorenin[4,44].

Measurements of renal artery and vein samples before and after intravenous diazoxide in patients with renal artery stenosis have shown that the renal vein/artery ratio for measured prorenin on the side of stenosis fell from a value not significantly different from 1.0 a value below 1.0, while the renal vein/artery ratio for active renin was significantly above 1.0 and rose after diazoxide[45].

A renal vein/artery ratio for prorenin below 1.0 combined with an elevated renal vein/artery ratio for active renin on the affected side has also been observed in another series of patients with renal artery stenosis[46]. These findings are consistent with conversion of plasma prorenin into active renin as it is passing through the kidney. However, parallel increments of both forms of renin have been measured in the renal vein after isoprenaline and frusemide in anaesthetized pigs[47].

Administration of the beta-adrenocepter antagonists, propranolol and metoprolol, causes a decrease of active renin in plasma and there are reports that this is associated with increased prorenin (3,48,49). Propranolol is also capable of preventing the rise of active renin and the fall in prorenin after diazoxide[45]. These findings suggest that the activation of prorenin might be under adrenergic control.

From the above studies it has also become clear that the plasma levels of naturally occurring active renin and prorenin are positively correlated under steady-state conditions both in normal subjects and in patients with different disorders. However, prorenin varies widely for a given level of active renin, possibly indicating varying degrees of activation.

Taken together there is suggestive evidence, but no proof, that the activation of prorenin *in vivo*, inside or outside the kidney, really occurs.

2.4.5. POSSIBLE ROLE IN VIVO FOR PLASMA KALLIKREIN AND PLASMIN IN PRORENIN ACTIVATION

It is often suggested that the coagulation and fibrinolytic cascades are continuously active *in vivo* and that both systems are in a dynamic equilibrium to maintain an intact and patent vascular bed. A similar balance may exist between the renin-angiotensin system and the kallikrein-kinin system with their opposite effects on vascular smooth muscle tone. The presence of circulating angiotensin I and II and circulating bradykinin is good evidence that the renin and kallikrein systems are active *in vivo*. Moreover, a close and positive correlation between plasma renin activity and bradykinin has been observed in normal subjects with widely different intakes of sodium and potassium and with adrenocorticotropic hormone-induced hyperaldosteronism[50]. Conversely, the evidence for continuous low grade coagulation and fibrinolysis is controversial.

It is possible that small quantities of kallikrein and plasmin are continuously formed not so much in the circulating plasma, but rather on the inner surface of blood vessels. At this site the activators might be less accessible and also less susceptible to circulating inhibitors. Binding of factor XII and the high molecular weight kininogen-prekallikrein complex to a negatively charged surface is known to enhance the reciprocal activation of factor XII and prekallikrein, so that the activation process can proceed in the presence of inhibitors in the surrounding fluid phase. In an analogous way plasmin molecules that are bound to fibrin and are actively degrading fibrin are inactivated by α2-antiplasmin

at a much slower rate than free plasmin, since they have both their active site and their α2-antiplasmin-binding sites protected from the inhibitor. The vascular plasminogen activator is also strongly bound to fibrin and this favours efficient activation of fibrin-bound plasminogen[31,36].

In the light of this knowledge we propose the following hypothesis of prorenin activation. Plasma prorenin is bound to the wall of certain blood vessels, perhaps including the glomerular capillaries. When conditions are favourable for low-grade activation of prekallikrein or plasminogen, enough activator is generated to convert blood vessel-bound prorenin into active renin. There is indeed, both direct and indirect evidence for the existence of vessel wall renin, which is capable of increasing smooth muscle tone via local angiotensin II formation (see the review by Swales[51]).

Evidence that the plasma kallikrein might be involved for the activation of prorenin *in vivo* is provided by the measurements of naturally occurring active and trypsin-activatable prorenin in subjects with Fletcher-trait (prekallikrein-deficiency). Such measurements have shown that the proportion of plasma renin that is in the active form is abnormally low in these subjects, whereas prorenin is abnormally high[22,23,52]. Moreover, a positive correlation has been reported between the concentration of plasma prekallikrein and the proportions of plasma renin that is in the active form in normal subjects with varying degrees of sodium depletion[53].

Strenuous exercise, β-adrenoceptor stimulation and oestrogens are known to stimulate the release of vascular plasminogen activator. The increase in fibrinolytic activity observed after intravenous injections of the diuretic, frusemide, probably also depends on the release of vascular activator[54]. The same stimuli are known to increase the renin activity of plasma. Interestingly, the effect of frusemide on the fibrinolytic activity of plasma was not seen in nephrectomized subjects, and it has therefore been suggested that the diuretic induces the liberation of fibrinolytic activator from the vessel wall in the kidney.

It is conceivable that the parallel stimulation of renin activity and the fibrinolytic activity is somehow related to the plasmin-mediated prorenin activation. Hedlin, Loh & Osmond[55] have found that the exercise-induced rise in plasma renin activity of women on oral contraceptive medication was associated with increased plasminogen activator and a rise in the proportion of active renin to prorenin. This proportion also increased after contraceptive medication alone. These data indeed indicate some link between fibrinolysis and prorenin activation.

Admittedly, the evidence so far that kallikrein and plasmin are involved in prorenin activation *in vivo* is only circumstantial, but the data that have been collected are encouraging and will stimulate investigators to persue this new approach to the renin-angiotensin system.

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3. PRORENIN-RENIN CONVERSION; ROLE OF INHIBITORS

3.1. PLASMA KALLIKREIN-MEDIATED ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM DOES NOT REQUIRE PRIOR ACIDIFICATION OF PRORENIN

3.1.1 ABSTRACT

Activation of prorenin in the neutral phase after pH 3.3 dialysis of plasma depends on clotting factor XII-initiated prekallikrein to kallikrein conversion. Acid dialysis may be necessary for destroying kallikrein inhibitors or rendering prorenin susceptible to attack by kallikrein. If the latter possibility proves true, it is difficult to see how the factor XII-kallikrein pathway could activate prorenin *in vivo*. Plasma prorenin was therefore separated from active renin and from the protease inhibitors α_2-macroglobulin, C_1-inactivator, α_1-antitrypsin, inter-α-trypsin inhibitor, and antithrombin III by gel filtration on Sephadex G-100 and affinity chromatography on Blue Sepharose CL-6B at neutral pH. The resulting prorenin preparation could be activated at pH 7.5 by highly purified human plasma kallikrein, which was prepared from prekallikrein by activation with active factor XII fragment β-factor XIIa. Activation proceeded at 4 and 37 C at a kallikrein concentration of 2 μg/ml, which is approximately 5% of the prekallikrein concentration in normal plasma. It appears that an acid-induced conformational change is not required for its activation by plasma kallikrein.

3.1.2. INTRODUCTION

Various enzymes circulate in plasma as inactive precursors. They are activated in important physiological processes, such as coagulation and fibrinolysis. Usually, the activation does not occur in circulating plasma but in close proximity to the blood vessel wall at sites where conditions favor rapid activation without interference by inhibitors, which are present in large quantities in the fluid phase[1,2]. A major proportion of renin in plasma is also in an inactive form. This form is often called prorenin, but it is not certain that inactive renin is a precursor of naturally occurring active renin.

Prorenin is converted *in vitro* to renin after acid treatment of plasma. Independent studies by Sealey et al.[3] and by our group[4,5] have demonstrated that endogenous activation of prorenin in the neutral phase after pH 3.3 dialysis

of plasma depends on clotting factor XII-initiated prekallikrein to kallikrein conversion.

It has recently been claimed that the acidification is necessary for rendering the prorenin molecule susceptible to attack by kallikrein[6]. Another possibility is that acid pretreatment is a necessary first step in prorenin activation, because various inhibitors of kallikrein and other proteases are inactivated in plasma at low pH. If it is true that an acid-induced conformational change of the prorenin molecule is essential for factor XII-kallikrein-mediated conversion to renin, it is difficult to see how this pathway of prorenin activation could be operative *in vivo*.

In view of this uncertainty, we have separated prorenin from protease inhibitors of plasma by nonaggressive methods and assessed the effect of purified plasma kallikrein on this preparation of prorenin. The experiments were carried out at neutral pH.

3.1.3. MATERIALS AND METHODS

Collection of plasma. Blood samples from five healthy male volunteers were collected into plastic tubes containing EDTA (final concentration, 5 mM). The blood was pooled and immediately centrifuged at 8,000 x g for 10 min. Plasma was stored at – 20 C. The concentration of renin and prorenin were 33 ± 2 and 230 ± 21 μU/ml, respectively (mean ± SD; n = 6).

Isolation of prorenin. Prorenin was separated from protease inhibitors and from renin by gel filtration of plasma followed by dye-ligand affinity chromatography. Plasma (4 ml) was applied to a 2.6 x 90-cm column of Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M Tris/HCl buffer, pH 7.0, containing 0.15 M NaCl and 1 mM EDTA. The same buffer was used for elution. The flow rate was adjusted to 10 ml/h, and 2.5 ml fractions were collected. The column was calibrated with the following molecular weight markers (Pharmacia): ribonuclease A (Mr 13,700), ovalbumin (Mr 43,000), and serum albumin (Mr 67,000). Blue dextran (Pharmacia) was used for determining void volume. Six fractions at the top of the prorenin peak were pooled and desalted on a 1.6 x 90-cm column of Sephadex G-25 (Pharmacia) with 0.02 M sodium phosphate buffer, pH 7.1. The desalted prorenin preparation (20 ml) was applied to a 1.6 x 24-cm column of Blue Sepharose CL-6B (Pharmacia) equilibrated with 0.02 M sodium phosphate buffer, pH 7.1. Elution was performed with the same buffer in three steps, i.e without added NaCl, with 0.2 M NaCl, and with 1.4 M NaCl. The flow rate was 50-60 ml/h, and 2.5-ml fractions were collected. Three fractions with the highest prorenin content were pooled and dialyzed for 4 h against 0.1 M sodium phosphate buffer, pH 7.5, containing 0.075 M NaCl and 1 mM EDTA. All of these procedures were carried out at 4 C.

Purification of plasma kallikrein. Plasma prekallikrein was isolated from normal human plasma according to the method of Bouma et al.[8]. Purification involved

several steps of ion exchange column chromatography, followed by Concanavalin A-Sepharose (Pharmacia) chromatography and sucrose density gradient centrifugation. This resulted in a 1,500-fold purification from plasma. The highly purified prekallikrein isolated in its precursor form consisted of two very similar apparent molecular weights near 85,000 on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. A goat antiserum raised against purified prekallikrein gave a single precipitation line in double immunodiffusion against human plasma and showed a reaction of identity with the single precipitation line of purified prekallikrein. No precipitation line was observed when unabsorbed antiserum was tested against Fletcher trait plasma, which was deficient in prekallikrein. The active factor XII fragment β-factor XII a was a gift of Dr. John Griffin (LaJolla,CA,USA). It gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent molecular weight of 28,000[9]. Prekallikrein was activated by β-factor XII a, and active kallikrein was separated from β-factor XII a by DEAE-Sephadex A-50 (Pharmacia) column chromatography, as described elsewhere[8]. The activated kallikrein gave two protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence of reducing agents. These protein bands had apparent molecular weight values identical to those seen for the two prekallikrein bands.

Assay of renin. Purified renin substrate was prepared from the plasma of nephrectomized sheep[10]. In the renin assays, it was used at a final concentration of 2.5 μM, expressed as the maximal quantity of angiotensin I which could be generated at pH 7.5 and 37 C in the presence of a large excess of human kidney renin, i.e. incubation of 10 μl substrate with 10,000 μU MRC renin standard (lot 68/356) for 24 h in a total volume of 1 ml. The renin assay has been described elsewhere[5]. Briefly, 0.1 to 0.5 ml samples were mixed with renin substrate, and the total volume was adjusted to 1.0 ml with 0.1 M sodium phosphate buffer, pH 7.5, containing 75 mM Na Cl and 1 mM EDTA. After the addition of 10 μl 8-hydroxyquinoline sulfate (0.34 M), 5 μl phenylmethylsulfonylfluoride (0.3 M) in ethanol, and 10 μl aprotinin (10,000 kallikrein-inhibiting units/ml; Trasylol, Bayer, Leverkussen, West Germany), the mixtures were incubated for 3 h at 37 C. Mixtures incubated at 4 C served as blanks. The reaction was stopped by heating for 10 min in a boiling water bath. The angiotensin I that had been generated during incubation was measured by RIA, and results are expressed as microunits per ml using the MRC renin standard.

Assay of prorenin. Prorenin was determined after its activation to renin by Sepharose-bound trypsin, as described previously[5]. Trypsin from bovine pancreas, twice crystallized (Sigma Chemical Co., St. Louis, MO; 12,000 N-α-benzoyl-L-arginine- ethylester (BAEE) units/mg protein), was covalently bound to CNBr-activated Sepharose (Pharmacia) according to the directions of the manufacturer. Sepharose-bound trypsin was diluted in 0.1 M sodium phosphate buffer, pH 7.5, containing 75 mM NaCl and 1mM EDTA. In a pilot study, aliquots of plasma pool and of some fractions (1.0 ml) of the prorenin peaks in the Sephadex G-100 and Blue Sepharose column eluates were incubated at 4 C with suspensions

(0.1 ml) of Sepharose-bound trypsin at final concentrations ranging from 0.01-1.0 mg trypsin/ml for various periods up to 72 h. Trypsin was removed by centrifugation. The amidolytic activity of the supernatants was tested with the chromogenic substrate N-benzoyl-L-isoleucyl-L-glutamylglycyl-L- arginine-p-nitroanilide (S2222, Kabi, Stockholm , Sweden). For this purpose, 0.1 ml of the supernatant of the incubates was mixed with 0.2 mM substrate (approximately 10 times the Km for trypsin) in 0.1 M Tris-HCL buffer, pH 8.2, in a total volume of 1.0 ml. The linear release of p-nitroaniline was followed for 1-2 min at 405 nm in a 1-cm semimicrocuvette at 37 C. With this method, the remaining trypsin activity in the supernatants was found to be less than 0.1% of the original activity in the incubates.

The renin activity generated by incubation with trypsin appeared independent of the concentration of the activator in the range of 0.01-1.0 mg/ml, unless the incubation was continued until a plateau was reached. At trypsin concentrations up to 0.3 mg/ml, there was no decrease in renin activity during incubation for 72 h. With higher concentrations of trypsin, some renin activity was lost when incubation was continued for such a long time. At trypsin concentrations of 0.2- 0.3 mg/ml, the activation was maximal within 12-24 h. Incubation for 24 h at 4 C with 0.3 mg/ml trypsin, which was bound to 7.5-10 mg Sepharose, was used as a routine method for activating prorenin. Renin was then assayed, as described above, by incubating the samples with sheep renin substrate for 3 h at 37 C. Less than 10% of the substrate was converted during this incubation, and generation of angiotensin I was linear with time with both trypsin-treated and untreated samples.

Assays of kallikrein and prekallikrein. Plasma kallikrein was measured using the chromogenic substrate H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide (S2302, Kabi)[11]. A 4.1 mM solution of this substrate in distilled water was prepared. Aliquots (0.1 ml) of the column eluates were diluted with 0.05 M Tris-HCL buffer, pH 7.8, containing 0.12 M NaCl to a volume of 0.8 ml. Then, 0.2 ml substrate solution was added so that the final concentration of substrate in the reaction mixture was 0.8 mM (Km for plasma kallikrein, 0.2 mM). The linear release of p-nitroaniline was followed for 1-2 min at 405 nm in a 1-cm semimicrocuvette at 37 C. Prekallikrein, which was activated by the active fragment of clotting factor XII (β-factor XII a), as described before, was used as a standard. The lower limit of detection was less than 10 ng. This corresponds with a kallikrein concentration of less than 100 ng/ml undiluted sample, which is about 0.2% of the concentration in normal plasma when all prekallikrein is converted to kallikrein[12].

Prekallikrein was determined after its activation with dextran sulfate[13]. Dextran sulfate (Pharmacia; Mr 500,000) was dissolved at a concentration of 25 mg/l in distilled water. Aliquots (0.05 ml) of the column eluates were mixed with 0.05 ml Fletcher trait plasma as a source of clotting factor XII and high molecular weight kininogen. These mixtures were incubated for 10 min at 0 C with 0.10 ml of the dextran sulfate solution in plastic tubes placed in ice water. The mixtures

were then diluted to a volume of 0.8 ml with 0.05 M Tris-HCL buffer, pH 7.8, containing 0.12 M NaCl, and after the addition of 0.2 ml of the solution of chromogenic substrate, kallikrein activity was measured as before.

Assays of plasmin and plasminogen. Plasmin was determined with the chromogenic substrate D-valyl-L-leucyl-L-lysine-p-nitroanilide (S2251, Kabi)[14]. A 3 mM solution of substrate in distilled water was prepared. Aliquots (0.1 ml) of the column eluates were diluted with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.12 M NaCl to a volume of 0.8 ml. Then, 0.2 ml of the substrate solution was added so that the final concentration of substrate in the reaction mixture was 0.6 mM (Km for plasmin, 0.3 mM). The linear release of p-nitroaniline was followed for 1-2 min at 405 nm in a 1-cm semimicrocuvette at 37 C. Plasmin (19 casein units/mg protein; Kabi) was used as a standard. The lower limit of detection was 20 ng. This corresponds with a concentration of 200 ng/ml undiluted sample, which is less than 0.1% of the concentration in normal plasma when all plasminogen has been converted to plasmin.

Plasminogen was determined after its activation by streptokinase[14]. Streptokinase (Streptase, Behring, Marburg-Lahn, West Germany) was dissolved at a concentration of 10,000 Christensen units/ml in distilled water. Aliquots (0.1 ml) of the column eluates were mixed with 0.1 ml 0.05 M Tris-HCl buffer, pH 7.4, containing 0.12 M NaCl. After the addition of 0.1 ml of the streptokinase solution, the mixtures were incubated for 10 min at 37 C. The activated column eluates were diluted to a volume of 0.8 ml with the Tris-HCl buffer, and after the addition of 0.2 ml of the solution of chromogenic substrate, plasmin activity was measured as before.

Immunological detection of protease inhibitors. Samples were tested for the presence of α_2-macroglobulin C_1-inactivator, α_1-antitrypsin, inter-α-trypsin inhibitor, and antithrombin III by radial immunodiffusion according to the method of Mancini et al.[15] using monospecific antisera (Behring). Aliquots (1 ml) of the column eluates were concentrated 10-fold in Amicon B-15 concentrators (Amicon, Lexington, MA, USA) before being applied to the immunodiffusion plates (Partigen plates, Behring). The immunoprecipitate rings were compared with those of a series of dilutions of standard plasma according to the directions of the manufacturer. The lower limit of detection of α_2-macroglobulin was less than 1 μg for the other inhibitors, it was less than 0.5 μg. α_2-Macroglobulin was applied to the immunodiffusion plates in 20-μl aliquots, and the other inhibitors were applied in 5-μl aliquots. The level of α_2-Macroglobulin, C_1-inactivator, α_1-antitrypsin, inter-α-trypsin inhibitor, and antithrombin III in the pooled plasma of five normal subject were 210, 23, 250, 40 and 27 mg/100 ml (means of two assays) respectively. Since all samples were concentrated 10-fold before assay, it can be calculated that α_2-Macroglobulin and α_1-antitrypsin were detected when they were present in the column eluates at concentrations as low as 1% of the concentration in normal plasma. C_1-Inactivator, inter-α-trypsin inhibitor, and antithrombin III were detectable when present in the eluates at concentrations as low as 5% of the concentration in normal plasma.

3.1.4. RESULTS

Elution patterns of the Sephadex G-100 and Blue Sepharose columns are shown in Figs. 1 and 2. The apparent molecular weight of prorenin on gel filtration was somewhat higher than that of active renin, i.e. 56,000 and 48,000 daltons, respectively. This agrees with the results of Sealey et al.[16]. Recovery of prorenin was better than 90% with Sephadex chromatography and better than 70% with Blue Sepharose chromatography. The peak fractions of prorenin from the Sephadex column that were pooled and subjected to Blue Sepharose chromatography comprised about 50% of the total quantity of prorenin recovered from the Sephadex column. The final recovery of prorenin after the two purification steps was 32%. The concentration of prorenin in the final preparation, as measured after maximal activation by Sepharose- bound trypsin, was 38 μU/ml, and the concentration of renin, as measured before activation by trypsin, was less than 0.5 μU/ml. With the functional assays we have used, prekallikrein, kallikrein,

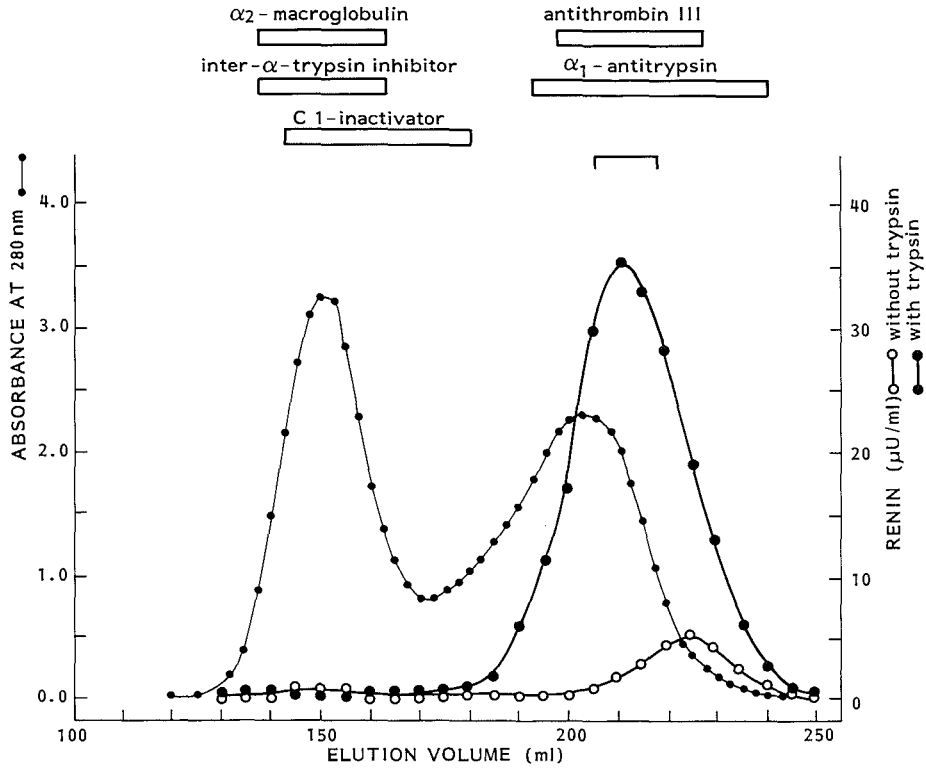

Fig. 3.1.1. Renin and prorenin in eluates from gel filtration of plasma on a Sepharose G-100 column. Renin activity was measured before and after activation of prorenin with Sepharose-bound trypsin. Protease inhibitors detected in 10-fold-concentrated fractions are indicated by bars. Brackets indicate fractions pooled for further purification.

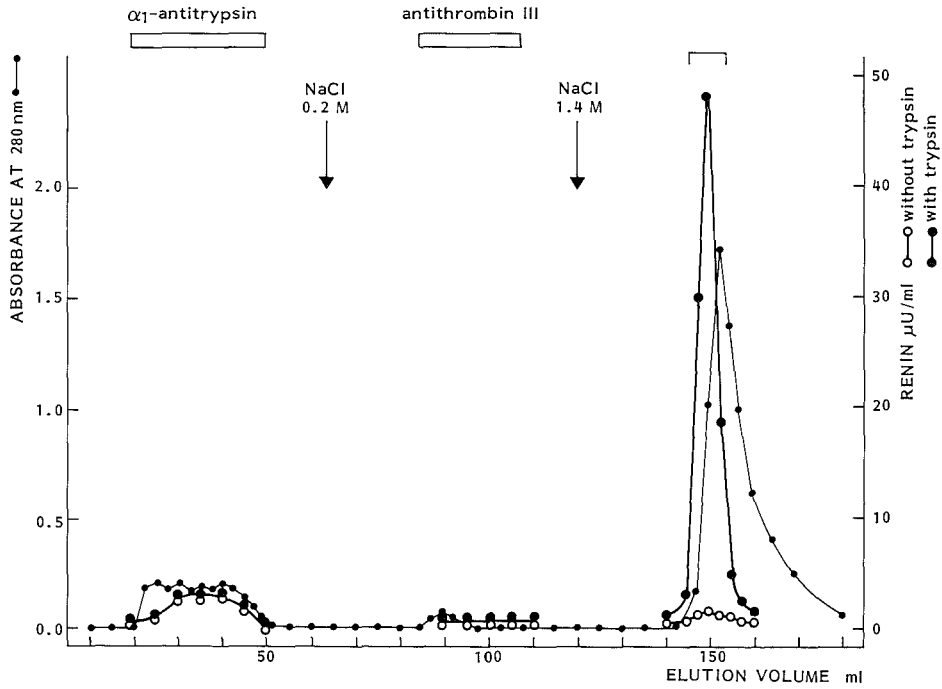

Fig. 3.1.2. Separation of renin and prorenin by affinity chromatography on a Blue Sepharose CL-6B column. The pooled fractions obtained after gel filtration of plasma (see Fig. 3.1.1.) were applied to the column. Renin activity was measured before and after activation of prorenin with Sepharose-bound trypsin. Renin did not bind to the column. Prorenin was eluted at NaCl 1.4 M. Protease inhibitors detected in 10-fold-concentrated fractions are indicated by bars. Brackets indicate fractions pooled for activation studies with plasma kallikrein and plasmin.

plasminogen, and plasmin could not be detected in the final preparation of prorenin. Immunoreactive α_2-Macroglobulin, C_1-inactivator, α_1-antitrypsin, inter-α-trypsin inhibitor, and antithrombin III were also not detectable in this preparation. One-milliliter aliquots of the final preparation of prorenin were incubated with the following activators: 1) Sepharose-bound trypsin (100 μl; final concentration, 0.3 mg/ml, 2) human plasmin (100 μl; final concentration, 0.1 mg/ml), and 3) human plasma kallikrein (20 and 5 μl: final concentrations, 8 and 2 μg/ml). The concentrations of plasminogen and prekallikrein in normal plasma are about 200 and 55 μg/ml[12,17], respectively. The incubations with activators were performed at 4 or 37 C for various periods, as indicated in Fig. 3 ; data in this figure are based on renin assays in which activated and nonactivated samples were incubated with sheep renin substrate for 3 h at 37 C, as described in Materials and Methods. Prorenin samples treated for 48 h at 4 C or for 60 min at 37 C with trypsin, plasmin, or kallikrein and untreated samples were also incubated for 1 and 2 h with sheep renin substrate. The

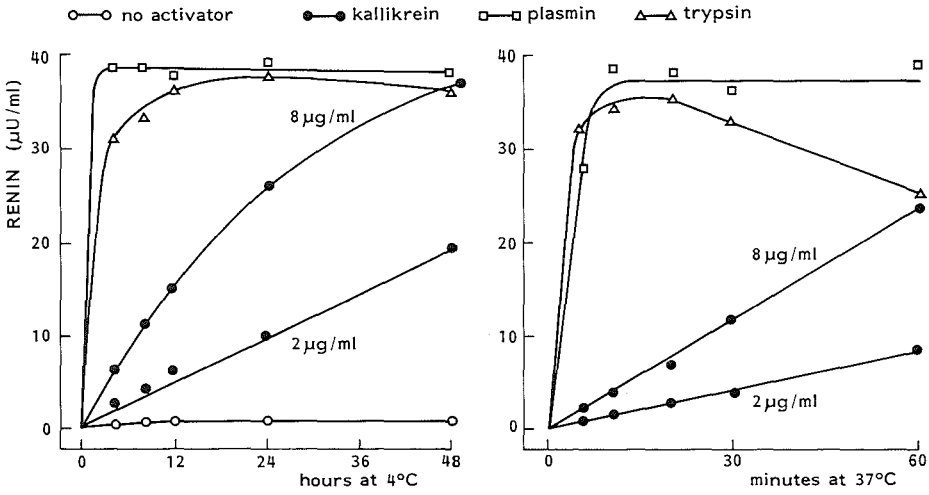

Fig. 3.1.3. Activation of partly purified prorenin (see Fig.3.1.2.) at pH 7.5 with plasma kallikrein, plasmin and trypsin at 4 C and 37 C. For details see the text.

generation of angiotensin I appeared linear with time both with the treated samples and the untreated samples.

As shown in Fig.3, the addition of human plasmin in a concentration which corresponds to the concentration of plasminogen in normal plasma led to an increase in renin activity at 4 C to a level which was close to the maximal activation obtained with Sepharose-bound trypsin. Activation to this maximum was also obtained after 48 h of incubation at 4 C with human kallikrein in a concentration which corresponds to about 16 % of the prekallikrein concentration in normal plasma. Prorenin activation proceeded more rapidly at 37 C. When the activators were incubated for 48 h at 4 C or 60 min at 37 C with 1 ml 0.1 M sodium phosphate buffer, containing 0.075 M NaCl and 1 mM EDTA, instead of prorenin, no renin activity could be detected. Measurements of kallikrein in kallikrein prorenin mixtures before and after incubation showed no loss of amidolytic activity during 48 h at 4 C or during 60 min at 37 C.

3.1.5. DISCUSSION

It has recently been reported that an acid-induced conformational change of the prorenin molecule is necessary before it can be converted to renin through attack by the neutral serine protease, renal kallikrein[6]. This claim was based on experiments in which renal kallikrein was incubated with mixtures of pH 3.3-pretreated plasma and untreated plasma. The percentage of renin that was activated appeared equal to the percentage of acid-pretreated plasma in the

mixture. This suggests that only the acid-treated prorenin is activated by kallikrein. However, the possibility that kallikrein inhibitors, which are known to be destroyed by acid treatment of plasma, were responsible for the result was not excluded. It should be noted that the kinetics of kallikrein inhibition in crude plasma are very complex: plasma contains a wide range of different inhibitors; some fast acting, others slow acting[18,19]. The question of whether acid treatment is required for kallikrein-mediated activation of prorenin is of more than academic interest. If it is true that a reduction of plasma pH to an unphysiological level is necessary for rendering the prorenin molecule susceptible to kallikrein attack rather than for removing inhibitors, one may doubt the significance of kallikreins as activators of prorenin *in vivo*.

In our experiments, the inhibitors were separated from prorenin by gel filtration and dye-ligand chromatography at neutral pH. The resulting prorenin preparation could be activated by plasma kallikrein at pH 7.5 without prior acid treatment of prorenin. It appears therefore, that an acid-induced conformational change of the prorenin molecule was not essential. We have used plasma kallikrein, which has a slightly different amino acid bond specificity from that of glandular kallikrein used in other laboratories[6,7,20]. It is possible that an acid-induced conformational change in prorenin is required for glandular kallikrein, but not for plasma kallikrein. In a previous study from our laboratory, however, glandular kallikrein was found to be able to activate prorenin in a partly purified preparation without prior acid treatment of prorenin[21]. Theoretically, it is also possible that the prorenin in our preparation was different, although prorenin is not known to change after Sephadex and Blue Sepharose chromatography.

Plasmin was also able to activate prorenin at pH 7.5 in our preparation. Since plasma kallikrein is known to activate plasminogen under certain circumstances[1], it is theoretically possible that in our experiments, kallikrein acted via activation of plasminogen. This, however, is unlikely because the concentrations of plasminogen and plasmin in our preparation of prorenin were 0.2 μg/ml or less, and when plasmin was added to this preparation in a final, concentration of 0.2 μg/ml, no increase in renin activity was observed during incubation for 48 h at 4 C or for 1 h at 37 C.

C_1-inactivator is the inhibitor in plasma that is known to inactivate active Factor XII, and both C_1-inactivator and α_2-macroglobulin important inhibitors of plasma kallikrein. C_1-inactivator is destroyed between pH 4.5-5.0. After treatment of plasma at this pH, kallikrein is formed at 4 C when the pH is restored to neutral. Kallikrein, however, is bound to α_2-macroglobulin and little activation of prorenin is observed under these circumstances. α_2-macroglobulin is destroyed below pH 4.0, and this coincides with the generation of uninhibited kallikrein and the formation of active renin after the pH has been restored[22]. These findings and the present results strongly suggest that the main function of the acid dialysis step in the activation of prorenin in plasma is to destroy inhibitors.

Activation of the factor XII-kallikrein pathway is known to occur upon contact

with negatively charged surfaces, including vascular basement membrane, despite the presence of inhibitors in the surrounding fluid[1,2]. In the fluid phase, however, active factor XII and kallikrein are rapidly inactivated. It is, therefore, conceivable that the blood vessel-bound prorenin rather than circulating prorenin is activated by this pathway.

The plasma of patients with the Fletcher trait (hereditary prekallikrein deficiency) has been reported to contain low quantities of renin, while plasma prorenin is abnormally high[16,23]. An inverse correlation between the plasma levels of prekallikrein and prorenin has also been reported in normal subjects in different state of sodium balance[24]. Such observations can be taken as evidence that activation of prorenin may occur *in vivo*, and that plasma prekallikrein is involved.

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3.2. ROLE OF INTRINSIC INHIBITORS IN ACID-ACTIVATION OF INACTIVE PLASMA RENIN (PRORENIN)

3.2.1. ABSTRACT

Inactive plasma renin is converted into an active form of renin during dialysis of plasma at 4 C against a pH 3.3-buffer. This form of renin is inactivated at neutral pH and 37 C. When plasma, after acid-dialysis, is kept at 4 C and neutral pH a different form of active renin is generated, which remains active. The irreversible activation of prorenin depends on factor XII-initiated kallikrein formation. C_1-inhibitor (C_1-INH) and α_2-macroglobulin (α_2-M) can be selectively denaturated by treatment of plasma at low pH values to which kallikrein and renin are resistant. After denaturation of C_1-INH at pH 4-5, factor XII becomes capable of activating prekallikrein. The kallikrein that is formed after restoration of pH is complexed with α_2M. This complex has little activity towards natural protein substrates including prorenin. α_2M is denaturated at pH 3-4 and, as a consequence, kallikrein that is formed after restoration of pH is not complexed with α_2M. This kallikrein is able to attack both high molecular weight kininogen and prorenin as manifested by the generation of bradykinin-forming and angiotensin-forming activities. These observations show a crucial role for C_1-INH and α_2M in prorenin activation and explain, at least in part, why the pH of the acid-treatment step has to be less than 4 before irreversible activation of prorenin at neutral pH can occur.

3.2.2. INTRODUCTION

The surface charge of a protein and therefore its tertiary structure will be affected by the pH of the medium in which it is dissolved. Treatment of plasma for 24 hours at pH 3.0-3.5 at 4 C leads to conversion of inactive renin or prorenin into an active form of renin that is inactivated at neutral pH and 37 C[1,2]. It has been suggested that acid-treatment induces a conformational change of the prorenin molecule or the dissociation of an inhibitor[1–3]. By this the active centre of the enzyme becomes accessible to renin substrate. When plasma, after acid-dialysis, is kept at neutral pH and 4 C a different form of active renin is generated. This form of renin remains active. The irreversible activation of prorenin depends on clotting factor XII-initiated kallikrein formation[4,5]. The two types of acid-activated renin, the reversibly activated form and the irreversibly activated form, can be separated by dye ligand affinity chromatography[6]. There is some controversy on whether the formation of *reversibly* activated renin is a necessary first step for *irreversible* activation by kallikrein[1,3,5,7]. Acid-dialysis also leads to denaturation of serine proteinase inhibitors. Destruction of this inhibitory capacity ensures survival of kallikrein in the neutral phase, which is then capable of attacking prorenin. This report focusses on the biochemical background of the acid-activation of prekallikrein and prorenin and on the role of plasma inhibitors in this process.

3.2.3. MATERIALS AND METHODS

Acid-activation. Two ml of normal plasma containing EDTA 5 mM was dialyzed for 24 hours at 4 C against buffers of pH 1.5-7.5[7]. pH was then restored by dialysis against a pH 7.5 phosphate buffer for 24 hours at 4 C. In some experiments pH was restored by adding NaOH 1 M.

Measurement of renin. The method is based upon RIA of angiotensin I generated during incubation with sheep renin substrate[7,8].

Measurement of plasma kallikrein. The chromogenic substrate H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide (S2303, Kabi, Sweden) was used[9]. For measuring kallikrein that is bound to α_2-macroglobulin the release of p-nitroaniline was followed in the presence of soybean trypsin inhibitor (SBTI, Sigma) in a final concentration of 2 mg/ml. Prekallikrein was measured after activation by dextran sulfate[9]. Kallikrein was also measured via its ability to cleave bradykinin from added high molecular weight kininogen (HMWK). Bradykinin was determined by RIA[10]. *Measurements of inhibitors.* C_1-inhibitor (C_1-INH) and α_2-macroglobulin (α_2M) were measured by radial immunodiffusion[8] and by functional assays[11,12].

3.2.4. RESULTS

Plasma was dialyzed at low pH followed by dialysis at pH 7.5 both for 24 hours at 4 C. The enzymes are more resistant to low pH than the inhibitors (Figs. 1 and 2). Prekallikrein is converted into kallikrein at pH 7.5 after treatment at pH 5.0. Prorenin is activated after treatment at pH below 4.0. The major inhibitors of kallikrein, C_1-INH and α_2M, are denaturated at pH-values below 5.0 and 4.0 respectively. When α_2M is denaturated plasma acquires the ability to activate prorenin.

In Fig. 3 the generation of kallikrein and renin in pH 3.3-pretreated plasma was followed for 24 hours after restoration of pH to neutral with 1 M NaOH. Prorenin is already present in an active form before the second dialysis step, but this acid-activated renin is rapidly inactivated at 37 C. In contrast, prekallikrein is not activated during pH 3.3-dialysis. Only after a lag period of about 4-8 hours is prekallikrein converted into kallikrein. When the acid-pretreated plasma is kept for 24 hours at neutral pH and 4 C prorenin is irreversibly activated.

As mentioned above prekallikrein is converted into kallikrein at pH 7.5 after treatment of plasma at pH below 5.0. However there is little formation of renin in pH 4.0-5.0-pretreated plasma. This may be explained by the fact that α_2M is resistant to this pH as illustrated in Fig. 2. Under these circumstances kallikrein-α_2M complexes are formed. The amidolytic activity of such complexes towards chromogenic substrates is known to be insensitive to SBTI, whereas kallikrein that is not bound to α_2M is very sensitive to SBTI[13]. Thus, by measuring kallikrein amidolytic activity both in the presence and absence of SBTI the bound and

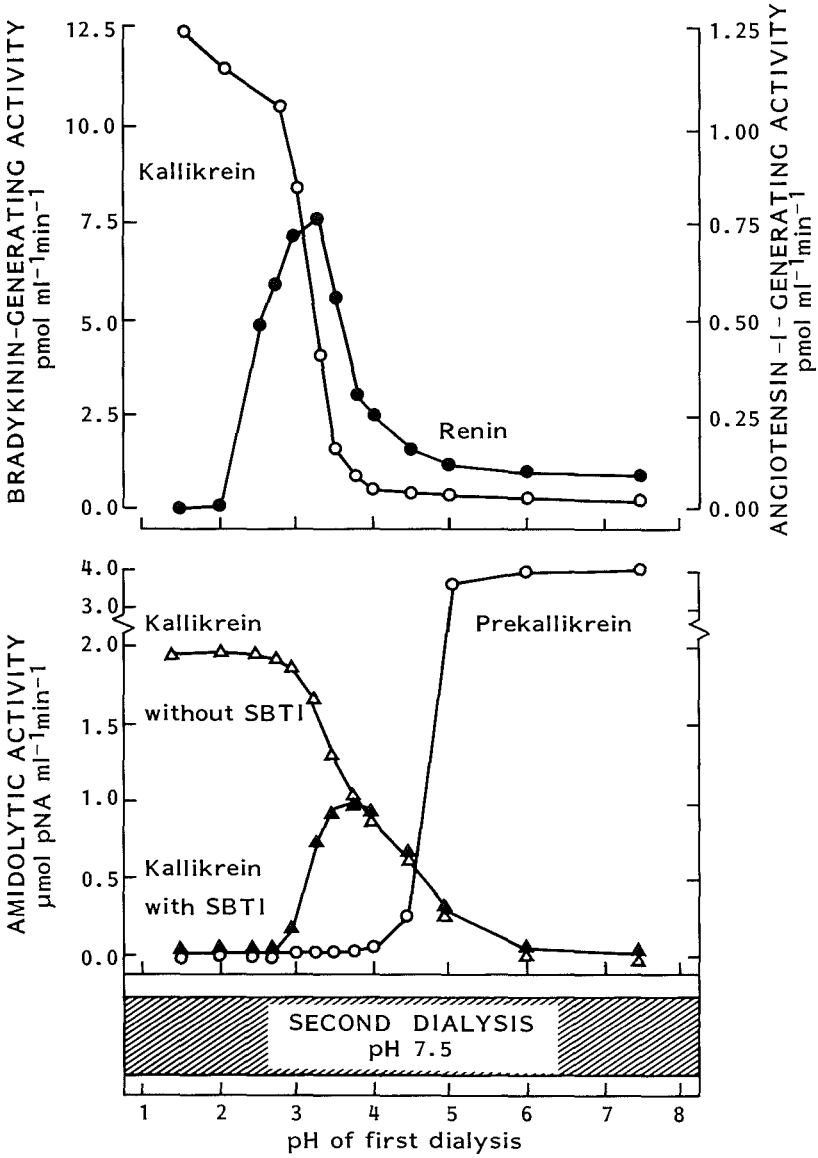

Fig. 3.2.1. Normal EDTA-plasma was first dialyzed for 24 h at 4 C against buffers of different pH and then against pH 7.5 buffer also for 24 hours. Renin was measured via its ability to form angiotensin I from sheep renin substrate. Kallikrein was measured via its ability to cleave p-nitroaniline from a chromogenic substrate and via its ability to cleave bradykinin from high molecular weight kininogen (HMWK). Kallikrein that is complexed with α_2-M is insensitive to soybean trypsin inhibitor (SBTI) and does not attack HMWK and prorenin. Kallikrein that is not complexed with α_2-M is sensitive to SBTI, attacks HMWK to form bradykinin and attacks prorenin to form active renin.

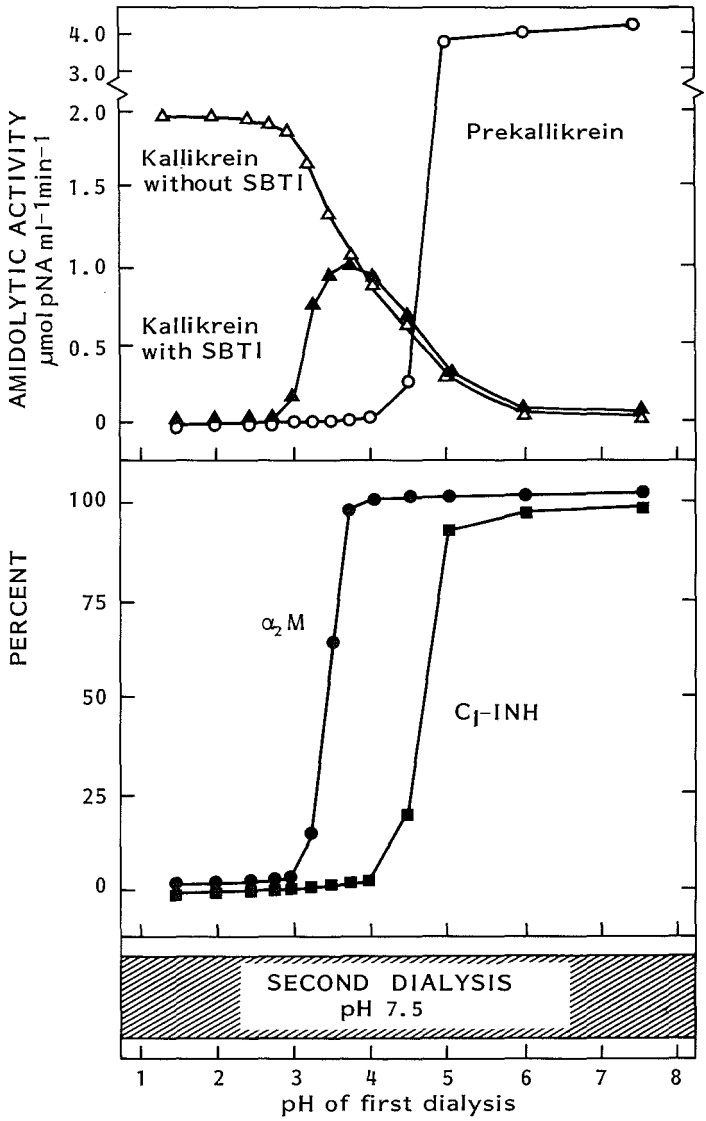

Fig.3.2.2. Normal EDTA- plasma was treated as in Fig. 3.2.1. Note the associations between the denaturation of C_1-inhibitor and the activation of prekallikrein and between the denaturation of α_2-macroglobulin and the formation of SBTI-sensitive kallikrein.

unbound forms of kallikrein can be discerned. As shown in Fig. 1, all kallikrein amidolytic activity in pH 4.0-5.0 pretreated plasma is complexed with α_2M. These complexes are not only insensitive to inhibition by SBTI but are also much less active towards natural protein substrates than towards the low molecular

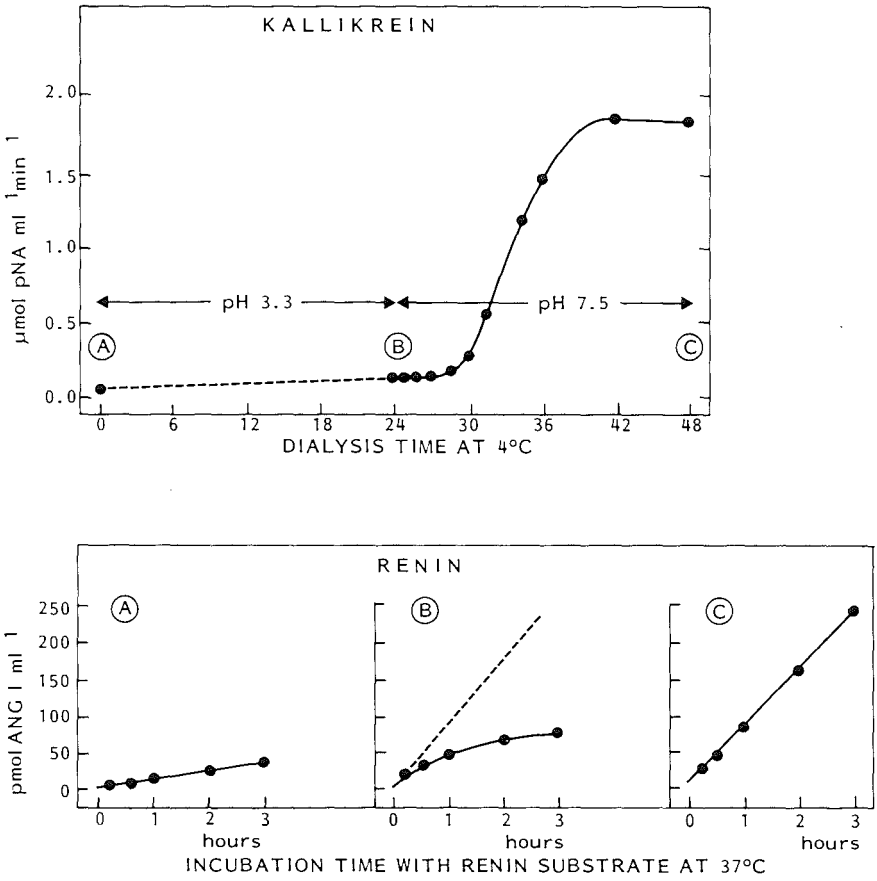

Fig.3.2.3. Normal EDTA-plasma was first dialyzed for 24 hours at 4 C against a pH 3.3 buffer. pH was then retored to 7.5 with NaOH 1 M followed by dialysis against pH 7.5 buffer also for 24 hours. Samples were taken just before the first dialysis step, just after restoration of pH with NaOH and finally at the end of the second dialysis step. These samples were incubated with sheep renin substrate and the formation of angiotensin I was followed for 3 h. A: Naturally occurring active renin. B: Renin activated by acidification *per se*; the generation of angiotensin I is not linear because of inactivation of renin. C: renin activated by kallikrein that is formed in acid- pretreated plasma.

weight chromogenic substrates[14]. Thus kallikrein complexed with α_2M is not capable of attacking HMWK to form bradykinin and is also not capable of activating prorenin (Fig. 1). After α_2M has been denaturated at pH below 4.0 unbound kallikrein is formed at pH 7.5. This can be detected as bradykinin-generating activity and is associated with prorenin activation.

3.2.5. DISCUSSION

C_1-INH is regarded as the most important inhibitor of activated clotting factor XII, and both C_1-INH and α_2M are the major inhibitors of plasma kallikrein. As shown in this study these inhibitors can be selectively denaturated by treatment of plasma at low pH-values to which kallikrein and renin are resistant. After denaturation of C_1-INH at pH 4.0-5.0 plasma acquires the ability to activate prekallikrein. Activation of prekallikrein in acid-pretreated plasma depends on factor XII[4,5]. Apparently, after C_1-INH has been destroyed, factor XII becomes capable of attacking prekallikrein. The kallikrein that is formed is complexed with α_2M. This complex is active towards artificial low molecular weight substrates but not towards protein substrates such as kininogen and prorenin.

α_2M is denaturated at pH 3.0-4.0 and, as a consequence, kallikrein that is formed after restoration of pH is not complexed with α_2M. This kallikrein is able to attack both HMWK and prorenin, which is manifested by the appearance of bradykinin generating and angiotensin-generating activities.

The active renin that is generated in acid-pretreated plasma by the factor XII-kallikrein pathway differs from the active renin that is formed by acidification *per se*. The latter is slowly inactivated at neutral pH at 4 C and more rapidly at 37 C, whereas the former remains active under these circumstances.

Our results strongly suggest that a major function of the acid-dialysis step in the activation of prorenin in plasma is to destroy inhibitors i.e. C_1-INH and α_2M. Experiments with semipurified plasma prorenin have shown that acid-pretreatment is not a *sine qua non* for activation by kallikrein and plasmin[9,15]. It is however possible that the prorenin molecule, after a conformational change for instance in acid milieu, becomes more susceptible to proteolytic attack. The course of events in acid-pretreated plasma can then be depicted as in Fig. 4.

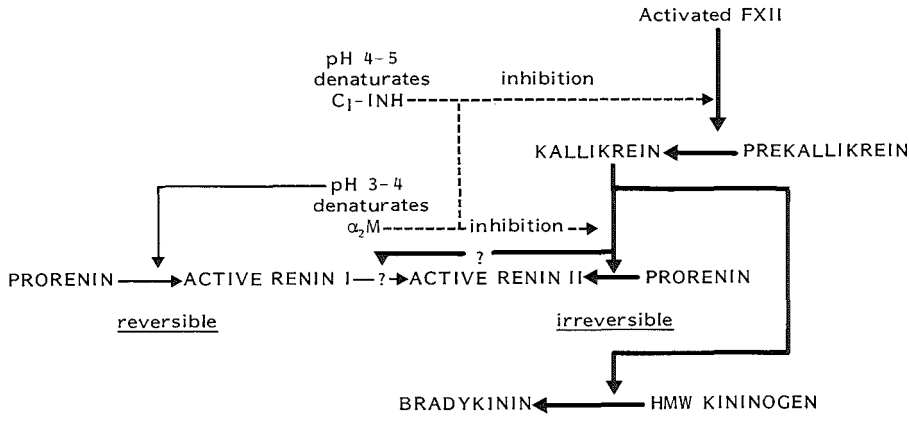

Fig.3.2.4. Biochemical reactions by which active renin is generated in acid-pretreated plasma.

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3.3. PRORENIN-RENIN CONVERSION BY THE CONTACT ACTIVATION SYSTEM IN HUMAN PLASMA: ROLE OF PLASMA PROTEASE INHIBITORS

3.3.1. ABSTRACT

Acid-pretreated normal human plasma generates renin activity at 0 C and neutral pH by the activation of prorenin. The activation is caused by kallikrein generated from prekallikrein by activated factor XII. Nonacidified plasma also generates renin at 0 C, but at a lower rate (cold-promoted activation). In normal plasma, 14 ± 1% of prorenin (mean ± SEM,n = 30) was activated during incubation at 0 C for 7 days (range 6% to 26%). Cold-promoted activation of prorenin was within the normal range in plasma deficient factor XI,X,IX,VIIIC,VII,V, prothrombin, or high molecular weight kininogen.

Cold-promoted activation of prorenin was less than 1% in plasma deficient in factor XII or prekallikrein. Reconstitution of these plasmas with highly purified factor XII or prekallikrein restored normal prorenin activation. Correction of high molecular weight kininogen deficiency had no effect. Thus cold-promoted activation of prorenin depends on the presence of factor XII and prekallikrein, whereas the other clotting factors are not essential. The influence of the inhibitors C_1-esterase-inhibitor, α_2-macroglobulin, antithrombin III, and α_1-antitrypsin on the activation of prorenin was studied in factor XII-deficient plasma from which one or more of these inhibitor had been selectively removed by immunoabsorption. Factor XII was subsequently added, and the generation of renin at 37 C was observed after complete factor XII-high molecular weight kininogen-mediated activation of prekallikrein induced by dextran sulfate. No activation of prorenin was observed at 37 C after depletion of C_1-esterase inhibitor, α_2-macroglobulin, antithrombin III, or α_1-antitrypsin. When prekallikrein was activated in plasma depleted of both C_1-esterase-inhibitor and α_2-macroglobulin, 6% of prorenin was activated in 2 hours at 37 C. After additional depletion of antithrombin III, the activation increased to 47%. These results indicate that the contact activation system is capable of activating prorenin in plasma at physiologic pH and temperature when the three most important kallikrein inhibitors, C_1-esterase-inhibitor,α_2-macroglobulin, and antithrombin III, are absent.

Abbreviations: C_1-esterase-inhibitor (C_1-INH), α_2-macroglobulin (α_2M), antithrombin III (AT III),α_1-antitrypsin (α_1AT), ethylenediaminetetra-acetic disodium salt (EDTA), phenylmethanesulfonylfluoride (PMSF), soybean trypsin inhibitor (SBTI), high molecular weight kininogen (HMWK).

3.3.2. INTRODUCTION

Human plasma contains a form of renin with little or no enzymatic activity. This 'inactive' renin resembles some proenzymes of the clotting and fibrinolytic cascades in that it can be converted to the active form by proteolytic enzymes

present in plasma (intrinsic activators) or in tissues (extrinsic activators)[1–5]. Inactive renin in plasma appears to be a more or less homogeneous molecular species with an apparent molecular weight of 56,000 on Sephadex gel filtration[6–8]. It is not known if activation occurs in vivo, but inactive renin that is activated *in vitro* has enzymatic and immunologic properties very similar, if not identical, to those of naturally occurring active renin in plasma and kidney[8–12]. Inactive renin is secreted by the kidney, and there is evidence that, with long-term stimulation of the secretion of active renin, the rate of secretion of inactive renin is increased as well[8,12]. Inactive renin in plasma may therefore tentatively be referred to as prorenin.

One of the intrinsic pathways of prorenin activation is the clotting factor XII-kallikrein pathway, which is initiated at 0 C in plasma that has been dialyzed against a pH 3.3 buffer followed by restoration of pH. The most important kallikrein inhibitors in plasma are C_1-INH, α_2M and AT III. C_1-INH also inactivates activated factor XII and the active 28,000 molecular weight factor XII fragment. These inhibitors are destroyed by acid, and this may promote activation of prorenin[13].

Exposure of plasma to low temperature (-4 C to 4 C) without prior acidification also increases its renin activity, but the activation proceeds more slowly than in acid- treated plasma[14,15]. Incubation of untreated plasma at low temperature is known to promote the activation of clotting factor VII, a process that depends on the presence of factor XII and activated prekallikrein[16]. The enzyme responsible for cold-promoted prorenin activation in nonacidified plasma has not been identified.

The factor XII-kallikrein pathway of prorenin activation links the renin-angiotensin system with the contact activation system. Evidence to suggest that this link is physiologically important can be derived from reports that in the plasma of patients with hereditary prekallikrein deficiency (Fletcher trait), active renin is abnormally low, whereas prorenin is abnormally high[2,3,17].

The issue is confounded by the observation that the addition of kaolin to nonacidified plasma failed to raise the renin activity, despite the fact that kaolin caused massive activation of the factor XII-kallikrein pathway[18]. This is in contrast to the rapid increase in renin activity seen when kaolin was added to plasma in the neutral phase after pH 3.3 dialysis. It has been postulated that an acid-induced reversible conformational change of the prorenin molecule is a necessary first step for subsequent activation of prorenin by plasma and tissue kallikrein[12,19].

The aim of our study was to clarify the role of the contact activation system and the involvement of plasma protease inhibitors in the activation of prorenin in nonacidified plasma both at 0 C and 37 C.

3.3.3. METHODS

Clotting factor deficient plasma. Plasma deficient in factor XII, prekallikrein, HMWK, or factor XI was purchased from Georg King, Overland Park, Kan.,

Table 3.3.1. Renin activity in clotting factor-deficient plasma.

Plasma			Renin μU/ml		
Clotting factor deficiency	Source	Lot No	No treatment	Trypsin 48 h (0 C)	pH 3.3, 24 h pH 7.5, 24 h (0 C)
None	H	-	27±6*	252±24*	248±20*
Prothrombin	B	500513A	19	153	151
Factor V	D	CF 5-46	23	198	151
	B	500315	19	229	219
Factor VIII:C	B	503844A	10	164	140
	H	-	18	228	220
Factor IX	D	003274	13	150	140
	B	A500821A	27	261	233
Factor X	B	504216A	22	241	220
	H	-	49	171	166
Factor XI	B	500310B	25	219	201
	GK	120569	23	141	144
	H	-	22	237	231
Factor XII	B	A500408A	22	193	34
	GK	120569	22	163	48
	H	1	24	257	44
	H	2	23	241	43
	H	3	22	206	43
	H	4	21	199	42
	H	5	41	221	59
Prekallikrein	GK	1701-520	12	544	34
	LJ	1	9.8	262	28
	LJ	2	9.5	269	30
HMWK**	GK	1603-139	10	203	193

Source of plasma: GK = George King; B = Behringwerke; D = Dade; LJ = La Jolla; H = own hospitals. Renin was measured in untreated plasma (naturally occurring active renin) and after treatment with trypsin (total renin) or acid-dialysis.

* Mean ± SD (n = 6).

** after correction of prekallikrein concentration to normal (50 μg/ml).

USA. Plasma deficient in factor XI,X,IX,VIIIC,VII, or V was obtained from Behringwerke AG, Marburg-Lahn, West Germany, and from Merz-Dade, Dudingen, Switzerland (Table 1). Plasma deficient in factor XII,XI,X or VIIIC was obtained from homozygous patients from our hospitals. Plasma from two added additional patients with prekallikrein deficiency was a gift from Dr.J.H.Griffin, Scripps Clinic and Research Foundation, La Jolla, Calif. The clotting activity of the respective factors in these plasma samples was less than 1% of normal. Human plasma, in which prothrombin had been lowered by immunoadsorption to less than 0.1% of normal, was obtained from Behringwerke.

Blood from 30 healthy volunteers was collected in tubes containing 0.1 volume of 0.13 M trisodium citrate as anticoagulant. Plasma was prepared by centrifugation at 3,000xg for 10 minutes at 22 C. All plasma was stored at -70 C.

Inhibitor deficient plasma. C_1-INH,α_2M, AT III, and α_1AT were isolated from

normal human plasma[20–23]. Monospecific goat antisera against α_2M and AT III were prepared[24]. Monospecific rabbit antisera against C_1- INH and α_1AT were obtained from Behringwerke. Immunopurified antibodies were prepared by affinity chromatography of the different antisera on a column of CNBr-activated Sepharose 4B (2 g) (Pharmacia Fine Chemicals AB, Uppsala, Sweden) to which either purified C_1-INH (5.6 mg), α_2M (10 mg), AT III (9,2 mg), or α_1AT (7,2 mg) was covalently bound. The antibodies were eluted with 3M KSCN in 0.01M sodium phosphate and 0.5M NaCl, pH 7.4, and after dialysis against 0.1M NaHCO3, pH 8.0, the antibodies were coupled to CNBr- activated Sepharose 4B. The immunopurified antibodies against C_1-INH, α_2M, AT III or α_1AT gave one precipitation line against normal human plasma, which showed a reaction of identity with the single line observed against human C_1-INH, α_2M, AT III, or α_1AT, respectively. Plasmas deficient in either C_1-INH, α_2M, AT III, or α_1AT were prepared by immunoadsorption of the respective inhibitors from a factor XII-deficient plasma, using columns of CNBr-activated Sepharose 4B to which immunopurified antibodies were coupled:9,4 mg anti-C_1-INH to 2 g Sepharose, 61 mg anti- α_2M to 7 g Sepharose, 22 mg anti-AT III to 3 g Sepharose, and 4.4 mg anti-α_1AT to 3 g Sepharose. Four milliliters of C_1-INH-deficient plasma, 4.5 ml of α_2M-deficient plasma, 5 ml of AT III-deficient plasma, and 4 ml of α_1-AT-deficient plasma were obtained. Plasma deficient in both C_1-INH and α_2M was prepared by successive immunoadsorption of α_2M and C_1-INH from the same factor XII-deficient plasma. Four milliliters of this plasma was applied to the anti-AT III column in order to obtain plasma deficient in all three inhibitors.

The inhibitor-deficient plasmas were completely deficient in the appropriate inhibitors as measured quantitatively by rocket immunoelectrophoresis[25]. Other serine protease inhibitors that were not removed, and prekallikrein, HMWK, renin, and prorenin remained at normal plasma levels (Table II). Normal plasma

Table 3.3.2. Concentrations of contact factors, inhibitors, and naturally occurring active renin and prorenin in factor XII-deficient plasma after depletion of inhibitors.

	Plasma proteins							
Deficiency	Pre-kallikrein μg/ml	HMWK μg/ml	C_1-INH μg/ml	α_2M μg/ml	ATIII μg/ml	α_1AT μg/ml	Renin μU/ml	Prorenin μU/ml
None	37	67	162	1.9	193	1620	41	180
C_1 inh	38	68	<1	1.8	144	1600	36	180
α_2M	39	66	187	<0.02	184	1580	38	186
ATIII	37	63	180	1.9	<1	1600	37	179
α_1AT	37	80	144	1.6	162	<20	36	180
C_1-INH and α_2M	35	69	<1	<0.02	146	1720	42	162
C_1-INH and α_2M and ATIII	36	67	<1	<0.02	1	1880	36	166

C_1-INH, α_2M, ATIII, and α_1AT were removed by immunoadsorption.
Source of factor XII-deficient plasma: own hospital (see also Table 1).

was also depleted of α_2M by chemical modification of the inhibitor with methylamine. One tenth ml of methylamine 0.4M in 1.0M Tris buffer, pH 8.1, was added to 0.9 ml normal plasma and incubated for 2 hours at 22 C[26]. Remaining α_2M was measured by its capacity to form an SBTI-resistant complex with trypsin[27]. For this purpose, the methylamine-treated plasma was diluted 1:250 with 0.05M Tris HCl buffer,pH 8.2, and prewarmed at 37 C. Trypsin (Sigma Chemical Co.,St.Louis, Mo, USA.), 10 μg in 0.1 ml Tris HCl buffer, was added to 0.5 ml of the diluted plasma. After incubation for 1 minute at 37 C, 0.1 ml SBTI (Sigma) 5 mg/ml was added, and 15 seconds later 0.2 ml of the tripeptide substrate N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide (S2222;Kabi,Stockholm,Sweden) 1.0 mM in distilled water was added, and the volume was adjusted to 1.0 ml with 0.05 M Tris HCl buffer, pH 8.2. The change in absorbance at 405 nm was followed in a 1 cm semimicrocuvette at 37 C, and the initial velocity was measured to give delta A/min. The result was read from a standard line of dilutions of the untreated plasma.

Factor XII, prekallikrein, and HMWK. Factor XII (150 μg/ml), prekallikrein (315 μg/ml), and HMWK (615 μg/ml) were isolated from normal plasma as described elsewhere[28,31]. The proteins were stored at -70 C in a buffer,pH 5.0, containing 5 mM sodium acetate, 0.15M NaCl,and 0.02% NaN3. Protein was measured with the Lowry[32] method, using bovine serum albumin as a standard.

Immunologic assays for contact factors and inhibitors. Monospecific goat antisera against factor XII, prekallikrein, and the light chain of HMWK were prepared as described elsewhere[28–31,33]. The preparation of immunopurified antibodies against C_1-INH,α_2M, AT III, and α_1AT is described above. The concentrations of the respective proteins in plasma, were measured by rocket immunoelectrophoresis[25].

Functional assay of kallikrein. Kallikrein amidolytic activity was measured as the initial rate of hydrolysis of the tripeptide substrate H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide (S2302;Kabi)[34]. A 4.1 mM solution of the tripeptide substrate in distilled water was prepared, and 20 μl of the test sample was added to 0.2 ml of the substrate solution. The volume was adjusted to 1 ml with 0.05M Tris HCl buffer, pH 7.8. The change in absorbance at 405 nm was followed in a 1 cm semimicrocuvette at 37 C, and the initial velocity was measured to give delta A/min. A second 20 μl test sample was mixed with 10 μl of SBTI 10 mg/ml at 37 C, and after 15 seconds it was assayed for remaining kallikrein activity[24,35]. Free kallikrein activity was calculated by subtracting the activity that was resistant to SBTI from the total kallikrein activity measured in the absence of SBTI.

Functional assay of renin and prorenin. Renin was measured via its ability to generate angiotensin I from partially purified renin substrate prepared from plasma of nephrectomized sheep, as described elsewhere[8,9]. Briefly, 0.25 ml of the substrate was added to 0.05 to 0.125 ml aliquots of a test sample, and the volume was adjusted to 0.5 ml with 0.1M sodium phosphate buffer, pH 7.5, containing EDTA,8-hydroxyquinoline sulfate, PMSF, and aprotinin as protease

inhibitors. The final concentration of renin substrate in the reaction mixture was 2.5 μM, expressed as the maximal quantity of angiotensin I that could be generated at pH 7.5 and 37 C in the presence of a large excess of human kidney renin. The concentration of homologous substrate in the incubation mixtures was less than 0.2 μM angiotensin I equivalents. In our assay system, this concentration of homologous substrate did not interfere with the reaction of renin with the heterologous substrate[9]. The reaction mixtures were incubated at 37 C for 15 and 30 minutes and for 1,2, and 3 hours. The renin-containing samples had been diluted in such a way that no more than 5% substrate was cleaved during incubation. Parallel incubations at 0 C served as blanks. Incubations of dilutions of the MRC human kidney renin standard (Lot 68/356; World Health Organization International Laboratory for Biological Standards, London, England) at 37 C and 0 C were run in each assay batch. In all samples, the generation of angiotensin I was linear for the first two hours. The reaction was stopped by adding 0.5 ml of 0.15 M NaCL, succeeded by heating for 10 minutes in a boiling water bath. The precipitate was removed by centrifugation. The concentration of angiotensin I in the supernatant was measured by RIA, and results are expressed as μU per milliliter using the MRC human kidney renin preparation as a standard.

Prorenin was measured after its activation by immobilized trypsin[2,8]. For this purpose 0.2 to 0.5 ml aliquots of a test sample were incubated with one-fifth volume of Sepharose-bound trypsin in a final concentration of 0.25 mg/ml trypsin for 24 and 48 hours at 0 C. In normal plasma, maximal activation of prorenin is obtained within 24 hours, and there is no further change after 48 hours. The degree of prorenin activation by immobilized trypsin at 0 C and neutral pH is the same as obtained with the intrinsic factor XII-kallikrein pathway at the same temperature and pH in pH 3.3-pretreated plasma[5,8]. This is an indication that the conditions for activating prorenin in our assay were optimal. The immobilized trypsin was removed by centrifugation, and the test samples were incubated with renin substrate for renin assay. Prorenin concentration was calculated as the difference between the renin concentration after activation with trypsin (total renin) and the concentration before activation (naturally occurring active renin).

3.3.4. RESULTS

Cold-promoted activation of prorenin.

Normal plasma. Plasma samples from 30 normal volunteers were kept at 0 C. Active renin and total renin were measured at various time intervals, as indicated in Fig.1. Total renin was 274 ± 17 μU/ml(mean ± SEM) at time zero and remained constant throughout the entire period of incubation at 0 C. The increase of active renin expressed as a percentage of prorenin at time zero was therefore used as an index of the degree of prorenin activation. Active renin increased progressively during cold exposure. It was 32 ± 4 μU/ml before

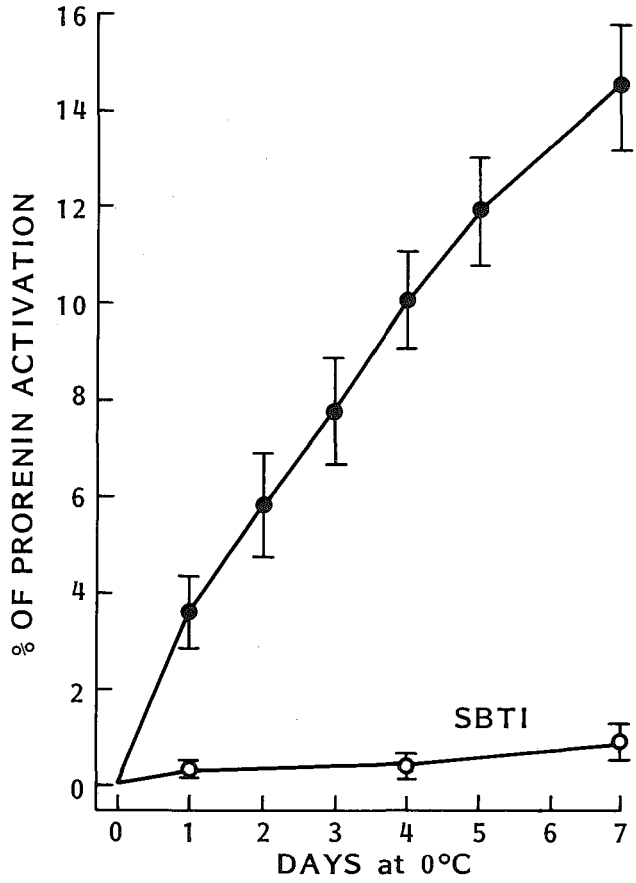

Fig. 3.3.1. Cold-promoted activation of prorenin in normal plasma(n = 30) in the absence (●) and presence (O) of SBTI (final concentration 1 mg/ml). Mean ± SEM.

cold exposure and 66 ± 6 μU/ml after 7 days ($p < 0.001$, paired t-test). During this period of 7 days, 14 ± 1% of prorenin was activated (range 6% to 26%). In the presence of SBTI, less than 1 % of prorenin was activated.

Clotting factor-deficient plasma. The concentration of naturally occurring active renin in these plasmas and the concentration of renin as measured after maximal activation by immobilized trypsin for 48 hours are given in Table I. The results with trypsin after 48 hours were not different from those after 24 hours. The plasmas (2 ml samples) were also activated by dialysis for 24 hours at 0 C against glycine HCL buffer, pH 3.3, succeeded by dialysis against sodium phosphate buffer, pH 7.5, again for 24 hours at 0 C[36]. Irreversible activation of prorenin occurs during the second dialysis step[2,3]. The results presented in Table I confirm that this process depends on the presence of both factor XII

and prekallikrein. After reconstitution of the plasma deficient in these factors with factor XII (final concentration 28 μg/ml) and prekallikrein (final concentration 50 μg/ml), the activation of prorenin after acid treatment reached the same maximum level as with trypsin. In the plasma deficient in factor XI,X,IX,VIIIC,VII,V, or prothrombin, the activation obtained via the intrinsic factor XII-kallikrein pathway after acid treatment also reached the same level as with exogenous trypsin. In plasma deficient in HMWK, the activation of prorenin after acid treatment was less than with trypsin, but this plasma had a low prekallikrein content (16 μg/ml), and after reconstitution of prekallikrein to normal (50 μg/ml), the degree of activation of prorenin obtained after acid treatment was the same as with trypsin. These results indicate that complete prorenin-renin conversion had occurred with trypsin not only in normal plasma but also in the deficient plasma.

In a subsequent series of experiments, the deficient plasma was kept at 0 C without any pretreatment, and both active renin and total renin were measured at various intervals. For comparing the results with those obtained in normal plasma, the change of active renin was expressed as a percentage of prorenin at time zero. By using this index, cold-promoted activation of prorenin was within the normal range in plasma deficient in factor XI,X,IX,VIIIC,VII,V, or prothrombin, but it was below normal in plasma deficient in factor XII or prekallikrein (Figs.2 and 3). Activation was in the lower end of the normal

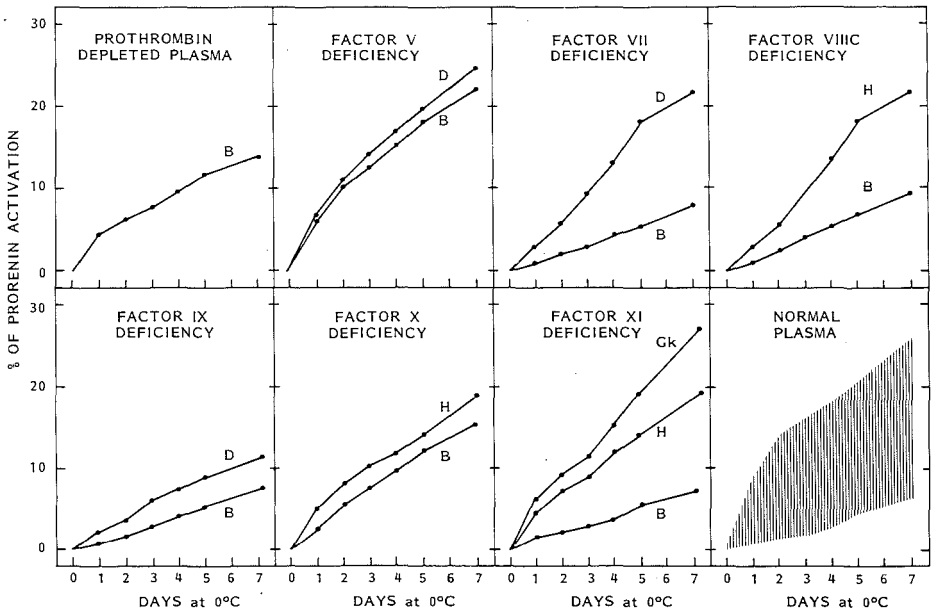

Fig. 3.3.2. Cold-promoted activation of prorenin in clotting factor-deficient plasma. The range for 30 normal plasma samples is presented. Source of plasma:GK = George King; B = Behringwerke; D = Dade; H = own hospitals. (see also Table 3.3.1)

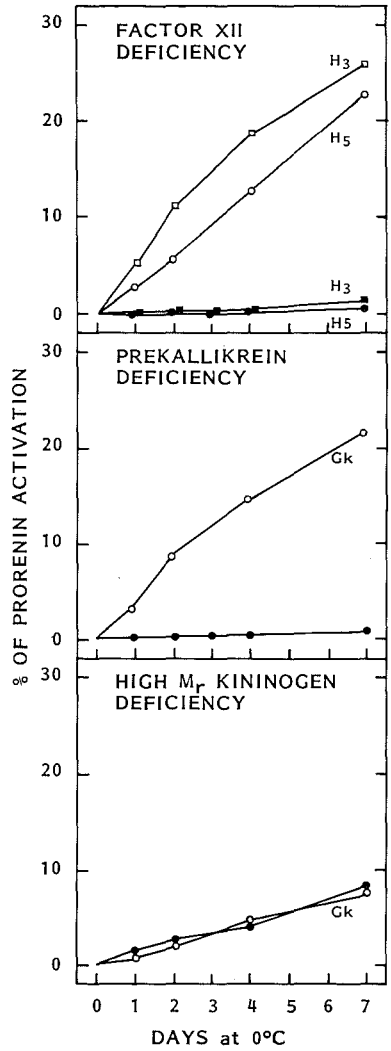

Fig. 3.3.3. Cold-promoted activation of prorenin in plasma deficient in contact factors before (■ ●) and after (□ O) reconstitution with the deficient factor. Final concentrations: factor XII 28 μg/ml; prekallikrein 50 μg/ml; high mol wt (Mr) kininogen 70 μg/ml. Source of plasma: GK = George King; H3, H5 = own hospitals (see also Table 3.1.1.).

range for plasma deficient in HMWK with low prekallikrein concentration corrected to normal (50 μg/ml). Reconstitution of the plasma deficient in factor XII or prekallikrein with factor XII (final concentration 28 μg/ml) and prekallikrein (final concentration 50 μg/ml) restored normal cold-promoted prorenin activation. Addition of HMWK (final concentration 70 μg/ml) to the HMWK-

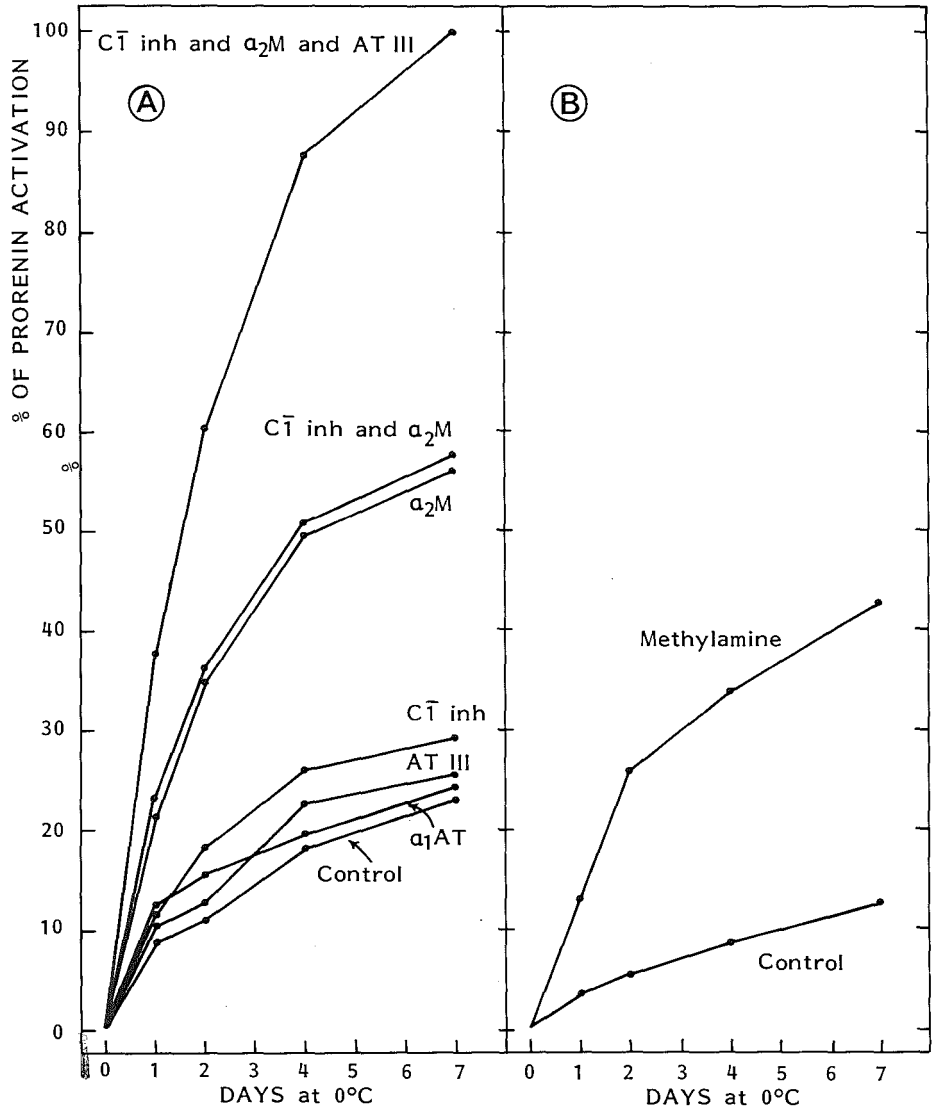

Fig. 3.3.4. Cold- promoted activation of prorenin in inhibitor-depleted plasmas. A, C_1-INH, α_2M, AT III and α_1-AT were removed from the same factor XII-deficient plasma by immunoadsorption. Factor XII (final concentration, 28 μg/ml) was added at time zero. B, α_2M actitivity was also removed from normal plasma by chemical modification using methylamine. Source of factor-XII deficient plasma: own hospitals (see also Table 3.3.1.)

deficient plasma had no additional effect (Fig.3). Therefore it appears that also in nonacidified plasma, the cold-promoted activation of prorenin depends on the presence of factor XII and prekallikrein, whereas the other clotting factors are not essential.

Inhibitor-deficient plasma. C_1-INH, α_2M, AT III, or α_1AT or combinations of these inhibitors were selectively removed, either by immunoadsorption or, in the case of α_2M, by chemical modification using methylamine. All inhibitor-deficient plasma was prepared from the same factor XII-deficient plasma. After the addition of factor XII (final concentration 28 μg/ml) ,these plasma samples were kept at 0 C for up to 7 days. Active renin and total renin were measured at various intervals (Fig.4). Total renin remained constant during the period of incubation at 0 C. As in the previous experiments, the change of active renin was expressed as a percentage of prorenin at time zero to provide an index of the degree of cold-promoted prorenin activation. In 7 days, 29% of prorenin was activated in C_1-INH-deficient plasma, 56% in α_2M-deficient plasma, 24% in AT III-deficient plasma, and 23% in α_1AT-deficient plasma, compared with 22% in control plasma. Activation of prorenin in plasma depleted of both C_1-INH and α_2M was not different from activation in plasma depleted of α_2M alone. In plasma that had been depleted of the three inhibitors, C_1-INH, α_2M, and AT III, 100% of prorenin was activated in 7 days. After chemical modification of α_2M by treatment of normal plasma with methylamine, 42% of prorenin was activated in 7 days compared with 12% in the untreated plasma (Fig.4). The results demonstrate the importance of these proteins, particularly α_2M, in inhibiting the activation of prorenin at 0 C.

Contact-induced activation of prorenin.

Dextran sulfate, molecular weight 500,000 is a potent inducer of contact activation. Addition of dextran sulfate to plasma in a final concentration of 12.5 μg/ml causes complete factor XII-HMWK-mediated activation of prekallikrein at 0 C within 10 minutes[37,38]. At 37 C, activation is not complete, probably because of the strong effect of inhibitors at this temperature. Therefore we activated the factor XII-kallikrein pathway in plasma with dextran sulfate at 0 C and incubated the activated plasma at either 0 C or 37 C. These experiments were carried out with factor XII-deficient plasma from which one or more inhibitors had been removed by immunoadsorption. The same factor XII-deficient plasma, but with the inhibitors still present, served as control. Immediately after factor XII (final concentration 28 μg/ml) had been added, 0.9 ml of the reconstituted plasma was mixed at 0 C with 0.1 ml dextran sulfate, 125 μg/ml, in distilled water. The plasma was kept at 0 C for 10 minutes, during which complete activation of prekallikrein took place, and then was divided into 0.5 ml aliquots. One aliquot was kept at 0 C for up to 2 hours, and the other was kept at 37 C, also for 2 hours. Active and total renin were measured at various intervals (Fig.5). Total renin remained constant during the entire period of incubation, and the change of active renin was expressed as a percent of prorenin at time zero. There was little change in the concentration of active renin at 0 C during the relatively short incubation period of 2 hours in either the control plasma or the inhibitor-deficient plasmas. There was also little activation at 37 C in the control plasma and in the plasmas deficient in C_1-INH, α_2M, AT III, or α_1AT. In 2 hours, 6 % of prorenin was activated in plasma depleted of both C_1-INH and α_2M, and 47% was activated in plasma

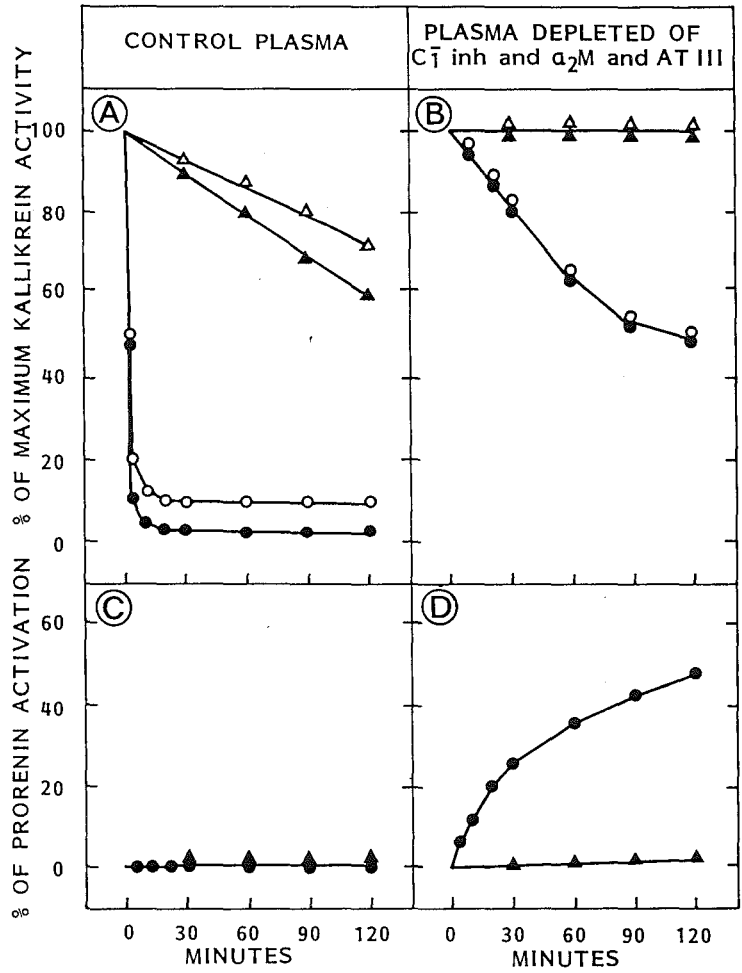

Fig. 3.3.5. Disappearance of kallikrein amidolytic activity (A and B) and activation of prorenin (C and D) at 37 C (O, ●)and 0 C (Δ Δ) after contact-induced activation of prekallikrein, in the presence (A and C) and absence (B and D) of the kallikrein-inhibitors C_1-INH, $\acute{\alpha}_2$-M and AT III. The inhibitors were removed from factor XII-deficient plasma by immunoadsorption. The same factor XII-deficient plasma, but with the inhibitors still present, was used as control. Factor XII (final concentration, 28 μg/ml) was added, and immediately thereafter the factor XII-high mol wt kininogen-kallikrein pathway was activated by incubation with dextran sulfate at 0 C for 10 minutes. The plasma was then kept at 0 C for 120 minutes or incubated at 37 C for 120 minutes. Renin was measured before and after activation with trypsin (active renin and total renin respectivily). Prorenin concentration was calculated as the difference between the total renin and active renin. Total renin remained constant during 120-minute incubation period. The change of active renin expressed as a percentage of prorenin at time zero was used as a measure of prorenin activation. Kallikrein activity was measured in the absence (total kallikrein) and presence of SBTI. The difference between total activity (O, Δ) and SBTI-resistant activity (α_2M-bound kallikrein) was taken as free kallikrein (●„ Δ). Source of factor XII-deficient plasma: our hospitals. (see also Table 3.3.1.)

that had also been depleted of AT III (Fig.5). Less than 1% of prorenin was activated in this triple- deficient plasma when factor XII was also absent. Thus it appears that prorenin can be activated by the contact activation system at 37 C in nonacidified plasma with the three most important kallikrein inhibitors selectively removed.

3.4.5. DISCUSSION.

Previous studies have demonstrated the importance of factor XII and plasma kallikrein in the activation of prorenin in acid-pretreated human plasma[2,3]. Our study describes the role of the contact activation system in the activation of prorenin that occurs when nonacidified plasma is kept at 0 C (cold-promoted activation), or when nonacidified plasma is incubated at 37 C after activation of the contact system with dextran sulfate (contact-induced activation). The activation process was studied in normal plasma, in plasma from patients with clotting factor deficiencies, and in plasma with one or more inhibitors of activated factor XII and kallikrein selectively removed.

Tatemichi and Osmond[39] have reported preliminary data showing that the cold-promoted increase of renin activity in two factor XII-deficient plasma samples was half the increase in two normal samples, whereas the increase in prekallikrein-deficient plasma was about equal to that in the normal plasma. However, the increase in renin activity was not expressed as a percentage of the total concentration of prorenin that was available for activation. These results are therefore difficult to interpret, even more so since prorenin in prekallikrein-deficient plasma is higher than in normal plasma[17,40]. In our study, factor XII-deficient plasma and prekallikrein-deficient plasma showed little or no cold-promoted increase in renin activity. Normal activation of prorenin was observed in such plasma after reconstitution with normal concentrations of factor XII and prekallikrein. The HMWK-deficient plasma we used contained about 30% of the normal concentration of prekallikrein as measured by clotting time assay and rocket immunoelectrophoresis. Plasma prekallikrein was therefore added to give a normal concentration of this protein. This plasma showed a significant degree of cold-promoted prorenin activation, which was not influenced by the addition of a normal concentration of HMWK. These results clearly show that the factor XII-kallikrein pathway is important for cold-promoted prorenin activation and that, under the conditions we have used, this pathway was similarly effective whether HMWK was present or not.

The possibility that other clotting factors are also involved cannot be excluded, but they appear to be less important than factor XII and plasma kallikrein because significant degrees of prorenin activation were observed in plasma deficient in factor XI,X,IX,VIIIC,VII,V, or prothrombin. The importance of factor XII and plasma kallikrein as opposed to the other clotting factors is also supported by the observations on prorenin activation in plasma that was dialyzed at pH 3.3 and then redialyzed at pH 7.5. Complete activation of prorenin

occurred during the second dialysis step in plasma deficient in HMWK after restoration of normal prekallikrein concentration, and in plasma deficient in either factor XI,X,IX,VIIIC,VII,V, or prothrombin; however, no increase in renin was observed in plasma deficient in either factor XII or prekallikrein.

It has previously been shown that in the factor XII-kallikrein pathway of prorenin activation in acid-pretreated plasma, it is kallikrein and not factor XII that is the more direct activator[2,3]. It has further been reported that active human plasma kallikrein, not activated bovine factor XII or the active human 28,000 molecular weight factor XII fragment, was capable of activating highly purified plasma prorenin[41]. Therefore, it is also likely that in the cold-promoted activation in nonacidified plasma, it is factor XII-activated prekallikrein rather than factor XII itself that causes prorenin activation.

Five inhibitors of plasma kallikrein with varying potencies have thus far been characterized in human plasma: C_1-INH, α_2M, AT III, α_1AT, and α_2-antiplasmin[42]. C_1-INH and α_2M have been shown to be the most important inactivators of kallikrein in plasma at 37 C. However, after these two inhibitors have been removed, kallikrein is bound to AT III and possibly other inhibitors[24,26]. At 0 C, α_2M appears to be a more potent inhibitor than C_1-INH[37]. C_1-INH is further considered to be the most important inhibitor of both activated factor XII and the active 28,000 molecular weight factor XII fragment. Our results are in agreement with these data. Selective removal of either C_1-INH, AT III, or α_1AT had little influence on the cold-promoted activation of prorenin. In contrast, the removal of α_2M resulted in a three times higher rate of prorenin activation. Depletion of both C_1-INH and α_2M had little or no additional effect, whereas the rate of activation was further increased by a factor of three after the plasma had also been depleted of AT III. In this triple-deficient plasma, 47% prorenin activation was observed during incubation at 37 C for 2 hours after rapid and complete factor XII-HMWK-mediated activation of prekallikrein with dextran sulfate at 0 C. During incubation at 37 C, kallikrein activity had a half-life of about 2 hours in this plasma (Fig.5). The half-life of kallikrein activity at 37 C in plasma deficient in C_1-INH is about 30 minutes, and it is about 60 minutes in plasma deficient in both C_1-INH and α_2M[24]. In spite of this, we observed no activation of prorenin at 37 C in C_1-INH-deficient plasma, and only 6 % in 2 hours in plasma deficient in both C_1-INH and α_2M. Thus a great excess of the enzyme kallikrein (50 μg/ml, approximately 0.6 μM) over the substrate prorenin (200 μU/ml, approximately 5 pM) was required for activation to be detected. This requirement may be necessitated by the extremely low concentration of substrate rather than a low affinity of the activating enzyme.

We conclude that the cold-promoted activation of prorenin in plasma depends on the contact activation system, and that this system is capable of activating prorenin in the plasma milieu at 37 C when the three most important kallikrein inhibitors, C_1-INH, α_2M and AT III, have been removed.

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4. PRORENIN-RENIN CONVERSION: A TWO-STEP PROCESS

4.1. TWO FORMS OF PLASMA RENIN AFTER ACTIVATION *IN VITRO* AND THEIR RELATION TO NATURAL PLASMA RENIN

4.1.1. ABSTRACT

Inactive renin in human plasma can be activated by pH 3.3-dialysis (generation of acid-activated renin), by clotting factor XII- mediated prekallikrein to kallikrein conversion after pH has been restored to neutral (generation of acid-kallikrein-activated renin) or by the addition of trypsin (generation of trypsin-activated renin). Natural active renin, acid-kallikrein-activated renin and trypsin-activated renin behave similarly during affinity chromatography on Blue-Sepharose CL-6B and during gel filtration on Sephadex G-100. They also show similar reaction kinetics with similar pH-optimum curves when acting on sheep renin substrate. Acid-activated renin is different. It is retained on Blue-Sepharose columns and it is inactivated at neutral pH during incubation at 37 C. This contrasts with the other forms of renin activated *in vitro* and with natural active renin. The pH-optimum curve of acid-activated renin, when acting on sheep renin substrate, is also different from that of the other forms of active renin. It is to be proven that the renins generated *in vitro* by neutral serine proteinases are identical with natural active renin, but clearly they bear more resemblance to natural renin than acid-activated renin does. Our preliminary conclusion is that acid-activated renin is a 'laboratory renin', which does not circulate in normal peripheral venous plasma.

4.1.2. INTRODUCTION

Inactive renin (prorenin) in human plasma can be activated by pH 3.3-dialysis of plasma (generation of acid-activated renin)[1], by clotting factor XII-mediated prekallikrein to kallikrein conversion after pH has been restored to neutral (generation of acid-kallikrein- activated renin)[2,3] or by the addition of trypsin (generation of trypsin-activated renin[2,4,5]. It is not known whether inactive renin is a precursor of naturally occurring active renin. It is also not known whether the forms of renin activated *in vitro* are identical with natural renin.

Some biochemical properties of the renins activated *in vitro* were therefore compared with those of natural renin.

4.1.3. METHODS

Collection of blood

Blood from five healthy male volunteers was collected into plastic tubes containing disodiumethylenediaminetetra-acetic acid (EDTA) in a final concentration of 5 mM. The blood was pooled and immediately centrifuged at 8,000 g for 10 min and plasma was stored at -20 C. Plasma was also obtained from a patient with Hageman trait (clotting factor XII less than 1% of normal) and a patient with Fletcher trait (plasma prekallikrein less than 1% of normal).

Activation procedures

Generation of acid-activated renin. EDTA-plasma was dialyzed against glycine/HCL buffer, pH 3.3, at 4 C for 24 h. pH was then restored to 7.5 with NaOH solution (1.0 M)[5,6].

Generation of acid- kallikrein-activated-renin.

EDTA-plasma was dialyzed against glycine/HCL buffer, pH 3.3, at 4 C for 24 h and then redialyzed against phosphate buffer, pH 7.5, again at 4 C, for 24 h[5].

Generation of trypsin-activated renin. EDTA-plasma was incubated with Sepharose-bound trypsin in a final concentration of 0.3 mg of trypsin/ml at 4 C for 24h[5].

Gelfiltration

Samples (4 ml) of activated and non-activated EDTA-plasma were applied to 2.6 cm x 90 cm columns of Sephadex G-100 (Pharmacia) equilibrated with Tris/HCL buffer (0.01 M, pH 7.5, containing NaCl (0.15 M). This buffer was also used for elution. Flow rate was 10-12 ml/h, and 2.5 ml fractions were collected. Void volume was determined with Dextran Blue (Pharmacia) and the columns were calibrated with the following molecular-weight markers (Pharmacia): ribonuclease A (Mr 13,700), ovalbumin (Mr 43,000) and human serum albumin (Mr 65,000). ^{14}C Ovalbumin (598 mCi/mmol) (Amersham) was used as an internal standard in each run. Gel filtration occurred at 4 C.

Affinity chromatography

Samples (4 ml) of activated and non-activated EDTA-plasma were applied to 1.6 cm x 24 cm columns of Blue-Sepharose CL-6B (Pharmacia). The columns were equilibrated with sodium phosphate buffer (0.02 mM), pH 7.1. Elution was performed with the same buffer in three steps i.e. without NaCl, with NaCl at 0.2 M and with NaCl at 1.4 M[7]. Flow rate was 60 ml/h and 2.5 ml fractions were collected. Chromatography occurred at 4 C.

Renin assay

The method was based upon radioimmunoassay of angiotensin I (ANG I) generated during incubation with sheep renin substrate in the presence of proteinase inhibitors and EDTA[5].

Kinetic studies

Aliquots (0.1 ml) of activated and nonactivated EDTA-plasma were incubated at 37 C with sheep renin substrate in concentrations ranging from 0.125 M

to 2.5 M in phosphate buffer, pH 7.5, in a final concentration of 1.0 ml. Incubation times were 15, 30 and 60 min to check for linearity of angiotensin I generation. Km was calculated from Lineweaver-Burk plots. For constructing pH-optimum curves, sheep renin substrate was dialyzed against phosphate buffers with pH values ranging from 4.5 to 8.5. Aliquots (0.1 ml) of activated and non-activated EDTA-plasma were incubated for 30 min at 37 C at these pH values.

4.1.4. RESULTS

Gel filtration and chromatografy

Natural renin, acid-kallikrein-activated renin and trypsin-activated renin had similar apparent molecular weights on Sephadex G-100 columns (Mr 48,000-54,000, three experiments). Estimations of molecular weight of acid-activated renin were hindered by inactivation of this form of renin at neutral pH (see below). The behaviour of acid-activated renin on Blue-Sepharose columns was clearly different from that of natural renin, acid-kallikrein-activated renin and trypsin-activated renin. Acid-activated renin was retained on the columns, whereas the other renins were not (Fig.1).

Reaction kinetics

The initial velocity of angiotensin I formation at pH 7.5 and 37 C from sheep renin substrate with non-activated normal plasma was 0.12 nmol of ANG I $min^{-1}.l^{-1}$. The initial velocities with acid-activated normal plasma, acid-kallikrein-activated normal plasma and trypsin-activated normal plasma were 1.16, 1.25 and 1.27 nmol of ANG I $min^{-1}.l^{-1}$ respectively (mean values of two experiments). Acid-activated renin was inactivated during prolonged incubation at pH 7.5 and 37 C (Fig.1). When acid-activated normal plasma was kept for 1 h at 37 C after pH had been restored to 7.5, the initial velocity of angiotensin I formation from sheep renin substrate at pH 7.5 and 37 C was reduced from 1.16 to 0.24 nmol of ANG I $min^{-1}.l^{-1}$. When, after 1 h storage at 37 C, the plasma was redialyzed at pH 3.3 for 24 h at 4 C, the initial velocity rose again to 0.84 nmol of ANG I $min^{-1}.l^{-1}$.

The initial velocity of angiotensin formation at pH 7.5 and 37 C from sheep renin substrate with non-activated factor XII-deficient plasma was 0.04 nmol of ANG I $min^{-1}.l^{-1}$. It was 0.25 nmol of ANG I $min^{-1}.l^{-1}$ with acid-activated factor XII-deficient plasma and it was 0.43 nmol of ANG I $min^{-1}.l^{-1}$ with trypsin-treated factor XII- deficient plasma (mean values of two experiments). The initial velocity of angiotensin I formation with non-activated prekallikrein-deficient plasma was 0.08 nmol of ANG I $min^{-1}.l^{-1}$. It was 1.48 nmol of ANG I $min^{-1}.l^{-1}$ with acid-activated prekallikrein-deficient plasma and 3.13 nmol of ANG I $min^{-1}.l^{-1}$ with trypsin-treated prekallikrein-deficient plasma (mean values of two experiments). From the results it appears that the generation of acid-activated renin does not depend on the factor XII—kallikrein pathway. Dialysis at pH 7.5 for 24 h after pH 3.3-dialysis of the deficient plasmas did not result in greater velocities of angiotensin I formation than with the non-activated deficient

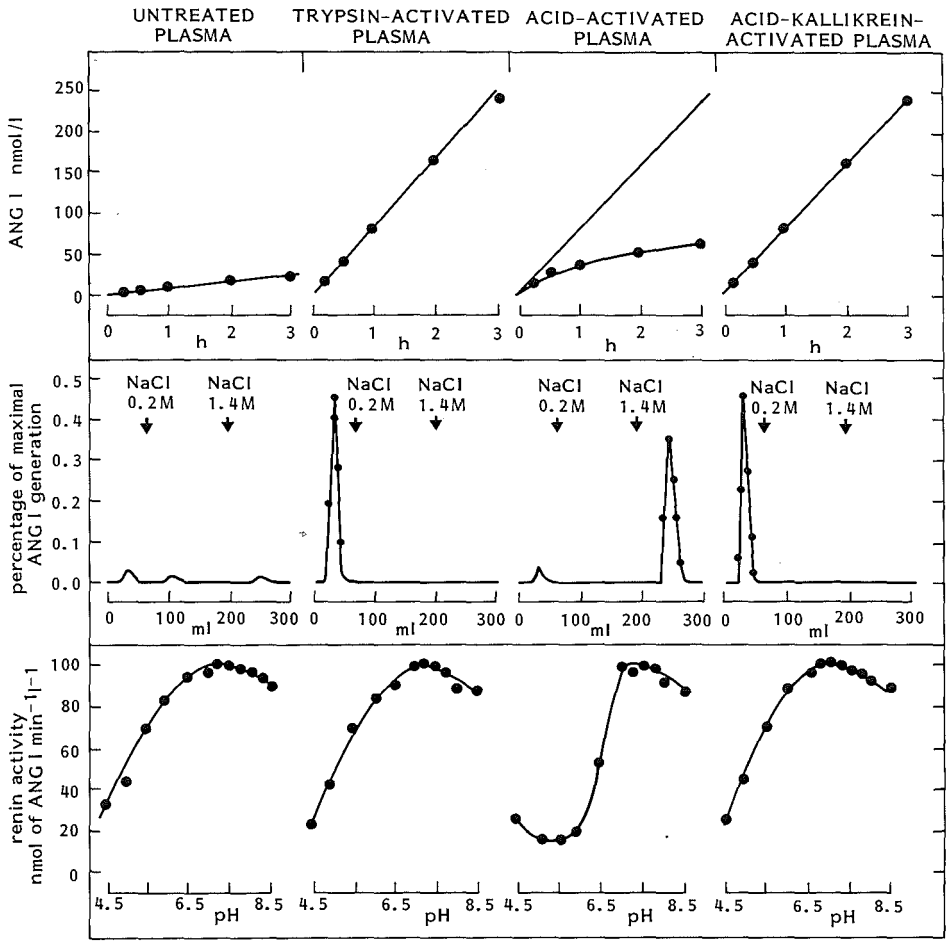

Fig. 4.1.1. Comparison between active renins in untreated plasma and activated plasmas. Natural active renin, trypsin-activated renin and acid-kallikrein-activated renin have similar pH optimum curves, when acting on sheep renin substrate (incubation at 37 C, pH 4.5- 8.5) (lower panel). These renins are not retained on dye-ligand chromatography (Blue Sepharose) columns (middle panel) and are not inactivated during incubation with sheep renin substrate at pH 7.5 and 37 C (upper pannel). Acid-activated renin has a different pH-optimum curve, is retained on Blue-Sepharose columns and is inactivated during incubation with sheep renin substrate at pH 7.5 and 37 C.

plasmas, since the factor XII-mediated kallikrein generation did not occur in these plasmas[2]. Km values for natural renin, acid-kallikrein-activated renin and trypsin-activated renin were 0.28, 0.25 and 0.24 μM of ANG I respectively (mean values of two experiments). Km for acid-activated renin could not accurately be determined because of rapid inactivation during incubation with sheep renin substrate at neutral pH. The pH-optimum curve for acid-activated renin appeared

somewhat different from the curves for natural renin, acid-kallikrein-activated renin and trypsin- activated renin (Fig.1).

4.1.5. DISCUSSION

The forms of active renin that are generated in plasma by acidification *per se* and by acidification followed by incubation at neutral pH at 4 C are different. They can easily be separated by dye-ligand chromatography, and their reactions with sheep renin substrate show different pH-optimum curves. The form of active renin that is generated by acidification *per se* is inactivated at neutral pH and 37 C, whereas the form of active renin that is generated by acidification followed by incubation at neutral pH and 4 C is not inactivated during subsequent incubation at neutral pH and 37 C. This agrees with the results of Leckie & McGhee[1].

The generation of active renin in plasma by acidification *per se* (acid-activated renin) does not depend on factor XII and plasma kallikrein, whereas the generation of active renin at neutral pH after acid-treatment depends on Factor XII-mediated kallikrein formation (acid-kallikrein-activated renin)[2]. Acid-kallikrein-activated renin and trypsin-activated renin behave similarly during dye-ligand chromatography and gel filtration. They also show similar reaction kinetics with similar pH-optimum curves, when acting on sheep renin substrate.

It is not certain that the active renins that have been generated *in vitro* by neutral serine proteinases are identical with natural active renin, but clearly they bear more resemblance to natural renin than acid-activated renin does. Our preliminary conclusion is that acid-activated renin is a 'laboratory renin', which does not circulate in normal peripheral venous plasma.

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4.2. TWO-STEP PRORENIN-RENIN CONVERSION. ISOLATION OF AN INTERMEDIARY FORM OF ACTIVATED PRORENIN

4.2.1. SUMMARY

Prorenin is an inactive form of the aspartic protease, renin. Like pepsinogen, it is activated at low pH. The kinetics of acid activation of prorenin were studied in human amniotic fluid and plasma and in preparations of purified prorenin isolated from amniotic fluid and plasma. Conversion of prorenin (pR) into active renin (R) appeared to be a two-step process involving the generation of an intermediary form of activated prorenin (pRa). The pR → pRa step is an acid-induced reversible change in the conformation of the molecule, and the pRa → R step is proteolytic.

pRa → R conversion occurred in amniotic fluid at low pH by the action of an endogenous aspartic protease. In plasma pRa → R conversion occurs after restoration of pH to neutral and is caused by the serine protease, plasma kallikrein. pRa → R conversion did not occur in purified preparations of prorenin. Thus, in contrast to pepsinogen, the acid-induced reversible conformational change is not followed by autocatalysis.

pRa of amniotic fluid and plasma could be separated from R by affinity chromatography on Cibacron Blue F3GA-agarose, and R but not pRa was detected by an immunoassay using monoclonal antibodies reacting with R and not with pR. The first-order rate constant for pR → pRa conversion depends on the protonation of a polar group (or groups) with pK ~ 3.4, the rate constant being proportional to the fraction of pR molecules that has this group protonated. This is analogous to the reversible acid-induced conformational change of pepsinogen that occurs before its proteolytic conversion into pepsin. kcat/Km for pRa → R conversion by plasmin and plasma kallikrein at pH 7.4 and 37 C was 7.8 x 10^6 and 5.2 x 10^6 M^{-1} min^{-1} respectively, which was about 50 to 70 times greater than for pR → R conversion. The susceptibility of pRa to proteolytic attack is high enough for the intrinsic factor XII-kallikrein pathway to cause rapid pRa → R conversion at 37 C even in whole blood with its abundance of serine protease inhibitors. Formation of pRa may occur *in vivo* in an acidic cellular compartment, such as exo- or endocytotic vesicles.

The abbreviations used are: pR,prorenin; pRa, intermediary form of activated prorenin; R, protease-activated prorenin or naturally occurring active renin.

4.2.2. INTRODUCTION

Renin is a key enzyme in the formation of angiotensin II, which regulates blood pressure and fluid and electrolyte balance. An inactive form of renin, prorenin, circulates in plasma. Acidification of human plasma at 4 C initiates a process by which prorenin is converted into active renin (EC 3.4.23.15) after pH has

been brought back to neutral. This process involves the inactivation of one or more protease inhibitors at low pH, followed by activation of prekallikrein by factor XII and conversion of prorenin into renin at neutral pH by the action of kallikrein itself or by other proteases activated by kallikrein[1–3]. The importance of inactivation of serine protease inhibitors for subsequent activation of prorenin was clearly demonstrated by the enhanced spontaneous prorenin-renin conversion at 4 C in plasma from which C_1-esterase inhibitor, α_2-macroglobulin and antithrombin III had been selectively removed by immunoadsorption[4].

However, renin is an aspartic protease, and, like pepsinogen, prorenin is activated by acidification independently of factor XII and kallikrein[5,6]. In contrast with the factor XII- kallikrein-mediated proteolytic prorenin-renin conversion at neutral pH *after* exposure of plasma to low pH, the activation of prorenin *during* exposure to low pH appears to involve a reversible process, since the activated prorenin is inactivated at neutral pH and is reactivated when again exposed to low pH[5,6].

Prorenin can be converted into active renin by plasma kallikrein without prior acidification[7,8], but it has been suggested that an acid-induced conformational change of the prorenin molecule causes it to become more susceptible to proteolytic activation by kallikrein[9]. If the latter possibility proves to be true, then the acid pretreatment of whole plasma serves at least two functions. First, it inactivates one or more protease inhibitors so that activation of the factor XII-kallikrein pathway is initiated at neutral pH. Second, it induces a conformational change of the prorenin molecule, by which it becomes active towards angiotensinogen to produce angiotensin I and by which it also becomes more susceptible to irreversible activation by serine protease.

In this paper we report the kinetics of acid activation of prorenin and the isolation of an intermediary form of activated prorenin, which indeed is more susceptible than native prorenin to proteolytic conversion into renin.

4.2.3. MATERIALS AND METHODS

Prorenin. Prorenin was isolated from normal human amniotic fluid. Amniotic fluid, free of contaminating blood, was collected from normal pregnant women. Benzamidine HCl, final concentration 10 mM, was added in order to prevent inadvertent activation of prorenin. It was also added to the buffers that were used during purification. All purification steps were carried out at 4 C. The first step was ion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala ,Sweden) at pH 7.4 using a linear gradient of NaCl up to 0.2 M[10]. The prorenin- containing fractions were pooled and subjected to dye-ligand chromatography on Cibacron Blue F3G-A agarose (Blue Sepharose CL- 6B, Pharmacia)[11]. The next step was antibody-affinity chromatography[12] on a column of CNBr-activated Sepharose 4B (Pharmacia) to which the renin antibody R 2-1-21 (Ciba-Geigy, Basel, Switzerland, *vide infra*) was coupled, 10 mg antibody to 2 g of dry Sepharose. The prorenin fraction that was eluted

from this column contained traces of active renin. They were removed by chromatography on a column of pepstatin-aminohexyl Sepharose 4B (Pharmacia), 30 mg pepstatin to 1 gram of dry Sepharose[13]. Prorenin does not bind to pepstatin, whereas renin is bound to this inhibitor. After this step highly purified human serum albumin (Behring AG, Marburg, West Germany), in a final concentration of 0.1%, was added in order to stabilize prorenin. The prorenin solution was dialyzed against 0.1 M sodium phosphate/0.05 M NaCl pH 7.4 and concentrated. It contained 16,000 pg prorenin per ml as measured with an assay using monoclonal antibodies directed against human kidney renin and reacting with both prorenin and naturally occurring active renin (*vide infra*). It contained less than 10 pg renin per ml as measured with an assay using monoclonal antibodies specific for active renin (*vide infra*). A similar procedure was used for isolating prorenin from human plasma. The concentration of prorenin in the final preparation was 450 pg/ml, and active renin was undetectable.

Activators of prorenin. Factor XII and prekallikrein were isolated from human plasma and purified according to previously published methods[14,15]. β-Factor XIIa and plasma kallikrein (both the two-chain molecule, Mr 85,000, and its enzymatically active light chain, Mr 28,000) were prepared from factor XII and plasma prekallikrein as described before[16–18]. Trypsin from bovine pancreas, twice crystallized, 12,000 N-α-benzoyl-L-arginine-ethylester (BAEE) units per mg protein, was from Sigma Chemical Co (St Louis, MO, USA). Pepsin A from porcine stomach, twice crystallized, 3,000 units per mg protein, was from Worthington (Freehold, NJ, USA). Human plasmin, 25 caseinolytic units per mg protein, was from KABI Diagnostica (Stockholm, Sweden). Thrombin, 64 NIH units per mg of protein, was from Hoffmann-La Roche Ltd (Basel, Switzerland. Urokinase 5,000 Ploug units per ml, was from Leo Pharmaceuticals (Ballerup, Denmark).

Monoclonal antibodies: Monoclonal antibodies R 3-27-6, R 3-36-16 and R 2-1-21 were donated by prof K Hofbauer and dr C Heusser (Ciba-Geigy). The characteristics of the R3 antibodies have been described elsewhere[19]. They react equally well with human kidney renin and human chorionic cell culture prorenin and also with prorenin, trypsin-activated prorenin and naturally occurring active renin of human plasma[20]. The antibodies recognize different epitopes on the renin molecule and do not inhibit each other in their binding to renin. They were used for the direct radioimmunoassay of total renin i.e. renin plus prorenin. The R2 antibody also reacts with both prorenin and active renin but with lower affinity. It was used for the purification of prorenin. Monoclonal antibodies 3E8 and 4G1 specific for active renin, and not reacting with prorenin, were donated by prof J Menard (INSERM,Paris,France) The characteristics of these antibodies were described by Galen et al.[21]. They react equally well with human kidney renin and both trypsin-activated prorenin and naturally occurring active renin of human plasma. These antibodies were used for the direct radioimmunoassay of either serine protease-activated prorenin or naturally occurring active renin.

Dye ligand chromatography. Naturally occurring active renin, acid-activated prorenin and protease-activated prorenin were separated on a 1 x 16 cm column of Blue Sepharose. The column was equilibrated with 0.02 M sodium phosphate buffer pH 7.2 containing 0.2 M NaCl. Elution was performed in two steps, i.e. with the phosphate buffer containing 0.2 M NaCl and with the phosphate buffer containing 1.4 M NaCl. The flow rate was 50-60 ml/h and 2.0-2.5-ml fractions were collected.

Activation of prorenin. Prorenin-containing samples were activated by acidification or by adding proteases. For acidification the samples were diluted with an equal volume of buffer. A 0.05 M glycine/HCl buffer containing 0.1 M NaCl was used in the pH 2.5-4.0 range, and a 0.1 M sodium citrate/sodium phosphate buffer containing 0.05 M NaCl was used in the pH 3.5- 5.0 range. Samples were neutralized by adding an equal volume of 0.1 M sodium phosphate buffer containing 0.05 M NaCl pH 8.0. Fine adjustment to pH 7.4 was made by adding 1 M NaOH or 1 M HCl. The phosphate buffer also contained protease inhibitors i.e. 4 μM phenylmethylsulfonyl fluoride (PMSF) and 40 μM soybean trypsin inhibitor (SBTI, from Sigma).

Direct RIA of naturally occurring active renin and activated prorenin. The total concentration of immunoreactive renin, i.e. active renin plus prorenin, was determined with a solid phase sandwich assay[19,20], by using the monoclonal antibodies R 3-27-6 and R 3-36-16. Antibody R 3-36-16 was labeled with ^{125}I. Results are expressed as pg/ml using highly purified human kidney renin (Ciba-Geigy), specific activity 700 Goldblatt units per mg of protein, as the standard. The lower limit of detection was about 20 pg/ml.

The direct RIA of naturally occurring active renin and protease-activated prorenin was carried out by using the monoclonal antibodies 3E8 and 4G1. Antibody 4G1 was labeled with ^{125}I. Details of the procedure were described elsewhere[21]. Results are expressed as pg/ml using the same renin standard as in the assay of total immunoreactive renin. The lower limit of detection was 10 pg/ml.

Indirect RIA of naturally occurring active renin and activate prorenin. This assay depends on the generation of angiotensin I from purified sheep renin-substrate, as described previously[22]. The samples were incubated with substrate for ten time periods ranging from 0 to 24 min. Angiotensin I (ANG I) was quantitated by RIA. The slope of the linear part of the ANG I generation curve was used for calculating renin activity. Renin activity is expressed as pmol ANG I ml^{-1} min^{-1}. Concentrations of naturally occurring active renin and activated prorenin are expressed as μU/ml using the MRC human kidney renin standard 68/356 (Medical Research Council, National Institute for Biological Standards and Control, London ,UK) as a reference. One μU MRC renin standard corresponds with 1.4 pg Ciba-Geigy renin standard.

4.2.4. RESULTS

Characteristics of acid-activated prorenin. Dialysis of amniotic fluid for 48 h at pH 3.3 and 0-4 C causes conversion of prorenin, pR, into a form of active renin that is very similar, if not identical, to naturally occurring active renin[23–25]. Both the naturally occurring active form of renin and the form that is generated in amniotic fluid after 48 h dialysis at pH 3.3 we designate here as R. However, when in the present study amniotic fluid was acidified either by dialysis at pH 3.3 or by dilution in low pH buffer in the presence of 4 μM pepstatin (pepstatin A, Sigma), an inhibitor of aspartic proteases, an activated form of prorenin was generated that was clearly different from naturally occurring active renin. This form we designate here as acid-activated prorenin, pRa. pRa was also generated after acidification of purified prorenin isolated from amniotic fluid and plasma, both in the presence and absence of pepstatin.

pRa and R of either amniotic fluid or plasma were readily separated by column chromatography on Blue-Sepharose, because, at neutral pH and low sodium concentration (0.2 M NaCl), pRa was retained on this column, whereas R was not (Fig.1). pRa was eluted at 1.4 M NaCl. Purified prorenin activated by incubation for 48 h at pH 7.4 and 0 C with either trypsin (Sepharose-bound trypsin, 10 μM)[22], plasmin (0.5 μM) or plasma kallikrein (Mr 85,000, 0.12 μM) behaved on the column in the same way as R. Purified prorenin activated by incubation for 48 h at pH 3.3 and 0 C with pepsin (0.1 μM) was also not bound to the column.

Previous acid activation experiments with purified human kidney prorenin have demonstrated that pRa differs from R also in the fact that the enzymatic activity of pRa progessively falls at neutral pH[26]. This was also observed in our acid activation experiments with *purified* amniotic fluid prorenin (Table 1). After acid treatment the activated prorenin was almost completely inactivated at neutral pH within 2 h at 37 C and after 72 hours at 0 C. This treatment had no effect on naturally occurring active renin of amniotic fluid nor did it affect the enzymatic activity of protease-activated prorenin. Similar results were obtained with purified plasma prorenin. Acid activation experiments with *whole* amniotic fluid and plasma demonstrated that after 48 h pR was converted into R in amniotic fluid but into pRa in plasma. pRa was also formed when pepstatin had been added to amniotic fluid or in a mixture of amniotic fluid and plasma (Table 2). Thus, in whole amniotic fluid a pepsin-like enzyme, which is inhibited by pepstatin and by plasma protein(s), appears to be involved in the formation of R.

A third way to distinguish pRa from R was by direct radioimmunoassay with antibodies that reacted with naturally occurring active renin from human kidney and plasma but not with native prorenin (Tables 1 and 2). This radioimmunoassay detected naturally occurring active renin of human amniotic fluid and plasma. It also detected purified prorenin of both amniotic fluid and plasma after it had been activated by protease but it did not detect pRa. Both pRa and R

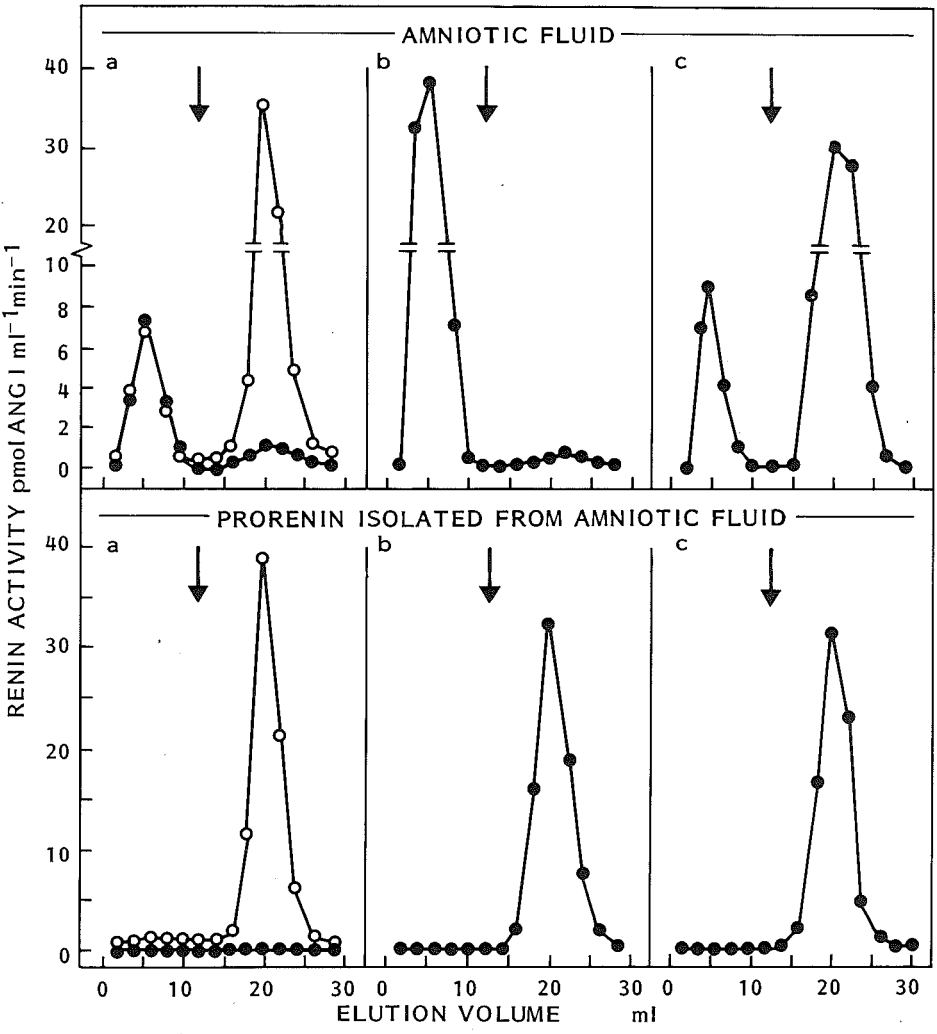

Fig. 4.2.1. Effects of various modes of treatment of prorenin (inactive) on its behavior during chromatography on Blue Sepharose CL-6B. a: no treatment; b: pH 3.3, 0 C,48 h, and chromatography immediately after adjustment of pH to 7.4; c: pH 3.3, 0 C, 48 h in the presence of 4 μM pepstatin, and chromatography immediately after adjustment of pH to 7.4. ●: assay of renin activity immediately after elution. O: assay of renin activity after treatment of the eluate with 0.5 μM plasmin, pH 7.4, 0 C, 48 h. Arrow: change to elution buffer containing 1.4 M NaCl. The first peak contains naturally occurring active renin (R) or proteolytically activated prorenin (R). The second peak contains native prorenin (pR) or non- proteolytically activated prorenin (pRa).

Table 4.2.1. Effect of various treatments on purified prorenin isolated from amniotic fluid and plasma

Treatment before assay	Indirect RIA active renin μU/ml		Direct RIA total renin pg/ml		Direct RIA active renin pg/ml	
	Amniotic fluid	Plasma	Amniotic fluid	Plasma	Amniotic fluid	Plasma
1 None	14	<1	16,270	270	<10	<10
2 Reversible activation-inactivation						
a) pH 3.3, 0 C, 48 h	9,400	400	14,900	280	<10	<10
b) as 2a in presence of pepstatin 4 μM	9,500	380	14,200	290	<10	<10
c) as 2a followed by pH 7.4,37 C,2 h	16	<1	15,400	280	<10	<10
d) as 2c followed by pH 3.3,0 C,48 h	8,900	380	15,300	260	<10	<10
3 Irreversible activation with aspartic protease						
a) pH 3.3, 0 C,48 h with pepsin 0.1 μM	9,200	410	15,900	290	15,200	270
b) as 3a followed by pH 7.4,37 C, 2 h	9,100	390	14,400	260	14,700	280
4 Irreversible activation with serine protease						
a) pH 7.4, 0 C, 48 h with plasmin 0.5 μM	10,200	400	16,100	270	15,200	270
b) as 4a followed by pH 7.4 , 37 C, 2 h,in presence of SBTI and PMSF	9,800	360	13,900	280	14,600	250
c) pH 7.4, 0 C, 48 h with plasma kallikrein (Mr 85,000) 0.5 μM	9,400	390	14,600	270	15,600	290
d) as 4c followed by pH 7.4, 37 C, 2h in presence of SBTI and PMSF	9,600	370	14,900	250	13,900	270

Indirect RIA: renin assay with angiotensin I antibodies, in which angiotensin I is generated from purified sheep renin substrate.
Direct RIA: renin assay with monoclonal renin antibodies.

were detected when using antibodies that reacted with naturally occurring active renin as well as with native prorenin.

Kinetics of acid-activation of prorenin. The acid-induced formation of pRa (pR → pRa) in purified preparations followed first-order kinetics. The observed rate constant (k*obs*) for pR → pRa conversion was not altered by dilution (Fig.2), indicating that the activation is a unimolecular process. Activation was not inhibited by pepstatin (Table 1), in a concentration that was high enough to block the action of pepsin (0.1 μM) on prorenin. k*obs* for the disappearance of pR during acid-treatment of amniotic fluid or plasma was not different from k*obs* in the purified preparations (Fig.2), which indicates that pR → pRa conversion also occurs in *whole* amniotic fluid and plasma.

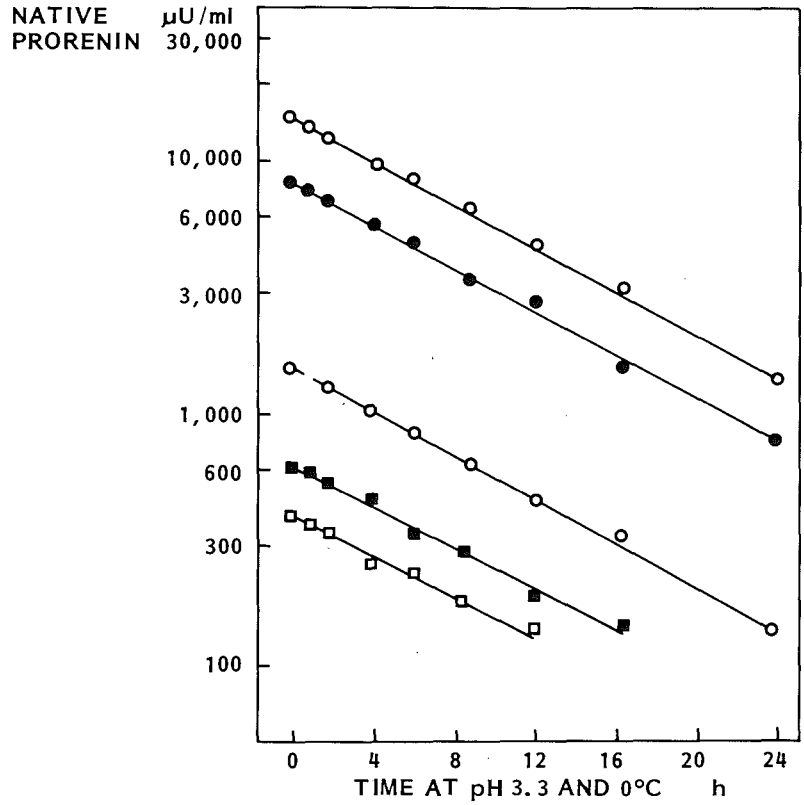

Fig. 4.2.2. Disappearance of prorenin (inactive) during acid-activation. Samples were brought to pH 3.3. At the time indicated, pH was restored to 7.4, and samples were immediately assayed for renin activity. Prorenin was determined as the difference between the renin activity after maximal activation of prorenin with 0.5 μM plasmin and the renin activity after acid-treatment. Results are expressed as microunits of the MRC kidney renin standard. Maximum activity after activation by plasmin was the same as after treatment at pH 3.3 and 0°C for 72 h. ●: amniotic fluid. ■: plasma. O: prorenin isolated from amniotic fluid, □: prorenin isolated from plasma.

A more detailed kinetic study on the acid-activation of prorenin at 0 C showed that k*obs* depends on the protonation of a polar group (or groups). For such a reaction k*obs* (h^{-1}) is given by the equation:

k*obs* = k . α, in which k = first-order rate constant of conversion of protonated species, α = fraction of molecules that has the polar group in the protonated form = $[H^+]$ (K + $[H^+]$), in which K = dissociation constant of group being protonated. Our measurements of k*obs* at different levels of pH (Fig.3) could be fitted in this model, giving a pK value of 3.4 (Fig.4).

The inactivation, at neutral pH, of pRa of amniotic fluid and plasma in purified preparations also followed first-order kinetics. The rate constant (k*obs*) at pH 7.4 and 37 C was 5.8 h^{-1} (mean of 3 experiments). It was independent of the initial concentration of pRa, which was equal to the concentrations of pR we had used in the acid-activation experiments shown in Fig.2. This is in accordance with a previously reported study on the inactivation of acid-activated human kidney prorenin[26]. Thus, there is good evidence that this is also a unimolecular process. Moreover, for prorenin of human kidney and plasma it has been demonstrated that both the activation at low pH and the subsequent inactivation at neutral pH are reversible processes, because acid-treated prorenin, after its inactivation at neutral pH, is fully reactivated when again exposed to low pH[5,27]. In the present study this was also found to be true for purified prorenin isolated from human amniotic fluid (Table 1).

Proteolytic conversion of acid-activated prorenin into renin. Our kinetic studies on the activation of prorenin in acidified amniotic fluid indicate that the activation proceeds in two steps (Figs.2-5). First, pRa is formed (pR → pRa), which is

Table 4.2.2. Effect of various treatments on prorenin in amniotic fluid and plasma.

Treatment before assay	Indirect RIA[a] active renin μU/ml			Direct RIA[b] active renin pg/ml		
	Amniotic fluid	Plasma	Amniotic fluid + plasma	Amniotic fluid	Plasma	Amniotic fluid + plasma
1. No treatment	480	22	260	680	32	360
2. Acid-treatment						
a) pH 3.3,0 C,48 h	8,100	240	4,160	11,300	44	440
b) as in 2a in presence of pepstatin 4 M	7,800			790		
c) as in 2a followed by pH 7.4, 37 C, 2h	7,900	34	320	10,700	37	420
d) as in 2b followed by pH 7.4, 37 C, 2h	530			720		

a Indirect RIA: renin assay with angiotensin I antibodies, in which angiotensin I is generated from purified sheep renin substrate.

b Direct RIA: renin assay with monoclonal renin antibodies.

then converted into active renin (pRa → R) by a pepsin-like enzyme. That pRa is an intermediary product is also indicated by the fact that it is converted by trypsin, plasmin or plasma kallikrein into a form of active renin that is very similar, if not identical, to naturally occurring active renin. The two active species, i.e. naturally occurring active renin and protease-treated pRa, behaved in the same way during chromatography on Blue-Sepharose. Both were not inactivated at neutral pH, and both were bound to antibodies that reacted with naturally occurring active renin and not with prorenin.

pRa is much more susceptible than native prorenin to proteolytic conversion

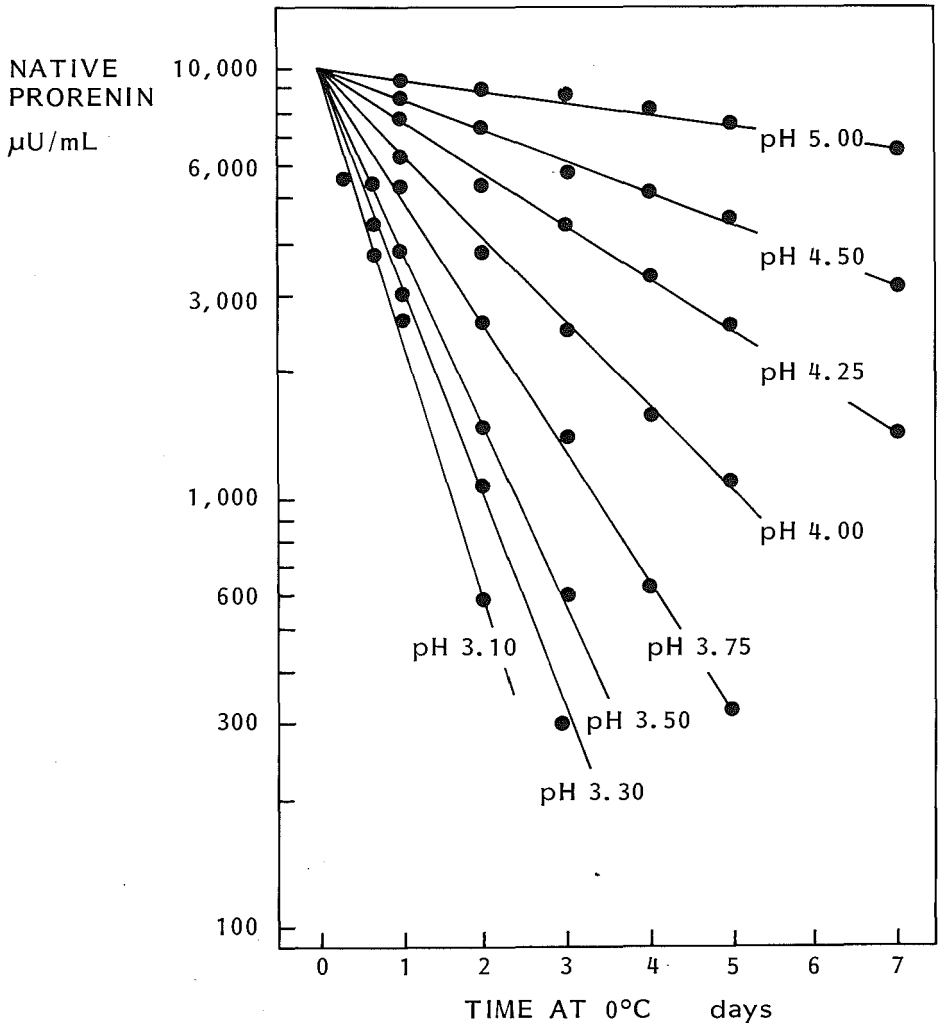

Fig. 4.2.3. pH-Dependence of kinetics of acid activation of prorenin (inactive) isolated from amniotic fluid. Prorenin was determined as described in the legends to Fig. 2. Disappearance of prorenin (pR — pRa) followed first-order kinetics. The observed rate constant (kobs) was highly pH-dependent. At pH <3.0 pR and/or pRa are destroyed.

Table 4.2.3. Kinetics of proteolytic conversion of native renin and the intermediairy acid-activated form of prorenin into active renin by serine proteases.

Serine Protease	kcat/Km M^{-1}. min^{-1}	
	Native prorenin	Acid-treated prorenin
Plasma kallikrein Mr 85,000	$1.2x10^5$	$52x10^5$
Plasma kallikrein (light chain) Mr 28,000	$0.7x10^5$	$48x10^5$
Plasmin	$1.2x10^5$	$78x10^5$
Trypsin	$18x10^5$	$960x10^5$

Concentration of serine protease ranged from 0.005 to 0.1 μM. Results are the means of 3 experiments.

into renin (Fig. 6). Conversion by either trypsin, plasmin or plasma kallikrein followed pseudo-first-order kinetics. The rate constant, which equals kcat/Km, was 50 to 70 times greater for pRa → R conversion than for pR → R conversion (Table 3). In contrast, kcat/Km for conversion of pRa that had been inactivated at neutral pH was not different from kcat/Km for conversion of native prorenin. Thrombin, urokinase and β-factor XIIa, in concentrations up to 0.1 μM, which is comparable to those we had used with the other serine proteases, had no effect neither on native prorenin nor on pRa. Figure 7 shows that pRa could be converted into renin by the intrinsic factor XII-kallikrein pathway even in whole blood. In this experiment purified amniotic fluid prorenin, after it had been treated at low pH, was added to blood at neutral pH. The factor XII-kallikrein pathway was then activated at 37 C by adding dextran sulfate (Mr 500,000) in a final concentration of 50 μg/ml[28]. This resulted in rapid pRa → R conversion.

4.2.5. DISCUSSION

This study confirms that prorenin is reversibly activated after acidification[5,6,26,27]. Our kinetic analysis indicates that the activation (pR → pRa) is an intramolecular process. It appears, therefore, that the activation does not depend on the interaction of pR with pRa or R (autoactivation) or with other enzymes. The process was not affected by pepstatin, indicating that the activation of prorenin was not caused by a contaminating aspartic protease. A previous study on the kinetics of inactivation of acid-activated prorenin at neutral pH showed that this is probably also an intramolecular process[26]. This implies that the inactivation (pRa → pR) is not caused by the interaction of pRa with an inhibitor.

The first-order rate constant for the acid-induced activation of prorenin is highly pH dependent. Our kinetic data suggest that the activation depends on

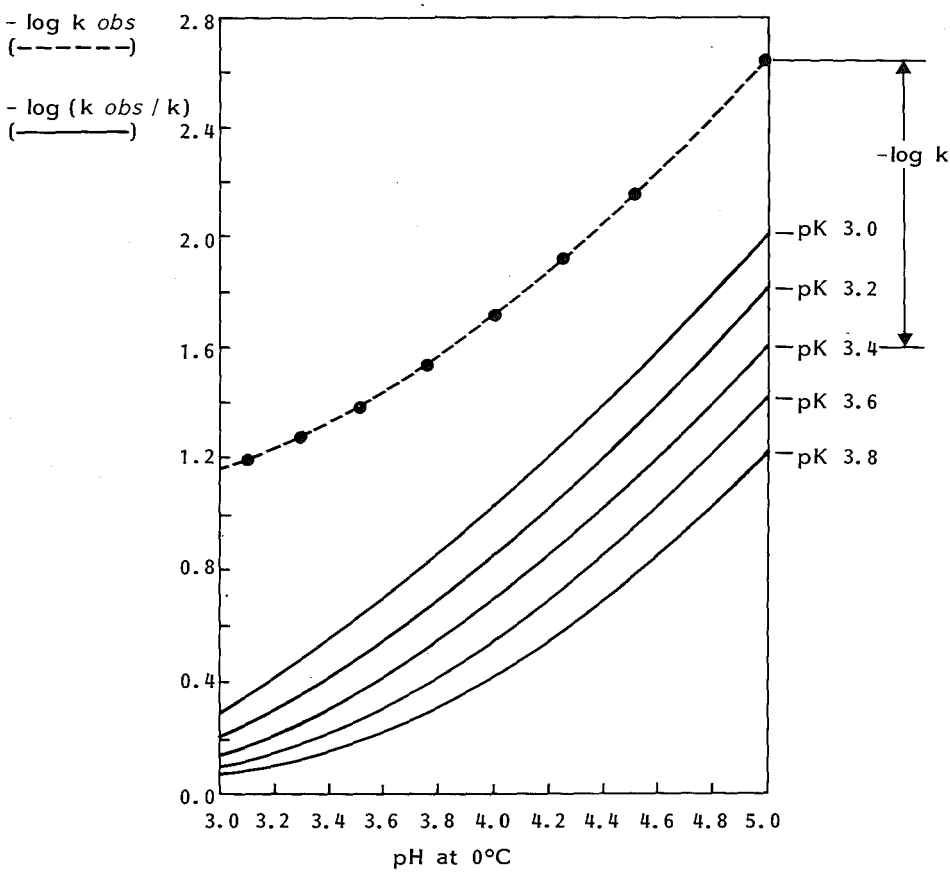

Fig. 4.2.4. pH-Dependence of kinetics of acid-activation of prorenin (inactive) isolated from amniotic fluid. Data from Fig.3 were used for calculating pK of the polar group (or groups) in prorenin that must be protonated for pR → pRa conversion to occur. Calculation was based on the the equation: k*obs* = k . α, in which k = first order rate constant of conversion of the protonated species, and α = fraction of molecules that has the polar group in the protonated form = $[H^+]/(K + [H^+])$ in which K = dissociation constant of the group being protonated. The equation can also be written as follows:

$$-\log (k_{obs}/k) = pH + \log(K + [H^+]).$$

The curves are constructed by computer. The *lower* group of curves describe -log(k*obs*/k) as a function of pH, each curve corresponding with a different value of pK. All curves that describe -log k*obs* as a function of pH and belong to the same pK are parallel. The uppur curve describes this function when pK = 3.4 and - log k = 1.03, so that k = 0.1 h^{-1}. Our data (●) showed a good fit with this curve.

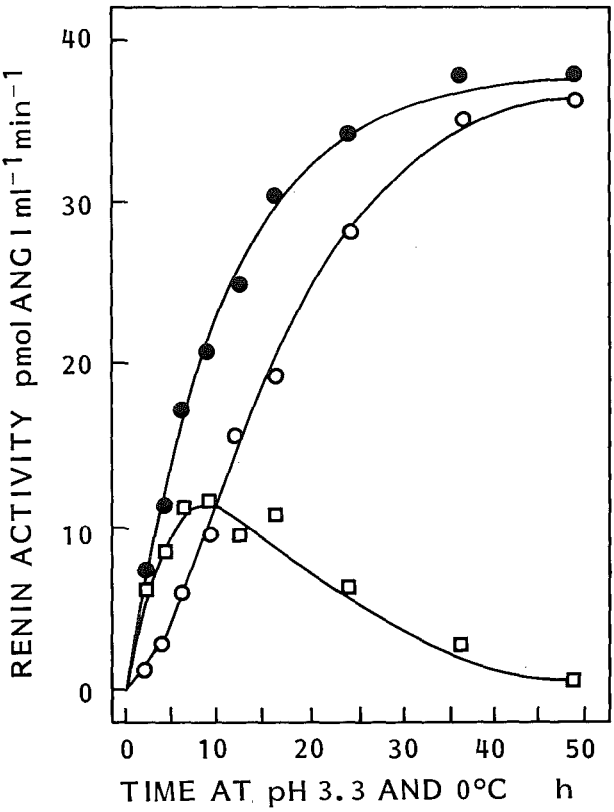

Fig. 4.2.5. Generation of an intermediary acid-activated form of prorenin in amniotic fluid. Amniotic fluid was kept at pH 3.3 and 0 C for up to 48 h. Renin activity was measured both immediately after restoration of pH to 7.4 and after incubation at pH 7.4 and 37 C for 2h. The immediate assay (●) measures both the intermediary reversibly acid-activated form of prorenin (pRa) and the irreversibly protease-activated form (R). The assay after 2h incubation at 37 C (O) measures only R. The difference between the results of the two assays (□) is therefore a measure of pRa. Results are the means of 6 experiments. The lines are computer-constructed according to the following reaction model:

$$R \xleftarrow{k3} pR \xrightarrow{k1} pRa \xrightarrow{k2} R,$$

in which k1, k2 and k3 are first-order rate constants with values of 0.095 h^{-1}, 0.130 h^{-1} and 0.002 h^{-1} respectively.

the protonation of one or more polar groups with a pK value of 3.4, which is followed by a conformational change that exposes the active site of the enzyme molecule. This is analogous to the effect of low pH on pepsinogen. Interactions between positively charged amino acid residues of the 'pro-part' polypeptide chain (activation peptide) and negatively charged residues of the 'enzyme-part'

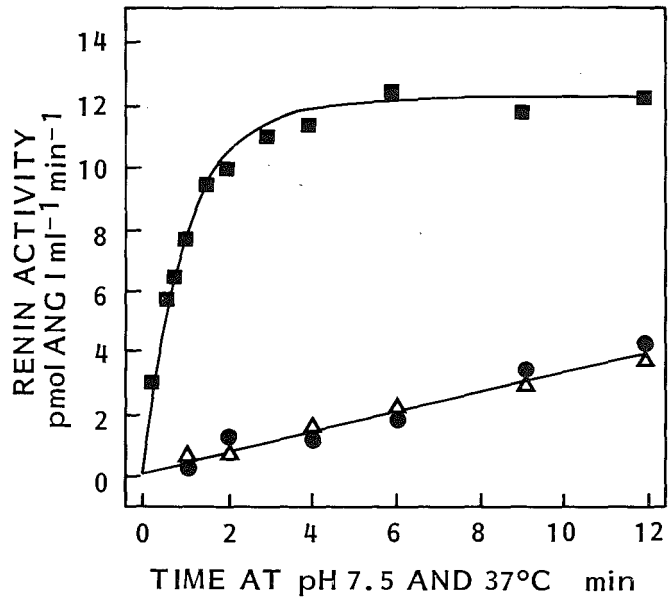

Fig. 4.2.6. Kinetics of proteolytic conversion of native prorenin (pR) and the intermediary acid-activated form of prorenin (pRa) into active renin (R). Prorenin isolated from amniotic fluid was used, and pRa was generated by incubation at pH 3.3 and 0 C for 72h. Generation of R from pR or pRa occurred during incubation with 0.01 μM trypsin (not immobilized) at pH 7.4 and 37 C.The reaction was stopped by 40 μM SBTI and 4 μM PMSF. Renin activity was measured after incubation at pH 7.4 and 37 C for 2h. This assay measures R but not pRa. : generation of R from pRa. : generation of R from pRa that had been converted into pR by incubation at pH 7.4 and 37 C for 2h. : generation of R from native pR. Results are the means of 3 experiments. The lines are constructed by computer according to the following reaction model:

$$R \xleftarrow{k2} pR \xrightarrow{k1} pRa \xrightarrow{k3} R,$$

in which k1, k2 and k3 are first-order rate constants with values of 0.095 min^{-1}, 0.980 min^{-1} and 0.024 min^{-1} respectively.

are thought to stabilize the pepsinogen molecule in its inactive form[29,30]. By protonation of negatively charged amino acid residues at low pH, the bonds are broken and the pro-part moves away from the bulk of the molecule, thereby uncovering the active site of the 'enzyme-part'. In the case of acid-activated prorenin, however, the molecule has little or no catalytic activity at low pH, despite the fact that its active site is exposed. This is because of the high pK-value of the carboxyl groups of the aspartic acid residues (Asp-38 and Asp-226, sequence numbering from Ref 31 and 32) in the active site of renin, as compared with the pK-value of the corresponding aspartic acid residues in pepsin (Asp-32 and Asp-215)[32]. The pH must be above 5.0 for Asp 38 in renin to be ionized ,which is a prerequisite for renin's catalytic activity.

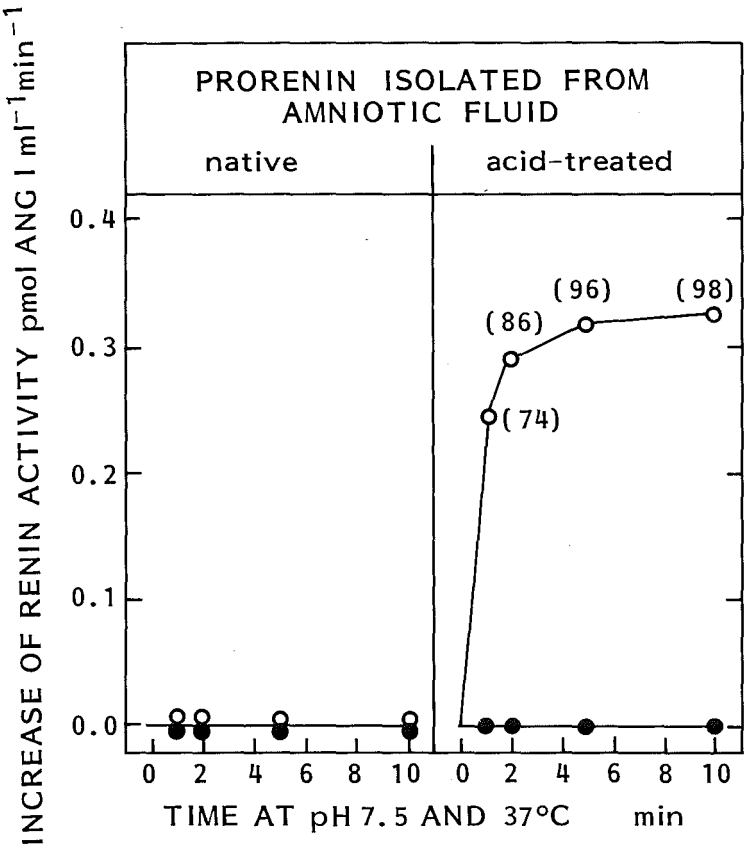

Fig. 4.2.7. Proteolytic conversion of native prorenin (pR) and of the intermediary acid-activated form of prorenin (pRa) into active renin (R) by the factor XII-kallikrein contact activation system in whole blood. Prorenin isolated from amniotic fluid was used, and pRa was generated by incubation at pH 3.3 and 0 C for 72h. After restoration of pH to 7.4, 50 μl pR or pRa (200 pg) was added to 1 ml of fresh, citrated, blood at 37 C. Immediately thereafter 50 l dextran sulfate (Mr 500,000) was added in a final concentration of 50 μg/ml, by which the factor XII-kallikrein pathway is activated. The mixture was incubated at 37 C for up to 10 min. The reaction was stopped by 40 μM SBTI and 4 μM PMSF. Renin activity was measured after incubation at pH 7.4 and 37 C for 2h. This assay measures R but not pRa. R was also measured by direct RIA using antibodies specific for active renin (figures in parentheses). O: with dextran sulfate. ●: with H_2O instead of dextran sulfate.

It has been reported that semipurified prorenin isolated from amniotic fluid is bound to the Cibacron Blue F3GA dye after acid-activation but not after activation by pepsin. We assume that this acid-activated form of prorenin is identical with pRa. Its affinity for Cibacron Blue F3 GA and other dye ligands, such as Procion Red HE3B (Matrix-gel red A, Amicon group, Lexington, MA, USA) (unpublished observations), can be used for separating it from naturally

occurring active renin and also from protease-activated prorenin. Our studies show that the presence of the pro part in pRa interferes, in the direct radioimmunoassay with the binding to antibodies that reacted with naturally occurring active renin and not with prorenin. This too can be used to distinguish pRa from naturally occurring active renin and protease-activated prorenin.

Activation of prorenin is a two-step process (Fig.8). The first step (pR → pRa) is reversible. The second step (pRa → R) is proteolytic. In amniotic fluid both steps occur at low pH, the second step being caused by an endogenous aspartic protease. The action of this protease was inhibited by pepstatin and also by contaminating plasma proteins. In plasma the pR → pRa step also occurs at low pH but the pRa → R step occurs after restoration of pH to neutral, and is initiated by the intrinsic serine protease factor XII-kallikrein pathway[1,2,4]. Franks et al.[34] reported that the activation of prorenin after overnight dialysis of amniotic fluid against pH 3.3 buffer at 4 C was completely reversible. In our study about half of the activated prorenin was in the pRa form after 12-h dialysis at pH 3.3. Franks et al.[34] used concentrated amniotic fluid and it is therefore possible that in their experiments the pRa → R step was inhibited by contaminating plasma protein(s).

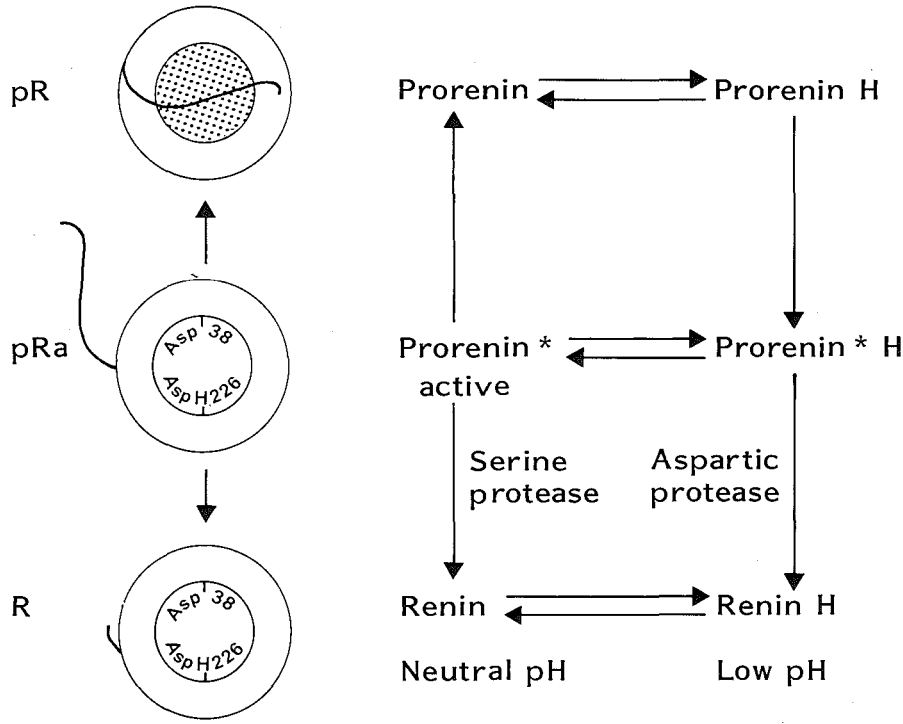

Fig. 4.2.8. Reaction steps involved in prorenin-renin conversion. H: protonated species. *: species with active site exposed. Asp 38 and Asp 226 are the two aspartic acid residues in the active site. Asp 226 but not Asp 38 must be protonated for rendering the molecule enzymatically active.

Our study supports the conclusion that the molecular conformation of prorenin is changed by acidification in such a way that the molecule becomes highly susceptible to proteolytic conversion into renin. It appears, therefore, that the acid-induced exposure of the catalytic site of prorenin, which causes *reversible* activation (pR → pRa), coincides with the exposure of cleavage sites susceptible to attack by aspartic and serine proteases that cause *irreversible* activation (pRa → R). One or more of these cleavage sites are so susceptible to proteolytic attack by serine protease that the intrinsic factor XII- kallikrein pathway of plasma is capable of generating renin at physiological pH and physiological temperature even in whole blood with its abundance of serine protease inhibitors.

Further studies are needed to answer the question of whether the effect of acid *in vitro* is only a test tube phenomenon or whether this has a physiological correlate. Given the low pH of secretory granules[35,36], one might speculate that renin is present in these granules in a form corresponding with the pRa form produced by acidification *in vitro* and that pRa is irreversibly converted into R by a protease before it is released into the circulation. It is also possible that the proteolytic activation of pRa occurs during its release, as soon as it enters a milieu where it is rapidly activated by a serine protease. Circulating prorenin might be taken up into endocytotic vesicles of the vascular endothelium. The formation of these vesicles is known to be followed by rapid acidification of its content to a pH as low as 5[37–41]. pH may be lower at sites near the proton pump at the inner side of the vesicle membrane. After its uptake prorenin might undergo the acid-induced conformational change, while it is transported by the vesicles to the luminal or contraluminal side of the endothelial cell. It might then be released into a milieu where it is rapidly activated by a serine protease.

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5. PHYSIOLOGICAL REGULATION OF PLASMA PRORENIN

5.1. RELEASE OF ACTIVE AND INACTIVE RENIN BY THE PORCINE KIDNEY

5.1.1. ABSTRACT

We studied the relative rates of release of active and inactive renin by the kidney in anesthetized pigs. Renin concentration was determined in arterial and renal venous plasma as follows: 1) before and after stimulation of renin release with isoproterenol or furosemide, 2) after suppression of renin release by extracellular fluid volume expansion, and 3) after administration of propranolol or indomethacin. Inactive renin was activated by dialysis of plasma at pH 3.3 for 24 hours. Renin concentration was estimated by radioimmunoassay determination of angiotensin I after a 3-hour incubation with excess homologous renin substrate. Following isoproterenol, the release of active renin increased from 8 ± 4 (SEM) to 58 ± 34 ng/min, and inactive renin increased from 53 ± 33 to 321 ± 136 ng/min. Similarly, furosemide stimulated the release of both active and inactive renin. Both forms of renin were suppressed by propranolol or indomethacin. Although changes in renin release following volume expansion were not statistically significant, the direction of change for both forms of renin was similar. Following logarithmic conversion of the rate of release, the plot of active vs. inactive renin formed a straight line. Values for active renin as a percentage of the total renin in simultaneously drawn arterial and renal venous plasma samples were not different. Thus, under the conditions of these experiments, release of active and inactive renin appears to be controlled by similar mechanisms. Both stimulation and suppression of renin result in parallel changes in release of the two forms. Data on relative amounts of active renin in arterial and renal venous plasma suggest that there is no systemic conversion of the two forms.

5.1.2. INTRODUCTION

Recent reports suggest the presence of an inactive form of renin in plasma and in renal tissue of both normotensive and hypertensive patients[1–4]. Studies from several laboratories have centered on that form of renin which is inactive at physiological pH but activated by treatment at acid pH[2,3]. This acid-activated or inactive renin has also been reported to be present in several species in addition

to man[5–10]. Whether inactive renin represents a true proenzyme or renin bound to an inhibitor remains unclear[11]. It also has been suggested that inactive renin may be the intrarenal storage form of the enzyme[8].

Acid-activated renin has been measured in systemic and renal venous plasma of man under varying conditions[2,3]. These data suggest that changes in the concentration of active and inactive renin in plasma may be dissociated under certain circumstances. However, no data are available relating the release of active and inactive renin in experimental animals. Therefore, studies were carried out to determine the rate of release of both forms of renin by the porcine kidney. The pig was selected as the experimental animal because acid-activated renin has been isolated from its kidney[5,6]. In addition, inactive renin has been reported to be released by the isolated perfused pig kidney[5]. To correlate the rate of release of active and inactive renin, renin release was stimulated with either isoproterenol or furosemide, and suppressed by volume expansion, propranolol, or indomethacin. By varying widely the rate of release of renin, we could better observe the relationship between release of the active and inactive forms.

5.1.3. METHODS

Surgical Procedures Experiments were carried out on pigs 45-57 days old weighing 3.0-13.5 kg. These pigs were small enough to study easily but still had mature renal function[12]. Anesthesia was induced with intraperitoneal pentobarbital sodium (35 mg/kg). Additional anesthetic was given during the experiment as required. The pigs were intubated and ventilated (Loosko Amsterdam Infant Ventilator) monitored and body temperature maintained with a heating pad and radiant heat lamp. A catheter was placed in the femoral artery to obtain arterial blood samples and to measure blood pressure using a strain gauge transducer. A catheter was placed in the external jugular vein for infusion of saline and drugs. A second catheter was placed in the bladder via a small midline abdominal incision.

The left kidney was exposed through a retroperitoneal flank incision and the left ureter cannulated. The renal artery and vein were carefully dissected free from the surrounding tissue, and a noncannulating electromagnetic flowmeter probe (Transflow 500 Electromagnetic Bloodflow Meter) was placed on the renal artery. A curved 22- gauge needle attached to a polyethylene catheter was inserted into the renal vein to obtain renal venous blood samples. A curved 27-gauge needle attached to a polyethylene catheter was inserted into the renal artery for infusion of saline or isoproterenol. A period of 45-60 minutes was allowed for stabilization following completion of surgery.

Experimental Protocols

Effects of Isoproterenol, Furosemide, Propranolol and Indomethacin. The following protocol was observed in six pigs. During a control period, two sets of arterial and renal venous blood samples were obtained 5 minutes apart. An infusion

of a solution of isoproterenol (1.0 μg/ml per kg) was started into the renal artery at a rate of 0.1 ml/min with a Braun infusion pump. The infusion rate was increased gradually until renal blood flow increased. After 5-7 minutes two more sets of arterial and renal venous blood samples were obtained 5 minutes apart. The infusion of isoproterenol then was stopped, and following a 45-60 minute recovery period a third collection of urine and two more blood samples were obtained. Following recovery from the isoproterenol, the pigs were given furosemide, 1 mg/kg,iv. As urine flow increased, isotonic saline containing KCL (4.5 mM) was infused into the jugular vein at a rate equal to urinary output. Ten minutes after the diuretic was given, two sets of arterial and renal venous blood samples were collected 5 minutes apart. A second injection of furosemide, 2 mg/kg, was given and the sampling repeated.

The effect of two inhibitors of renin secretion then was determined. Immediately after sampling, following the second dose of furosemide, propranolol, 1 mg/kg, was given intravenously, and after 10 minutes two sets of blood samples were obtained. Indomethacin, 2 mg/kg was given intravenously and a final set of blood samples obtained.

Effects of Volume Expansion, Furosemide, and Indomethacin In five pigs the response to volume expansion and furosemide was determined. After control blood samples were obtained, the pigs were given isotonic saline equivalent to 5% body weight, infused over 30 minutes. The infusion rate then was adjusted to equal urine flow rate. When an apparent steady state urine output was reached, two sets of arterial and renal venous blood samples were drawn 5 minutes apart. The pigs were then given furosemide, 2 mg/kg per hr, as a constant intravenous infusion and the saline infusion increased to match urine flow rate. When urinary output had stabilized, blood samples again were obtained. The pigs then received indomethacin, 2 mg/kg, and a final set of samples was drawn.

Analytical Techniques and Data Handling.

All blood samples were collected in tubes chilled on ice and containing disodium ethylenediaminetetraacetic acid (EDTA 5mM) . Blood samples were centrifuged within 5 minutes of collection and the plasma removed. The red blood cells then were reconstituted to the original hematocrit in saline and returned to the pig. Plasma for renin determinations was stored at -20 C until assayed. Hematocrit was determined by the micro method.

Renin in arterial and renal venous plasma was estimated as described by Derkx et al[2]. Plasma was dialyzed at either pH 4.5 or 3.3 for 24 hours at 4 C and then returned to pH 7.4 by dialysis for another 24 hours. After dialysis, plasma was incubated with excess homologous substrate, and the angiotensin I generated was determined by radioimmunoassay. The renin concentration dialyzed at pH 4.5 was active renin. Following dialysis at pH 3.3, the concentration of renin was greater, representing the sum of active renin and acid-activated renin, i.e.,total renin. Inactive renin was calculated as the difference between total renin and active renin. Renin substrate was prepared according to the method of Skinner[13].

The substrate was found to be free of renin and angiotensinase activity. Incubation of substrate with renin resulted in zero order kinitics. The concentration of renin in plasma was expressed as nanograms of angiotensin I generated per milliliter per hour (ng/ml/h). Renin release was calculated as the product of the venous-arterial difference and the renal plasma flow. Mean arterial blood pressure, heart rate, and mean renal blood flow were taken directly from digital readout.

Statistical analysis was carried out using analysis of variance and t-test for paired and unpaired comparisons. The level of significance was set 5 %. Because significant heterogeneity of variance in renin release existed, all data were converted to logarithms prior to statistical analysis.

5.1.4. RESULTS

Effects of isoproterenol, furosemide, propranolol, and indomethacin.

The release of total renin increased following intrarenal arterial infusion of isoproterenol (Fig.1). This increase in the release of total renin was due to an increase in the rate of release of both active and inactive renin. During perfusion of isoproterenol renal blood flow increased from 73 ± 10 to 94 ± 6 ml/min,

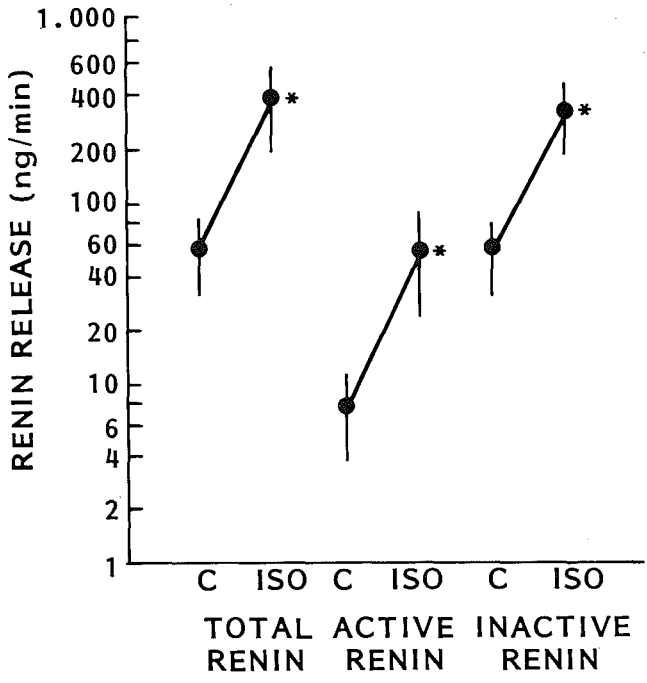

Fig.5.1.1. Effects of isoproterenol on the release of total, active, and inactive renin. C = control; ISO = isoproterenol; n = 5. *Significantly different from control ($p < 0.05$).

whereas mean systemic blood pressure decreased from 114 ± 3 to 107 ± 4 mm Hg. Both changes were significant.

Following the first dose of furosemide (1 mg/kg) there was an increase in the release of total renin from the kidney (Fig.2). As with isoproterenol, the increase was due to to changes in the release of both active and inactive renin. Although renal blood flow increased in each pig, the change from 87 ± 8 to 95 ± 9 ml/min was not statistically significant. No consistent changes in mean arterial pressure were noted.

The second injection of furosemide (2 mg/kg) did not result in any further increase in the release of total, active, or inactive renin (Fig.2). No significant changes in hemodynamics were noted, although blood flow averaged 102 ± 9 ml/min after the second dose of diuretic.

The systemic administration of propranolol resulted in a significant decrease in the release of total renin (Fig.3). This change again was accompanied by

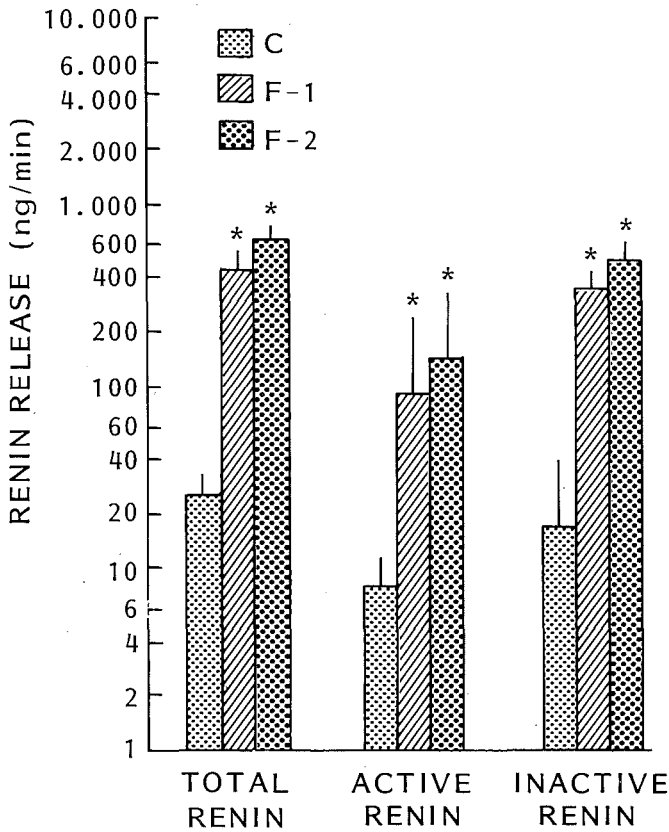

Fig.5.1.2. Effects of furosemide on the release of total, active, and inactive renin. C = control; F-1 = furosemide, 1 mg/kg; F-2 = furosemide, 2 mg/kg; n = 5. *Significantly different from control ($p < 0.05$).

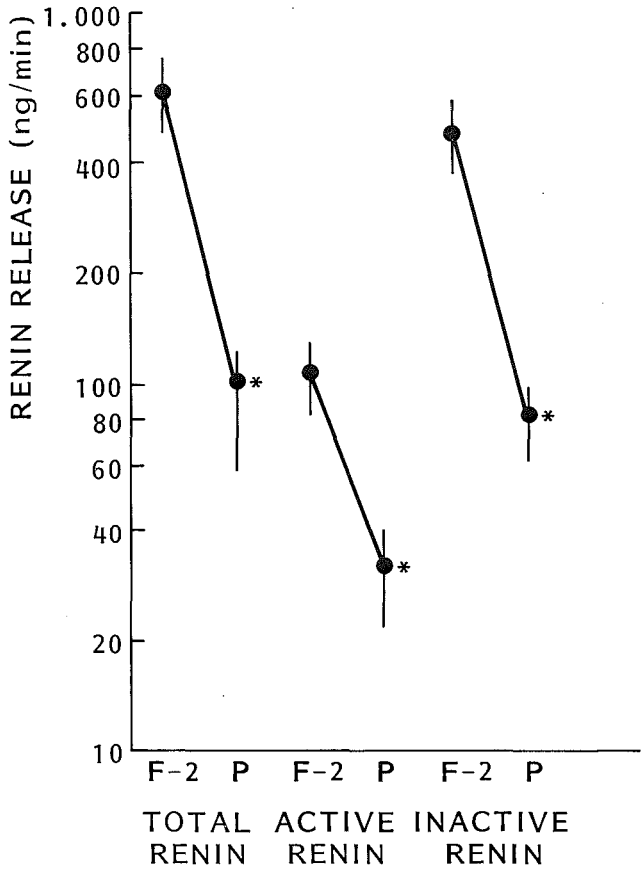

Fig.5.1.3. Effects of propranolol on the release of total, active, and inactive renin. F-2 = furosemide, 2 mg/kg; P = propranolol, 1 mg/kg; n = 5. *Significantly different from F-2.($p < 0.05$).

a significant change in both the active and inactive forms of the enzyme.

The administration of indomethacin produced a further fall in the rate of release in total renin from 104 ± 47 to 37 ± 20 ng/min. Active renin decreased from 32 ± 9 to 9 ± 3 ng/min, and inactive renin decreased from 74 ± 47 to 39 ± 15. Although these changes were not statistically significant, they were seen in five of the six pigs. The effects of isoproterenol, furosemide, propranolol, and indomethacin on renal blood flow, and the concentration of renin in renal venous plasma are shown in Table 1. Although each drug tended to cause a change in blood flow, the concentration of renin in renal venous plasma increased with isoproterenol and furosemide and decreased with propranolol and indomethacin.

Table 5.1.1. Renal blood flow (RBF) and renal venous renin (RVR) concentration in pigs during control periods (C) and following isoproterenol (ISO), furosemide (F), propranolol (P), and indomethacin (IM).

	C	ISO	C	F-1	F-2	P	IM
RBF	73	94	87	95	102	85	75
(ml/min)	(10)	(6)	(8)	(9)	(9)	(8)	(7)
T-RVR	4.8	12.3	4.2	14.1	19.2	9.2	4.4
(ng/ml)	(0.4)	(4.9)	(1.0)	(3.0)	(3.6)	(1.4)	(5.9)
A-RVR	0.8	1.9	0.8	2.0	3.3	1.6	0.8
(ng/ml)	(0.1)	(0.8)	(0.2)	(0.5)	(0.8)	(0.3)	(0.2)
I-RVR	4.1	10.3	3.4	12.0	14.9	7.6	3.6
(ng/ml)	(0.3)	(3.7)	(0.8)	(2.8)	(3.4)	(1.3)	(0.5)

Values are mean (SEM in parentheses); n = 5. F-1 = furosemide 1 mg/kg, F-2 = furosemide 2 mg/kg: T = total renin; A = active renin; I = inactive renin.

Effects of volume expansion, furosemide, and indomethacin.

In this group of pigs, the treatments resulted in changes in release of total, active, and inactive renin (Table 2). However, because of the wide variation among animals, the changes were not statistically significant.

Table 5.1.2. Renin release (ng/min) under control conditions and following saline volume expansion, furosemide, and propranolol.

	Control	Saline	Furose-mide	Propra-nolol
Total	121	77	1035	621
	(57)	(42)	(664)	(342)
Active	23	14	190	47
	(8)	(8)	(87)	(47)
Inactive	98	57	937	574
	(44)	(38)	(538)	(334)

Values are mean (SEM in parentheses); n = 5.

Relation of release of active and inactive renin

When the logarithm of the rate of release of active and inactive renin was plotted, the relative proportions of the two forms of the enzyme remained constant (Figs.4 and 5). This relationship appears to hold regardless of the treatment. This finding is supported by the data demonstrating that the relative concentration of active renin in either arterial or renal venous plasma is not changed by treatment (Table 3). In addition, there was no significant difference between the relative concentration of active and inactive renin in arterial and renal venous blood (Table 3).

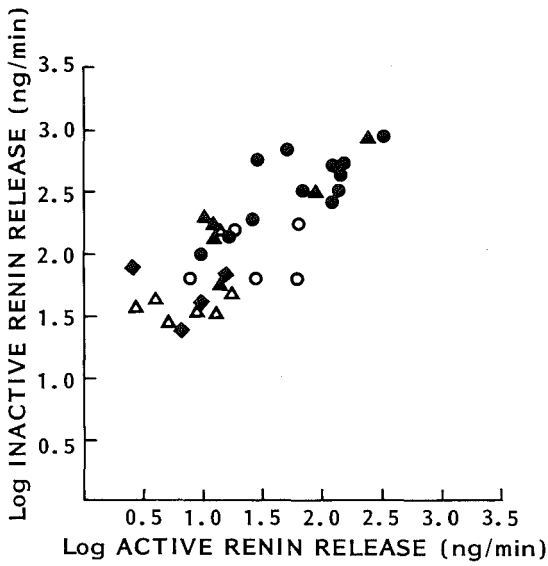

Fig.5.1.4. The relation between the release of active and inactive renin in pigs during control periods (Δ) and receiving isoproterenol (Δ), furosemide (●), propranolol (O), and indomethacin (◆). Correlation coefficient = 0.82;n = 5.

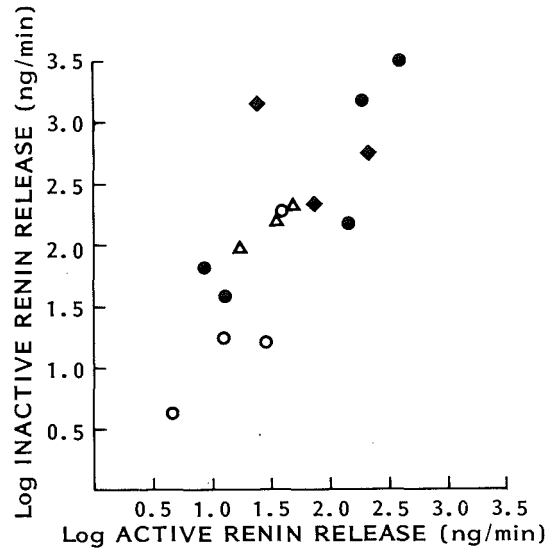

Fig.5.1.5.The relation between the release of active renin and inactive renin in pigs during control periods (Δ) and treated with saline volume expansion (O), furosemide (●), and indomethacin (◆). Correlation coefficient = 0.77; n = 5.

Table 5.1.3. Percent active renin in arterial and renal venous plasma.

	Control	Isopro-terenol	Furo-semide	Propra-nolol
Arterial	19.6	16.6	16.1	18.4
	(3.9)	(2.5)	(2.5)	(5.3)
Renal venous	16.7	14.4	17.4	19.5
	(0.9)	(1.7)	(2.3)	(2.6)

Values are mean (SEM in parentheses); n = 5.

5.1.5. DISCUSSION

An acid-activated form of the enzyme renin has been demonstrated in kidneys of several animal species including the pig[5,6,7,8,10]. The studies of Leckie and McConnell (1975) suggest that inactive renin may be coupled to an inhibitor, which is destroyed by acidification. Most studies on experimental animals to date have concentrated on the estimation of tissue renin content, although James and Hall (1974) did measure acid-activated renin in dog plasma.

Boyd (1974) has demonstrated that porcine kidney contains at least two forms of renin. Renin A had a molecular weight of 40,000 and was more active than renin B, which had a molecular weight of 60,000. Renin B could be converted to renin A by acidification. Furthermore, he demonstrated stimulation of release of renin A and B in response to isoproterenol in the isolated perfused kidney. Therefore, it appeared reasonable to carry out experiments in the anesthetized pig.

In the present studies, the plasma concentration and release by the kidney of two forms of renin were determined. Several authors have demonstrated that renin is activated by dialysis of human plasma at pH 3.3[2,3], and by dialysis of renin extracted from pig and rabbit kidneys at similar pH[5,6,7]. In the studies reported here, a comparison of plasma renin concentration at pH values above 4.5 to concentrations at 4.5 and 3.3 was not made. However, in view of the reported data and the low concentration of active renin in renal venous plasma under all conditions (Table 1), it is unlikely that there was any significant activation at pH 4.5 in these studies.

Stimulation of renin release from the intact pig kidney resulted in increases in both active and inactive forms of the enzyme (Fig.1 and 2), whereas suppression of release caused both to decrease (Fig.3). Determination of renin release over a wide range of values indicates a consistent relationship between the release of active and inactive renin under the conditions of these studies(Figs.4 and 5). Furthermore, the change in concentrations of both active and inactive renin in renal venous plasma indicates that the effects of the drugs on renin release were at least partially independent of renal blood flow (Table 1).

The plasma concentration of active and inactive renin has been determined under varying conditions in man. Both Derkx et al. (1976) and Weinberger

et al. (1977) found that acute stimulation or suppression of renin release could result in opposite changes in the active and inactive forms. Weinberger et al. (1977) reported that, in normal subjects, saline infusion suppressed active but not inactive renin, whereas furosemide caused both to increase, although the relative increase inactive renin was greater. Derkx et al. (1976) reported that isoproterenol increased active but not inactive renin concentration in plasma, and propranolol suppressed active but elevated inactive renin. These authors also found that the plasma concentrations of active and inactive renin were directly related.

There are several possible explanations for the differences between the present results and those reported for man. First, studies were carried out in anesthetized animals. Whereas there is no evidence that anesthesia will qualitatively modify the response of renin release to the drugs used, anesthesia cannot be eliminated as a cause for the differences. Second, although there may be species differences, available data do not suggest significant qualitative differences in the control of renin release in man and in other animal species.

Third, it has been suggested that inactive renin may represent a storage form of the enzyme[8]. Therefore, the rate of release of either active or inactive renin could be related to the state of intrarenal synthesis and storage of renin in an inactive form and to the activity of mechanisms for conversion of inactive to active renin in the kidney. Such a mechanism is suggested by the differences in concentration of active and inactive renin in renal venous plasma from stenotic and nonstenotic kidneys and peripheral venous plasma[3]. The nature of the mechanisms which could possibly lead to interconversion of the two forms of renin are unknown. The amount of intrarenal renin present in the the inactive form may be related to a balance between synthesis and release[8]. However, the reason that the inactive form is released and is not all converted first to active renin is unknown. Further physiological and biochemical studies will be required to clarify these issues specially.

A fourth possible source for the differences between the results of these studies and those obtained for man may be related to the interconvension of active and inactive renin in plasma and/or the relative rates of metabolism of the two forms of the enzyme. The failure to find any change in the relative concentration of active renin in simultaneously obtained arterial and renal venous plasma samples (Table 2) suggests that there is no major interconversion of the two forms in plasma. However, changes could be masked by differences in the rate of catabolism of the two forms of the enzyme.

In the present studies the biochemical differences in the forms of renin were not determined. Although it has been demonstrated by several authors that acidification appears to activate an inactive form of renin[2,3,5,7], there is still confusion concerning the molecular nature of the product. Until further biochemical clarification is available, the possibility remains that the activated enzyme may not be renin but may act nonspecifically on angiotensinogen to form angiotensin I[14].

We conclude from these studies that both the active and acid- activated form of inactive renin are released from the kidney of the pig. The mechanisms controlling release of both forms of the enzyme in this animal model appear to be similar. The physiological significance of inactive renin remains obscure, and additional studies will be required to clarify its importance in regulation of the renin-angiotensin system.

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5.2 RELEASE OF ACTIVE AND INACTIVE RENIN BY THE PIG KIDNEY DURING DEVELOPMENT

5.2.1. ABSTRACT

The release of both active and acid-activatable (inactive) renin was determined in piglets ranging in age from 3 to 53 days. The concentration of active renin in arterial plasma during control periods decreased from 4.4 ± 1.1 ng ANG I/ml/h in 3-5 day-old animals to 0.7 ± 0.1 ng ANG I/ml/h in 42-53 day-old animals, while the rate of release of active renin was unchanged (11.8 ± 5.1 ng/min and 7.5 ± 3.4 ng/min,respectively). Both isoproterenol and furosemide stimulated release of renin in all age animals while propranolol supressed renin release. The data indicate that the qualitative control of renin release is similar in animals of various ages and stages of development. However, the differences in the concentration of renin in plasma of newborn and more mature animals may depend on factors other than release of renin by the kidney. Newborn animals release both active and inactive renin from the kidney.

5.2.2. INTRODUCTION

Although plasma renin activity and angiotensin II concentration have been reported to be higher in fetal and newborn than in adult animals and humans[9,11,12,17], recent studies suggest that control of the release of renin from the kidney is qualitatively similar in animals of different ages[15,17]. The elevated plasma renin activity of younger animals could be due to several factors including: (a) a more rapid rate of release of renin from the newborn kidney; (b) a smaller volume of distribution of renin in the extracellular space in younger animals; (c) a more rapid rate of catabolism of renin in the mature animals, and (d) a change in the form of renin from a more to a less active state in the mature animal. While changes in plasma renin activity with maturation have been reported, none of the studies have actually estimated the rate of release of renin by the kidney in the same animals. In addition, in studies on the control of renin release the actual rate of release from the kidneys was not estimated. Changes in release were inferred from changes in plasma renin activity[15,17].

The present studies were carried out in anesthetized piglets during the first 8 weeks of life in order to further define the control of renin release and plasma renin activity during maturation. In addition to the measurement of the concentration of renin in plasma and the rate of release of renin by the kidney, the plasma concentration and release of an inactive form of the enzyme was also determined. Observations were made under control conditions, following stimulation of renin release with isoproterenol or furosemide and suppression of renin release with propranolol. The results suggest that factors other than the rate of release of renin from the kidney may be important in the regulation of plasma renin concentration in newborn animals. The data also indicate, as

previously suggested[15,17], that control of renin release is qualitatively similar in animals of various ages.

5.2.3. METHODS

Surgical procedures.

Experiments were carried out on pigs divided into three age groups: 3-5, 19-23 and 42-53 days. The younger animals were taken from the sow 1h prior to starting the experiment. Anesthesia was induced with intraperitoneal pentobarbital sodium (25 mg/kg in 3-5-day-old animals and 35 mg/kg in older animals). Additional anesthetic was given during the experiment as required. The animals were intubated and ventilated (Loosko Amsterdam Infant Ventilator). Rectal temperature was monitored and body temperature maintained at 37 C with a heating pad and radiant heat lamp. A catheter was placed in the femoral artery to obtain arterial blood samples and to measure blood pressure using a strain-gauge pressure transducer. A second catheter was placed in the femoral vein for infusion of a solution of inulin (3g/dl) at 0.05 ml/min/kg resulting in plasma concentrations of inulin of 25-35 mg/dl. A third catheter was placed in the external jugular vein for infusion of saline and drugs, and a fourth placed in the bladder via a small midline abdominal incision.

The left kidney was exposed through a retroperitoneal flank incision and the left ureter cannulated. The renal artery and vein were carefully dissected free from the surrounding tissue and a noncannulating electromagnetic flowmeter probe (Transflow 500 Electromagnetic Bloodflow Meter) placed on the renal artery. A curved 22-gauge needle attached to a polyethylene catheter was inserted into the renal vein to obtain renal venous blood samples. A curved 27-gauge needle attached to a polyethylene catheter was inserted into the renal artery for infusion of isotonic saline or isoproterenol. Renal blood flow was monitored during insertion of the needles, and no change in blood flow was noted. A period of 45-60 min was allowed for stabilization following completion of surgery.

Experimental protocols.

In order to study the effects of various stimuli on renin secretion the following protocols were carried out on 5 animals in each of the three age groups. During a control clearance period, two sets of arterial and renal venous blood samples were obtained 5 min apart. Infusion of a solution containing isoproterenol (1 μg/ml/kg) was started into the renal artery at a rate of 0.1 ml/min using a Braun infusion pump. The rate of infusion was gradually increased until renal blood flow increased. After 5-7 min a second timed urine collection was started and two more sets of arterial and renal venous blood samples were obtained 5 min apart. The infusion of isoproterenol was then stopped, and following a 45- to 60-min recovery period a third collection of urine and two more blood samples were obtained.

Following recovery from the isoproterenol the animals were given furosemide

(1 mg/kg i.v.). As urine flow increased, saline was infused into the jugular vein at a rate equal to urinary output. 10 min after giving the diuretic, a timed urine sample and two sets of arterial and renal venous blood samples were again collected. A second injection of furosemide (2 mg/kg) was given and the sampling repeated.

The effect of an inhibitor of renin secretion, propranolol, was then determined. Propranolol (1 mg/kg) was given intravenously and after 10 min urine and blood samples were obtained as previously described.

Analytical techniques and data handling

All blood samples were collected in tubes chilled on ice and containing EDTA (5 mM). Blood samples were centrifuged within 5 min of collection and the plasma removed. The red blood cells were then reconstituted in saline and returned to the animal. Plasma for renin determinations was stored at -20 C until assayed.

Plasma and urine were analyzed for inulin using the method of Schreiner[14]. Hematocrit was determined by the micro method. Total and active renin in arterial and renal venous plasma was estimated as described by Derkx et al.[4] Plasma was dialyzed at either pH 4.5 or 3.3 for 24h at 4 C and then returned to pH 7.4 by dialysis for another 24h. Plasma dialyzed at both pH 3.3 and 4.5 was incubated with excess homologous renin substrate and the angiotensin I generated determined by radioimmunoassay[6]. The renin concentration of plasma dialyzed at pH 4.5 was designated active renin. Following dialysis at pH 3.3 renin concentration was higher (total renin) representing the sum of the active and acid-activated renin. Inactive renin was calculated as the difference between total renin and active renin. The renin substrate was prepared according to the method of Skinner[16]. The substrate was found to be free of renin and angiotensinase activity. Incubation of substrate with renin resulted in zero order kinetics. The concentration of renin in plasma was expressed as nanograms of angiotensin I generated per milliliter per hour (ng ANG I/ml/h).

Mean arterial blood pressure, heart rate and mean renal blood flow were taken directly from a digital readout. Glomerular filtration was estimated from the clearance of inulin and renal vascular resistance was calculated as the mean arterial blood pressure divided by the renal blood flow and expressed as mm Hg/ml/min. Renin release was calculated as the product of the venous-arterial renin concentration differences and the renal plasma flow. Statistical analysis was carried out using analysis of variance and t test for paired and unpaired comparisons. The level of significance was set at 5%. Since significant heterogeneity of variance existed in values for renin secretion and renin concentration between animals, statistical analyses were performed on log values as previously reported[2].

5.2.4. RESULTS

The total renin concentration in arterial plasma during control periods was highest

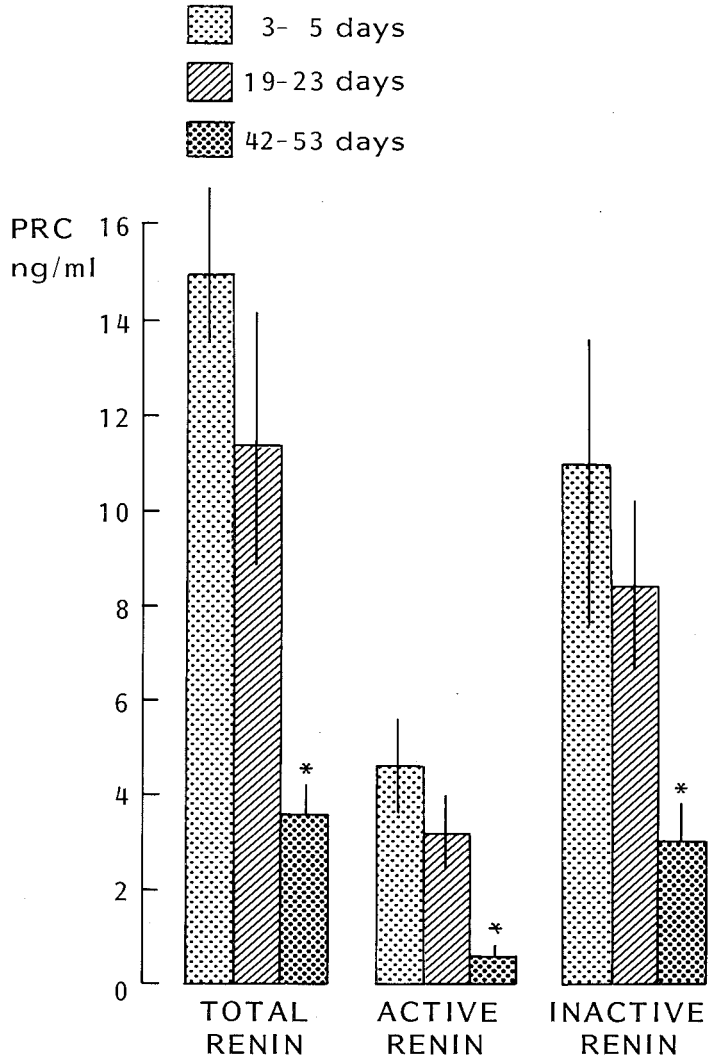

Fig. 5.2.1. Age-related changes in plasma renin concentration (PRC) for total renin, active renin and inactive renin during control periods in anesthetized piglets. Mean ± SEM; n = 5.* = Significantly less than 3- to 5 and 19- to 23-day-old animals ($p < 0.05$)

in the youngest animals and decreased with maturity (Fig.1). This change in total renin concentration was due to a decrease in the concentration of both active and inactive renin. Determination of the rate of release of active and inactive renin during control periods (Fig. 2) demonstrated an insignificant rise between 3-5 and 19-23 days, a time when the arterial renin concentration was beginning to decline (Fig 1). By 42-53 days renin release had returned to values

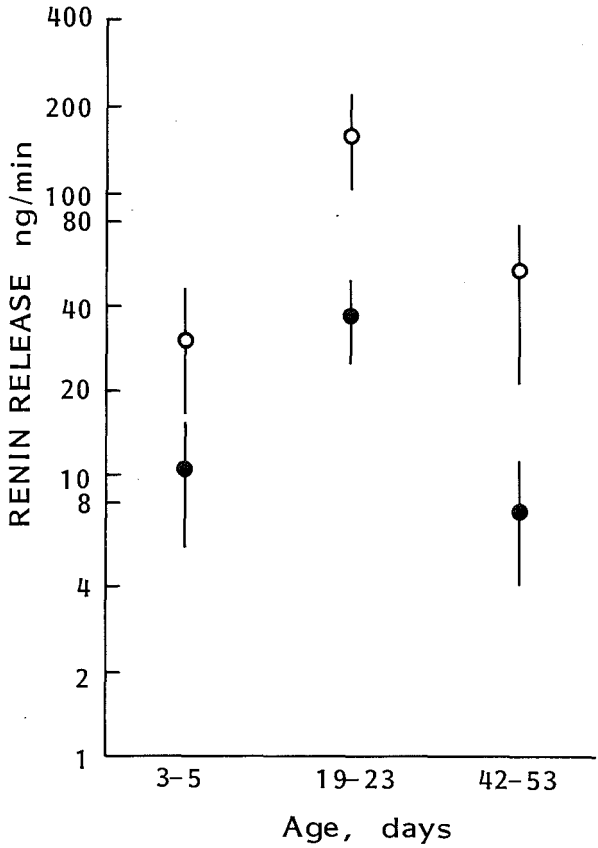

Fig. 5.2.2. Release of active renin (●) and inactive renin (O) during control periods in piglets of three age groups. Mean ± SEM; n = 5.

which were the same as those in the 3- to 5-day-old animals while plasma renin concentration was 4-6 times less than that during the first 3 days of life. Overall, during the first 53 days after birth no significant changes in the rate of renin release was found (Fig. 2).

Renin release was stimulated with two drugs. Isoproterenol infused directly into the renal artery resulted in an increase in the release of both active and inactive renin in all three age groups (Fig. 3). Following isoproterenol the animals were allowed to recover and were then given intravenous furosemide (Table I). Since the rate of renin release following recovery in 1-3 to 5-day-old animal did not return to the control, these animals had a higher mean value in the prefurosemide periods than the control values prior to isoproterenol (Fig.1). The response to furosemide was more variable than that of isoproterenol (Table I). Furosemide (1 mg/kg) increased the release of active renin in all three age

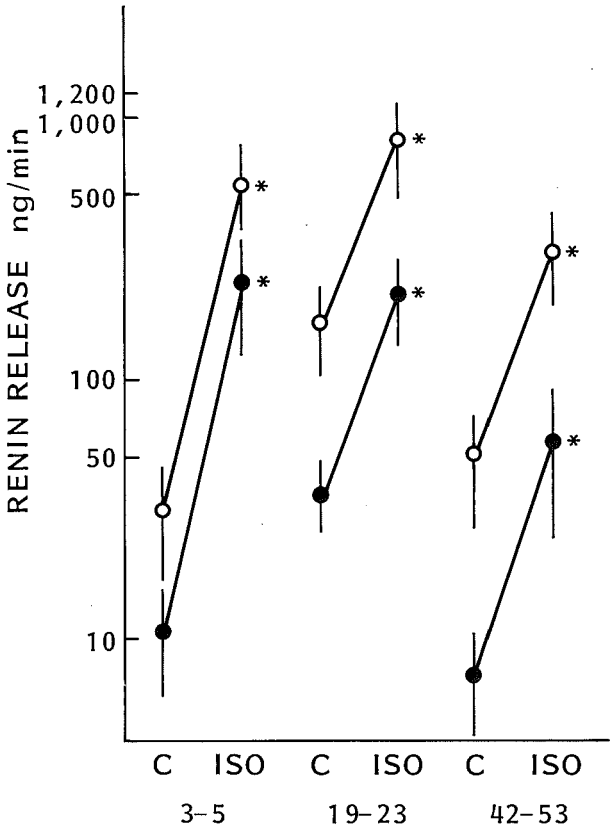

Fig. 5.2.3. Rate of release of active renin (●) and inactive renin (O) in animals in the three age groups. C = Control; ISO = isoproterenol. Mean ± SEM.* = Significantly different from control ($p < 0.05$).

groups. While the rate of release of inactive renin was increased in all three age groups, this change was not statistically significant in the youngest animals. Furthermore, the second injection of furosemide (2 mg/kg) failed to significantly increase the rate of renin release further in any age group.

Propranolol was then given to inhibit the release of renin and resulted in a decrease in all three age groups (Fig.4). Again the change in 3- to 5-day-old animals was not statistically significant because of the variation between animals.

When the relative concentration of active renin in arterial plasma was calculated (active renin concentration/total renin concentration), active renin accounted for 31 ± 2% of the total renin in 3- to 5-day-old animals, 28 ± 2% in the 19- to 23-day- old animals and 20 ± 5% in the animals with an age of 42-53 days. This latter value in the older animals was significantly less than that in the two younger groups.

Table 5.2.1. Release of renin in piglets in the three groups following furosemide. Values are given in ng/min.[1]

Age, days	Active renin			Inactive renin		
	Pre-F	F-1	F-2	Pre-F	F-1	F-2
3-5	45	140*	282*	153	741	637
	(27)	(56)	(78)	(82)	(524)	(439)
19-23	52	414*	789*	204	1014*	2782*
	(23)	(113)	(209)	(64)	(489)	(1334)
42-53	8	61*	107*	17	349*	483*
	(4)	(20)	(39)	(13)	(87)	(180)

Pre-F = Prefurosemide; F-1 = furosemide 1 mg/kg i.v.; F-2 = furosemide 2 mg/kg i.v.
[1] Mean values; standard errors of the mean are given in parenthesis; n = 5.
* Significantly different from control ($p < 0.05$).

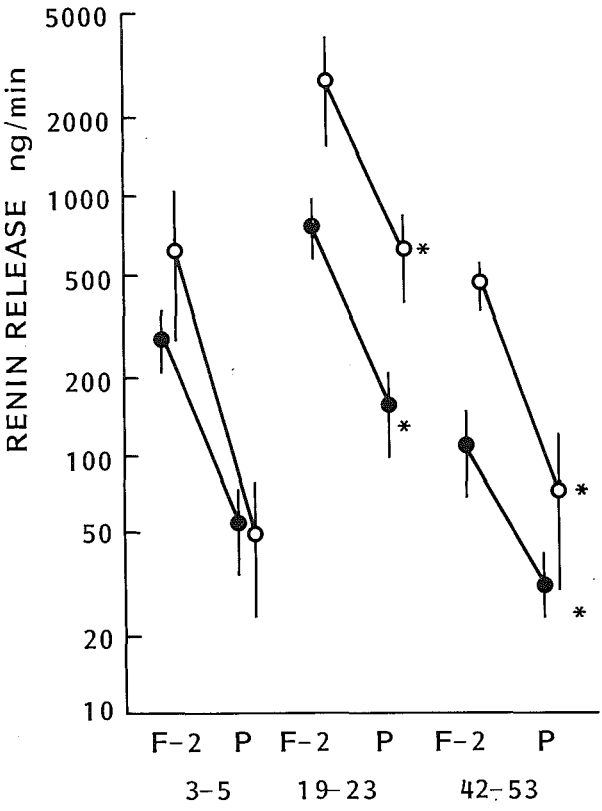

Fig. 5.2.4. Rate of release of actice renin (●) and inactive renin (O) in animals in the three age groups. F-2 = furosemide 2 mg/kg;P = propranolol. Mean ± SEM.* = Significantly different from control ($p < 0.05$).

Over the age range of animals used in this study, kidney weight increased from 10.4 ± 0.9 g in the 3- to 5-day-old animals to 25.7 ± 2.0 g in the 42- to 53-day-old animals. This change was associated with an increase in body weight from 2.9 ± 0.6 to 11.9 ± 0.8 kg, respectively. Control values for mean systemic blood pressure, glomerular filtration rate and renal blood flow increased with increasing age of the animals (Fig.5). Systemic blood pressure appeared to approach a plateau earlier than glomerular filtration rate or renal blood flow. Renal resistance decreased from 6.1 ± 1.9 mmHg/ml/min in 3- to 5-day-old animals to 2.7 ± 0.2 in 19- to 23-day-old animals and 1.7 ± 0.3 mmHg/ml/min in 42- to 53-day-old-animals.

5.2.5. DISCUSSION

The present experiments demonstrate that the concentration of both active and inactive renin is higher in the arterial plasma of newborn piglets (4.4 and 10.7 ng/ANG I/ml/h, respectively) and decreases to 0.7 and 3.0 ng ANGI/ml/h by 53 days of age (Fig.1). However, while arterial plasma renin concentration decreased, the rate of release of both forms of the enzyme by the kidney was not suppressed (Fig.2). These results indicate that factors other than a change in the rate of release of the enzyme may be related to the fall in plasma renin concentration observed during the first 8 weeks of extrauterine life in the piglet. Several additional possibilities which might explain the changes in the amount of renin in the plasma include: (a) an increase in the volume of distribution of renin as plasma volume increases with age; (b) an increase in the rate of catabolism of renin with maturation, and (c) a shift in the form of renin from a more to less active enzyme.

No data have been reported on the catabolism or half-life of renin in the newborn animals. Since the liver is a major site of catabolism of renin[13], maturation of hepatic function could be involved in control of the concentration of renin in plasma of newborn animals. However, preliminary observations from this laboratory (unpublished data) suggest that the rate of catabolism of renin during this period of development does not change. Plasma volume changes during development in both humans and piglets[3,18]. Talbot and Swenson[18] demonstrated a 6-fold increase in total plasma volume of the piglet between birth and 6 weeks of age. This change in plasma volume could not account for the fall in renin concentration in the present study. During the period of the present study, body weight increased fourfold while renin concentration in arterial plasma decreased by a similar amount (Fig. 1). While the body weight may only indirectly reflect changes in plasma volume, the changes in weight support a possible relationship between renin concentration and volume.

The fact that arterial plasma renin concentration is higher in the newborn than in the adult animal, while rates of renin secretion are similar, suggest that the responsiveness of the kidney to the inhibitory effect of angiotensin II in the feedback control of renin secretion may be less in the newborn. At the

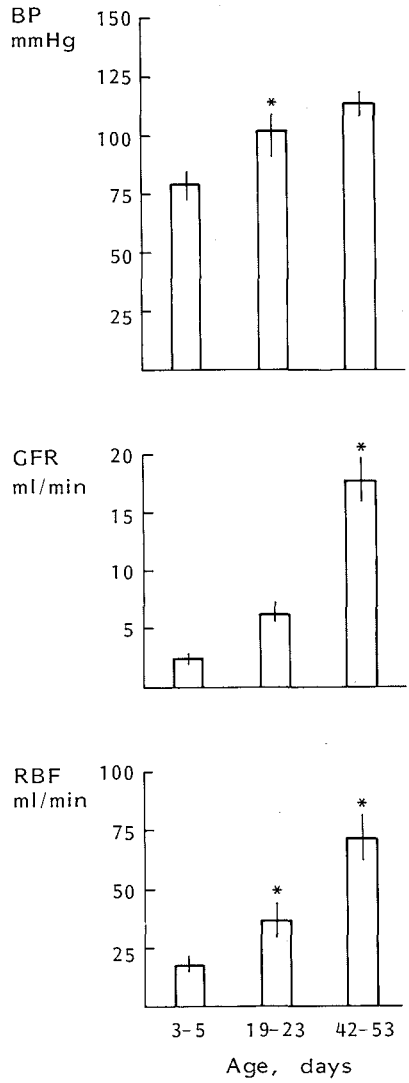

Fig. 5.2.5. Age-related changes in mean systemic blood pressure (BP), glomerular filtration rate (GFR) and renal blood flow (RBF) during control periods in anesthetized piglets. Mean ± SEM; n = 5.* Significantly greater than previous age animals ($p < 0.05$).

present time no data is available on the sensitivity of the newborn kidney to circulating angiotensin II.

In addition to the usual form of renin (active renin) an acid-activated form of the enzyme was also determined in the present experiments. This form of renin has been isolated form plasma and kidneys of animals of several species[1,4,10].

While the physiological significance of multiple forms of renin is not clear, the present studies demonstrate that an acid-activatable, inactive renin is present in plasma of newborn piglets and is released by the kidney. Furthermore, the decrease of the total renin concentration as active renin from 30 to 20% could account, in part, for the observed changes in plasma renin concentration with maturation. However, the finding does not explain a similar decline in the concentration of inactive renin without a change in the release of this form of the enzyme (Figs. 1,2).

In the present experiments renin release was estimated directly, rather than relying on determination of changes in the plasma renin activity as an index of release. The response to stimulation with isoproterenol and furosemide and the suppression with propranolol were qualitatively similar in immature (3-5 day) and mature (45-53 day) animals.

Previous studies describing developmental changes in the renin-angiotensin system[9,12,17] and the capacity of the kidney from the immature animal to release renin[15,17,19] have depended on measurements of arterial plasma renin activity as the index of renin release. Over a wide range of ages in the developing animals, plasma renin activity can be increased by a variety of stimuli[15,17,19] also suggesting that the control of renin release is qualitatively similar in the immature and in the mature animals. Thus, the present studies confirm these observations by direct measurement of renin release.

Finally, the physiological significance of the renin-angiotensin system and the relatively elevated concentrations of renin and angiotensin II in the newborn remain to be clarified. The increase in renal blood flow and in glomerular filtration rate and decrease in renal vascular resistance with maturation are well documented in this study (Fig 5) and in others[5,7]. The data of Jose et al.[8] suggest that the renin-angiotensin system is not involved in the control of renal hemodynamic in newborn animals. However, changes in response to angiotensin II and effects of this hormone on age-related changes in renal function are still possible and need to be studied.

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5.3. ASYNCHRONOUS CHANGES IN PRORENIN AND RENIN SECRETION AFTER CAPTOPRIL IN PATIENTS WITH RENAL ARTERY STENOSIS

5.3.1. SUMMARY

An assay of plasma prorenin was developed in which the conversion to renin occurred under apparently optimal conditions. Some characteristics of the assay were 1) prorenin was activated by Sepharose-bound trypsin at 4 C; 2) the concentration of activator was not critical provided that incubation was prolonged until renin activity had reached a plateau; and 3) this plateau was stable and had the same height as after maximal activation with acid, pepsin, plasmin or urokinase. Maximal activity with Sepharose-bound trypsin at 4 C was higher than with cryoactivation, and optimal conditions were more readily reproduced than with trypsin at 37 C or with acid-activation. The assay was used for measurements in peripheral and renal vein plasma after captopril in hypertensive patients with unilateral renal artery stenosis. Peripheral renin rose within 30 minutes after a first dose of captopril, 50 mg orally, and it remained high with chronic treatment. In contrast, peripheral prorenin fell initially and rose after 4 hours. These changes in peripheral plasma were related to changes in the secretion rates of the two forms of renin from the affected kidney. Thus chronic, but not acute, stimulation of renin release was associated with an increased secretion rate of prorenin. The late rise in prorenin is probably an indication of enhanced synthesis in the kidney, so that more prorenin is available for conversion. The data suggest that prorenin is indeed a biosynthetic precursor of renin and that, at least under certain circumstances, a major proportion of circulating prorenin originates from the kidney.

5.3.2. INTRODUCTION

About 80% of the renin in normal human plasma is thought to circulate in an inactive form[1–3]. Inactive renin is often called 'prorenin' because it can be converted *in vitro* to active renin[4]. It is not certain that prorenin is a precursor of naturally occurring renin, however.

Activation of the factor XII-kallikrein pathway causes irreversible prorenin-to-renin conversion after dialysis of plasma against acid followed by restoration of pH to neutral (acid-activation)[5,6] and possibly also in plasma that is stored at low temperature (cryoactivation)[7]. Prorenin can also be activated by adding trypsin or pepsin[9,10] to plasma. These exogenous activators act independently of factor XII and kallikrein.

Prorenin is measured by functional assays in which prorenin is converted to renin. The difference in renin activity before and after activation is taken as a measure. The implicit assumptions are that all prorenin molecules are converted and that one molecule of activated prorenin has the same enzymatic

activity as one molecule of naturally occurring renin. Cryoactivation, however, often leads to incomplete prorenin-to-renin conversion[3]. Acid activation appears more complete but careful adjustment of pH is of critical importance[11,12]. For trypsin activation, high concentrations of trypsin are required to overcome the inhibitors in plasma but such high concentrations may destroy renin[3,13,14]. Trypsin may also attack renin substrate and may interfere with the radioimmunoassay of angiotensin I, the final step in the assay of renin. Soybean trypsin inhibitor (SBTI) has therefore been used to prevent this. However, some commercial SBTI preparations appear to have angiotensinase activity resulting in considerable loss of angiotensin I during the assay[15].

The present paper describes an assay of prorenin in which it is activated by trypsin that is bound to Sepharose. The immobilized activator can quantitatively be removed by centrifugation. Optimal conditions were worked out for activation in plasma and the results were compared with maximal acid activation and with maximal activation by pepsin, plasmin and the plasminogen activator urokinase. The behaviour of naturally occurring active renin on gel filtration and dye affinity chromatography columns was compared with that of prorenin activated by immobilized trypsin. Some enzymatic properties of the two forms of renin were also compared. The assay was then applied to a study of the effects of angiotensin converting enzyme inhibition by captopril on peripheral and renal vein plasma prorenin in patients with unilateral renal artery stenosis.

5.3.3. MATERIALS AND METHOD

Reagents

Trypsin from bovine pancreas, 2 × crystallized, was purchased from Sigma, St. Louis., Missouri. Its specific activity was 12,000 α-N-benzoyl-L-arginine-ethyl ester (BAEE) units/mg of protein. Pepsin A, specific activity 3,200 units/mg of protein, was also from Sigma. Activator-free highly purified human plasmin, specific activity 19 casein units/mg of protein, was a product of Kabi, Stockholm, Sweden. Urokinase was the urokinase reference standard of Leo, Copenhagen, Denmark. Aprotinin (Trasylol) was obtained from Bayer, Leverkusen, West Germany. Ile-5-Angiotensin I (ANGI) from Serva, Heidelberg, West Germany, was dissolved in Tris/acetate buffer of pH 7.5 (vide infra) and stored at -20 C at a concentration of 40 μM. Its strength was tested by bioassay in the rat against Val-5-Angiotensin II (Hypertensin) from Ciba- Geigy, Basel, Switserland. When allowance was made for the impurities present in the Ile-5-ANGI preparation as stated by the manufacturer, the pressor activities of Val-5-AII and Ile-5-ANGI on a molar base were found to be equal[11]. Renin substrate was prepared from sheep plasma, which was taken 4-5 days after bilateral nephrectomy[16]. It was dissolved in phosphate buffer pH 7.5 and stored at -20 C at a concentration of 5 μM, expressed as the maximal quantity of Ile-5-ANGI equivalents that could be generated in the presence of a large excess of renin. Renin substrate prepared from human plasma[16] was also used in some experiments. A human kidney renin standard, lot MRC 68/356, was kindly

supplied by the WHO International Laboratory for biological Standards, London, England. This standard was dissolved in phosphate buffer pH 7.5 containing 0.35% bovine serum albumin. It could be stored at -20 C at a concentration of 10,000 μU/ml for up to 3 months without loss of activity. ^{125}I-labeled Ile-5-ANGI and anti-Ile-5-ANGI rabbit antiserum were prepared as described previously[11]. Radioactive ANGI in 0.05 M acetic acid, containing 0.1% bovine serum albumin, 0.01% thiomersalate, 0.001% neomycin sulfate and 0.1 M NaCl, was stored in 0.25 ml portions at -20 C.

CNBr-activated Sepharose 4B, Sephadex G-100, Blue Sepharose CL-6B, Blue Dextran 2000, and the molecular weight markers for gel chromatography, ribonuclease A (Mr 13,700), chymotrypsinogen A (Mr 25,000), ovalbumin (Mr 43,000), and human serum albumin (Mr 67,000) were purchased from Pharmacia, Uppsala, Sweden. ^{14}C-ovalbumin (Mr 46,000) and ^{14}C-bovine serum albumin (Mr 69,000) were also used as molecular weight markers and were obtained from the Radiochemical Centre, Amersham, England.

Buffer solutions

Phosphate Buffer, pH 7.5. This buffer contained 12.2 mM Na H_2 PO_4, 86.7 mM NA_2 HPO_4, 75.9 mM NaCl and 1.0 mM disodium ethylenediaminetetraacetate (EDTA).

Glycine/HCl Buffers, pH 3.3 or pH 4.0. These buffers contained 50 mM glycine, 94.9 mM NaCl, and 5.1 mM EDTA. The pH was adjusted with concentrated HCl.

Tris/Acetate Buffer, pH 7.5. This buffer contained 0.1 M Tris, 0.35% bovine serum albumin, 0.1% lysozyme, and 0.2% neomycin sulfate. The pH was adjusted with glacial acetic acid.

Preparation of plasma

Blood was collected in chilled plastic tubes containing EDTA in a final concentration of 5 mM. It was centrifuged at 3,000 $\times$ g and 4 C immediately after collection. Plasma was kept frozen at -20 C before use.

Preparation of immobilized trypsin and pepsin

The enzymes were covalently bound to CNBr-activated Sepharose 4B in a ratio of 30- 40 mg of protein per g of dry Sepharose according to the directions of the manufacturer. In this manner more than 97% of protein was bound to Sepharose. Sepharose-bound trypsin was diluted in phosphate buffer pH 7.5. Suspensions of Sepharose- bound pepsin were stored in glycine/HCl buffer pH 3.3.

Activation of prorenin

Activation by Immobilized Trypsin. Dilutions of Sepharose-bound trypsin (100 μl, 0.05 - 4.0 mg trypsin) were added to 1 ml plasma. The mixtures were incubated at 37 and 4 C for various time periods as indicated in the results section. Trypsin

was removed by centrifugation. The supernatants were checked for amidolytic activity with the chromogenic substrate N-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-p-nitroanilide (S2222, Kabi,Stockholm , Sweden). For this purpose 0.1 ml of the supernatant of the incubates was mixed with 0.2 mM substrate (about 10 times Km for trypsin) in 0.1 M Tris/HCL buffer pH 8.2 in a total volume of 1.0 ml. The linear release of p-nitroaniline was followed for 1-2 minutes at 405 nm in a 1-cm semi-microcuvette at 37^0. With this method the remaining trypsin activity in the supernatants was found to be less than 0.1% of the original activity in the incubates.

Acid activation. Plasma samples (2 ml) were dialyzed against glycine/HCl buffer pH 3.3 for 24 h at 4 C, followed by dialysis against phosphate buffer pH 7.5, containing 6% polyvinylpyrrolidone, again for 24 hours at 4 C. Polyvinylpyrrolidone had been added to the buffer to prevent dilution of the plasma due to colloid-osmosis. The dialysis bags were emptied in calibrated plastic tubes and rinsed with phosphate buffer pH 7.5 and the volume was adjusted to 2 ml with the same buffer.

Activation by immobilized pepsin. Plasma samples (2ml) were dialyzed against glycine/HCl buffer pH 3.3 for 24 h at 4 C. To 1 ml of the dialyzed plasma was added Sepharose-bound pepsin (100 μl,0.3 mg pepsin). The mixture was incubated at 32 C for various time periods, as indicated in the Results. Pepsin was removed by centrifugation and pH was restored to 7.5 with 1 M NaOH.

Activation by plasmin and urokinase. Plasmin was dissolved at a concentration of 20 casein units/ml in phosphate buffer pH 7.5. Urokinase was dissolved in this buffer at a concentration of 1000 Ploug units/ml. The plasma samples were dialyzed against glycine/HCl buffer pH 4.0 for 24 hours at 4 C and pH was restored to 7.5 with 1 M NaOH. The activator solutions (100 μl) were added to 1 ml of the pH 4.0-pretreated samples and incubated at 4 C for various time periods as indicated in the Results.

Assay of naturally occurring renin and prorenin that is activated in vitro

For this assay, 0.10-0.25-ml samples were added to 0.5 ml of renin substrate, and the volume was adjusted to 1.0 ml with phosphate buffer pH 7.5. The final concentration of renin substrate in the mixture was 2.5 μM Ile-5-ANGI equivalents, which corresponds to about 10 times Km (see Results). After addition of protease inhibitors, i.e., 10 μl of 0.34 M 8-hydroxyquinoline sulfate, 5 μl of 0.28 M phenylmethylsulphonylfluoride in ethanol, and 10 μl aprotinin (10,000 kallikrein-inhibiting units/ml), the mixtures were incubated at 37 C. The incubation time was 3 hours except when stated otherwise. The renin-containing samples had been diluted in such a way that no more than 5% of the substrate was cleaved during incubation. Parallel incubations at 4 C served as blanks. Incubations of dilutions of standard human kidney renin at 37 C and 4 C were run in each assay batch. The concentration of homologous substrate in the incubation mixtures was less than 0.2 μM Ile-5-ANGI equivalents. Previous studies have shown that this concentration of homologous substrate did not

interfere with the reaction of renin and the heterologous substrate[11]. The reaction was stopped by adding 1 ml of 0.15 M NaCl followed by heating for 10 minutes in a boiling water bath. The precipitate was removed by centrifugation. The concentration of ANGI in the supernatant was measured by radioimmunoassay, using ^{125}I-labeled Ile-5-ANGI and rabbit anti-Ile-5-ANGI antiserum[11]. Renin concentration is expressed as microunits of the renin standard per ml (μU/ml). Prorenin was measured as the difference between the renin concentration after activation of the test sample ('total renin') and the concentration before activation. For routine measurements in plasma, 1.0 ml plasma was incubated with 100μl Sepharose-bound trypsin in a final concentration of 0.25 mg trypsin/ml for 24 hours at 4 C. The reasons why this procedure was selected are presented and discussed in the results and discussion sections.

Interassay variability was evaluated by weekly measurements of naturally occurring active renin and prorenin in a normal plasma pool(stored at -20 C during a 9-month period. The mean value of naturally occurring active renin was 27 μU/ml (36 assays) with a standard deviation of 3 μU/ml (coefficient of variation 11%). The mean value of 'total renin' was 254 μU/ml, with a standard deviation of 26 μU/ml (coefficient of variation 10%). The mean value of prorenin was 227 μU/ml with a standard deviation of 24 μU/ml(coefficient of variation 11%). In normal plasma the contribution of naturally occurring active renin to total renin is small but it becomes greater after stimulation of renin release. The coefficient of variation of prorenin measurements then also becomes greater since prorenin is taken as the difference between total renin and naturally occurring active renin. In 17 healthy men (aged 24-45 years) who were recumbent for at least 1 hour before blood sampling, naturally occurring renin had a mean value of 23 μU/ml (antilog of arithmetic mean after logarithmic transformation of data) with a range of 14 to 43 μU/ml. The mean value of prorenin was 196 μU/ml with a range of 138 to 312 μU/ml; the mean value of the proportion of renin that was in the active form was 10.9%, with a range of 4.3% to 17.5%.

Gel filtration

Untreated plasma (2ml) or trypsin-activated plasma (2ml) was applied to 2.6x90 cm columns of Sephadex G-100 equilibrated with 0.01 M Tris/HCl buffer pH 7.0 containing 0.15 M NaCl and 1 mM EDTA. The same buffer was used for elution. Flow rate was adjusted to 10 ml/hr and 1.5-ml fractions were collected. The columns were calibrated with ribonuclease A, chymotrypsinogen A, human albumin and ovalbumin. Blue dextran 2000 was used for determining void volume. ^{14}C-ovalbumin and ^{14}C-BSA were used as internal standards. Gel filtration was carried out at 4 C.

Affinity chromatography

Untreated plasma (4 ml) or trypsin- activated plasma (4 ml) was applied to 1.6x25 cm columns of Blue Sepharose CL-6B equilibrated with 0.02 M phosphate buffer pH 7.1. Elution was performed with this buffer in 3 steps, i.e., without

added NaCl, with 0.2 M NaCl and with 1.4 M NaCl added to the buffer.[17] Flow rate was 50 to 60 ml/hr and 2.5-ml fractions were collected. Affinity chromatography was carried out at 4°C.

Studies in patients Fifty-four hypertensive patients were studied after they had given their informed consent. All had unilateral renal artery stenosis as demonstrated by renal arteriography. Treatment was stopped at least 3 weeks before the studies began. The patients were recumbent for at least one hour before blood sampling.

Group 1 (n = 14) Renal arteriography had already been performed before this study. The patients received a first dose of captopril, 50 mg orally. Peripheral venous blood was sampled from an indwelling needle for renin and prorenin measurements before and at different time intervals after captopril. Renin before captopril was 81 μU/ml (antilog of arithmetic mean after logarithmic transformation of data) with a range of 19 to 250 μU/ml, and prorenin was 190 μU/ml with a range of 77 to 410 μU/ml. The patients were then treated with captopril, 50 mg 3 times a day, and blood was taken after 1, 2, and 4 weeks 1-2 hours after the morning dose. Group 2 (n = 15) Blood was sampled from both renal veins and from the abdominal aorta before and 30 minutes after a first dose of captopril, 50 mg, just before renal arteriography. Because some time elapsed between sampling of the renal vein of one side and sampling at the other side, two aortic samples were taken each at exactly the same time that a renal vein sample was collected. Peripheral vein renin before captopril was 51 μU/ml (range 16-190 μU/ml) and prorenin was 170 μU/ml (range 57-320 μU/ml).

Group 3(n = 15) Blood was sampled from both renal veins and from the abdominal aorta 16 hours after a first dose of captopril, 50 mg, just before renal arteriography. This time interval was chosen because studies in Group I had shown that peripheral prorenin was significantly increased after 16 hours. Peripheral vein renin before captopril was 58 μU/ml (range 15-480 μU/ml) and prorenin was 130 μU/ml (range 20-490 μU/ml).

Group 4(n = 10) Blood was sampled from both renal veins and from the abdominal aorta while the patients were taking captopril, 50 mg three times a day, for 2 weeks. Sampling occurred 1-2 hours after the last 50 mg dose and just before renal arteriography. Peripheral vein renin before captopril was 66 μU/ml (range 30-360 μU/ml) and prorenin was 210 μU/ml (range 72-520 μU/ml).

5.3.4. RESULTS

Activation of plasma prorenin by immobilized trypsin: selection of optimal conditions.

Normal plasma pool was incubated at 4 C and 37 C with Sepharose-bound trypsin at concentrations ranging from 0.05 to 0.50 mg per ml of plasma at 4 C and from 0.05 to 4.0 mg/ml at 37 C. The results at 4 C and 37 C were markedly different (Fig.1). First, the reaction velocity was higher at 37 C than

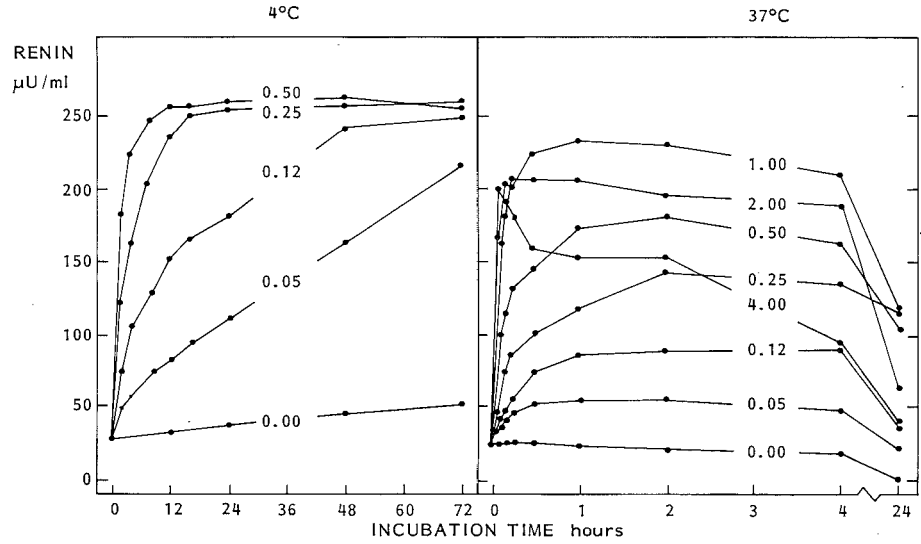

Fig. 5.3.1. Activation of prorenin in a pool of normal plasma by immobilized trypsin at 4 C (left) and 37 C (right). The concentrations of trypsin, in mg/ml of plasma, are indicated. Results are the means of three experiments.

at 4 C, but for a given trypsin concentration, the maximum level of renin activity ultimately obtained was higher at 4 C than at 37 C. Second, with trypsin concentrations ranging from 0.12 to 0.50 mg/ml, this maximum of renin activity was independent of trypsin concentration during incubation at 4 C and not at 37 C. Third, very high concentrations of trypsin were required at 37 C for approaching the maximal renin activity obtained at 4 C but these high concentrations also caused inactivation or destruction of renin.

As shown in figure 1, with the use of 0.25 mg trypsin per ml of plasma at 37 C, activation had reached its maximum after 2 hours of incubation, and at that time the renin activity was about half the maximum obtained with the same concentration of trypsin at 4 C. With this concentration of trypsin the generation of active renin at 37 C had stopped because not enough uninhibited trypsin was left. This was demonstrated by the following experiments. Plasma (1 ml) was incubated with Sepharose-bound trypsin (100 μl) in a concentration of 0.25 mg trypsin per ml of plasma for 4 hours at 37 C. After incubation the plasma was separated from the activator by centrifugation. The supernatant was transferred to a new tube and renin was measured; it was 140 μU/ml as compared to 32 μU/ml before incubation with trypsin. The supernatant was mixed with fresh Sepharose-bound trypsin, again in a concentration of 0.25 mg/ml, whereas the trypsin pellet was resuspended in fresh plasma (1 ml). The mixtures were then incubated for 24 hours at 4 C and renin was measured. Newly added trypsin caused further generation of active renin to its maximal

value of 250 μU/ml in the supernatant but addition of fresh plasma to the precipitate was without effect. In contrast, at 4 C maximal activation of prorenin was obtained while active trypsin was still present. This was shown as follows: plasma (1 ml) was incubated with Sepharose-bound trypsin (100μl) in a concentration of 0.25 mg/ml for 24 hours at 4 C. Plasma was then separated from the activator by centrifugation. The supernatant was transferred to a new test tube and renin was measured; it was 240 μU/ml as compared to 34 μU/ml before incubation with trypsin. Fresh plasma (1 ml) was added to the trypsin pellet and mixed. Renin was generated during subsequent incubation at 4 C, whereas no further activation occurred after addition of fresh trypsin to the supernatant. It is therefore very likely that at 4 C all the prorenin that could be converted by trypsin was indeed converted.

Recovery of prorenin and renin during incubation with immobilized trypsin

The possibility that some loss of prorenin has occurred during trypsin treatment at 4 C cannot be excluded but the fact that the same maximum of renin activity was reached irrespective of the trypsin concentration in the incubate argues against such a loss of prorenin. Renin activity decreased with prolonged incubation with trypsin at 37 C but not at 4 C, thereby indicating that active renin was inactivated or destroyed at 37 C. This was substantiated by the following experiment (Fig.2). Standard human kidney renin (1,000 μU/ml) in phosphate buffer pH 7.5 was incubated at 4 C and 37 C with Sepharose-bound trypsin in a concentration of 0.25 mg/ml. After various time intervals trypsin was removed by centrifugation, and renin activity in the supernatant was determined. Incubation for 24 hours at 4 C had practically no effect on renin activity but at 37 C renin was rapidly inactivated or destroyed. It should be noted that these incubates are free of trypsin inhibitors, so that the concentration of uninhibited trypsin is higher under these circumstances than when the same quantity of trypsin is added to plasma. As shown in figure 2, at 37 C some destruction of renin occurred in the absence of trypsin. This may be due to a change in pH; in some examples it rose during 24 hours of incubation at 37 C from 7.4 to maximally 8.1. Renin has been reported to be destroyed at this temperature at alkaline pH[3].

Recovery of Angiotensin I in trypsin-treated plasma

Aliquots of normal plasma (1 ml), to which Ile-5-ANGI had been added in a final concentration of 0.04 μM, were incubated for 24 h at 4 C with immobilized trypsin (100 μl) in a concentration of 0.25 mg/ml or with phosphate buffer 7.5 (100 μl). After incubation the immobilized trypsin was removed by centrifugation and 0.5 ml of the plasma samples was incubated with 0.5 ml of sheep renin substrate in the presence of protease inhibitors as described in the methods section. The recovery of added Ile-5- ANGI was 98 ± 10.1% in trypsin-treated plasma and 98 ± 9.3% in untreated plasma (mean ± SEM, n = 5).

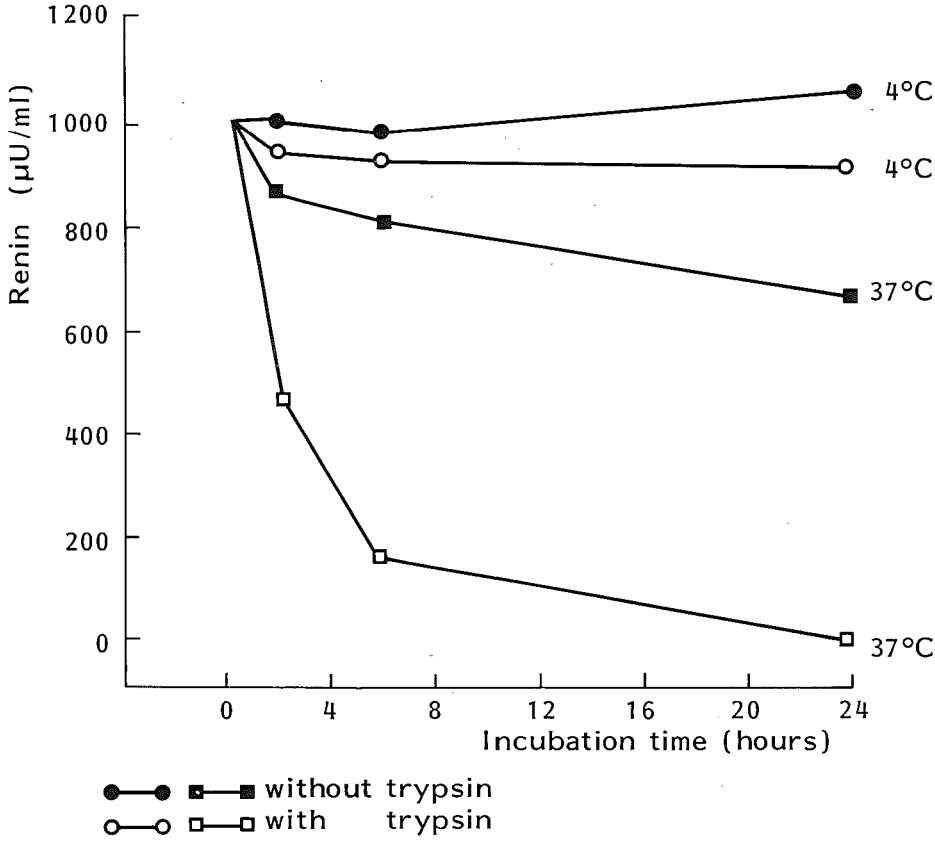

Fig. 5.3.2. Inactivation or destruction of human kidney renin (MRC standard) by immobilized trypsin (0.25 mg/ml of incubate) at 4 C (closed squares) and at 37 C (open circles). Controls without trypsin are shown as closed symbols.

Comparison between trypsin activation of prorenin and other activation procedures

The following procedures were compared (Fig. 3): 1) incubation of normal plasma pool at 4 C with immobilized trypsin (0.25 mg/ml); 2) dialysis for 24 hours at pH 3.3 and 4 C followed by dialysis at pH 7.5 again at 4 C;3) dialysis for 24 hours at pH 4.0 and 4 C followed by restoration of pH to 7.5 with 1 M NaOH and incubation at 4 C with plasmin (2 casein units/ml) or with urokinase (100 Ploug units/ml); and 4) dialysis for 24 hours at pH 3.3 and 4 C followed by incubation at 32 C with immobilized pepsin (0.3 mg/ml) and subsequent restoration of pH to 7.5 with 1 M NaOH. The maximum levels of renin activity obtained with each of these procedures appeared not different. This is an indication that all the prorenin that could be converted to active renin by proteolytic attack was indeed converted.

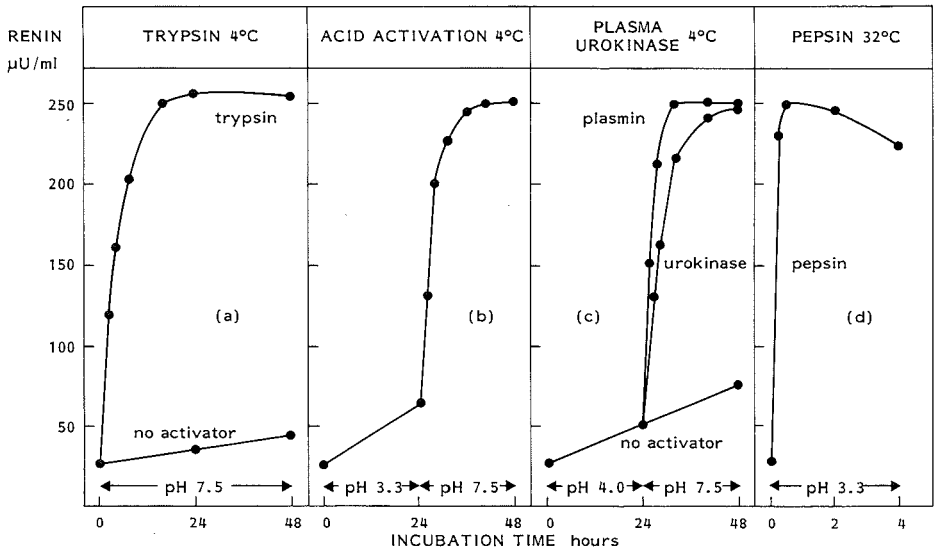

Fig. 5.3.3. Comparison between various activation procedures:(a) incubation of a pool of normal plasma with immobilized trypsin (0.25 mg/ml of plasma) at 4 C;(b) dialysis at pH 3.3 followed by dialysis at pH 7.5 both at 4 C; (c) incubation with plasmin (2 casein units/ml or urokinase (100 Ploug units/ml) at 4 C following by dialysis at pH 4.0 and restoration of pH by 1 M NaOH; and (d) incubation wih pepsin (0.3 mg/ml) at pH 3.3 and 32 C followed by restoration of pH by 1 M Na OH. Results are the mean of three experiments.

Comparison between trypsin-activated prorenin and naturally occurring renin

Gel filtration. The eluates were treated with trypsin for measuring prorenin. The conditions were the same as for plasma, i.e., incubation with immobilized trypsin (0.25 mg/ml) for 24 hours at 4 C. We have not rigorously checked whether the conditions chosen for plasma were also appropriate for measuring prorenin in the column eluates. They probably are because, when normal plasma was treated with immobilized trypsin (0.25 mg/ml) for 24 hours at 4 C *before* it was subjected to chromatography, the quantity of renin in the eluate was the same as when native plasma was applied to the column and the eluate was *subsequently* treated with trypsin. This quantity was 15 times higher than the quantity of renin that was recovered from the column, when trypsin-treatment had been omitted both *before* and *after* chromatography. The factor of 15, as found in the column eluates, was the same as the ratio between total renin and naturally occurring active renin in the plasma itself.

When plasma was activated prior to chromatography, only active renin was recovered from the column, with its peak appearing at the same elution volume as the peak of naturally occurring active renin (Fig.4). Mr of naturally occurring active renin was 49,000 and Mr of prorenin was 56,000 (mean value of three experiments).

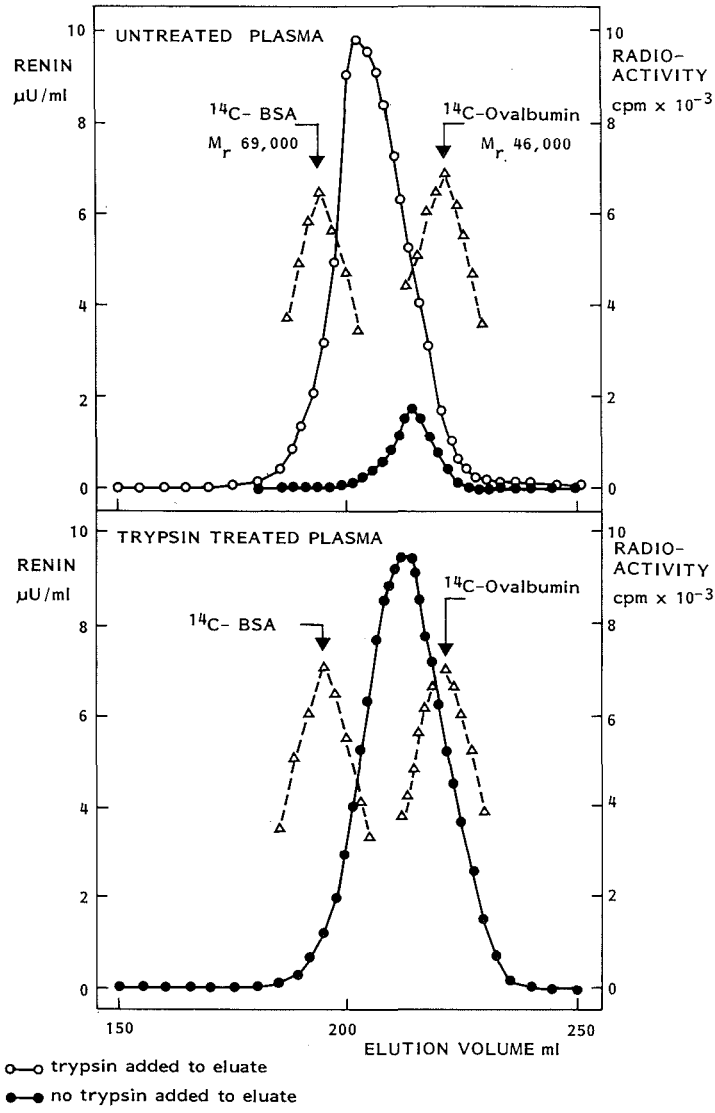

Fig. 5.3.4. Gel filtration on Sephadex G-100 of untreated normal plasma and of plasma treated with immobilized trypsin (0.25 mg/ml) for 24 hours at 4 C. The molecular weight markers ^{14}C bovine serum albumin (Mr 69,000) and ^{14}C-ovalbumin (Mr 46,000) were used as internal standards. Renin was determined in the eluate before (closed circles) and after (open circles) incubation with immobilized trypsin. Calculated Mr-values; plasma prorenin 56,000, plasma renin 49,000 and human kidney renin (MRC standard) 42,000

Dye-ligand affinity chromatography

The eluates were treated with immobilized trypsin in the same way as after gel filtration. When native plasma was subjected to affinity chromatography, the bulk of active renin passed through the column, while prorenin was eluted with 0.2 M NaCl. The quantity of renin that was recovered after trypsin-treatment of the eluate was about 15 times higher than before trypsin-treatment, which agrees with the ratio between total renin and naturally occurring active renin in the plasma itself. This suggests that the method of trypsin-treatment was appropriate for measuring prorenin in the column eluates.

When plasma had been activated by trypsin prior to chromatography, all the renin appeared to pass through the column, since no renin was detected both before and after trypsin treatment of the eluates at 0.2 M NaCl and at 1.4 M NaCl (Fig.5).

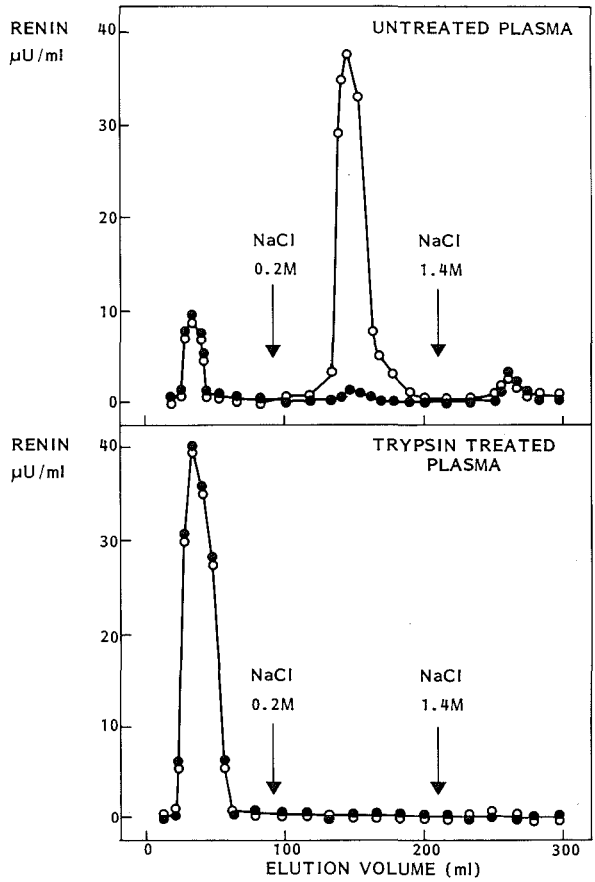

Fig. 5.3.5. Affinity chromatography on Blue Sepharose of untreated normal plasma and of plasma treated with immobilized trypsin (0.25 mg/ml) for 24 hours at 4 C. Renin was determined in the eluate before (closed circles) and after incubation with immobilized trypsin (open circles)

Enzyme kinetics of naturally occurring renin and prorenin that is activated by trypsin

Under the conditions of the renin assay the generation of ANGI from sheep renin substrate by both forms of renin proceeded linearly with time and was proportional to the concentration of active renin. The activity of human kidney renin (MRC standard) was not influenced by the addition of untreated or trypsin-treated normal plasma, indicating that substances interfering with the reaction between renin and its substrate were absent. Active renin was isolated from plasma of a patient with hypertension and renal artery stenosis. The concentration of naturally occurring renin in this plasma was very high, 1,200 μU/ml. Renin was isolated by Sephadex G-100 gel filtration and further purified by DEAE-Sepharose ion exchange chromatography[10,18] followed by affinity chromatography on Blue-Sepharose[17]. The preparation was free of renin substrate and prorenin. Prorenin was isolated from normal plasma by Sephadex-G-100 gel filtration followed by chromatography on Blue Sepharose. The preparation was free of renin substrate and renin. Prorenin was then activated with immobilized trypsin as described above. The renin preparations were incubated with sheep renin substrate for 1 hour at 37 C and ANGI that had been formed was determined for constructing Lineweaver-Burk plots (Fig.6). Km-values for naturally occurring plasma renin and standard human kidney renin and for plasma prorenin that was activated by trypsin appeared similar; they ranged from 0.21 to 0.28 μM.

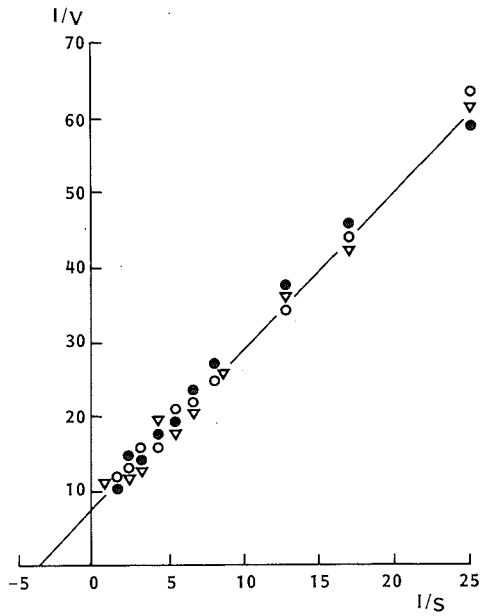

Fig. 5.3.6. Enzyme kinetics of naturally occurring active plasma renin (closed circles), trypsin-activated prorenin (open circles) and human kidney renin (MRC standard, triangles). The renins were incubated at 37 C with sheep renin substrate for 5, 10, 20, 30, and 60 minutes. v = initial velocity in μM angiotensin I per hour. s = substrate concenration in μM angiotensin I-equivalents

pH-Optimum curves of naturally occurring renin and prorenin that is activated by trypsin

The sources of naturally occurring renin and of prorenin that was activated by trypsin were the same as in the previous experiment. The renin preparations and sheep renin substrate were dialyzed for 24 hours at 4 C against phosphate buffers with pH values ranging from 4.5 to 9.0. The renin preparations were then incubated with sheep renin substrate or with human renin substrate at these various pH-values for 1 hour at 37 C. The concentrations of sheep substrate in these incubates was 2.5 μM ANGI equivalents/ml, and the concentration of human substrate was 0.4 μM ANGI equivalents/ml. The pH-optimum curves of naturally occurring renin and of prorenin that was activated by trypsin appeared not different (Fig.7). The pH-optimum was 7.5 for the reaction with sheep substrate and 5.8 with human substrate.

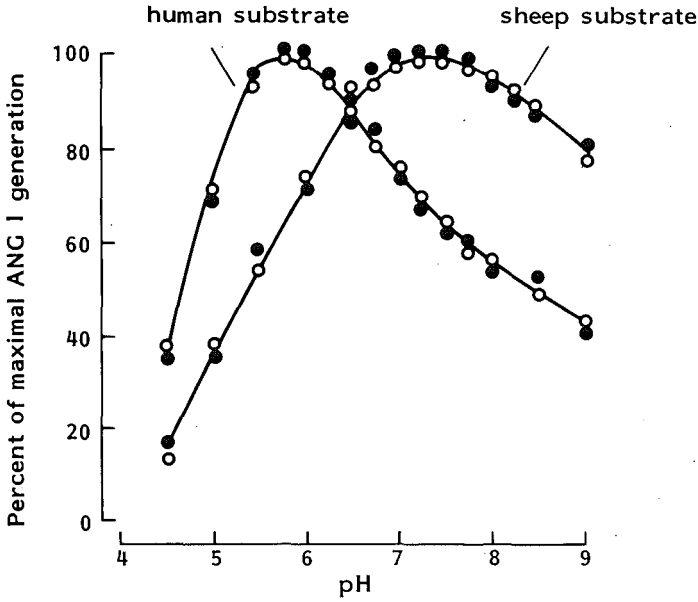

Fig. 5.3.7. Optimum pH curves for naturally occurring active plasma renin (closed circles) and trypsin-activated prorenin (open-circles) with human renin substrate and with sheep renin substrate. Results are the mean of two experiments.

Measurements of plasma renin and prorenin in patients

Group 1 Results are shown in Figure 8. Renin in peripheral plasma rose within 30 minutes after the first dose of captopril and reached a peak value after 1-2 hours. It remained high with chronic treatment. Prorenin fell initially ($p < 0.05$; paired t-test) but with long-term treatment it reached a value as high or higher than renin.

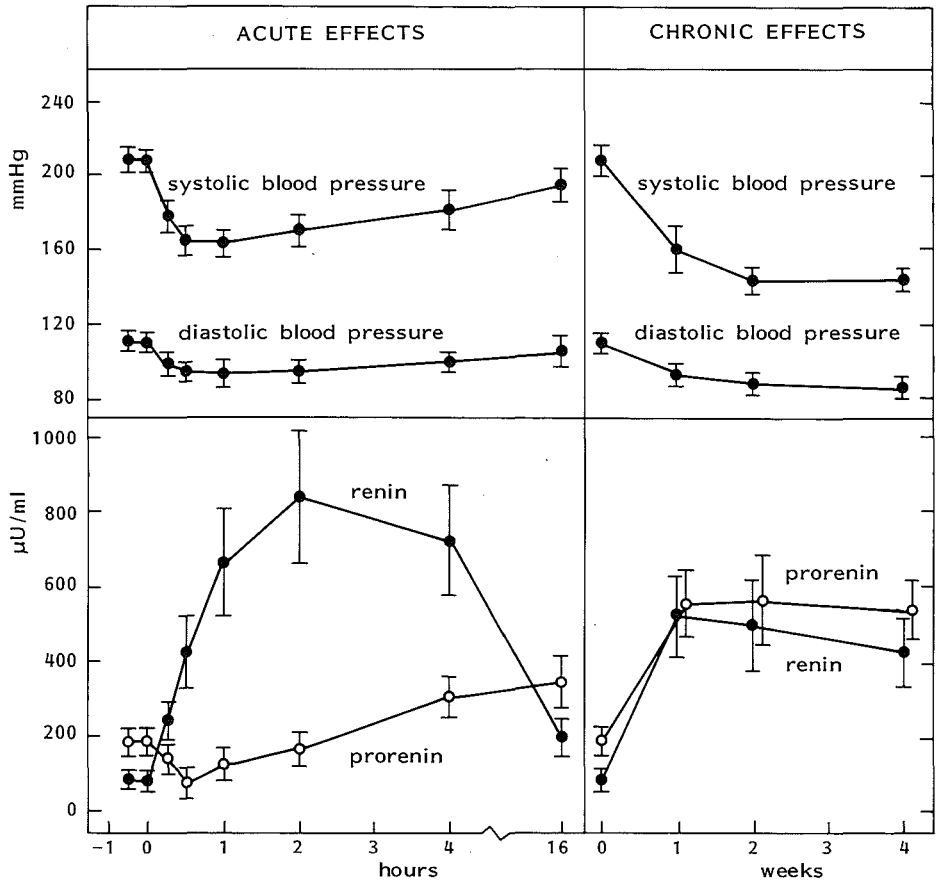

Fig. 5.3.8. Effects of a first dose of captopril, 50 mg orally, and of chronic captopril treatment, 50 mg three times a day, on blood pressure and on renin and prorenin in peripheral plasma. Prorenin at 30 and 60 minutes after the first dose of captopril was significantly below the control value ($p < 0.05$, paired t test). At 4 and at 16 hours it was above control ($p < 0.001$). During chronic treatment blood was sampled 1 to 2 hours after the morning dose of captopril. Prorenin in these samples was 3 times higher than control ($p < 0.001$). Values in normal plasma ($n = 17$) are 23 µU/ml (range 14-43 µU/ml) for renin and 196 µU/ml (range 138-312 µU/ml) for prorenin.

Groups 2, 3 and 4 Basal values of peripheral vein renin and prorenin were comparable in the three groups (see Methods section). Blood pressure fell from 208±6/112 ±5 mmHg to 164±10/94±6 mmHg 30 minutes after captopril in Group 2. Blood pressure was 211±7/110±6 mmHg before captopril and 198±8/104±7 mmHg 16 hours after captopril in Group 3. In Group 4 blood pressure was 202±9/109±4 mmHg before captopril and 143±6/91±5 mmHg 1-2 hours after the last dose of captopril. Data collected at the time of renal vein sampling

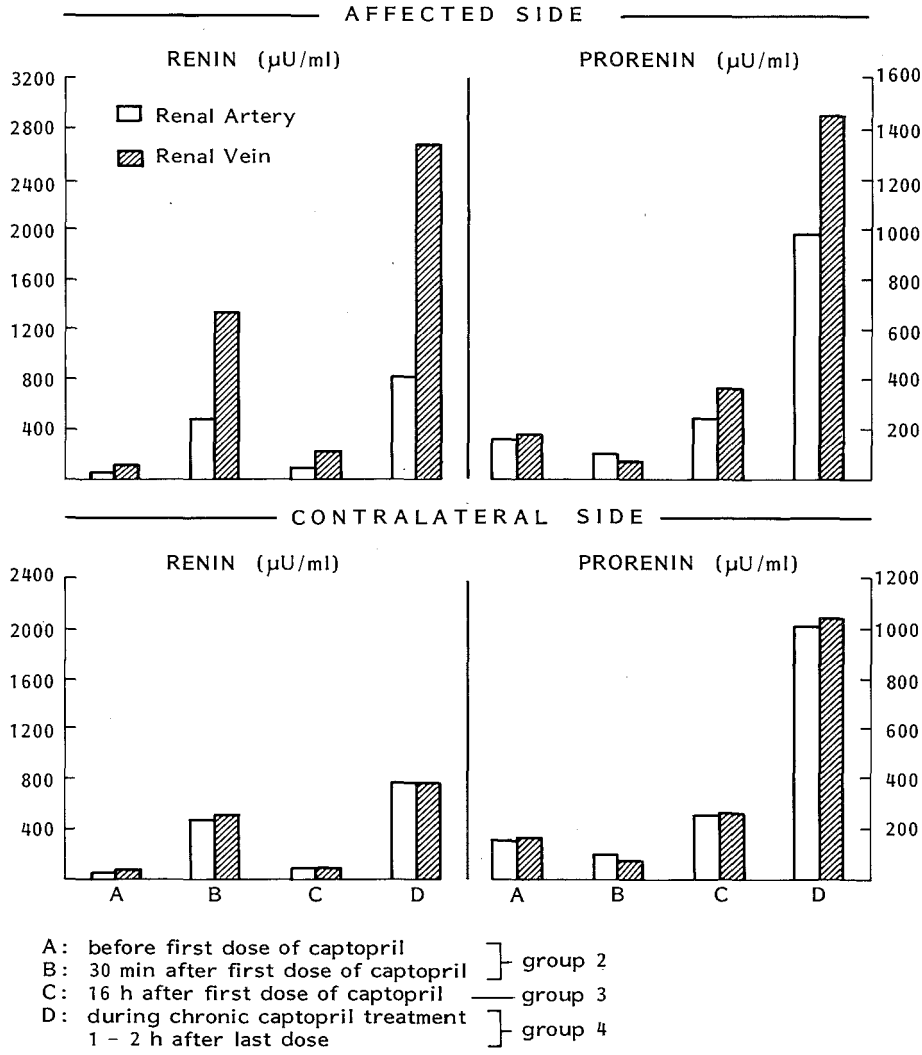

Fig. 5.3.9. Effects of a first dose of captopril, 50 mg orally, and of chronic captopril treatment, 50 mg three times a day, on plasma renin and prorenin in the renal vein and the abdominal aorta. Statistics of these data and details on the study design are presented in Tables 5.3.1. and 5.3.2.

are shown in figure 9 and in table 1. Renin in the renal vein on the affected side, but not contralaterally, was higher than in the aorta before captopril and 30 minutes after a first dose of captopril ($p < 0.001$, Group 2), and at 16 hours after a first dose of captopril ($p < 0.001$, Group 3), and also with chronic captopril treatment ($p < 0.001$, Group 4). Prorenin in the renal vein on the affected side was significantly higher than in the aorta before and 16 hours after a first dose

Table 5.3.1[a] Renin and prorenin in plasma of renal vein and aorta in patients with renal artery stenosis.

			^{10}Log renin (μU/ml)				^{10}Log prorenin (μU/ml)			
			Affected side		Contralateral		Affected side		Contralateral	
Patient group	Study design	Blood sampling time	Aorta	Renal vein	Aorta	Renal vein	Aorta	Renal vein	Aorta	Renal vein
Group 2 (n = 15)	Captopril 50 mg single dose	Before first dose of Captopril	1.73 (0.10)	2.09 (0.10)	1.72 (0.09)	1.79 (0.09)	2.23 (0.04)	2.26 (0.06)	2.21 (0.06)	2.21 (0.06)
		30 min after first dose of Captorpil	2.66 (0.11)	3.12 (0.13)	2.70 (0.11)	2.71 (0.10)	2.02 (0.09)	1.93 (0.20)	2.01 (1.14)	1.89 (0.19)
Group 3 (n = 15)	Captopril single dose	16 h after Captopril	2.02 (0.15)	2.37 (0.19)	2.01 (0.15)	2.01 (0.15)	2.41 (0.13)	2.57 (0.14)	2.41 (0.12)	2.42 (0.12)
Group 4 (n = 10)	Captopril 50 mg tid for 2 weeks	1-2 h after last dose of Captopril	2.91 (0.13)	3.42 (0.12)	2.89 (0.12)	2.89 (0.13)	2.99 (0.06)	3.16 (0.06)	3.01 (0.05)	3.02 (0.05)

For details see Methods section. Values are mean (SEM in parentheses).

Table 5.3.1[b] Renal vein-to-aorta ratio of renin and inactive renin.

Patient group	Study design	Blood sampling	Renal vein-to-aorta ratio of renin: Affected side	Renal vein-to-aorta ratio of renin: Contra-lateral	Renal vein-to-aorta ratio of prorenin: Affected side	Renal vein-to-aorta ratio of prorenin: Contra-lateral
Group 2 (n = 15)	Captopril 50 mg single dose	Before first dose of Captorpil	2.38*** (0.21)	1.16 (0.09)	1.19* (0.08)	1.04 (0.08)
		30 min after first dose of Captopril	3.25*** (0.50)	1.05 (0.05)	1.14 (0.26)	1.04 (0.13)
Group 3 (n = 15)	Captorpil single dose	16 h after Captopril	2.72*** (0.50)	1.00 (0.02)	1.53** (0.16)	1.03 (0.03)
Group 4 (n = 10)	Captopril 50 mg tid for 2 weeks	1-2 h after last dose Captropil	3.39*** (0.48)	1.00 (0.03)	1.50** (0.11)	1.03 (0.02)

For details see Methods section. Values are mean (SEM in parentheses)
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the difference from 1.00.

of captopril ($p < 0.05$ and $p < 0.01$ in Groups 2 and 3 respectively), and also with chronic captopril treatment ($p < 0.01$, Group 4). Contralaterally the prorenin levels in the renal vein and aorta were not different neither before captopril nor after captopril. High levels of renin were measured in the aorta 30 min after a single dose of captopril and with chronic treatment. High levels of prorenin were measured in the aorta with chronic captopril. This is in agreement with the measurement of peripheral vein renin in Group 1.

5.3.5. DISCUSSION

Methodological Aspects

For the results of functional assays of prorenin to be valid, conversion of the prorenin in the test sample should be complete and the enzymatic activities of equimolar quantities of activated prorenin and naturally occurring renin should not differ. These criteria are likely to be met when immobilized trypsin is used for prorenin activation in human plasma under the assay conditions we have worked out. Several lines of evidence support this conclusion. First, identical results were obtained with acid-activation, with activation by pepsin, plasmin, and urokinase and with immobilized trypsin, provided that optimal conditions were selected. A plateau of maximal renin activity was obtained, and the level of this plateau had the same height with each of these procedures.

Second, Km-values and pH-optimum curves for the reactions of trypsin-activated prorenin and naturally occurring renin with sheep renin substrate did not appear different. Third, the behaviour of trypsin-activated prorenin on Sephadex-G-100 and Blue Sepharose chromatography columns was similar to that of naturally occurring renin. The Mr-values of naturally occurring active renin (49,000) and prorenin (56,000) as estimated by gel filtration, are in agreement with those reported by others[19,20] and confirm that the Mr of these plasma renins is greater than the Mr of human kidney renin (42,000).

Maximal activation of prorenin was observed, when plasma was incubated with trypsin at 4 C. Other workers have incubated at 37 C[14]. Our results indicate that it is difficult if not impossible, to obtain complete activation of prorenin at this temperature. Moreover, the results at 37 C are strongly dependent on the concentration of trypsin and on the incubation time. At 4 C these variables are less critical, provided that incubation is prolonged until renin activity has reached a plateau. At 37 C a larger proportion of added trypsin is inhibited by plasma than at 4 C, and at 37 C the remaining uninhibited trypsin causes progressive inactivation or destruction of renin. Both the inhibition of trypsin, which leads to incomplete activation of prorenin, and the inactivation of renin by trypsin are the cause of measuring falsely low prorenin values. Low values have also been obtained after cryoactivation. It is therefore not surprising that the results of prorenin measurements reported in the literature are widely different. A literature search showed that the measured percentage of renin in normal plasma is highest with cryoactivation (35%-53%),[8,21–23] whereas it is intermediate with acid-activation (20%-50%),[1,2,10,11,13,22,24–32] and lowest with trypsin (12%-

34%)[3,12,14,33]. In our study, 11% of the renin in normal plasma was in the active form.

The methodological difficulties are amplified when prorenin is measured after maneuvers that are known to increase circulating renin. The accuracy of renin assays expressed as absolute values is inversely correlated to the height of the measured value. Thus, the higher the renin, the less accurate is its measurement. This causes problems particularly when the difference in renin before and after activation of plasma is small. It explains why there is confusion on whether, after certain stimuli, increments in circulating renin are associated with decrements in prorenin[1,2,25,27,30,31,34,35].

Changes in Prorenin after Captopril

As shown by the present study on patients with renal artery stenosis, stimulation of renin release by angiotensin converting enzyme inhibition with captopril causes a precipitous rise in circulating renin with initially no change or even fall in prorenin. Later also prorenin begins to rise. A transient fall in plasma prorenin after captopril has recently been reported[36–37], whereas earlier studies had failed to demonstrate such a fall[38,40].

Before stimulation with captopril we found a renal vein-to-aorta prorenin ratio of 1.19±0.08 (mean±SEM) on the affected side and of 1.04±0.08 contralaterally; the value on the affected side was just significantly different from 1.00 ($p < 0.05$). Also in earlier studies renal secretion of prorenin was difficult to demonstrate under basal conditions[2,11,25,27,41]. It has therefore been postulated that prorenin is formed by extrarenal inactivation of intrarenally produced renin. However, since prorenin appears to have a longer plasma half-life than renin[11], a relatively low secretion rate of prorenin may suffice to maintain a relatively high plasma level. It is possible therefore that the venoarterial difference in prorenin across the kidney is often too small to detect with an assay that has an accuracy that is not better than 10%.

After acute stimulation with captopril the secretion of prorenin by the affected kidney was not significantly increased, despite a tenfold rise in the secretion of renin. With chronic stimulation however, the venoarterial difference in prorenin became large enough to be easily detectable. Thus it appears that the kidney is indeed capable of secreting prorenin. This finding is probably not an artifact since secretion could be demonstrated on the affected side but not contralaterally. In fact, our data indicate that the changes in peripheral prorenin concentration after captopril are a consequence, at least in part, of corresponding changes in the rate of prorenin secretion from the affected kidney. Both the concentration of prorenin in peripheral plasma and its secretion are increased with prolonged stimulation of renin release but not with acute stimulation. An extrarenal source of plasma prorenin, however, cannot be entirely ignored since low to normal concentrations of prorenin are present in the plasma of nephrectomized subjects[11,25,42].

This pattern changes in renin and prorenin after captopril is similar to that of insulin and proinsulin following an oral glucose load; insulin rises within a few minutes but proinsulin begins to rise not earlier than after 1-2 hours[43]. The late rise in proinsulin is a manifestation of an increased rate of synthesis in the pancreas. More prohormone is then available for conversion to the active hormone before it is released into the circulation. These points of resemblance between the two hormonal systems should not distract from the fact that under normal basal conditions the concentration of insulin in peripheral plasma is 8-9 times higher than that of proinsulin, whereas the reverse is true for renin and prorenin.

Our results do not answer the question whether or not prorenin is a storage form of renin but they do suggest that prorenin enters the circulation either by leakage from the juxtaglomerular cells or by corelease before it is converted to renin. In conclusion, these data indicate that prorenin is indeed a biosynthetic precursor of renin and that under some circumstances, if not mostly, a major proportion of prorenin in the circulation originates from the kidney.

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5.4. RENAL VEIN IMMUNOREACTIVE RENIN IN PATIENTS WITH RENAL ARTERY STENOSIS OR ESSENTIAL HYPERTENSION

5.4.1. ABSTRACT

In 36 patients with unilateral renal artery stenosis and in 24 with essential hypertension the plasma levels of total immunoreactive renin, and enzymatically active renin were measured in both renal veins (V) and in the aorta (A) by direct RIA by using monoclonal renin antibodies. Active renin and trypsin-activatable inactive renin were also measured by indirect RIA with angiotensin-I antibodies. The V/A ratio for the different forms of renin calculated from the results of direct and indirect RIA were not different. The V/A ratio of active renin for the kidney with the stenotic artery was 3.04 ± 0.28 (mean ± sem) with direct and 3.02 ± 0.25 with indirect RIA. The contralateral ratio was 1.04 ± 0.02 with the direct and 1.05 ± 0.02 with the indirect RIA. In essential hypertension it was 1.28 ± 0.04 with direct RIA and 1.28 ± 0.04 with indirect RIA.

Chronic treatment with captopril had no influence on this ratio in both patients groups. The V/A ratio of total immunoreactive renin was lower than that of active renin and this ratio had lost discriminative power for lateralization. This ratio was significantly greater than one on the affected side in renal artery stenosis but not contralaterally and in essential hypertension. This study shows that renin activity after trypsin-activation of plasma is an accurate measure of the total renin concentration,i.e. active renin plus prorenin. It also shows that a kidney with a stenotic artery secretes inactive renin, which is immunologically related to active renin and is likely to be prorenin.

Direct RIA for measuring active renin is technically more simple than indirect RIA. Direct RIA however is somewhat less sensitive. For measuring the V/A ratio for active renin in patients with renal artery stenosis this can be overcome by stimulating the renin-angiotensin system for instance by captopril.

5.4.2. INTRODUCTION

Split renal vein sampling for demonstrating increased production of renin by the kidney supplied by a stenotic artery is helpful in the diagnosis of renovascular hypertension. The renal vein-to-artery (V/A) ratio of renin on the affected kidney versus the contralateral kidney is considered to be a useful index of the functional importance of the stenosis[1,2]. Furthermore a high ratio on the affected side with a suppressed ratio contralaterally is believed to predict operative curability[1,3]. A variety of stimulatory maneuvers has been employed to increase the sensitivity of this index by treating the patient with diuretics, vasodilatators and angiotensin-converting enzyme inhibitors[4–9].

In the studies reported so far, renin was determined by the enzyme kinetic method in which angiotensin I, generated from renin substrate *in vitro*, is measured

by RIA (indirect RIA). Monoclonal antibodies with high affinity for both active renin and inactive renin and monoclonal antibodies that only recognize active renin are now available and such antibodies can be used for direct RIA of renin. The purpose of the present study was to compare the results of direct RIA with those of indirect RIA in order to evaluate whether or not direct renin RIA is useful in the diagnostic work-up of patients with renal artery stenosis.

5.4.3. MATERIAL AND METHODS

Patients. Thirty-six hypertensive patients with unilateral renal artery stenosis and 28 with essential hypertension were studied. Renal artery stenosis was caused by atherosclerosis in 32 and by fibromuscular dysplasia in 4 patients. Eighteen patients with unilateral renal artery stenosis and 14 with essential hypertension were off therapy for at least two weeks before the study. The remainder of the patients were on captopril, 50 mg t.i.d. Blood was sampled from both renal veins and from the abdominal aorta. Since plasma renin may change during the course of the catheterisation procedure and since some time elapses between the samplings at the two sides, we prefer to measure the renal vein-to-aorta (V/A) renin ratio for each kidney instead of measuring the ratio between the veins of the two kidneys. The arterial samples were collected at exactly the same time as the corresponding renal vein samples. No radiocontrast drugs were used before or during the sampling procedure. Blood was collected in tubes containing 0.1 vol 0.13 M trisodium citrate. The samples were immediately centrifuged at room temperature and, plasma was stored at – 20 C.

Renin measurements. The plasma concentrations of enzymatically active renin (active renin) and inactive renin (prorenin) was measured by an enzyme kinetic assay[9,10]. In this assay plasma is incubated at 37 C with saturating amounts of sheep renin substrate, and angiotensin I that is formed is quantitated by RIA (indirect RIA). For measuring prorenin by indirect RIA plasma was incubated at 0 C with immobilized trypsin before assay, in order to convert prorenin into active renin[9]. Total immunoreactive renin (active renin and prorenin) and active renin were also measured by direct RIA, using monoclonal antibodies that were reacting with both active renin and prorenin or by antibodies that only recognize active renin. Total immunoreactive renin was measured using the monoclonal antibodies R 3-27-6 and R 3-36-16 (Ciba-Geigy, Basle, Switzerland)[11]. Details of the procedure have been described elsewhere[11,12]. Active renin or trypsin activated inactive renin were measured by using the monoclonal antibodies 3E8 and 4G1 (Diagnostic Pasteur, Marnes-la-Coquette, France) as described elsewhere[13]. Results of direct RIA are expressed as picograms per ml, using highly purified human kidney renin (Ciba-Geigy), specific activity 760 Goldblatt Units per mg of protein, as the standard.

5.4.4. RESULTS

The plasma levels of active renin and total renin in the aorta in patients with renal artery stenosis and in essential hypertensive subjects measured with the enzyme kinetic assay (indirect RIA) and with the immunological assay (direct RIA) are summarized in Table 1.

Table 5.4.1. Total renin and active renin in plasma of aorta measured with direct and indirect RIA.

Patient group	Active renin		Total renin	
	Direct RIA pg/ml	Indirect RIA μU/ml	Direct RIA pg/ml	Indirect RIA μU/ml
Essential hypertension				
no therapy	25.2 (10* -79.1)	17.9 (5.9 -54.3)	141 (32.0–620)	106 (23.8–470)
captopril 50 mg tid	102 (10* -1650)	76.9 (94.8–1221)	470 (72.1–3070)	343 (54.1–2170)
Renal artery stenosis				
no therapy	72.0 (10* -376)	52.6 (10.1–274)	331 (105 -1040)	238 (75.6–751)
captopril 50 mg tid	376 (42.1–3370)	273 (31.0–2390)	1040 (22.4–4830)	763 (161 -3630)

Mean values and 95% confidence intervals (in parentheses) are shown. Data were analysed after logarithmic transformation of the results to obtain a Gaussian distribution. Direct RIA of active renin was measured by monoclonal antibodies specific for active renin, direct total renin was determined with monoclonal antibodies that recognize active renin and prorenin. *lower limit of detection 10 pg/ml.

Table 5.4.2. Comparison of results of direct and indrect RIAs of renin.

Source of plasma	Trypsin treatment	Direct RIA total renin pg/ml	Direct RIA active renin pg/ml	Indirect RIA active renin μU/ml
Essential hypertension	no	184±17	18.7±3.7	14.2±1.2
	yes	181±19	189 ±20	139 ±10
Renal artery stenosis	no	398±36	92.5±6.7	54.8±5.1
	yes	402±29	396 ±39	290 ±26

Mean and SD of five experiments.

For measuring total renin with the indirect RIA plasma is treated with Sepharose-bound trypsin[8]. This treatment may lead to degradation of renin or activated prorenin. We therefore have measured total immunological renin and active renin with direct RIA in a plasma pool of patients with essential hypertension and renovascular hypertension before and after trypsin treatment of plasma. The result of the direct RIA for total renin was not altered by trypsin treatment of plasma (Table 2).

The V/A ratios for active renin and total renin calculated from the results from direct and indirect RIA are not different (Fig.1 and Table 3). The V/

Table 5.4.3. Renal vein-to-aorta ratio of active renin, total renin and inactive renin.

Patient group	Captopril	Active renin		Total renin		Inactive renin	
		Direct	Indirect	Direct	Indirect	Direct	Indirect
Essential hypertension							
Right side	no	1.28±0.04	1.27±0.05	1.07±0.02	1.06±0.02	1.03±0.02	1.02±0.02
	yes	1.30±0.04	1.31±0.04	1.11±0.03	1.10±0.03	1.03±0.02	1.04±0.02
Left side	no	1.27±0.04	1.29±0.04	1.08±0.02	1.08±0.02	1.04±0.02	1.05±0.03
	yes	1.29±0.04	1.31±0.05	1.12±0.03	1.13±0.03	1.07±0.02	1.09±0.04
Renal artery stenosis							
Affected side	no	3.04±0.28	3.02±0.25	1.71±0.11	1.74±0.10	1.25±0.06	1.23±0.05
	yes	3.05±0.27	3.07±0.27	2.27±0.19*	2.24±0.18*	1.59±0.16*	1.64±0.14*
Contralateral side	no	1.04±0.02	1.05±0.02	1.03±0.02	1.03±0.02	1.03±0.03	1.02±0.03
	yes	1.05±0.02	1.04±0.02	1.04±0.03	1.04±0.03	1.04±0.03	1.05±0.04

Mean ± SEM are presented. Direct RIA: active renin was measured with a monoclonal antibody specific for active renin, total renin was measured with monoclonal antibodies that recognize active renin and prorenin. Indirect RIA was measured with angiotensin I antibodies. In this assay total renin was measured after trypsin-treatment of plasma. Inactive renin is calculated as the difference between total renin and active renin. *difference $p < 0.01$ from no treatment (unpaired t-test). Captopril dosage was 50 mg t.i.d. for three to four weeks.

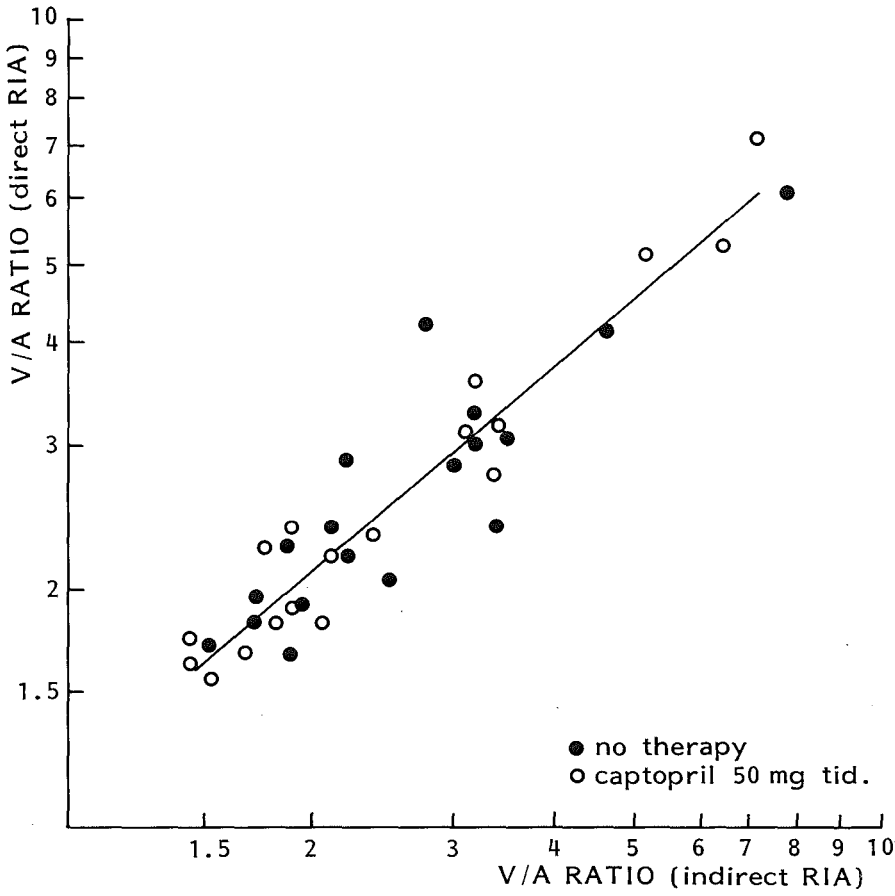

Fig.5.4.1. Renal vein-to-aorta ratio for active renin measured by the direct and indirect RIA on the affected side in patients with unilateral renal artery stenosis.

A ratios for active renin in the kidney with the stenotic artery calculated from the results of the direct RIA or the indirect RIA were elevated as compared to the ratios in essential hypertension, and stimulation of the renin angiotensin system with captopril had no effect on these ratios. V/A ratios of active renin on the contralateral side in patients with renal artery stenosis were suppressed, also when the patients were treated with the converting enzyme inhibitor.

The V/A ratios for active renin measured with either the direct or indirect RIA on the affected side in patients with renal artery stenosis were higher than the V/A ratios for total renin (active renin and prorenin) on this side (Table 3). The V/A ratios for prorenin were significantly greater than one on the affected side in patients with renal artery stenosis but not contralaterally and in essential hypertension. During captopril treatment the V/A ratio for prorenin on the affected side rose significantly from 1.25 to 1.59 (Table 3).

5.4.5. DISCUSSION

An elevated V/A renin ratio for the kidney with the stenotic renal artery and a suppressed renin ratio contralaterally is considered to be good index of the functional importance and is also believed to predict operative curability[1-3]. Therefore renin measurements are of crucial importance in the diagnostic work-up of patients with suspected renovascular hypertension.

Most investigators use PRA measurements for calculating the V/A renin ratio's. However PRA measurements depends not only on the concentration of active renin but also on the concentration of endogenous renin substrate and the contribution of each of the two components has not been precisely quantitated. It is conceivable that high concentrations of active renin in the plasma of the renal vein of the affected kidney leads to erroneously low PRA levels due to renin substrate exhaustion. This will lead to a relatively low V/A ratio for PRA on the affected side. We therefore prefer measurements of active renin concentration in which saturating amounts of exogenous renin substrate are added and the generation of angiotensin I solely depends on renin concentration.

This study shows that the V/A ratios for active renin measured with the direct RIA are not different from those measured with the indirect RIA. The use of monoclonal antibodies has some advantages 1) the method is independent of endogenous or exogenous substrate, 2) the generation step of angiotensin I, with problems such as recovery and pH of the incubation mixture, is not longer necessary, 3) there is no need for a centrifuge and water bath and, 4) one technician can easily handle up to a 100 samples in one day. The indirect RIA for renin normally takes two days. A disadvantage is the relatively low level of detection of about 10 pg/ml, which leads to a relatively high intra- and interassay variability (the intra-assay coefficient of variation is 16% for a plasma pool with an active renin concentration of 21.2 pg/ml ($n = 6$) and only 3.6 % for a pool with a mean value of 66.4 pg/ml ($n = 6$). This problem can easily be solved when renal vein sampling is done during stimulation of the renin-angiotensin system by captopril.

Stimulation of the renin-angiotensin system by captopril, leading to a 6-fold increase in the peripheral level of active renin, does not influence the V/A renin ratios. Why is this so ? It is often implied that the high renin ratio is caused by a high secretion rate of renin from the affected kidney and a low secretion rate contralaterally. However, a high ratio is not necessarily caused by such a difference in secretion rate but is an indication of diminished renal plasma flow on the affected side rather than an index of increased renin secretion[2,7]. It is interesting to note that despite captopril treatment the V/A ratio for active renin on the contralateral side in patients with unilateral renal artery stenosis is still suppressed. A suppressed ratio in untreated patients could be explained by elevated angiotensin II levels which will inhibit renin secretion contralaterally. During converting enzyme blockade with the captopril dose we have used angiotensin II levels are low[14]. Thus it is unlikely that the suppressed ratio is

caused by elevated angiotensin II levels. This phenomenon therefore remains unexplained.

Normally about 90 percent of the renin in plasma circulates in the enzymatically inactive form (prorenin). For the indirect RIA of plasma prorenin, we activated the samples with immobilized trypsin at 0 C. The two important questions about the validity of the method are, 1) is active renin or activated prorenin degraded into inactive fragments and, 2) are all prorenin molecules in the sample converted into active renin. Our results of the direct RIA for total renin was not altered by trypsin-treatment of the plasma, therefore it is unlikely that naturally occurring active renin or activated renin are destroyed during the activation step with trypsin. With our method of trypsin treatment prorenin activation in plasma reached a plateau independent of the trypsin concentration over a wide range of concentrations[9]. Km for naturally occurring active renin is not different from that of trypsin-activated prorenin[9]. The Vmax, expressed as the catalytic activity per unit weight of renin is also the same for both renins[12]. Thus the renin activity after trypsin-activation of plasma is an accurate measure of the total renin concentration that is active renin plus prorenin. In some reports, Skinner's method[15] was used for measuring plasma total renin concentration: plasma was dialyzed for 24 h at pH 3.3 and 4 C followed by 24 h at pH 7.5 and 4 C before the indirect RIA to convert prorenin to active renin. The V/A ratio on the affected side was greater for active renin or PRA than for total renin[16–19]. A reason for this discrepancy could be the method of activation of prorenin. The degree of prorenin to active renin conversion is critically dependent on pH and duration of dialysis. In our hands e.g., acid-activation of prorenin in plasma (24-h dialysis at 4 C against a pH 3.3 buffer followed by dialysis for 24 h against a pH 7.5 buffer) is too short in many plasma samples to activate prorenin completely. However, even with the use of monoclonal antibodies that recognize both prorenin and active renin , V/A ratios of total renin are much lower than the V/A ratios for active renin. Therefore the V/A ratios of total renin on the affected side in patients with renal artery stenosis have less discriminative power for lateralization of the disease.

The present study also demonstrates that immunoreactive renin (active renin and prorenin) is secreted by the kidney with the stenotic artery especially when the renin-angiotensin system is stimulated by a converting enzyme inhibitor. This confirms an earlier study of our group[9] and other reports[16,20] that the kidney with the stenotic artery secretes inactive renin, which is immunologically related to active renin and is likely to be prorenin.

In summary direct RIA of active renin in plasma of renal veins and aorta for calculating the V/A ratios is a powerful tool for diagnosis and lateralization in patients with suspected renal artery stenosis.

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5.5. ACTIVATION OF PLASMA PRORENIN BY PLASMINOGEN ACTIVATORS *IN VITRO* AND INCREASE IN PLASMA RENIN AFTER STIMULATION OF FIBRINOLYTIC ACTIVITY *IN VIVO*.

5.5.1. ABSTRACT

Plasminogen can be activated by intrinsic activators that circulate in plasma in a precursor form, by extrinsic activator originating from tissues or the vessel wall and by the exogenous activators, urokinase and streptokinase. Tissue activator and vascular activator are probably identical. Dialysis of plasma against pH 4.0 buffer causes denaturation of the plasmin inhibitors, α_2-antiplasmin and C_1-inhibitor, while α_2-macroglobulin is left intact. Incubation of pH 4.0 pretreated plasma with urokinase or streptokinase at pH 7.5 led to activation of plasminogen and prorenin. Incubation of a plasma fraction, which contained plasminogen and prorenin but no α_2-antiplasmin and renin, with highly purified tissue plasminogen activator also led to activation of prorenin. Incubation of a plasma fraction, which contained plasminogen and prorenin but no α_2- antiplasmin and renin , with highly purified tissue plasminogen activator also led to activation of prorenin. The vasopressin analoque, 1-des-amino-8-D-arginine vasopressin (DDAVP), is a potent stimulant for the release of extrinsic activator into the blood stream. After infusion of DDAVP, 0.4 μg/kg, into normal subjects, parallel increments in plasma fibrinolytic activity and renin were observed. Infusion of DDAVP into patients with type IV hyperlipoproteinaemia had little effect on plasma fibrinolytic activity and the response of plasma renin was also subnormal. These observations warrant further studies on a possible role for plasminogen activators in prorenin activation in vivo.

5.5.2. INTRODUCTION

Two intrinsic activators of prorenin have been identified: plasma kallikrein and plasmin[1]. These activators are present in plasma precursor form. Acidification of plasma to pH 3.0-3.5 causes denaturation of C_1-inhibitor (C_1-INH) and α_2-macroglobulin (α_2M), the major inhibitors of kallikrein. By this treatment plasma acquires the ability to convert its prorenin into active renin by kallikrein that is formed by factor XII-initiated activation of prekallikrein[2–4]. However, contact activation of the intrinsic factor XII-kallikrein pathway by kaolin without prior treatment of plasma with acid does not lead to activation of prorenin. Similarly, the addition of the plasminogen activators, streptokinase and urokinase, to untreated plasma does not lead to activation of prorenin. The most important inhibitor of plasmin is α_2-antiplasmin but C_1-INH and α_2M are also effective plasmin inhibitors.α_2-Antiplasmin is denaturated by acid as are C_1-INH and α_2M.

We have studied the effects of adding plasmin, streptokinase or urokinase to plasma pretreated with buffers of various pH. We have also studied the effect

of adding highly purified tissue plasminogen activator to a plasma fraction, which contained plasminogen and prorenin but was free of α_2-antiplasmin and active renin.

Tissue plasminogen activator and vascular plasminogen activator are likely to be identical. This activator is released into the blood by various stimuli[5]. Some of these, for instance physical exercise and certain vasodilator substances, also stimulate the release of renin. A particularly powerful stimulus for the release of vascular activator is the vasopressin analogue , 1-desamino-8-D-arginine vasopressin (DDAVP)[6]. It has antidiuretic activity but its vasoconstrictor activity is much less than that of L- arginine vasopressin. We have administrated DDAVP to normal volunteers and to patients with type IV hyperlipoproteinaemia. In such cases the release of vascular plasminogen activator is less responsive to DDAVP than in normals[7]. Because of this we thought it might be of interest to compare the effects of DDAVP on plasma renin in these patients and in normals.

5.5.3. METHODS AND SUBJECTS

Measurement of plasmin, plasminogen and α_2-antiplasmin

Plasmin was measured via its amidolytic action on the chromogenic substrate D-valyl-leucyl-l-lysine-p-nitroanilide (S2251, Kabi,Sweden)[8,9]. The release of p-nitroaniline was followed in a spectrophotometer. Plasminogen was determined as amidolytic activity of the streptokinase-plasminogen complex. Measurement of α_2-antiplasmin was based on the rapid inhibitory action of this plasma protein on the amidolytic activity of added plasmin.

Measurement of fibrinolytic activity

An euglobulin fraction was prepared from plasma and fibrinolytic activity of this fraction was determined by the fibrin plate method[10,11].

Measurement of renin and prorenin

Renin was measured via its ability to cleave angiotensin I from sheep renin substrate. Angiotensin I was determined by radioimmunoassay. Prorenin was measured in the same way after complete conversion into active renin by immobilized trypsin[8].

Normal subjects and patients

Nine healthy volunteers and nine subjects with type IV hyperlipoproteinaemia received DDAVP (Ferring, Denmark), 0.4 μg/kg intravenously, over 10 minutes. The subjects were lying in bed for at least one hour before the infusion was started. Blood samples were taken at various time intervals as indicated under 'Results'. EDTA-plasma was used for determinations of plasmin, plasminogen,α_2-antiplasmin, renin and prorenin. Citrate-plasma was used for measurement of fibrinolytic activity.

5.5.4. RESULTS

Effects of acid-pretreatment of plasma on plasminogen,α_2-antiplasmin and prorenin

Normal plasma was dialyzed for 24 h at 4 C against buffers of pH 4.0-7.5[3]. pH was then restored by dialysis against a pH 7.5 phosphate buffer for 24 hours at 4 C. As shown in Fig.1 ,α_2-antiplasmin was denaturated at pH 5-6 but there was no evidence that plasminogen and prorenin were activated; consumption of streptokinase activatable plasminogen and trypsin-activatable

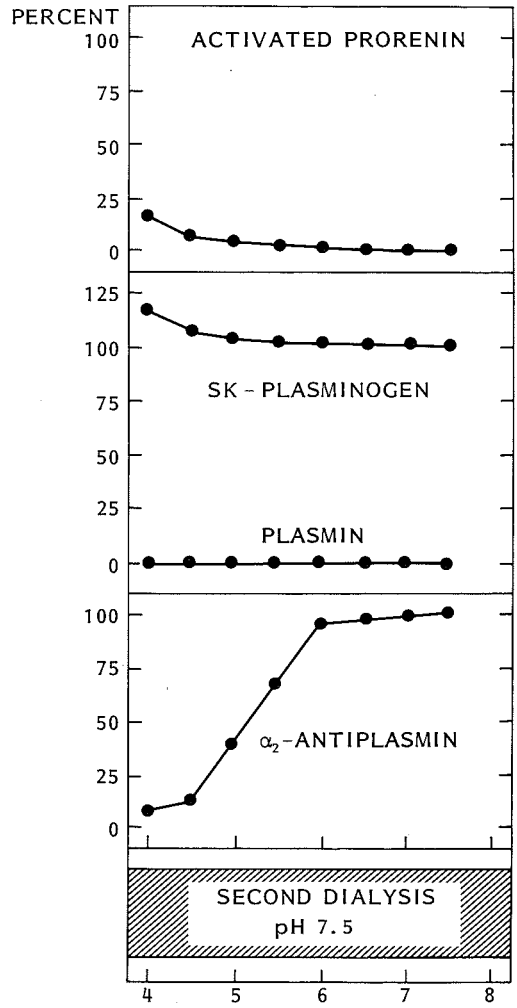

Fig. 5.5.1. Normal EDTA-plasma was first dialysed for 24 hours at 4 C against buffers of different pH and then against pH 7.5 buffer also for 24 hours. The levels of plaminogen and prorenin after maximal activation in non-dialyzed plasma by streptokinase (SK) and immobilized trypsin respectively were taken as 100%. Also for α_2-antiplasmin the level in non-dialyzed plasma was taken as 100%.

prorenin was undetectable, and plasmin and renin activities were not generated. Thus, plasma pretreated at pH 4.0 could serve as a source of prorenin and plasminogen with little or no α_2-antiplasmin activity. Pretreatment of plasma at pH 4.0 also destroys C_1-INH but leaves α_2M intact[3].

Effects of plasmin, streptokinase and urokinase on prorenin in pH 4.0- pretreated plasma

When untreated plasma was incubated at pH 7.5 with plasmin (final concentration 2 casein units/ml) or urokinase (100 Ploug units/ml) little activation of prorenin occurred. There was some activation with streptokinase in high concentration (1000 Christensen units/ml). This contrasts with the results when pH 4.0-pretreated plasma was incubated with these activators at pH 7.5 (Fig.2).

Effects of highly purified tissue plasminogen activator on a semipurified fraction of plasma prorenin

Highly purified plasminogen activator isolated from human uterine tissue[12] was a gift of Dr Kluft (Gaubius Institute, TNO, Leiden, The Netherlands). Prorenin was isolated from normal plasma by affinity chromatography on columns packed with Blue-Sepharose CL-6B (Pharmacia,Sweden). Prorenin was eluted with phosphate buffer pH 7.1 containing 0.2 mol/l NaCl. Plasminogen appeared in the same fraction but active renin and α_2-antiplasmin were undetectable. When this fraction was incubated with the tissue activator (final concentration 10 Ploug units/ml), prorenin was progessively activated (Fig.3).

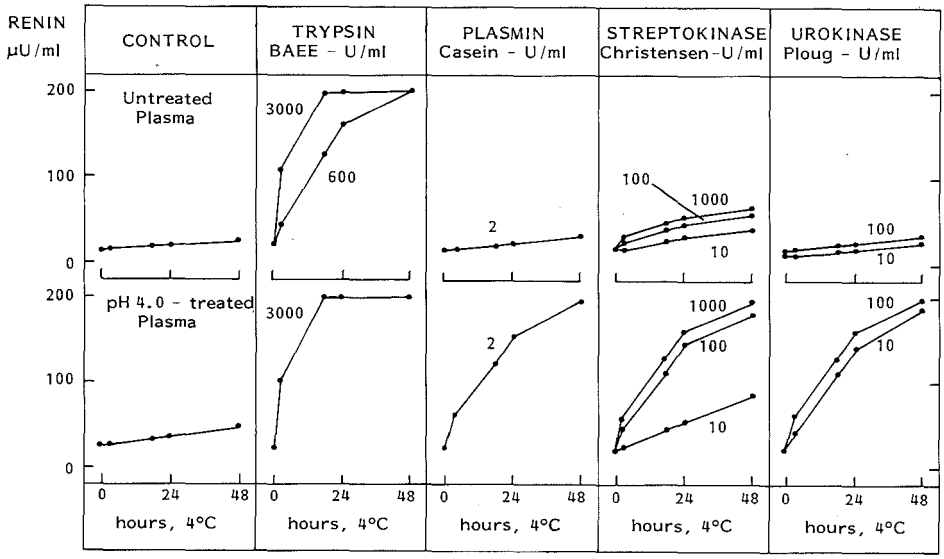

Fig. 5.5.2. Normal EDTA-plasma was pretreated at pH 4.0 as in Fig.1 and incubated with activators at pH 7.5. Results are compared with those obtained without acid-pretreatment.

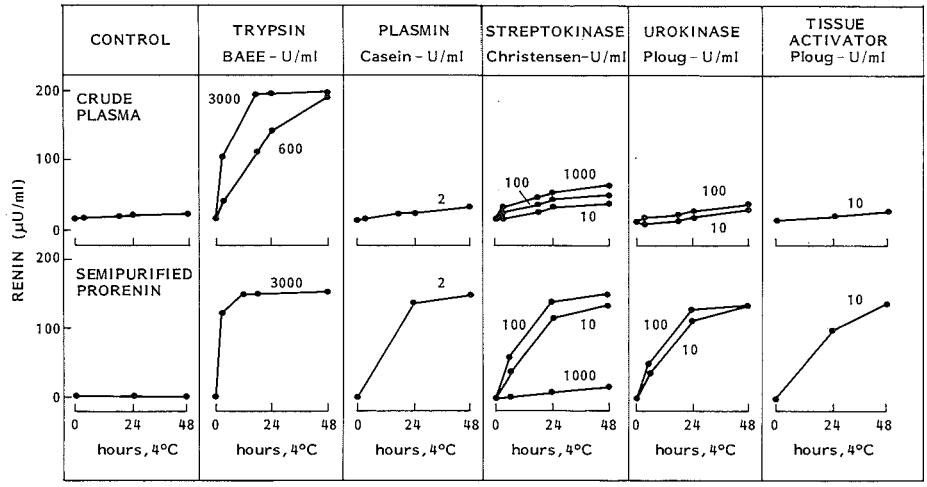

Fig. 5.5.3. A prorenin-containing fraction was isolated from plasma by dye-ligand chromatography (see text). This fraction also contained plasminogen but active renin and α_2-antiplasmin were undetectable. Results are compared with those obtained in crude plasma.

It is of interest that in semipurified prorenin high concentrations of streptokinase were less effective than low concentrations. At low concentrations a relatively small proportion of plasminogen is complexed with streptokinase. These complexes convert the remaining uncomplexed plasminogen into plasmin, which then activates prorenin. Streptokinase-plasminogen complex is a good activator of plasminogen but towards prorenin it may be less potent than plasmin. Besides being less potent than plasmin these complexes are also less sensitive to inhibitors including α_2-antiplasmin[13]. These differences between the active streptokinase-plasminogen complex and plasmin may explain why in untreated plasma high concentrations of streptokinase are more effective in prorenin activation than low concentrations, whereas the reverse is true in semipurified prorenin fractions. Urokinase converts plasminogen into plasmin but does not form complexes with plasminogen.

Effects of DDAVP in normal subjects and in patients with type IV hyperlipoproteinaemie

Fig.4 shows the results in eleven healthy volunteers in whom the effects were followed for 60 minutes. Systolic arterial pressure did not change significantly but diastolic pressure fell by about 10 mm Hg ($P<0.05$) during the first 20 minutes after DDAVP infusion had been started. Plasma fibrinolytic activity rose markedly and this was accompanied by a parallel increase in plasma renin. A significant change in prorenin was not observed.

In Fig.5 the effects in nine normal subjects and in nine patients with type

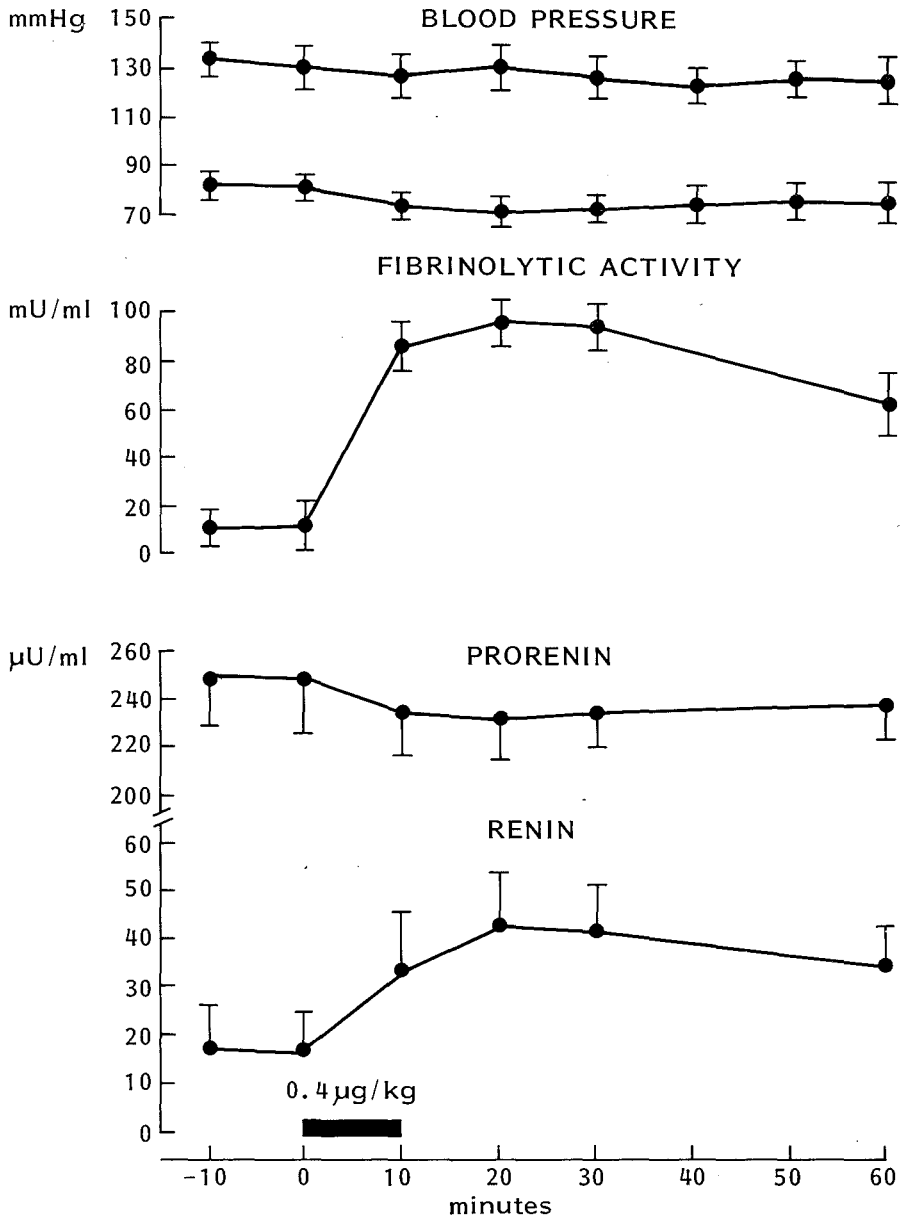

Fig. 5.5.4. Effects (mean±SEM) of DDAVP, 0.4 μg/kg i.v., in eleven normal subjects.

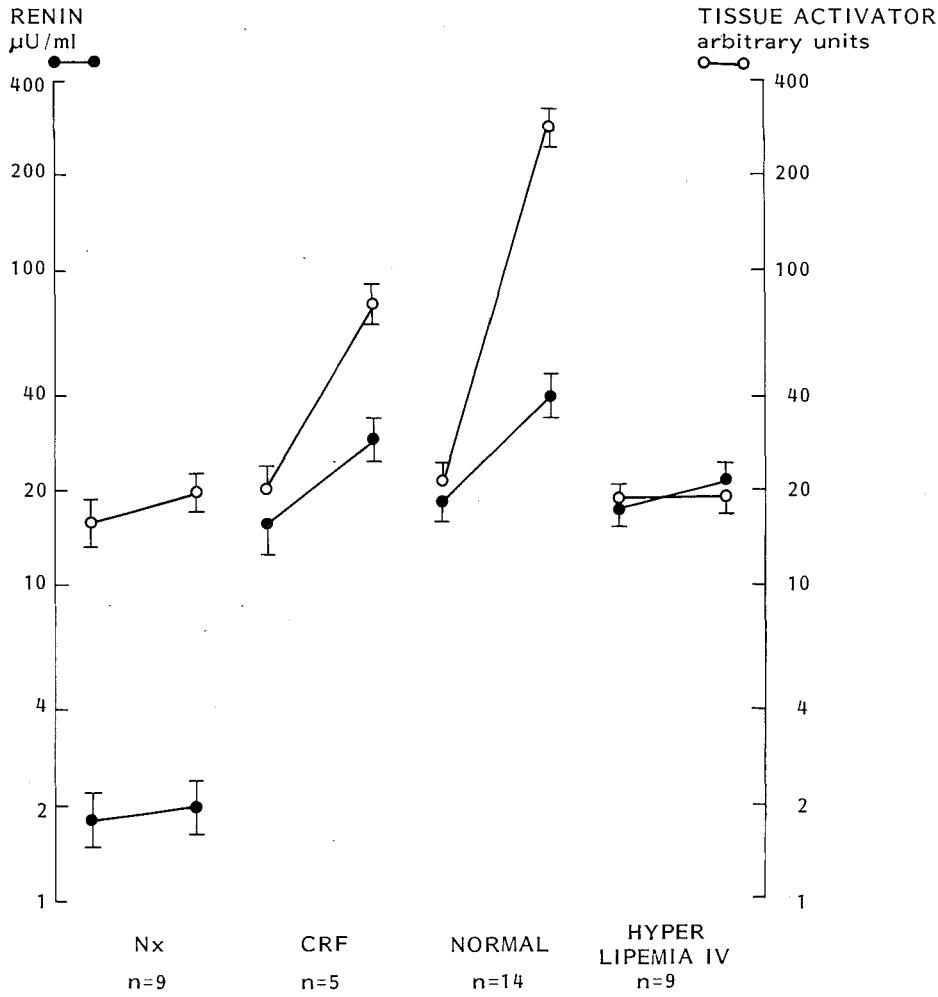

Fig. 5.5.5. Effects (mean±SEM) of DDAVP, 0.4 μg/kg i.v. 10 minutes, in nine normal subjects and in nine patients with type IV hyperlipoproteinaemia. Blood was sampled just before and 30 minutes after DDAVP infusion had been started. N_x = nephrectomized patients, CRF = chronic renal failure.

IV hyperlipoproteinaemie are compared. DDAVP was infused in the same way as in Fig. 4, and blood samples were taken just before DDAVP and 30 minutes after the infusion had been started. In the patients with hyperlipoproteinaemie plasma fibrinolytic activity did not change after DDAVP and plasma renin in these patients was also less responsive than in the normal subjects. Again there were no significant changes in prorenin.

5.5.5. DISCUSSION

Plasminogen activation may occur by an intrinsic pathway involving the plasma proteins factor XII, prekallikrein and high molecular weight kininogen and possibly other plasma components as well. An extrinsic pathway also exists in which the activator originates from tissue or from the vessel wall and is released into the blood by certain stimuli. Finally plasminogen can also be activated by an exogenous pathway in which activators , streptokinase and urokinase, are added to plasma or are infused into patients for therapeutic purposes. Diurnal variations in the so-called fibrinolytic activity of plasma are mainly caused by changes in the level of extrinsic activator. The increased fibrinolytic activity of plasma after physical exercise and after DDAVP also reflects a higher content of extrinsic activator, due to stimulation of the release of this activator into the bloodstream[11].

Our experiments in vitro demonstrate that not only the exogenous activators, streptokinase and urokinase, but also the extrinsic tissue plasminogen activator, which is probably identical with vascular activator, are capable of activating prorenin. The studies in normal subjects and patients show parallel increments of the fibrinolytic and renin activities of plasma after DDAVP. This of course does not imply that the rise in fibrinolytic activity is the cause of the rise in renin. It is however interesting that analogous results have been obtained with cultures of islets of Langerhans from rat pancreas[14]. By raising the glucose content of the medium the release of insulin and the release of plasminogen activator were stimulated in a parallel fashion. There is further some biochemical evidence that proinsulin can be cleaved by plasmin to yield an insulin-like fragment. Our studies have been performed in small groups of subjects and need to be extended to elucidate the possible role of plasmin and plasminogen activators in prorenin activation.

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5.6. IMMUNOREACTIVE RENIN, PRORENIN AND ENZYMATICALLY ACTIVE RENIN IN PLASMA DURING PREGNANCY AND IN WOMEN TAKING ORAL CONTRACEPTIVES.

5.6.1. ABSTRACT

Direct RIA of renin with monoclonal renin antibodies and indirect RIA with angiotensin I antibodies were performed in plasma of 44 pregnant women, 44 women taking an oral contraceptive (OC) and 54 normal women. The following parameters were measured: immunoreactive renin, naturally occurring enzymatically active renin (active renin), trypsin-activatable inactive renin (prorenin), plasma renin activity (PRA), and renin substrate.

Immunoreactive renin (mean; 95% confidence interval) was significantly higher in pregnant women (1090; 420-2800 pg/ml,third trimester) than in normal women (248; 101-562 pg/ml,$P<0.001$), and lower in OC-treated women (131; 41-415 pg/ml,$P<0.001$). Prorenin and active renin also were increased in pregnant women and decreased in OC-treated women. The fraction of renin that was in the active form was lower in pregnant women (4.8; 1.4-18%) than in OC-treated women (8.8; 3.0-25%,$P<0.001$) and in normal women (9.1; 2.9- 29%,$P<0.001$). Renin substrate was increased to comparable levels in pregnant women and OC-treated women, but PRA was increased in pregnant women and normal in OC-treated women.

The maximum velocity per unit weight of renin was the same for active renal renin as for active plasma renin and trypsin- activated plasma prorenin. Maximum velocity and Km-values measured in mixtures of purified active renin and renin substrate and the concentrations of active renin and renin substrate measured in whole plasma were entered into the Michaelis-Menten equation for calculating PRA. The calculated values were similar to the measured results in all three groups ,indicating that PRA was determined by the molar concentrations of enzyme and substrate. Thus, we found no evidence of unknown substances in plasma interfering with the enzyme-substrate reaction. The percentage of circulating renin in the active form was much lower during pregnancy than in other conditions where the renal release of active renin is stimulated and where prorenin is as high as during pregnancy. This suggests that a smaller fraction of prorenin is intrarenally converted into active renin before its release into the circulation or that a larger fraction of circulating prorenin is of extrarenal origin. The finding that PRA is normal during OC treatment suggests that the estrogen-induced increase in renin substrate is compensated for by suppressed renal release of active renin.

5.6.2. INTRODUCTION

Both pregnancy and the use of oral contraceptives (OC) induce impressive changes in the renin-angiotensin system, but the pathophysiological consequences of these

changes are not clear. Part of the uncertainty is related to the fact that measurements of the various components of the renin-angiotensin system are not always easy to interpret. There is general agreement that both the plasma concentration of renin substrate (angiotensinogen)[1–5] and the plasma renin activity (PRA)[1–3,6,7] are increased during pregnancy. Renin substrate is also increased in women taking OC, but it is uncertain whether PRA is altered by OC[8–18]. PRA depends on the concentration of renin and renin substrate, and the contribution of each of the two components has not been precisely quantitated.

A large proportion of renin circulates in plasma in an enzymatically inactive form (prorenin). In previous studies, it was often not clear whether only active renin or both active renin and prorenin were measured and to what extent activation of prorenin had occurred during storage and handling of the plasma samples. Measurements of prorenin during pregnancy have yielded widely different results depending on the method of measurement[1,3,19,20]. Data on prorenin in women taking OC are lacking.

In the studies reported so far, renin was determined by the enzyme kinetic method, in which angiotensin I (ANG I) generated from renin substrate *in vitro* is measured by bioassay or RIA. The enzyme kinetic assay of inactive renin depends on the conversion *in vitro* of prorenin to active renin. Antibodies with high affinity for both the active and inactive forms of renin are now available, and such antibodies can be used for the assay of immunoreactive renin.

Here, we report measurements of immunoreactive renin, enzymatically active and inactive renin, PRA, and renin substrate in plasma of women who were pregnant or taking OC. In this paper we refer to measurements of renin by RIA of *in vitro* generated angiotensin I as indirect RIA, as opposed to the direct RIA, which makes use of antibodies against renin. The assays were improved to be able to compare the results of the indirect assays with those of the direct assay and to express results in terms of catalytic activity per unit weight of renin. This, in turn, enabled us to examine whether the measured changes in PRA can be explained by changes in the molar concentrations of renin and renin substrate or whether other factors, such as a change in the kinetic constants of the reactants or the presence of interfering substances in plasma, are also involved.

5.6.3. MATERIALS AND METHODS

Subjects. Caucasian pregnant women (n = 44) referred to the obstetric outpatient clinic of our hospital were studied. All were in the last trimester of pregnancy, between 30 and 38 weeks gestation (mean 34.8 weeks). Their age ranged from 19-39 yr (mean, 30 yr). Seventeen women were primigravida.

Women taking OC (n = 44; 21-33 yr old; mean, 25 yr) and normal women (n = 54; 20-37 yr old; mean, 27 yr), who were hospital personnel or students were also studied. The former had been taking OC for at least 6 months. The OC contained 30 or 37.5 μg ethinyl estradiol as the estrogen and 0.15 mg

levonorgestrel or 0.15 mg desogestrel as the progestagen [Microgynon 30, (Schering, Weesp, Netherlands), Stederil 150/30 (Wyeth, Hoofddorp, Netherlands), Marvelon (Organon, Oss, Netherlands)] . The normal women were studied in the first 7-12 days of the menstrual cycle. All were normotensive, were eating unrestricted sodium diets, and were not taking any medication, with the exception of some pregnant women who used ferrous sulfate and/or a multivitamin preparation.

Blood sample collection

An indwelling catheter was inserted into the left antecubital vein, and the women rested quietly in the recumbent position for 45 min before blood samples were drawn. The pregnant women were lying on the left side. Blood was collected in tubes containing either 0.1 vol 0.13 M trisodium citrate or 0.1 vol 0.13 M trisodium citrate to which soybean trypsin inhibitor (Sigma, St Louis, MO; USA, 10 mg/ml), had been added. The blood samples were immediately centrifuged at 3000 × g for 10 min at room temperature, and plasma was stored in 1 ml aliquots at -70 C.

Various forms of renin: terminology

The following terms are used to designate the various forms of renin and the different types of renin assay.

Active renin, the naturally occurring enzymatically active species;

Prorenin, the enzymatically inactive species that is converted into active renin by trypsin;

Immunoreactive renin, the form recognized by monoclonal renin antibodies;

Direct RIA, assay of renin with monoclonal renin antibodies;

Indirect RIA, assay of renin based on the generation of angiotensin I (ANG I) *in vitro*, quantitated using antibodies against ANG I;

Concentrations of active renin and prorenin, these were measured by the indirect RIA; ANG I was generated at saturating amounts of exogenous renin substrate, and prorenin was activated by trypsin before assay; and *PRA*, this was measured by the indirect RIA; ANG I was generated from endogenous renin substrate.

Renin measurements

Direct RIA. The concentration of immunoreactive renin in plasma was determined with a solid phase sandwich assay using two monoclonal antibodies, R 3-27-6 and R 3-36-16 (Ciba-Geigy,Basel,Switzerland), as described by Hofbauer et al.[21]. The two antibodies react equally well with active human kidney renin and chorionic cell culture prorenin.

Antibody R 3-27-6 was coupled to microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA, USA). The coupling buffer was 0.057 M carbonate-bicarbonate, pH 8.6, containing 0.1 M NaCl and 0.2 % sodium azide. Antibody R 3-27-6 was dissolved in this buffer in a final concentration of 10 μg/ml. The antibody-containing coupling buffer (150 μl) was pipetted into each well of the

microtiter plate and incubated for 2 h at 37 C in a moist chamber. After washing the plates with 0.1 M sodium phosphate, pH 7.5, containing 0.05 M NaCl, (phosphate buffered saline, PBS), the remaining protein-binding sites in the wells were blocked by adding 250 μL PBS containing 1% BSA and 0.2% sodium azide (PBS-BSA) and by incubation for 1 h at 37 C, followed by washing with PBS.

Monoclonal antibody R 3-36-16 was labeled with ^{125}I. The specific activity was 16 mCi/mg protein. Renin-free plasma was prepared from normal plasma by immunoadsorption on a column of CNBr-activated Sepharose 4 B (Pharmacia Fine Chemicals AB,Uppsala,Sweden) to which the monoclonal antibody R 3-36-16 had been coupled (5 mg antibody to 2 g Sepharose).

For the assay, 50 μl PBS-BSA and either 50 μl serial dilutions of highly purified human kidney renin (Ciba-Geigy); 20 to 20,000 pg/ml in renin-free plasma) or 50 μl of the unknown sample were added to the wells together with 50 μl of labeled antibody (100,000 cpm/50 μl). Purified human kidney renin (MRC standard no 68/356, Medical Research Council,National Institute for Biological Standards and Control,London,UK) was also run in each assay. All samples were assayed in quadruplicate. The mixture were incubated at 37 C for 2 h, followed by incubation at 4 C for 30 min in a moist chamber. The plates were thoroughly washed with PBS, and the wells were cut and counted in a scintillation counter.

Results were derived from a standard curve (Fig.1) with subtraction of the blank and are expressed as picograms per ml plasma, using the Ciba-Geigy renin preparation as a standard. Interassay variability (coefficient of variation) was determined by repeated measurements in pooled plasma from pregnant women , women taking OC, and normal women (8%,20% and 17% respectively; $n = 8$). The concentration of immunoreactive renin (mean) in these plasma pools were 1035, 121, and 278 pg/ml. Thus, the interassay variability was rather high when the level of immunoreactive renin was relatively low, due to the relatively high blank. Repeated freezing and thawing of the plasma samples leads to erroneously low results. We, therefore, froze these samples only once and assayed them immediately after thawing.

Indirect RIA. The assay was described previously[22]. The concentration of active renin was measured in plasma samples containing soybean trypsin inhibitor at a final concentration of 2 mg/ml. The plasma was thawed rapidly at 37 C before assay to prevent inadvertent activation of prorenin. The concentration of prorenin was measured in samples that did not contain soybean trypsin inhibitor, after activation by incubating plasma with immobilized trypsin at 0 C. Purified prorenin was activated by trypsin after addition of human serum albumin (Behringwerke AG, Marburg, West Germany) in a final concentration of 30 mg/ml. In this way, degradation of renin by trypsin was prevented. Aliquots (0.05 to 0.25 ml) of the unknown sample were added to 0.50 ml of purified sheep renin substrate and the volume was adjusted to 1.0 ml with PBS. After addition of protease inhibitors, *i.e.* EDTA, 8-hydroxyquinoline sulfate, phenylmethylsulfonylfluoride,

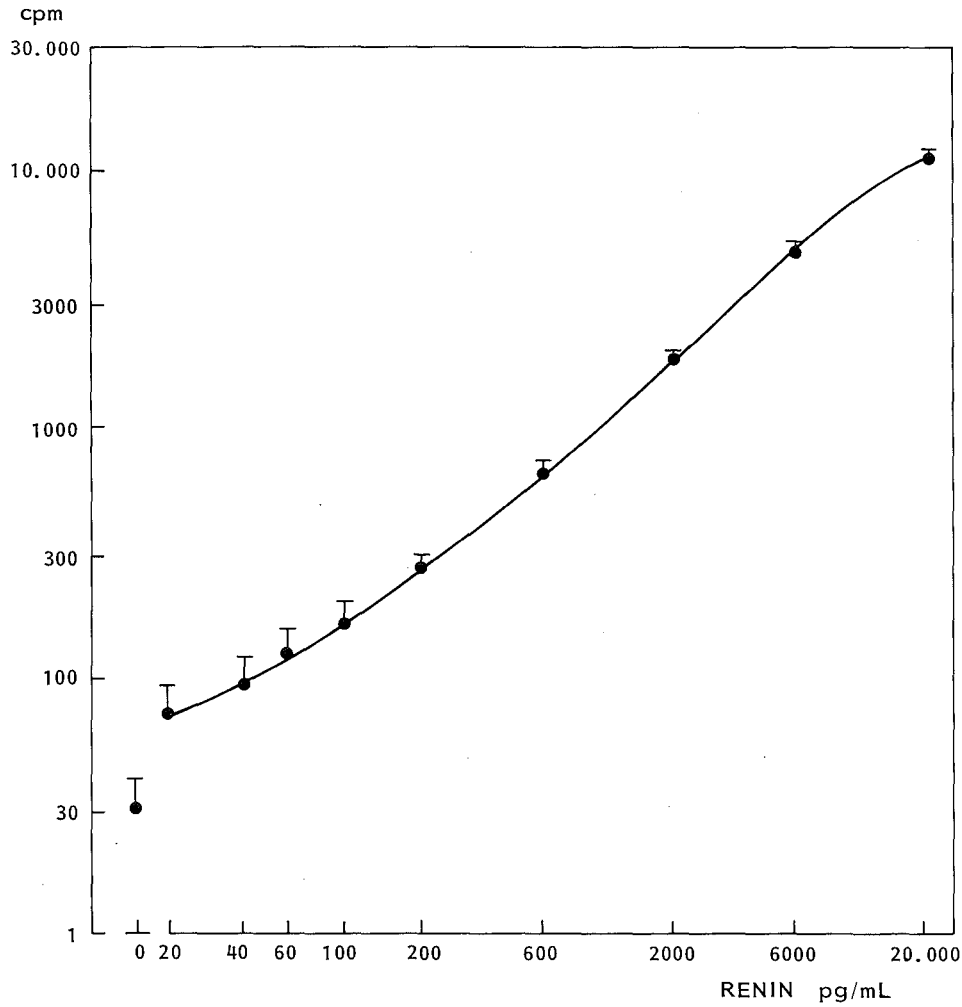

Fig. 5.5.1. Direct radioimmunoassay of renin. Human kidney renin (Geigy) was added to plasma from which renin (prorenin and renin) had been removed by immunoadsorption. Data are the mean (± SD) of eight experiments.

and aprotinin[22] the samples were incubated at 37 C for 0, 10, 30, 60, 120 and 180 min. Endogenous renin substrate might influence the results, particularly when its level is high as in plasma of pregnant women and women taking OC. Under the conditions of our assay, the maximum velocity (Vmax) of the reaction of active renin with sheep renin substrate was 3 times the Vmax of the reaction with human renin substrate; Km values were 240 pmol/ml and 1200 pmol/ml respectively (see below). The concentration of sheep renin substrate was 1500 pmol/ml, and the maximum concentration of human renin substrate was 1250

pmol/ml. By using the Michaelis-Menten equation for reactions involving one enzyme and two substrates, it can be calculated that the contribution of human renin substrate to ANG I formation was less than 4%. By adding known amounts of MRC human kidney renin standard to plasma of pregnant women and women taking OC, we could demonstrate that the rate of ANG I generation was not significantly influenced by the presence of endogenous renin substrate (see Results). Generation of ANG I was linear for the whole 180-min incubation period. The concentration of ANG I was measured by RIA. The concentrations of active renin and prorenin are expressed as microunits of MRC human kidney renin standard per ml plasma. The prorenin concentration was calculated as the difference between the result of the indirect RIA after activation by trypsin and the result before activation.

Plasma samples containing soybean trypsin inhibitor were also used for measuring PRA. They were rapidly thawed at 37 C and incubated in the presence of protease inhibitors, without the addition of sheep renin substrate, for 0, 10, 30, 60, 120, and 180 min. The ANG I formed was measured by RIA. Only the linear part of the ANG I generation curve was used. Results are expressed as picomoles ANG I generated by 1 ml plasma during 1 h.

In most laboratories, blood for measuring renin has been collected in chilled tubes kept on ice for some time before centrifugation and storage in the freezer before the incubation step of the indirect assay. It is now known that prorenin is activated by exposure of plasma to low temperature (cryoactivation). Cryoactivation is enhanced in plasma of pregnant women and women taking OC[23,24]. Large increments in renin activity may occur in such plasma samples when they are kept at -20 C and remain liquid, as they occasionally do. The effect of cryoactivation is illustrated in Fig. 2, which shows the results of an experiment in which blood samples were kept on melting ice for 2 h and subsequently stored at -20 C. There was a significant increase in PRA in plasma of pregnant women and women taking OC, but not in plasma of normal women . The activation was largely prevented by prior addition of soybean trypsin inhibitor[25]. Skinner[26] pretreated the plasma samples for PRA measurement by dialysis at pH 4.5 for 24 h, followed by dialysis at pH 7.5 for 24 h, both at 4 C. With this procedure, we found a 3-to 5-fold increase of PRA in plasma of pregnant women and women taking OC compared with a 1.5- to 2-fold increase in plasma of control women. For the present study we, therefore, collected blood samples in an anticoagulant fluid containing soybean trypsin inhibitor, centrifuged them immediately at room temperature, stored the plasma samples at -70 C, thawed them once at 37 C, and assayed them immediately after thawing.

Measurement of renin substrate

The concentration of renin substrate in plasma was determined as the maximal quantity of ANG I generated during incubation at 37 C and pH 7.5 with an excess of active renin. Aliquots (5 to 40 μl) of plasma were incubated with purified active renin from human amniotic fluid (see below) in a final con-

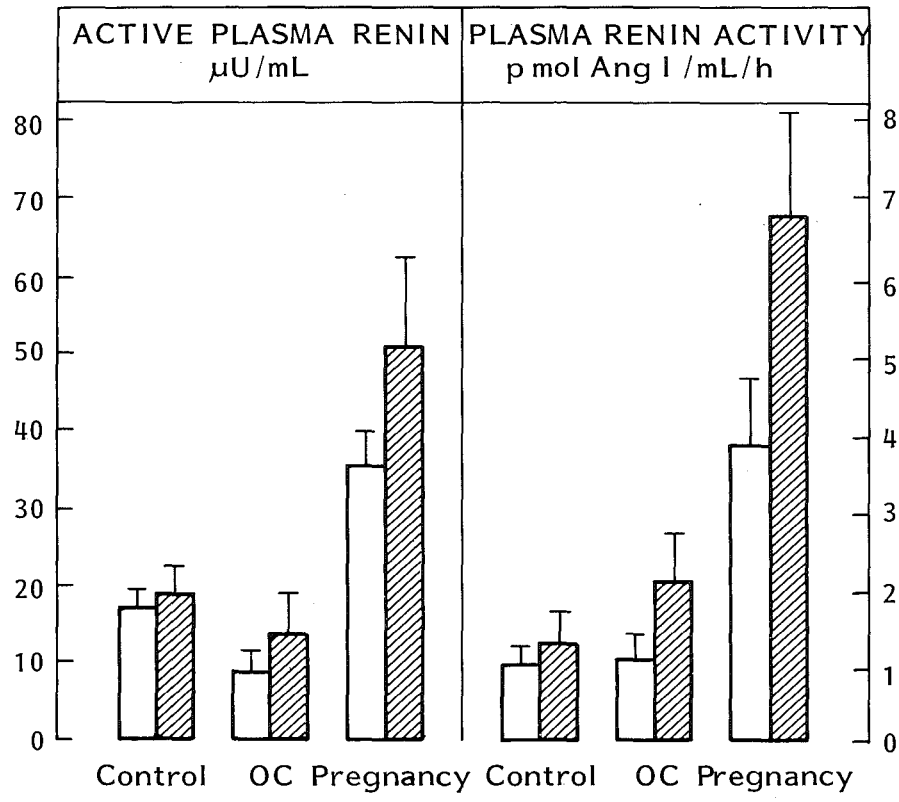

Fig. 5.6.2. Activation of prorenin during collection and handling of blood samples. Open bars: Blood was collected in anti-coagulant containing soybean trypsin inhibitor (see text) and immediately centrifuged at room temperature; the plasma was snap frozen and stored at -70 C. Hatched bars: Blood was collected in anti-coagulant without soybean trypsin inhibitor and kept at 0 C for two hours, and then centrifuged at 4 C; the plasma was stored at -20 C. Data are the mean ± SEM of six plasma samples. Results of the two methods of sample handling are different ($p < 0.001$, by paired t-test) for OC-treated and pregnant women but not for normal women.

centration of 40,000 pg/ml. The incubation volume was 1 ml. Results are expressed as picomoles per ml plasma.

Enzyme kinetic studies

These studies were performed by using semipurified preparations of active renin from plasma, amniotic fluid and kidney, and trypsin-activated preparations of purified prorenin from plasma and amniotic fluid. Active renin and prorenin were isolated from normal human plasma and amniotic fluid using a four-step procedure including two affinity chromatography steps. Benzamidine-HCl at a final concentration of 0.01M was added to the equilibration and elution buffers to prevent inadvertent activation of prorenin. The first step was ion exchange

chromatography on DEAE-Sepharose (Pharmacia) at pH 7.5 using a linear gradient of NaCl up to 0.2 M. This caused partial separation of prorenin and active renin. The second and third steps were affinity chromatography on Blue Sepharose CL-6B (Pharmacia)[27] and on pepstatin-aminohexyl Sepharose CL-6B (Pharmacia)[28]. The last step was gel filtration on Sephadex G-100 (Pharmacia). The final preparations of active renin and prorenin were dialyzed against PBS and concentrated. They were calibrated against highly purified human kidney renin (Ciba- Geigy) with the direct RIA. The renin preparations had no detectable prorenin, and the prorenin preparations contained less than 0.5% active renin. The preparations also had no renin substrate and angiotensinase activity.

For the enzyme kinetic studies, purified active renin or trypsin-activated purified prorenin was diluted in PBS and incubated with sheep or human renin substrate at 37 C for 2, 5, 10, 20, 30 and 60 min. The final concentration of active renin or trypsin-activated prorenin in the incubation mixture was 500 pg/ml. Reaction velocity was measured with the indirect RIA of renin at 10 different concentrations of renin substrate ranging from 25-750 pmol/ml for sheep renin substrate and from 125-2500 pmol/ml for human renin substrate. These concentrations were chosen in such a way that an even distribution of reciprocal values was obtained. The linear part of the ANG I generation curve, at which no more than 4 % of substrate (s) was hydrolyzed, was used for calculating reaction velocity (v). Samples were run in triplicate. Km and Vmax were determined by the linear plot of s/v against s using the statistical method of Wilkinson[29].

5.6.4. RESULTS

Results of renin measurements in semipurified preparations by direct and indirect RIAs showed a relationship that was the same for active renin isolated from human plasma, amniotic fluid, and kidney as for trypsin-activated semipurified prorenin from plasma and amniotic fluid (Tables 1 and 2). This relationship also was not significantly different from that for trypsin-activated prorenin in whole plasma from pregnant women, women taking OC, or normal women. The results of the direct RIA were not altered by trypsin treatment of the samples (Table 1). The renin preparations, *i.e.* highly purified active kidney renin, semipurified active renin of plasma and amniotic fluid, and trypsin-activated semipurified prorenin of plasma and amniotic fluid, had reaction kinetics that were similar in terms of Km and Vmax (Table 2).

Pregnancy and the use of OC caused a 3- to 4-fold increase in the plasma concentration of renin substrate, but the effects on the plasma concentrations of immunoreactive renin, prorenin, and active renin concentration were different in the two situations (Fig.3 and Table 3). Immunoreactive renin, prorenin, and active renin were increased during pregnancy compared with those in normal women. In contrast, they were all decreased in women taking OC.

In pregnant women, PRA was 4-5 times higher than in normal women. This increase was due to the increased concentration of renin substrate and by the

Table 5.6.1. Comparison of results of direct and indirect RIAs of renin.

Source of renin	Trypsin treatment	Number of assays	Direct RIA pg/ml	Indirect RIA μU/ml	Ratio indirect RIA/ direct RIA μU/pg
Amniotic Fluid					
semipurified prorenin	no	3	36,400	171	
	yes	3	34,500	20,400	0.6
semipurified active renin	no	3	4,530	2,630	0.6
	yes	3	4,610	2,810	0.6
Plasma					
semipurified prorenin	no	6	874±79	4.4 ±0.3	
	yes	6	890±84	542± 47	0.6±0.1
semipurified active renin	no	6	85±16	52±4.9	0.6±0.2
	yes	6	81±13	48±5.1	0.7±0.1
plasma pool control women	no	8	278±46	15±1.1	
	yes	8	292±47	190±12	0.7±0.1
plasma pool OC-users	no	8	121±24	8.6 ±0.6	
	yes	8	131±26	98±9.8	0.7±0.1
plasma pool pregnant women	no	8	1,035±84	40±3.9	
	yes	8	1,140±93	678±53	0.7±0.1

Mean ± SD

Table 5.6.2. Enzyme kinetic parameters.

Source of human renin	Trypsin treatment	Vmax/pg renin pmol Ang I/h		Km pmol/ml	
		Sheep substrate	Human substrate	Sheep substrate	Human substrate
Kidney					
MRC standard	no	0.22	0.08	240	1,250
Geigy standard	no	0.20	0.07	230	1,200
Amniotic fluid					
semipurified active renin	no	0.21	0.06	230	1,280
semipurified prorenin	yes	0.20	0.07	250	1,150
Plasma					
semipurified active renin	no	0.19	0.07	240	1,160
semipurified prorenin	yes	0.21	0.08	260	1,220

Measurements were made in triplicate.

2-fold increase in the concentration of active renin (Table 3). Experiments in which known quantities of human kidney renin (MRC standard) were added to plasma of pregnant women and normal women, demonstrated that for a given quantity of added active renin, PRA in pregnancy was about 1.5 times the PRA in normal women (Fig.4). PRA in women taking OC was not different from that in normal women, but, again, for a given quantity of added active renin, PRA in women on OC was 1.5 times PRA in control women. Thus,

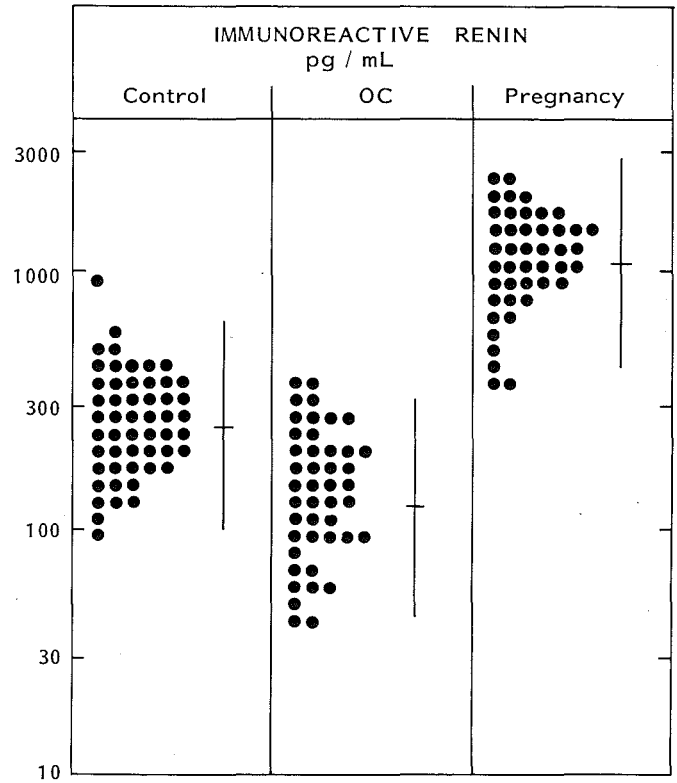

Fig. 5.6.3. Plasma concentrations of immunoreactive renin in normal women (control), women taking OC, and pregnant women. Mean (horizontal line) and 95% confidence intervals (vertical line) are shown. The results in OC-treated and pregnant women are different from those in normal women ($p < 0.001$, by unpaired t test).

the 3- to 4-fold increase of renin substrate in pregnant women and women taking OC caused PRA to increase by a factor of 1.5. In contrast, measurements of the plasma concentration of active renin, made in the presence of saturating amounts of sheep renin substrate, were not influenced by the level of endogenous substrate.

5.6.5. DISCUSSION

Methodological aspects

For the indirect RIA of plasma prorenin, we activated the samples with immobilized trypsin at 0 C. There are two important questions about the validity of the method. Are all prorenin molecules in the sample converted into active renin? Is active renin or activated prorenin degraded into inactive fragments?

Table 5.6.3. Concentrations of renin substrate and different forms of renin and PRA.

Source of plasma	Renin-substrate pmol/ml	Prorenin μU/ml	Active renin μU/ml	PRA		Fraction of renin that is active (%)
				measured pmolAngI/ml.h	calculated pmolAngI/ml.h	
Normal women	1545	151	15.4	0.88	1.04	9.1
(n = 54)	(745–2340)	(62–357)	(4.7–59)	(0.24–3.25)	(0.27–3.95	(2.9–29.0)
OC-users	4560*	94*	8.9*	1.02	1.02	8.8
(n = 44)	(1940–7620)	(33–268)	(2.434)	(0.26–3.82)	(0.30–3.66)	(3.0–25.1)
Pregnant women	5130*	645*	32.8*	4.36*	3.90*	4.8*
(n = 44)	(3050–7210)	(263–1583)	(10–103)	(1.25–16.6)	(1.20–12.7)	(1.4–18.0)

Mean values and 95% confidence interval (in parentheses) are shown. Statistical analysis was performed after logarithmic transformation of the results to obtain a Gaussian distribution. Results are expressed as the antilog. For calculating PRA, the plasma concentration of active renin, which is expressed as microunits of the MRC human kidney renin standard per ml plasma (μU/ml), was converted into picograms per ml by dividing by 0.7 (see Table 1). The concentrations of active renin and renin substrate were entered in the Michaelis-Menten equation, and calculations were based on Vmax = 0.08 pmolAngI/h per pg of active renin and Km = 1,200 pmol/ml (see Table 2). The measured and calculated values of PRA were not significantly different. OC = oral contraceptives.

* $P < 0.001$ vs. normal women (by unpaired t-test).

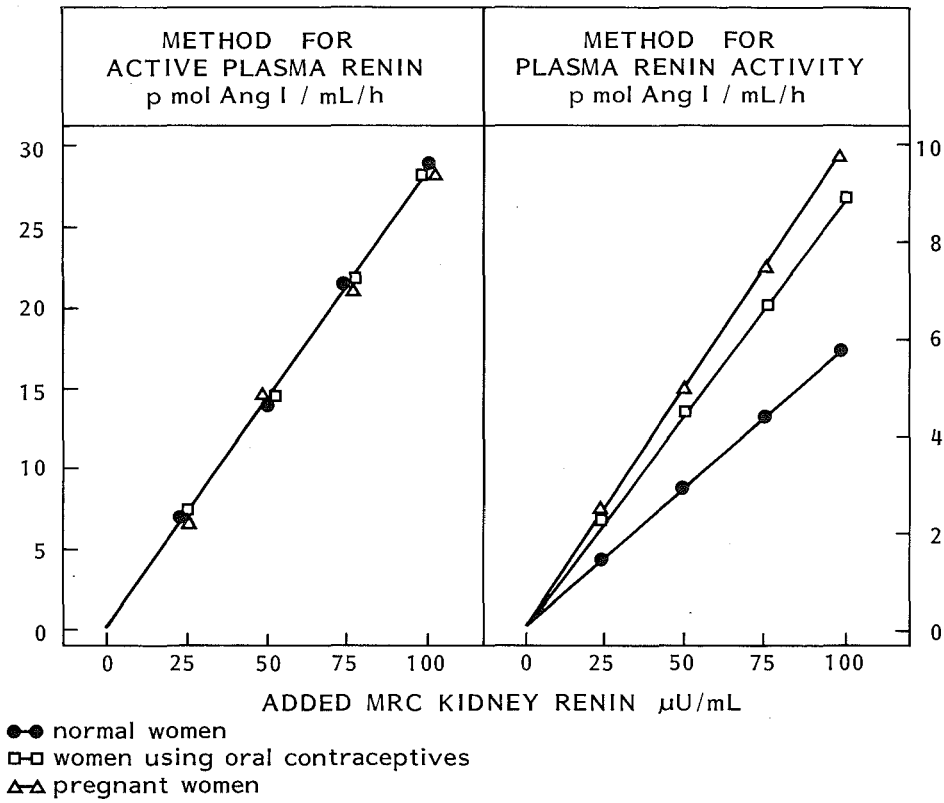

Fig. 5.6.4. The effect of the addition of active kidney renin to plasma on the result of two types of indirect RIA of renin, i.e. assay of the concentration of active renin using exogenous renin substrate and assay of PRA using endogenous renin substrate. The date are presented as mean values. ●, Plasma of normal women (mean renin substrate concentration, 1360±181 (±SEM) pmol/ml; n = 5). □, Plasma of OC-treated women (renin substrate concentration, 4280±618 pmol/ml; n = 5). Δ, Plasma of pregnant women (renin substrate concentration, 5310±431 pmol/ml; n = 5). For each quantity of added renin, PRA was higher in OC treated and pregnant women than in normal women ($p<0.001$, by unpaired t test).

By comparing the results of indirect RIA with those of direct RIA, we found that after trypsin treatment of plasma prorenin, the maximum enzymatic activity per unit weight of renin at saturating concentrations of renin substrate was very similar, if not identical, to the enzymatic activity of active plasma renin and active kidney renin. There was also no decrease in immunoreactivity after trypsin treatment. With our method of trypsin treatment prorenin activation in plasma reached a plateau independent of the trypsin concentration over a wide range of concentrations[22]. These data lend support to the conclusion that the difference between the result of indirect RIA before and after trypsin treatment

is indeed a valid measure of the prorenin content of plasma. In some reports, Skinner's method[26] was used for measuring plasma prorenin; plasma was dialyzed for 24 h at pH 3.3 and 4 C before the indirect RIA to convert prorenin to active renin[1,3]. The degree of prorenin to active renin conversion, however, is critically dependent on pH and duration of dialysis. In our hands, dialysis for 24 h was too short for many plasma samples to obtain maximal conversion.

A special problem in measuring active renin is caused by the enhanced cryoactivation of prorenin in plasma of pregnant women and women taking OC[23,24]. This might be related to elevated levels of factor XII and prekallikrein in these women[24,30,31], whereas the concentration of C_1-inactivator, which is an important inhibitor of factor XII and plasma kallikrein, is decreased[24,32]. This may lead to erroneously high values of active renin, particularly in pregnancy, where a high concentration of prorenin is available for cryoactivation. The overestimation is even greater for PRA measurements because of the high concentration of renin substrate in pregnancy and women taking OC.

Effects of pregnancy and OC on the plasma concentrations of renin substrate, immunoreactive renin, prorenin, and active renin

We found that both during pregnancy and with the use of OC, plasma concentration of renin substrate were increased to approximately 4500-5000 pmol/ml, a value also reported by others[1–6,8–13,15,17,18]. Our data confirm other reports that PRA and prorenin and active renin concentrations are increased during pregnancy[1–3,6,7,19,20], but two qualifications have to be made. Firstly, our PRA results in pregnant women are lower than those in other reports, related, in part, to the fact that in our assay ANG I was generated during incubation at physiological pH, whereas others used pH 5-6, closer to the pH optimum of the reaction of active renin with human renin substrate. However, our results are also lower than those of investigators who incubated their samples at the same pH as we did[1,3]. Inadvertent activation of prorenin during storage and handling of the plasma samples is likely to be the cause of this discrepancy. Secondly, our prorenin values are higher than those of previous studies, probably due to more complete activation. That the prorenin to active renin conversion was indeed complete is confirmed by our observation that the enzymatic activity per unit weight of renin in trypsin-treated plasma did not differ from the activity of purified active plasma renin.

In pregnant women, the fraction of renin in the active form was 4.8%. This is much higher (30-50%) than that in conditions in which the renal release of active renin is stimulated and plasma levels of prorenin are as high as during pregnancy[22,33]. This finding may indicate that a smaller fraction of prorenin is converted intrarenally to active renin before its release into the circulation or that a larger fraction of circulating prorenin is of extrarenal origin.

Vmax and Km values measured in mixtures of purified active renin and renin substrate and the concentrations of active renin and renin substrate measured in whole plasma were entered into the Michaelis-Menten equation for calculating

PRA. Calculated PRA values in normal women were not significantly different from the actual measurements. This was also true in pregnant women. At any rate, we found no evidence for the existence of plasma factors in pregnancy modulating the reaction between renin and its substrate.

PRA has been reported to be increased in women taking OC[8–15]. It is, however, important to note that PRA increases during the luteal phase of the normal menstrual cycle[9,14,34,35]. Yet, PRA was higher in women taking OC than in women not taking OC and in the luteal phase of the menstrual cycle[9,14]. In some other studies[16–18], including a survey of a large number of women[18], no difference in PRA was found between women taking OC and women not taking OC. PRA in women taking OC was within the normal range in our study, and the mean value was not different from the mean value in normal women during the first 7-12 days of the menstrual cycle. The increased PRA in women taking OC reported in some previous studies might be related to the higher estrogen content of the older OC preparations. Another explanation might be the overestimation of PRA in women taking OC due to inadvertent activation of prorenin during the storage and handling of the plasma samples. The finding in previous studies[9,11,36] that the renin activity of acid-pretreated plasma, in which part of the prorenin is activated, is reduced in women taking OC can be explained by the fact that the concentrations of both prorenin and active renin were decreased in these women. Accordingly, the plasma concentration of immunoreactive renin also was lower. The Km for the hydrolysis of renin substrate by active renin is probably not altered by the use of OC[37]. The Michalis-Menten equation predicts that PRA in women taking OC will not be different from PRA in normal women. Our results are in agreement with this prediction. In other words, the normal PRA levels in women taking OC can be fully accounted for by the lower concentration of active renin and the higher concentration of renin substrate. There was no evidence for other factors, besides changes in the molar concentrations of enzyme and substrate, affecting PRA.

In summary, immunoreactive renin, prorenin, and active renin were increased during pregnancy and decreased in women taking OC. Stimulation of renin release during pregnancy may be a defense mechanism against one or more vasodilator factors, possibly prostaglandins[38]. The fact that, in contrast with other conditions in which the release of renin by the kidney is stimulated, prorenin is more elevated than active renin may indicate that in pregnancy, a larger fraction of circulating prorenin is of extrarenal origin. Suppression of renin release by OC is perhaps a physiological adaptation to enhanced formation of ANG II by the estrogen-induced increase in renin substrate. Our measurements of various components of the renin-angiotensin system explain most of the discrepancies reported in the literature and may serve as a background for a more accurate analysis of the role of these components in the hypertensive states related to pregnancy and the use of OC.

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5.7. HIGH CONCENTRATIONS OF IMMUNOREACTIVE RENIN, PRORENIN AND ENZYMATICALLY ACTIVE RENIN IN HUMAN OVARIAN FOLLICULAR FLUID

5.7.1. SUMMARY

Prorenin (enzymatically inactive) and renin (active) were measured by radioimmunoassay, using monoclonal antibodies reacting either with both prorenin and renin or with renin alone, in pre-ovulatory follicular fluid (FF) from women in an *in vitro* fertilization programme who were stimulated with human menopausal/human chorionic gonadotrophin. The concentration of prorenin in FF was 40 times higher than in plasma taken at the time of FF collection; renin in FF was 10 times higher. The plasma concentration of prorenin, but not of renin, in these women was higher than in non-stimulated women in the late follicular phase of the menstrual cycle. The concentration of renin substrate and angiotensin-converting enzyme in FF was 60 percent of that in plasma. Contamination of blood, which may occur at the time of FF collection, was less than 5 percent. Prorenin in FF was irreversibly converted into renin after adding trypsin or by endogenous serine protease using procedures that also cause conversion of prorenin in plasma. These results support the hypothesis that the increased plasma level of prorenin in women whose ovulation is stimulated for the collection of oocytes has originated from the ovary and is under gonadotrophic control. This may also be true for the increase of plasma prorenin that has been observed in non-stimulated women during the luteal phase of the cycle and in early pregnancy.

5.7.2. INTRODUCTION

There is growing evidence that the plasma level of prorenin is under gonadotrophic control. In normal ovulating women prorenin is increased by a factor of 1.5 in the first week of the luteal phase[1], and by a factor of 5-6 in pregnancy, the increase in early pregnancy being proportional to the increase of chorionic gonadotrophin in plasma[2]. An increase of plasma prorenin comparable to that in pregnancy has been reported in a case-study of a woman in an *in vitro* fertilization programme who received gonadotrophin injections[3]. Ovarian follicular fluid from such women contains renin-like activity[4]. The findings suggest that under those circumstances part of the prorenin in plasma may have originated from the ovary.

It is not known whether prorenin is present in follicular fluid nor whether the renin-like activity of follicular fluid is caused by the hydrolytic action of renin itself or whether other proteases capable of generating angiotensin I are involved. Here we report on measurements of prorenin (enzymatically inactive) and renin (active) in follicular fluid by direct radioimmunoassays using monoclonal antibodies reacting either with both prorenin and renin or with renin alone.

5.7.3. MATERIALS AND METHODS

Patients

Fifteen white European women in an *in vitro* fertilization programme were included in the present study. Their age ranged from 30 to 39 years (mean 34 years); they had normal ovulatory cycles but suffered from tubal abnormalities. Follicular development was stimulated with human menopausal gonadotrophin (Pergonal, Serono Laboratories, Rome, Italy), i.e. 225 I.U. of follicle-stimulating hormone and 225 I.U. of luteinizing hormone intramuscularly, once a day from day 3 or 4 of the cycle. Human chorionic gonadotrophin (Pregnyl, Organon, Oss, The Netherlands), 10,000 I.U., was given intramuscularly when the largest follicle was 17 mm in diameter. Follicle puncture was performed 35-36 hours later. Oocytes were removed from the follicular fluid and contamination with blood was estimated by hemoglobin measurement, the follicular fluid was stored at -20 C. At the time of follicle puncture a blood sample was drawn from a forearm vein and collected in plastic tubes containing 0.1 volume of 0.13 M trisodium citrate as anticoagulant. Blood was centrifuged and plasma was stored at -20 C.

Fifteen women aged between 28 and 37 years (mean 33 years), selected from hospital personal or students, acted as controls. They were in the 9th-14th day of the menstrual cycle. All patients and control subjects were normotensive. They had an unrestricted sodium intake and were not on any medication.

Direct radioimmunoassay of active renin and prorenin

The concentration of total immunoreactive renin, i.e. active renin and prorenin, was determined with a solid-phase sandwich assay[5,6]. The two monoclonal antibodies R 3-27-6 and R 3-36-16 (Ciba-Geigy, Basle, Switzerland) that were used in this assay react equally well with active human kidney renin and chorionic cell culture prorenin as with prorenin, trypsin-activated prorenin and naturally occurring active renin of human plasma. Antibody R 3-27-6 was coupled to microtiter plates. Antibody R 3- 36-16 was labeled with ^{125}I. Results are expressed as picograms per ml using highly purified human kidney renin (Ciba-Geigy), specific activity 760 Goldblatt units per mg of protein, as the standard. The sensitivity of the assay was about 20 pg/ml.

The direct radioimmunoassay of naturally occurring active renin or serine protease-activated prorenin used the monoclonal antibodies 3E8 and 4G1. The first antibody was bound to magnetizable gel beads (Magnogel, Diagnostics Pasteur, Marnes La Coquette, France). The second antibody was labeled with ^{125}I. Details of the procedure have been described[7]. The antibodies react equally well with active human kidney renin as with naturally occurring active renin and trypsin-activated prorenin of human plasma. Results are expressed as picogram per ml using the same renin standard as in the assay of total immunoreactive renin. The lower limit of detection was 10 pg/ml. The concentration of prorenin was calculated as the difference between the results of

the direct immunoassay of total renin and the direct immunoassay of naturally occurring active renin.

Indirect radioimmunoassay of active renin and prorenin.

In this assay renin was measured by its capacity to generate angiotensin I from purified sheep renin substrate as described previously[8,9]. Angiotensin I was quantified by radioimmunoassay. Renin concentration is expressed as microunits of the MRC human kidney renin standard per ml[10]. Prorenin has to be activated before it can be measured by the indirect radioimmunoassay. This was done by adding Sepharose-bound trypsin in a final concentration of 0.25 mg/ml[9]. Prorenin concentration was calculated as the difference between renin concentration after trypsin-activation and renin concentration before activation.

Assay of renin substrate.

The concentration of renin substrate was measured as the maximal quantity of angiotensin I generated after addition of an excess of semipurified human kidney renin[8,9]. Results are expressed as picomoles per ml.

Assay of angiotensin-converting enzyme

This assay was carried out as described before[11]

5.7.4. RESULTS

Four components of the renin-angiotensin system, i.e. prorenin, renin, renin substrate and angiotensin-converting enzyme, are present in follicular fluid (Table 1, Fig. 1). The most striking finding was the very high concentration of total

Table 5.7.1. Different forms of renin in follicular fluid and plasma.

Source	Method of assay	Total renin	Active renin	Prorenin	Active total renin ratio %
Plasma of control women	Immunological (pg/ml)	228 (90–471)	21.1 (7.1–32.4)	207 (112–445)	9.2 (4.0–21.4)
	Enzyme kinetic (μU/ml)	173 (67–352)	15.4 (6.7–24.3)	158 (58.1–343)	8.9 (3.8–24.6)
Plasma of women treated with gonadotrophin	Immunological (pg/ml)	472** (251–886)	25.7 (12.7–41.8)	443** (230–885)	5.9* (2.1–9.9)
	Enzyme kinetic (μU/ml)	359** (191–674)	20.5 (10.2–41.4)	338** (174–645)	6.0* (1.9–10.1)
Follicular fluid of women treated with gonadotrophin	Immunological (pg/ml)	16930 (7130–40190)	243 (100–592)	16670 (6710–39680)	1.5 (1.1–2.1)
	Enzyme kinetic (μU/ml)	12140 (3890–39590)	194 (80–474)	12110 (3810–39130)	1.6 (1.2–2.0)

Statistical analysis was performed after logarithmic transformation of results. Results (mean value and 95 percent confidence interval) are expressed as the antilogs. *$p < 0.01$, **$p < 0.001$ for difference from control women (unpaired t-test).

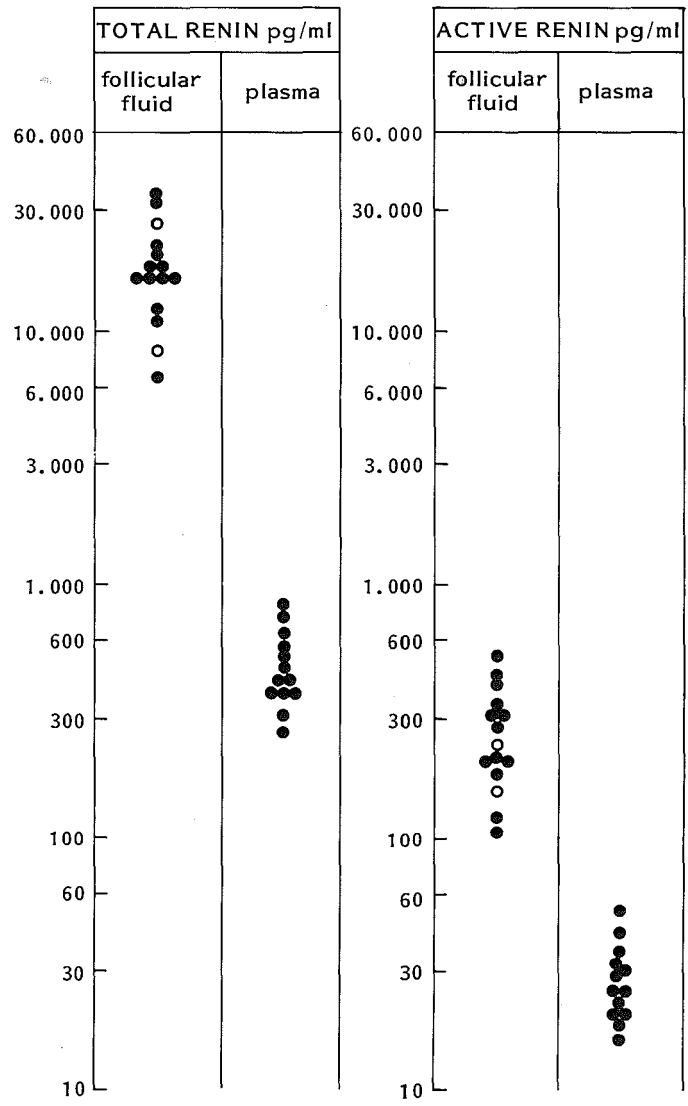

Fig.5.7.1. Total renin and enzymatically active renin, as measured with renin-antibodies reacting with either both prorenin and active renin or with active renin alone, in follicular fluid and plasma of women treated with gonadotrophin. Plasma was taken at the time of follicular fluid collection. Open circles: no plasma samples were taken from these women.

renin, 98 percent of which is prorenin. The concentration of prorenin in follicular fluid was about 40 times higher than in plasma that was taken at the time of follicle puncture. That we are indeed dealing with renin and prorenin, and

Table 5.7.2. Prorenin-renin conversion in follicular fluid.

Treatment of follicular fluid	Renin assay		
		Immunological	
	Enzyme kinetic μU/ml	Total renin pg/ml	Active renin pg/ml
1. None	360	23,600	610
2. Dialysis 48 h, pH 3.3, 0C, followed by rapid neutralization by dilution in 0.1 M phosphate buffer pH 7.5 containing SBTI 1 mg/ml and PMSF 3 mM and immediate assay: reversible activation.	20,900	24,000	3,700
3. As in no. 2 and incubation at 37 C for 2 h in the presence of SBTI and PMSF prior to assay: reversible activation-inactivation.	2,600	20,800	3,200
4. Dialysis 48 h, pH 3.3, 0C, followed by dialysis 48 h, pH 7.5, 0C: irreversible activation by endogenous serine protease.	21,880	21,500	22,170
5. Dialysis 48 h, pH 3.3, 0C, followed by dialysis 48 h, pH 7.5, 0C, in the presence of SBTI and PMSF: irreversible activation by endogenous serine protease in the presence of inhibitors.	2,900	21,000	3,400
6. Addition of trypsin-Sepharose 0.25 mg/ml, incubation 48 h, pH 7.5, 0C: irreversible activation by exogenous serine protease.	19,900	22,700	20,400

Results are mean values of two experiments. PMSF = phenylmethylsulfonylfluoride, SBTI = soybean trypsin inhibitor.

not with other aspartic proteases capable of generating angiotensin was demonstrated by the use of the monoclonal antibodies specific for both prorenin and renin (antibodies R 3-27-6 and R 3-36-16) and the antibodies that only recognized active renin (antibodies 3E8 and 4G1).

Prorenin in follicular fluid was irreversibly activated by Sepharose-bound trypsin or by 48 h dialysis at pH 3.3 followed by 48 h dialysis at pH 7.5 both at 0 C (Table 2). Dialysis at pH 3.3 followed by rapid neutralization also leads to activation of prorenin but in this case the activation was reversible, i.e. the activated prorenin was inactivated at 37 C within two hours. Similar observations

Table 5.7.3. Renin-substrate and angiotensin-converting enzyme in follicular fluid and plasma.

	Renin substrate pmol/ml	Angiotensin-enzyme converting mU/ml
Plasma of control women	1785 (985–2840)	13.5 (7.0–20.0)
Plasma of women treated with gonadotrophin	2234 (1076–4639)	13.8 (7.2–19.4)
Follicular fluid of women treated with gonadotrophin	1462** (695–3076)	8.3* (4.1–16.6)

Statistical analysis was performed after logarithmic transformation of results. Results (mean and 95 percent confidence interval) are expressed as the antilogs.
*$p < 0.01$, **$p < 0.001$ for difference from plasma of women treated with gonadotrophin.

were made after acid-treatment of plasma[13–15]. Irreversibly activated prorenin was detected by the assay in which the antibodies 3E8 and 4G1, specific for active renin, were used. Reversibly activated prorenin was not detected by this assay. Both the irreversibly and reversibly activated forms of prorenin were bound to the antibodies R 3-27-6 and R 3-36-16. Irreversible activation of prorenin also occurs in acid-treated plasma after pH has been restored to neutral. This depends on the proteolytic action of plasma kallikrein, which is generated from prekallikrein by clotting factor XII[16,17]. Formation of an endogenous serine protease causing irreversible activation of prorenin at neutral pH may also have occurred in acid-pretreated follicular fluid. This was confirmed by the addition of SBTI and PMSF to acid-treated follicular fluid after restoration of pH to neutral; irreversible prorenin activation was blocked by these serine protease inhibitors (Table 2).

Renin substrate and angiotensin-converting enzyme were present in follicular fluid in a concentration about 60 percent of that in plasma (Table 3). The concentration of hemoglobin was 0.3 mM (SD 0.1mM) ,which is less than 5 percent of the hemoglobin concentration in blood. It is therefore unlikely that the presence of renin substrate and angiotensin-converting enzyme in follicular fluid is caused by contamination of blood at the time of follicle puncture.

The plasma prorenin concentration in the women stimulated with gonadotrophin was 2-3 times higher than in control women, whereas the concentration of naturally occurring active renin was not significantly different (Table 1).

5.7.5. DISCUSSION

This study demonstrates the presence of prorenin and renin as well as renin substrate and angiotensin-converting enzyme in pre-ovulatory follicular fluid

in women stimulated by injections of gonadotrophin. The concentration of these components of the renin-angiotensin system in follicular fluid is too high for explaining their presence by contamination with blood at the time of follicular fluid collection. Prorenin was 40 times and renin was 10 times higher in follicular fluid than in plasma. Because of the magnitude of this difference, it seems likely that prorenin is secreted by follicle cells and that its presence is not merely caused by transudation of plasma and subsequent concentration.

From a case report[3] it appears that the rise of plasma prorenin, caused by gonadotrophin injections for inducing ovarian stimulation, occurs mainly in the first days after ovulation. Our results also indicate that the pre-ovulatory plasma levels of prorenin in gonadotrophin-stimulated women are higher than in non-stimulated women. These findings are further evidence that the ovary produces prorenin and that part of it may reach the circulation.

The presence of prorenin, renin, renin substrate and angiotensin-converting enzyme in follicular fluid poses a question concerning the role of the renin-angiotensin system in the follicle. Follicle and corpus luteum development require extensive neovascularization[18]. Rabbit corpus luteum, mouse ovarian extract and human and porcine follicular fluid have been shown to initiate angiogenesis[19–22]. Angiotensin II stimulates new collateral vessel formation in ischaemic areas[23], and corneal implants of this peptide in rabbits initiate the local formation of new blood vessels[24]. Thus local prorenin-to-renin conversion and angiotensin formation may be important for the vascularization of the developing follicle and corpus luteum. It may also play a role in the vascularization of the area of implantation of the developing embryo since the elevated plasma concentration of prorenin in the luteal phase of the cycle rises further when ovulation is followed by pregnancy[2].

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5.8. SOURCE OF PLASMA PRORENIN IN EARLY AND LATE PREGNANCY. OBSERVATIONS IN A PATIENT WITH PRIMARY OVARIAN FAILURE

5.8.1. ABSRACT

The plasma levels of enzymatically active renin (active renin) and inactive renin (prorenin) were measured in a woman with primary ovarian failure, in whom pregnancy was established by the induction of an artificial cycle and in vitro fertilization of a donated oocyte fertilized with the sperm of her husband. The results were compared with those in 9 normal pregnant women. Pre-pregnancy plasma active renin and prorenin levels were normal in the patient. Active renin rose two-fold during pregnancy both in the patient and in the normal pregnant women. In the first eight weeks of pregnancy prorenin rose by 156 μU/ml in the patient, whereas it rose by 869$\pm$169 μU/ml (mean$\pm$SD) in the normal pregnant women. Thus, the rise in prorenin in our patient was much less than normal. Prorenin remained abnormally low throughout the pregnancy, which lasted 40 weeks. Therefore, the high prorenin levels that occur normally during pregnancy may depend on normal ovarian function.

Amniotic fluid prorenin in the patient was similar to that in normal pregnant women, and was 75 times times higher than in plasma. This finding suggests that prorenin production by the chorionic cells was normal in the patient and that chorionic prorenin does not contribute in any major degree to the level of prorenin in maternal plasma.

Because of these findings and in the light of recent evidence that the ovary secretes prorenin and produces high plasma prorenin levels in women with hyperstimulated cycles, we conclude that the ovary is the main source of the elevated plasma prorenin of pregnant women.

5.8.2. INTRODUCTION

During pregnancy the levels of enzymatically active renin (active renin), inactive renin (prorenin) and renin substrate (angiotensinogen) in maternal plasma are all increased[1–4]. As a result angiotensin II formation is increased and aldosterone secretion is stimulated. This may be a physiological response to maintain normal blood pressure, by counteracting vasodilator mechanisms operative in pregnancy[5].

The kidney is the major, if not the only, source of active renin in plasma of men and non-pregnant women, and this is believed to be the case in pregnant women as well[6]. The kidney is also an important source of prorenin[7,8], but there is evidence that, at least in women who are in the luteal phase of the menstrual cycle, part of the circulating prorenin originates from the ovary[9]. In pregnancy it is not known whether maternal plasma prorenin originates mainly from the kidney, the ovary, the placenta or other organs. Here we report measurements of active renin and prorenin in a patient with primary ovarian failure, in whom

pregnancy was achieved by the induction of an artificial cycle and *in vitro* fertilization of a donated oocyte fertilized with the sperm of her husband.

5.8.3. SUBJECTS AND METHODS

Patient and normal subjects. At the time of embryo transfer the age of the patient with primary ovarian failure was 36 yr. Her menarche occurred when she was 14 yrs old, but she then became amenorrheic after a few irregular cycles. Her plasma FSH and LH levels were above 100 mIU/ml and serum estradiol levels were below 0.01 ng/ml. At the age of 29 yr, laparotomy was performed. The ovaries were atrophic; one ovary was removed for histological examination. This showed absence of follicles and reduced stromal cellularity. Her karyotype was 46, XX.

Exogenous estrogen (17 β-estradiol tablets) and progesterone (suppositories) therapy were given to mimic the steroid profile of the normal ovulatory cycle[10]. On day 14 of the cycle, she received four embryos three days after *in vitro* fertilization. The donor oocytes were fertilized with the sperm of the patient's husband. In vitro fertilization and embryo transfer were performed as described by Navot et al.[10].

Substitution therapy was maintained for 12 weeks (from day 1 of the cycle). By the eighth week the fetus was appropriately developed for gestational age, as judged by ultrasonography. Pregnancy progressed normally. The patient's blood pressure remained normal, 105-115 mm Hg systolic and 65-75 mm Hg diastolic. After 40 weeks of pregnancy a healthy girl weighing 3550 g was born by vaginal delivery. The placenta, which was delivered 10 min later, weighed 600 g and looked normal. Parturition was uncomplicated.

Plasma 17 β-estradiol, progesterone, β-hCG, active renin and prorenin were measured before embryo transfer and during and after pregnancy. For comparison measurements of active renin and prorenin were also made, during pregnancy and 6 – 8 weeks after delivery, in 9 normal women, aged 28 to 38 yr. Their pregnancies were uncomplicated and, apart from ferrous sulfate and/or multivitamin preparations, they received no drugs. Active renin and prorenin were also measured in amniotic fluid obtained at delivery in both the patient with primary ovarian failure and the normal women.

Measurements of hormones. Venous blood samples were taken after the subjects had been in the left lateral recumbent position for 30 min. The plasma concentration of progesterone was measured by RIA according to De Jong et al.[11]. The plasma levels of 17 β-estradiol and β-hCG were measured by using commercially available RIA kits (Estradiol kit, Diagnostic Products Corporation, Los Angeles, Ca, and β-hCG kit, IRE, Fleurus, Belgium). In the assay for the β-hCG subunit, 1 mg β-hCG standard equaled 790 IU MRC 75/551 standard (Medical Research Council, National Institute for Biological Standards and Control, London, United Kingdom). The mean normal value between weeks 7 and 11 of pregnancy was 246±47 (SD) ng/ml (n = 15).

Plasma active renin and prorenin concentrations were measured by *indirect* RIA. In this assay, angiotensin I generated by incubation of plasma with an excess of sheep angiotensinogen under standardized conditions was quantitated by RIA[4,7]. The results are expressed as μU MRC standard 68/356 (active renin from human kidney) per ml plasma. Plasma prorenin was measured by *indirect* RIA after it had been converted into active renin by immobilized trypsin at 0 C[7]. For conversion in amniotic fluid we incubated the samples with immobilized trypsin after addition of human serum albumin in a final concentration of 30 mg/ml. The difference between the results of *indirect* RIA before and after treatment with trypsin was taken to be a measure of prorenin concentration.

Plasma and amniotic fluid may contain substances interfering with the renin-angiotensinogen reaction. Active renin was, therefore, not only measured by *indirect* RIA but also by *direct* RIA, using monoclonal antibodies, which react with active renin but not prorenin[12,13]. The validity of the indirect RIA of prorenin was confirmed by measuring total immunoreactive renin (active renin plus prorenin) by direct RIA, using monoclonal antibodies, which react with both active renin and prorenin[4,14]. The results of the direct assays of active renin and total immunoreactive renin are expressed as pg of the Ciba-Geigy standard (active renin from human kidney, Ciba-Geigy, Basel, Switzerland) per ml sample. One μU MRC standard equaled 1.41 pg Ciba-Geigy standard.

5.8.4. RESULTS

Before substitution therapy the patient's plasma estradiol level was below 0.01 ng/ml. In the first four weeks of substitution therapy the plasma estradiol and progesterone levels were similar to those of normal ovulatory cycles (Fig.1). Plasma estradiol reached a value of 1.1 ng/ml on day 8. Plasma progesterone rose to 9.9 ng/ml on day 18, four days after embryo transfer, and it was 9.2 ng/ml on day 21. During this period there was little change in the plasma active renin and prorenin levels. Plasma β-hCG was 25.4 ng/ml on day 30 and it reached a peak value of 437 ng/ml in week 8. After 12 weeks, when the estrogen and progesterone therapy was discontinued, the placenta was able to maintain high levels of these steroids in maternal plasma.

Plasma active renin rose from 15-20 μU/ml in the first 10 weeks to 30-35 μU/ml in weeks 35-40. Plasma prorenin began to rise after four weeks from a value of 140 μU/ml on day 30 to a peak value of 287 μU/ml in week 9. It then declined and reached a plateau of about 200 μU/ml after 12 weeks.

The change in the patient's active renin levels during pregnancy resembled that in the normal women. In contrast, the rise of prorenin was much smaller than in the normal women (Fig.2). The increments of prorenin in the first eight weeks in the normal women ranged from 439 to 1,040 μU/ml (mean 869$\pm$169 (SD) μU/ml,n = 9), whereas in our patient it rose by 156 μU/ml. Throughout pregnancy, from week 8 onwards, the patient's plasma prorenin was below the 95% confidence interval of the normal women. Active plasma renin, but not

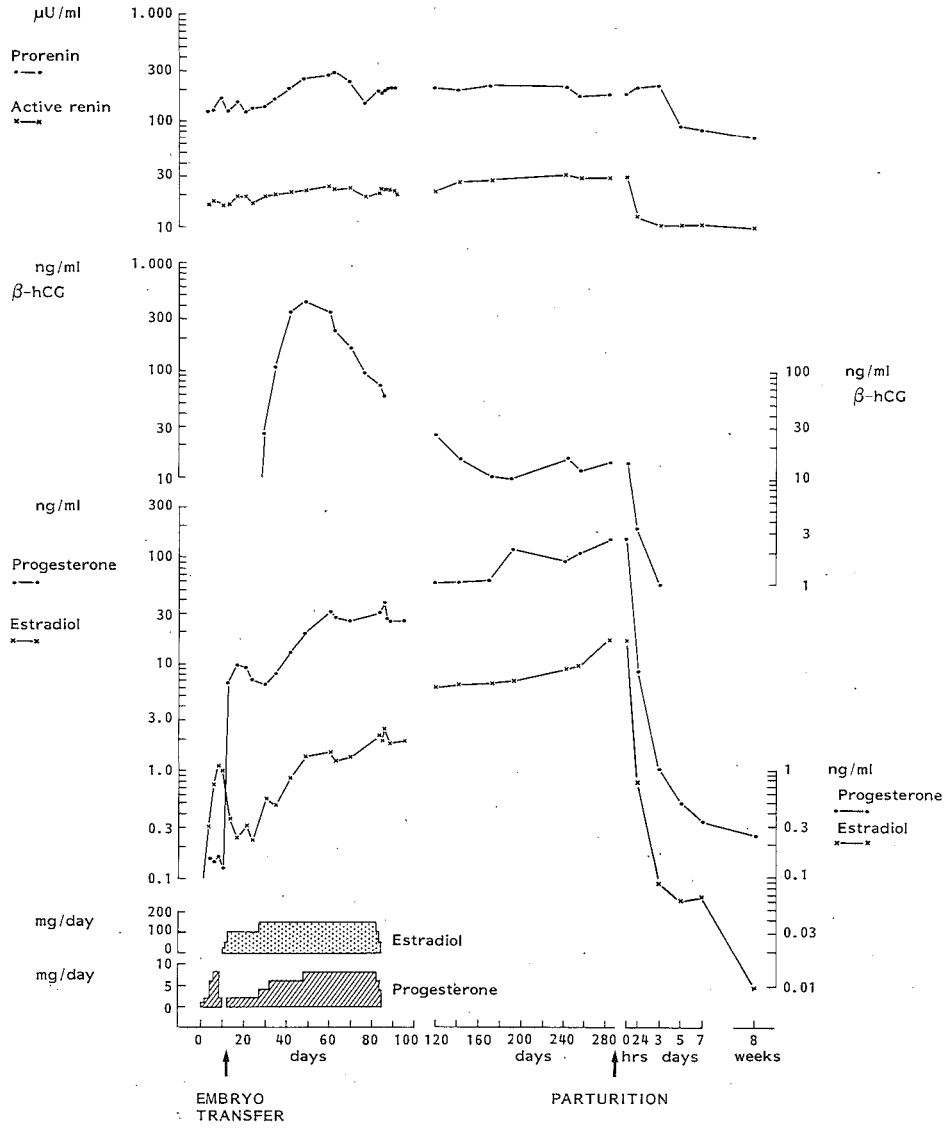

Fig.5.8.1. Substitution therapy with estradiol and progesterone and plasma levels of these steroids and β-hCG, active renin and prorenin during and after pregnancy in a patient with primary ovarian failure. The renin and prorenin measurements were done by indirect RIA.

prorenin, fell during the first 24h following delivery both in the patient and in the normal women. During the following five days prorenin fell to pre-pregnancy levels.

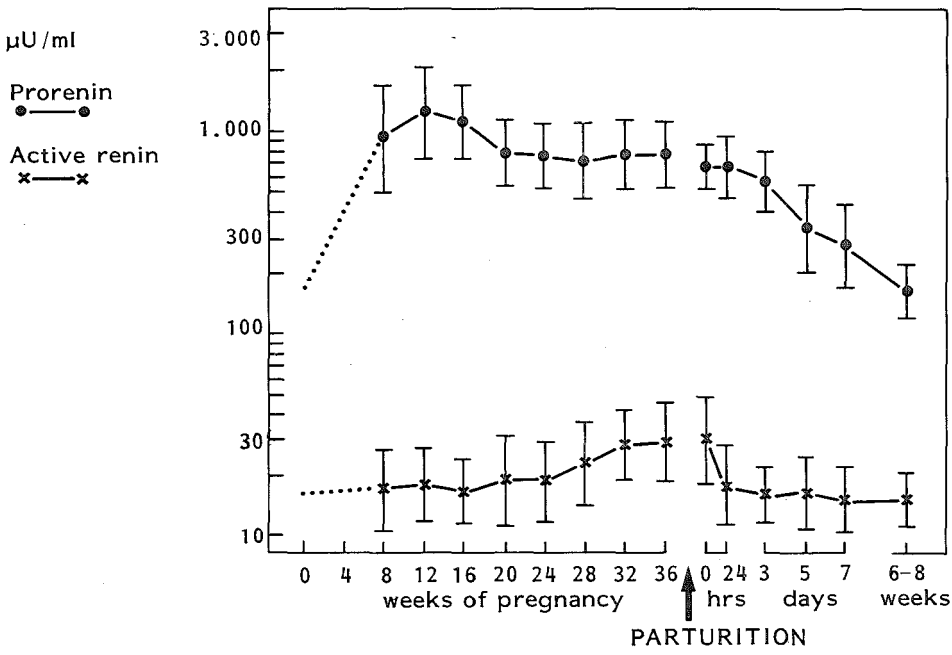

Fig.5.8.2. Mean (±SD) plasma active renin and prorenin levels measured by indirect RIA, during and after pregnancy, in nine normal women.

Both active renin and prorenin in the amniotic fluid of our patient were slightly above the upper 95% confidence limit of the values in the normal pregnant women (Table 1), but in one of the normal women active renin was nearly as high as in the patient (1,170 μU/ml), while prorenin was higher (18,300 μU/ml) (Table 1).

The ratio between the results of the direct and indirect RIAs was the same for active renin as for total renin (active renin plus prorenin), both in plasma and in amniotic fluid (Table 1). This finding indicates that the trypsin-induced prorenin to active renin conversion was complete and that the renin-angiotensinogen reaction was not modified by unknown substances in plasma or amniotic fluid.

5.8.5. DISCUSSION

Plasma active renin in a patient with primary ovarian failure increased two-fold during pregnancy, an increase similar to that in normal women. The rise of plasma prorenin in the first trimester of pregnancy, however, was much smaller in our patient than in normal women, in whom prorenin increased five- to six-fold, as has been described by others[3]. Furthermore, the patient's plasma prorenin

Table 5.8.1. Levels of active renin and total renin (active renin plus prorenin) in pregnancy plasma and amniotic fluid measured by direct and indirect RIA.

Subjects	Number of subjects	Total renin			Active renin		
		Direct RIA pg/ml	Indirect RIA μU/ml	Ratio μU/pg	Direct RIA pg/ml	Indirect RIA μU/ml	Ratio μU/pg
Normal women							
Plasma, week 8 of pregnancy	6	1630* (1240–2140)	1140 (896–1450)	0.70±0.11**	24.6 (15.3–39.6)	17.5 (10.6–28.9)	0.71±0.12
Plasma, week 36 of pregnancy	6	957 (536 –1710)	689 (399–1190)	0.72±0.12	50.8 (27.4–94.1)	34.1 (14.4–81.0)	0.67±0.08
Plasma, week 6-8 after parturition	6	249 (115–538)	192 (56.9–378)	0.77±0.12	21.1 (5.8–76.5)	15.4 (4.6–52.0)	0.73±0.11
Amniotic fluid	7	7680 (2710–21800)	5410 (1890–15500)	0.70±0.09	254 (90.9–710)	168 (57.4–492)	0.66±0.10
Patient with primary ovarian failure							
Plasma, day 1 of pregnancy		243	148	0.61	22.1	16.8	0.76
Plasma, week 9 of pregnancy		423	309	0.73	30.0	22.2	0.74
Plasma, week 12 of pregnancy		368	216	0.71	30.0	21.0	0.70
Plasma, week 34 of pregnancy		324	243	0.75	46.6	31.2	0.67
Plasma, week 8 after parturition		126	82	0.65	15.9	10.2	0.64
Amniotic fluid		25400	17150	0.68	1810	1290	0.71

* Mean value and 95% confidence limits (in parentheses); statistical analysis was performed after logarithmic transformation of results to obtain Gaussian distribution and results are expressed as antilog.

** Mean value and SD.

remained abnormally low (220 μU/ml) in the second and third trimesters of pregnancy. In an earlier study of 44 normal women, we found a mean prorenin level of 645 μU/ml (95% confidence limits 263 to 1,583 μU/ml) in the third trimester of pregnancy[4], which corresponds with the results of this study. It appears, therefore, that the high prorenin levels that normally occur in pregnancy may depend on normal ovarian function.

Since chorionic cells are known to produce prorenin, one might expect the placenta to be a major source of prorenin in maternal plasma[15]. At term the concentration of prorenin in amniotic fluid is about 10 times higher than in plasma[1,2]. It is possible that the ovary is indirectly involved by stimulating the placenta to secrete prorenin. In our patient amniotic fluid prorenin was not lower than in the normal women, which suggests that chorionic prorenin does not contribute in any major degree to the in maternal plasma prorenin levels.

The disappearance rate of plasma prorenin after delivery in the normal women was much lower than the disappearance rate we previously found after bilateral nephrectomy[16]. Maternal plasma prorenin did not fall until the second day after parturition both in the normal women and in the patient with primary ovarian failure, again suggesting that the placenta is not a major source of maternal plasma prorenin. However, we cannot exclude the possibility that the plasma half-life of non-renal prorenin is longer than the plasma half-life of renal prorenin.

Because of the findings of this study and in the light of recent reports that the ovary secretes prorenin[17] and that plasma prorenin in women with hyperstimulated cycles may rise to levels as high as in pregnancy[18], it is conceivable that the ovary is the main source of the elevated prorenin in plasma of pregnant women.

High prorenin concentrations in follicular fluid obtained at the time of oocyte collection in women with hyperstimulated cycles have been reported[13,17], and local production of prorenin is postulated to play a role in the development of the corpus luteum[9,17,18]. The finding in our patient with primary ovarian failure that plasma prorenin was abnormally low, not only in the first trimester of pregnancy but also in the second and third trimester, is in accordance with previous reports that the corpus luteum continues to be hormonally active during the whole period of pregnancy[19].

The stimulus for the large increase of maternal plasma prorenin in pregnancy is not known. Plasma prorenin is suppressed in women taking estrogen-containing contraceptives[4]. Progesterone on the other hand may stimulate the renal secretion of active renin and perhaps also of prorenin, because it acts as an aldosterone antagonist[20]. However, the changes in prorenin in normal pregnant women did not closely follow the changes in progesterone that are known to occur during pregnancy. After prorenin had reached its peak value at about 12 weeks, it fell until week 20 , whereas progesterone is known to rise continuously from week 12 onwards[21], a rise that also occurred in our patient with ovarian failure.

The increase of plasma prorenin in women with hyperstimulated cycles and in normal pregnancy may be due to gonadotropic stimulation[3,18]. A positive

correlation has been reported between plasma prorenin and the changes in plasma hCG during the five weeks following conception[3]. In our patient with primary ovarian failure the rise of prorenin, albeit small, closely followed the β-hCG peak in the first weeks of pregnancy, which lends some support to the hypothesis that also in this patient the rise of prorenin early in pregnancy was due to stimulation by chorionic gonadotropin.

Note: After completion of this paper we were informed by Dr J. Sealey from New York, NY, about similar results in a patient with primary ovarian failure, who became pregnant after embryo transfer and was followed for five weeks[22].

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6. SAMENVATTING

6.1. DOEL VAN HET ONDERZOEK

De hoogte van de bloeddruk wordt voor een groot deel bepaald door het renine-angiotensinesysteem. Dit systeem is samengesteld uit een aantal enzymen en enzymsubstraten die zich in plasma bevinden en oefent zijn bloeddrukverhogende werking uit door de vorming van angiotensine II, het biologisch actieve eindproduct (Fig.1.). Hoe belangrijk dit systeem is blijkt uit het feit dat antagonisten van angiotensine II en remmers van de enzymen die bij de vorming van angiotensine II zijn betrokken de bloeddruk verlagen. Remmers van het enzym dat zorgt voor de omzetting van angiotensine I in angiotensine II ('angiotensin-converting enzyme'), worden op het ogenblik op grote schaal toegepast bij de behandeling van patienten met hoge bloeddruk.

Op theoretische gronden ligt het voor de hand om de plasmaspiegel van angiotensine II te hanteren als maat voor de activiteit van het renine-angiotensine systeem *in vivo*. Practisch stuit dit echter op problemen omdat de concentratie van angiotensine II in plasma zeer laag is, enkele picogrammen per ml, en omdat de bij de radioimmunoassay gebruikte antilichamen behalve met angiotensine II ook met in het plasma aanwezige afbraakproducten van dit peptide reageren.

Voor klinische toepassingen is de bepaling van de zogenaamde plasmarenine-activiteit (PRA) nog steeds de meest gebruikte maat voor de vorming van angiotensine II *in vivo*. Bij bepalingen van PRA wordt het plasma van de patient gedurende een bepaalde tijd geincubeerd bij 37 C. Tijdens deze incubatie *in vitro* wordt door de hydrolytische werking van het protease, renine, op reninesubstraat (angiotensinogeen) het biologisch niet actieve peptide, angiotensine I, gevormd. Plasma heeft angiotensinase activiteit, waardoor angiotensine I wordt afgebroken. Dit wordt voorkomen door angiotensinaseremmers aan het plasma toe te voegen. Deze stoffen remmen echter ook het eveneens in plasma aanwezige 'angiotensin-converting enzyme', dat het decapeptide, angiotensine I, omzet in het octapeptide angiotensine II, zodat de reactie stopt bij de vorming van angiotensine I. Het gevormde angiotensine I kan vervolgens worden bepaald met behulp van radioimmunoassay.

Onder normale omstandigheden ligt de concentratie van reninesubstraat in plasma op het niveau van de Km-waarde, hetgeen betekent dat de snelheid waarmee angiotensine I wordt gevormd niet alleen afhankelijk is van de concentratie van renine maar ook van die van reninesubstraat. In bepaalde

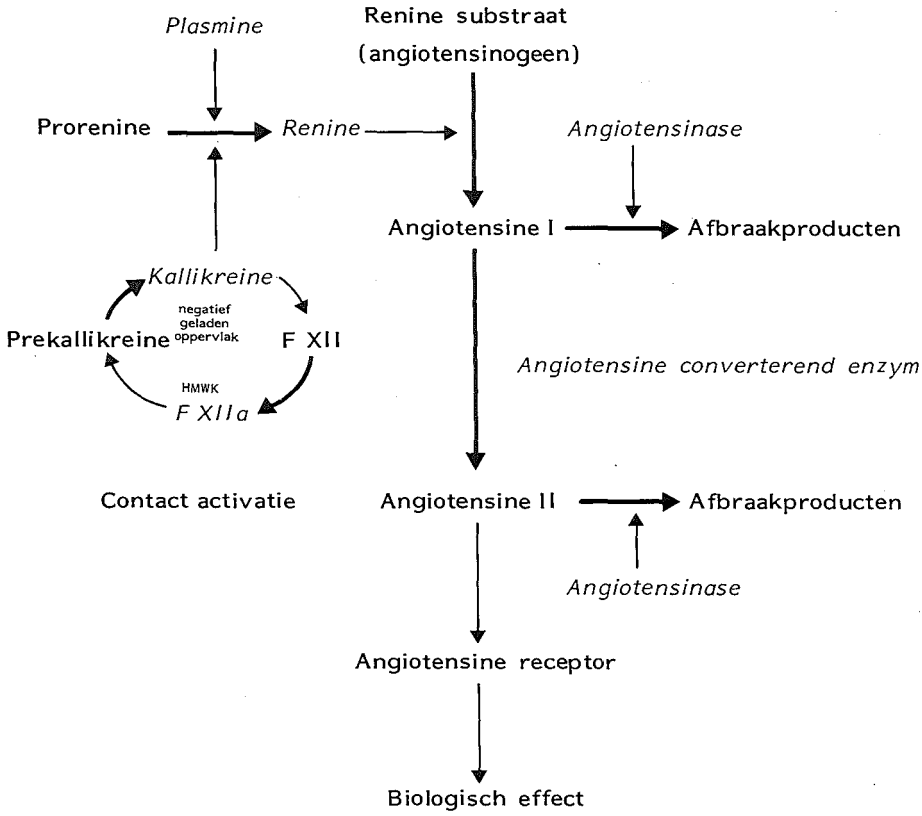

Fig.6.1. Componenten van het renine-angiotensine systeem. FXII = stollingsfactor XII. FXIIa = geactiveerde FXII. HMWK = Kininogeen met hoog molecuul gewicht.

gevallen, namelijk wanneer de substraatconcentratie sterk afwijkt van de norm, wordt de interpretatie van PRA bepalingen bemoeilijkt. Om de reactiesnelheid *in vitro* onafhankelijk te maken van de substraatconcentratie zijn bepalingsmethoden ontwikkeld, waarbij renine voor incubatie wordt gescheiden van het substraat of het endogene substraat tevoren wordt gedenatureerd. Hierna wordt een grote overmaat min of meer gezuiverd substraat toegevoegd, zodat het enzym renine verzadigd is met substraat.

De hoeveelheden angiotensine I die bij deze bepalingen van de plasmareninconcentratie (PRC) worden gevormd zijn groter dan bij bepalingen van PRA. Dit verschil berust echter niet alleen op de aanwezigheid van een overmaat aan exogeen substraat. Bij de meeste van de bovengenoemde PRC bepalingen wordt het plasma aangezuurd (pH 3-4) om het endogene reninesubstraat te denatureren. Daarna wordt de pH van het plasma weer hersteld en wordt substraat toegevoegd. Uit experimenten van Lumbers en Morris[1] bleek dat de renine-activiteit van

menselijk vruchtwater na aanzuren veel hoger was dan voor aanzuren; tijdens incubatie bij 37 C bij neutrale pH werd na aanzuren per tijdseenheid meer angiotensine I gevormd dan voor aanzuren, ondanks het feit dat ervoor gezorgd was dat de substraatconcentratie in het incubatiemengsel gelijk was. Een dergelijk resultaat werd ook verkregen wanneer, in plaats van vruchtwater, menselijk plasma werd gebruikt. Dit bleek te berusten op de activatie van een enzymatisch inactieve vorm van renine. Verondersteld werd dat dit z.g. inactieve renine een pro-enzym is (prorenine) en dat de activatie (zuur-activatie) tot stand komt door beperkte proteolyse onder invloed van een protease werkzaam bij lage pH. De mogelijkheid dat inactief renine niet zozeer een pro-enzym is maar een enzym-remmercomplex dat bij lage pH uiteenvalt is ook geopperd[2]. Activatie van inactief renine kan echter ook optreden bij neutrale pH wanneer plasma bij 0-4 C wordt bewaard (cryo-activatie).

Het is duidelijk dat deze waarnemingen belangrijke consequenties hebben voor de technische uitvoering en de interpretatie van plasmareninebepalingen. Wat ons echter intrigeerde was, dat ongeveer 90% van het bij gezonde personen in plasma aanwezige renine zich in de enzymatisch inactieve vorm bevindt en dat het renine-angiotensinesysteem in dit opzicht enigszins vergelijkbaar is met andere proteolytische systemen in plasma, zoals het stollingssysteem en het fibrinolytische systeem. Tot voor kort werd het renine-angiotensinesysteem opgevat als een circulerend hormonaal systeem. In feite berust de huidige interpretatie van PRA bepalingen, waarbij de *in vitro* generatie van angiotensine I in plasma geacht wordt een maat te zijn voor de *in vivo* generatie van angiotensine I (en II), op deze zienswijze. Het onderzoek dat in dit proefschrift is beschreven werd uitgevoerd met de gedachte dat het renine-angiotensinesysteem niet alleen zijn fysiologische werking uitoefent door de proteolytische activiteit van circulerend renine op circulerend reninesubstraat, maar dat misschien ook locale activatie van prorenine optreedt en dat bij deze activatie enzymen betrokken kunnen zijn die deel uitmaken van het stollingssysteem en het fibrinolytische systeem.

Het proefschrift bestaat uit twee delen, namelijk biochemische *in vitro* experimenten en fysiologisch onderzoek met behulp van dierexperimenten en bepalingen van prorenine in plasma van gezonde personen en patienten met verschillende aandoeningen en in menselijk vruchtwater en ovariele follikelvloeistof.

6.2. ACTIVATIE VAN PRORENINE *IN VITRO*

6.2.1. Zuur-activatie en cryo-activatie

Bij de bepaling van PRC volgens Skinner[3] wordt plasma voor incubatie met een overmaat exogeen reninesubstraat gedurende 24 uur gedialyseerd bij 0-4 C tegen een pH 3.3-buffer en vervolgens, eveneens gedurende 24 uur, bij 0-4 C tegen een pH 7.4-buffer. Onze kinetische studies toonden aan dat de renine-activiteit direct na het herstel van de pH naar pH 7.4 hoger was dan voor aanzuren.

De grootste toename van de renine- activiteit trad op gedurende de 24 uur durende dialyse bij pH 7.4. Deze activatie bij pH 7.4 kon worden geremd door toevoeging van serineproteaseremmers, zoals aprotinine (Trasylol), soyaboon-trypsineremmer en benzamidine. Experimenten met plasma afkomstig van patienten met een erfelijke deficientie van hetzij stollingsfactor XII (Hageman-factor) hetzij plasma-prekallikreine (Fletcher-factor) toonden aan dat beide factoren onontbeerlijk zijn voor de activatie van prorenine in aangezuurd plasma, nadat de pH is hersteld. Geactiveerde factor XII (factor XIIa) en kallikreine zijn serineproteasen die in aangezuurd plasma na herstel van de pH worden gevormd uit respectivelijk factor XII en prekallikrein. Verder onderzoek m.b.v. gezuiverd actief β-factor XIIa (lichte keten met Mr 28.000 die de actieve groep bevat) en plasmakallikreine (zowel het gehele molecuul met Mr 85.000 als de lichte keten met Mr 28,000) toonden aan dat of kallikreine zelf of door kallikreine geactiveerde enzymen verantwoordelijk zijn voor de activatie van prorenine in met zuur voorbehandeld plasma en dat de functie van factor XII beperkt is tot de activatie van prekallikreine, waardoor kallikreine wordt gevormd. Kininogeen met hoog molecuulgewicht, Fitzgerald factor, (HMWK, Mr 110.000), dat nodig is voor de snelle activatie van factor XII en prekallikreine in de aanwezigheid van een negatief geladen oppervlak (contactactivatie), is niet essentieel voor de toename van de renine-activiteit van met zuur voorbehandeld plasma: de renine-activiteit nam normaal toe in plasma van een patient met een erfelijke deficientie van HMWK.

Hoewel plasmakallikreine onder bepaalde omstandigheden in staat is plasminogeen te activeren en plasmine op zijn beurt in staat is de renine-activiteit van met zuur voorbehandeld plasma te verhogen, bleek de door factor XII-kallikreine veroorzaakte activatie van prorenine in met zuur voorbehandeld plasma normaal te verlopen, ook na selectieve verwijdering van plasminogeen.

Het is bekend dat, wanneer plasma bij 0-4 C wordt bewaard, factor XII en prekallikreine worden geactiveerd. Wij vermoedden daarom dat dit de oorzaak was van de eerder vermelde z.g. cryo-activatie van prorenine in plasma bij neutrale pH. Inderdaad bleek deze cryo-activatie van prorenine niet op te treden in het plasma van patienten met een erfelijke deficientie van factor XII of prekallikreine. Weer bleek HMWK niet essentieel te zijn voor dit fenomeen. Met behulp van plasma afkomstig van patienten met een erfelijke deficientie van factor V, VII, VIIIC, IX, X en XI en met behulp van plasma, waaruit prothrombine door immunoadsorptie was verwijderd, kon worden aangetoond, dat al deze stollingsfactoren, in tegenstelling tot factor XII en plasmakallikreine, geen belangrijke invloed hadden op de stijging van de renine-activiteit in plasma dat bij 0-4 C wordt bewaard.

De cryo-activatie van prorenine in plasma is een langzaam proces. Dit proces verliep veel sneller in plasma waaruit de belangrijkste remmers van factor XII en kallikreine, nl. C_1-esterase remmer, α_2-macroglobuline en antithrombine III, d.m.v. immunoadsorptie waren verwijderd. Wanneer aan een dergelijk plasma het polyanion dextraansulfaat (Mr 500.000) wordt toegevoegd, treedt bij 0-4

C snelle en volledige activatie van prekallikreine op. Deze z.g. contactactivatie van prekallikreine is afhankelijk van de aanwezigheid van factor XII en HMWK, waarbij dextraansulfaat als een negatief geladen oppervlak fungeert. Wanneer vervolgens het plasma enkele minuten na deze maximale contactactivatie werd verwarmd tot 37 C, nam ook de renine-activiteit toe. Dit gebeurde niet wanneer de bovengenoemde serineproteaseremmers niet tevoren uit het plasma waren verwijderd.

We kunnen dus vaststellen dat de toename van de renine-activiteit in plasma bij neutrale pH, zowel met als zonder voorbehandeling met zuur, afhankelijk is van de activatie van factor XII en prekallikreine. In beide gevallen wordt de activatie van factor XII en prekallikreine waarschijnlijk bevorderd door denaturatie of inactivatie van serineproteaseremmers. Wanneer plasma werd aangezuurd tot pH 3.3 en gedurende 24 uur op deze pH werd gehouden bij 0-4 C, was inderdaad na herstel van de pH tot 7.4 geen activiteit van C_1-esterase remmer en antithrombine III meer aantoonbaar. De activiteit van α_2-macroglobuline was verminderd maar niet geheel verdwenen. Verder is het bekend dat de activiteit van deze serineproteaseremmers bij 0-4 C veel geringer is dan bij 37 C.

Het onderzoek naar de activatie van prorenine *in vitro* werd uitgebreid met experimenten met gezuiverd prorenine geisoleerd uit menselijk vruchtwater en plasma. Bij de zuivering werd gebruik gemaakt van kolomchromatografie over de ionenwisselaar DEAE-Sepharose, van affiniteitschromatografie over een kolom van Cibacron Blue gebonden aan Sepharose en over een kolom van een aan Sepharose gebonden monoclonaal renine-antilichaam dat reageerde met zowel prorenine als actief renine. Sporen actief renine werden verwijderd d.m.v. chromatografie over een kolom van aan Sepharose gebonden pepstatine. Pepstatine is een remmer van aspartylproteasen, inclusief enzymatisch actief renine. Prorenine wordt niet gebonden aan pepstatine. Door aan preparaten van gezuiverd prorenine hoog gezuiverd plasmakallikreine (zowel het intacte molecuul als de lichte keten) of plasmine of trypsine toe te voegen kon worden aangetoond, dat elk van deze stoffen in staat was prorenine om te zetten in enzymatisch actief renine. Gezuiverde factor XIIa (α-factor XIIa, het hele molecuul met Mr 80.000),β-factor XIIa (actieve lichte keten met Mr 28.000), thrombine en urokinase waren daartoe niet in staat, althans niet in de concentraties van maximaal 0.1 μM. De conversie geschiedde bij 37 C en neutrale pH en voorbehandeling met zuur was niet noodzakelijk.

6.2.2. Activatie met en zonder proteolyse

Intussen werd door andere onderzoekers aangetoond dat prorenine kan worden geactiveerd door aanzuren tot pH 3.3, ook wanneer de bovengenoemde serineproteasen afwezig zijn[2,4]. Deze onverwachte bevinding konden wij bevestigen. Dit stelde ons voor de vraag naar het verband tussen de z.g. zuur-activatie in plasma en vruchtwater enerzijds en de door serineprotease veroorzaakte activatie

anderzijds. Een van de mogelijkheden was dat prorenine niet zozeer een inactief pro-enzym is maar een complex van actief renine en een remmer en dat dit complex in zuur milieu uiteenvalt, terwijl de remmer vervolgens, wanneer de pH hersteld is, door een serineprotease wordt geinactiveerd. Hiermee in overeenstemming is het feit dat de zuur-activatie in gezuiverde preparaten van prorenine een reversibel proces is: in neutraal milieu daalt de renine-activiteit weer tot de oorspronkelijke waarde voor aanzuren. Ook deze inactivatie is een reversibel proces, omdat na opnieuw aanzuren de renine-activiteit weer stijgt. Ook in plasma blijkt zuur-activatie een reversibel proces te zijn, althans wanneer activatie van factor XII en prekallikreine wordt voorkomen door toevoeging van serineproteaseremmers, zoals aprotinine, soyaboon-trypsineremmer of benzamidine.

Kinetisch onderzoek over de reactie tussen renine en renine-substraat, waarbij gebruik gemaakt werd van gezuiverd actief renine geïsoleerd uit menselijk vruchtwater, plasma en nierweefsel, van gezuiverd prorenine geïsoleerd uit menselijk vruchtwater en plasma en van gezuiverd reninesubstraat geïsoleerd uit menselijk plasma of schapeplasma, toonde aan dat het door trypsine geactiveerde prorenine van vruchtwater en plasma dezelfde enzym-kinetische eigenschappen (Km en Vmax) heeft als het natuurlijk voorkomende actieve renine van vruchtwater, plasma en nierweefsel. Ook was er geen verschil in pH-optimum. Inmiddels hebben andere onderzoekers aangetoond dat een monoclonaal antilichaam gericht tegen het pro-segment van renaal prorenine, de biosynthetische voorloper van actief renaal renine, reageert met prorenine in plasma, terwijl het niet reageert met het actieve renine in plasma[5,6]. Het is dus zeer aannemelijk dat de proteolytische activatie van prorenine berust op afsplitsing van dit prosegment van de rest van het molecuul. Inactief renine kan daarom met recht prorenine worden genoemd.

Om meer inzicht te verkrijgen over de rol van proteasen bij de zuur-activatie van prorenine werd een meer gedetailleerd onderzoek verricht naar de kinetiek van dit proces, zowel in plasma en vruchtwater als in preparaten van gezuiverd prorenine. Daarbij bleek dat de zuur-activatie van prorenine (pR) in vruchtwater in twee stappen verloopt. Eerst wordt een intermediaire vorm van geactiveerd prorenine (pRa) gegenereerd: pR → pRa. Deze wordt vervolgens omgezet in een vorm van geactiveerd prorenine die identiek is aan of in ieder geval zeer sterk gelijkt op natief renine (R): pRa → R. Beide reacties vinden in vruchtwater plaats bij lage pH.

De Vmax van de reactie van pRa met reninesubstraat verschilt niet voor die van de reactie van R met substraat. De beide actieve vormen, pRa en R, zijn echter in andere opzichten duidelijk verschillend van elkaar. Ten eerste verliest pRa, in tegenstelling tot R, zijn enzymatische activiteit bij pH 7.4. Ten tweede wordt pRa, in tegenstelling tot R, gebonden aan 'Cibacron Blue'-Sepharose. Ten derde kon pRa niet, en R wel, worden aangetoond met behulp van een radioimmunoassay waarbij gebruik gemaakt werd van monoclonale renine-antilichamen met affiniteit voor R maar niet voor pR. De omzetting van pRa

in R is een proteolytisch proces en vindt in vruchtwater plaats onder invloed van een aspartylprotease. In plasma vindt de omzetting van pRa in R niet plaats tijdens de behandeling met zuur maar pas na herstel van de pH, en wel onder invloed van factor XII-kallikreine.

Kinetisch onderzoek toonde verder aan dat de pR → pRa stap, die bij lage pH plaats vindt, een unimoleculair proces is, evenals de inactivatie van pRa in neutraal milieu (pRa → pR). De zuur-activatie is dus niet het gevolg van dissociatie van een complex van renine en een renineremmer. De eerste-orde reactiesnelheidsconstante voor het proces pR → pRa is afhankelijk van de protonering van een in een bepaald aminozuurresidu (of residuen) aanwezige atoomgroep (of groepen) met een pKa van ongeveer 3.4. Deze reactieconstante is evenredig met het percentage pR moleculen, waarbij deze groep is geprotoneerd. Dit komt overeen met de activatie van pepsinogeen. Interactie van positief geladen aminozuurresiduen in het 44 aminozuren tellende N-terminale pro-segment van varkenspepsinogeen en negatief geladen carboxylgroepen van aminozuren in het enzym-segment houden het pepsinogeenmolecuul in zijn enzymatisch inactieve vorm. Na aanzuren worden deze electrostatische bindingen verbroken en ondergaat het molecuul een reversibele conformatieverandering, waarbij het catalytisch apparaat wordt geëxponeerd. Dus na aanzuren wordt het activeringspeptide losgemaakt van zijn oorspronkelijke positie waarin het de actieve groep blokkeert. De proteolytische activering van pepsinogeen vindt daarna plaats. De pRa → R conversie onder invloed van plasma kallikreine, plasmine of trypsine verloopt ongeveer 50-70 maal sneller dan de activatie van natief prorenine (pR → R) door deze serineproteasen. Dus pRa is veel gevoeliger dan pR voor proteolytische omzetting in R.

Op grond van deze resultaten zijn we nu in staat ons een duidelijker beeld te vormen van de activatie van prorenine *in vitro*. Dit beeld kan als volgt worden samengevat. Cryo-activatie van prorenine in plasma berust op beperkte proteolyse van prorenine, waarbij actief renine wordt gevormd (pR → R). Deze proteolyse komt tot stand door de werking van kallikreine dat op zijn beurt is gevormd uit prekallikreine door de werking van geactiveerd factor XII. Deze processen treden bij lichaamstemperatuur niet of nauwelijks op, vanwege de inhiberende werking van de in plasma aanwezige remmers van factor XII en kallikreine. Bij de zuur-activatie ondergaat het prorenine een niet-proteolytische, reversibele, verandering in conformatie, waarbij het katalytische apparaat van het molecuul wordt geëxponeerd en een binding kan aangaan met het reninesubstraat. Het actieve centrum heeft echter bij lage pH, in tegenstelling tot met zuur geactiveerd pepsinogeen, geen katalytische activiteit. Dit komt doordat de aspartylresiduen (Asp-38 en Asp-226), die deel uitmaken van het actieve centrum, een hogere pK-waarde hebben dan de overeenkomstige aspartylresiduen bij pepsine (Asp-32 en Asp-215). Voor de katalytische activiteit van renine is het noodzakelijk dat Asp-38 in de geïoniseerde vorm is, terwijl Asp-226 in de niet-geïoniseerde vorm dient te zijn. Dus het bij zuur gevormde pRa dient op neutrale pH te worden gebracht alvorens het in staat is uit reninesubstraat angiotensine I te

vormen. In met zuur behandeld plasma worden echter, na terugkeer van de pH, factor XII en prekallikreine geactiveerd, waarschijnlijk omdat remmers van factor XII en kallikreine na zuur-behandeling gedeeltelijk zijn gedenatureerd. Omdat pRa zeer gevoelig is voor proteolytische omzetting in R door kallikreine, wordt, na het neutraliseren van aangezuurd plasma, snel actief renine gevormd (pRa → R) en is er, althans onder optimale omstandigheden, geen vorming van prorenine (pRa → pR). Terwijl in plasma de omzetting pRa → R na herstel van de pH plaats vindt, gebeurt dit in aangezuurd vruchtwater voor herstel van de pH door de werking van een endogeen aspartylprotease.

In vivo vindt de activatie van pepsinogeen in de maag bij pH 2-3 plaats. Het is mogelijk dat de vorming van de intermediaire actieve vorm van prorenine (pRa) *in vivo* gebeurt in bepaalde intracellulaire compartimenten met lage pH, zoals secretiegranula en endo- of exocytotische blaasjes[7]. De membraan van endocytotische blaasjes (receptosomen), die betrokken zijn bij de receptor-afhankelijke endocytose, bezit een actieve protonpomp, die ervoor zorgt dat de pH binnen deze blaasjes daalt tot 5 of misschien lager. Waarschijnlijk is de pH in de nabijheid van de protonpomp zelfs nog lager. In een dergelijk milieu is de vorming van pRa mogelijk. De intermedaire vorm is uiterst gevoelig voor beperkte proteolyse, waardoor er lokaal renine kan worden gevormd. Het is mogelijk dat een deel van het locaal gevormde renine afkomstig is van circulerend prorenine[8–10].

6.3. REGULATIE VAN PRORENINE *IN VIVO*

6.3.1. Directe en indirecte radioimmunoassay van prorenine

Bij de meeste onderzoekingen die in dit proefschrift zijn beschreven werd het reninegehalte van plasma en andere lichaamsvloeistoffen bepaald met behulp van een enzym-kinetische methode. Daarbij wordt de snelheid, waarmee angiotensine I onder gestandaardiseerde omstandigheden *in vitro* wordt gegenereerd, gemeten met behulp van radioimmunoassay (indirecte RIA). Voor de bepaling van prorenine met behulp van de indirecte RIA is het noodzakelijk dat prorenine eerst volledig wordt omgezet in enzymatisch actief renine. Bovendien mogen prorenine, geactiveerd prorenine en het natuurlijk voorkomende actieve renine niet door de voor activatie van prorenine gebruikte behandeling van het plasmamonster worden gedenatureerd. Tenslotte moet het *in vitro* geactiveerde prorenine dezelfde enzym-kinetische eigenschappen bezitten als het natuurlijk voorkomende renine.

De omzetting van prorenine in het enzymatisch actieve renine *in vitro* kan op verschillende manieren plaats vinden: 1) door bewaren van plasma en andere lichaamsvloeistoffen bij lage temperatuur, 2) door zuur-behandeling en 3) door toevoeging van proteolytische enzymen.

Zoals besproken in het eerste gedeelte van de samenvatting, zijn de cryo- en zuur-activatie in plasma afhankelijk van de aanwezigheid van factor XII en

prekallikreine. Wanneer deze stollingsfactoren afwezig zijn of wanneer hun concentraties laag zijn, zijn deze methoden niet bruikbaar. De cryo-activatie verloopt langzaam, ook in normaal plasma, waardoor cryo-activatie niet geschikt is voor praktische toepassing. Na een week is slechts 15% van het in plasma aanwezige prorenine bij 0 C geactiveerd. De zuur-activatie van prorenine verloopt weliswaar sneller en volledíger, maar een nadeel is dat de hoogte van de pH erg kritisch is. Beneden pH 3.1 worden prorenine en actief renine gedenatureerd ook bij 0 C, terwijl er boven pH 4.0 bij 0 C nauwelijks activatie optreedt. Een veel gebruikte methode om prorenine te activeren is die door toevoeging van trypsine aan plasma. De concentratie van trypsine en de duur van de incubatie zijn hierbij van grote invloed. Bij een te hoge concentratie of bij te lang incuberen worden prorenine en actief renine gedenatureerd. Wij hebben aanvankelijk minder aggressieve serineproteasen gebruikt, zoals plasmine en urokinase. Het nadeel van deze methode is dat activatie van prorenine met deze enzymen alleen optreedt wanneer van te voren antiplasmine uit het plasma is verwijderd. Wij hebben een methode ontwikkeld, waarbij volledige omzetting van prorenine in het actieve renine plaats vindt zonder enig verlies van prorenine, van geactiveerd prorenine of van het reeds tevoren aanwezige actieve renine. Voor deze methode wordt plasma bij 0 C geincubeerd met aan Sepharose gebonden trypsine. Hiermee stijgt de renine-activiteit tot een plateau, waarvan de hoogte onafhankelijk is van de gebruikte trypsineconcentratie, althans in het bereik van 2 tot 20 μM. Bij een trypsineconcentratie van 10 μM treedt volledige activatie op binnen 24 uur. Het trypsine kan eenvoudig worden verwijderd door centrifugeren.

In de laatste twee jaar zijn monoclonale antilichamen beschikbaar gekomen, die een grote affiniteit bezitten voor zowel actief renine als prorenine. Ook zijn antilichamen beschikbaar die alleen de actieve species herkennen. Met behulp van deze antilichamen is het mogelijk het z.g. immunoreactieve renine te bepalen (directe RIA). Door de uitkomsten van de directe en indirecte RIA met elkaar te vergelijken, zowel in plasma als in preparaten van gezuiverd prorenine en actief renine, konden wij bevestigen dat de door ons toegepaste indirecte RIA van prorenine, waarbij het prorenine m.b.v. geïmmobiliseerd trypsine bij 0 C wordt omgezet in het actieve renine, een goede maat is voor het proreninegehalte van plasma.

Tijdens het verwerken en bewaren van de plasmamonsters kan onbedoeld cryo-activatie van prorenine optreden. Dit leidt bij de indirecte RIA tot foutief hoge waarden van actief renine en te lage waarden van prorenine. Het bleek ons dat dit vooral het geval was bij plasma afkomstig van vrouwen die een oraal anticonceptivum gebruiken of zwanger waren. Dit hangt waarschijnlijk samen met de verhoogde concentratie van factor XII en/of prekallikreine in het plasma van deze vrouwen. Daar komt nog bij dat bij zwangeren de concentratie van C_1-esterase remmer in het plasma verlaagd is. Bovendien is bij deze vrouwen de proreninеconcentratie verhoogd, zodat meer prorenine beschikbaar is voor activatie. Door bij het afnemen van bloed en bij het verwerken en bewaren van de plasmamonsters speciale voorzorgsmaatregelen in acht te nemen kon deze onbedoelde activatie van prorenine grotendeels worden voorkomen.

6.3.2. Concentratie van prorenine in plasma en andere lichaamsvloeistoffen onder fysiologische en pathologische omstandigheden

Gegevens over het gehalte aan prorenine en enzymatisch actief renine in plasma van gezonde personen en patienten met verschillende aandoeningen zijn samengevat in Tabel 1. Plasma van patienten, bij wie de nieren waren verwijderd, bevatte vrijwel geen actief renine. Dus het circulerende actieve renine is vrijwel

Table 6.1. Plasmaspiegels van actief renine en prorenin bij normale personen met verschillende aandoeningen.

	n	Actief renine μU/ml	Prorenine μU/ml	Actief renine % van totaal
Normalen	112	17.7 (4.8–65.3)	171 (77.2–377)	9.2 (3.2–26.7)
Anefrische patienten	37	1.0 (0.2–6.1)	61.0 (13.4–277)	1.6 (0.4–5.9)
Conn syndroom	14	0.58 (0.11–3.0)	37.3 (17.9–77.4)	1.5 (0.4–6.5)
Normale vrouwen bij gebruik van orale anticonceptiva	47	8.8 (2.3–33.2)	91.8 (32.7–258)	8.6 (3.1–24.4)
Essentiele hypertensie	68	13.8 (3.0–63.3)	115 (40.4–330)	10.0 (2.8–36.7)
Renovasculaire hypertensie	68	52.7 (10.0–277)	165 (50.2–542)	23.1 (8.1–66.1)
Essentiele hypertensie bij gebruik van captopril 3dd 50 mg	45	65.5 (3.4–1260)	241 (42.9–1350)	19.7 (3.0–68.1)
Renovasculaire hypertensie bij gebruik van captopril 3 dd 50 mg	46	463 (63.6–3360)	593 (200–1750)	41.1 (20.6–81.9)
Bartter syndroom	9	923 (352–2430)	690 (635–4480)	54.7 (32.7–91.5)
Prekallikreine-deficientie	8	11.4 (4.5–29.0)	339 (171–669)	3.3 (1.1–9.4)
Zwangeren in het eerste trimester	9	17.5 (10.4–28.9)	1120 (898–1410)	1.6 (0.8–2.0)
Zwangeren in het derde trimester	50	31.7 (10.0–100)	634 (263–1530)	4.7 (1.2–18.6)
Prorenine producerende ovariumtumor	1	1010	120000	0.8

De resultaten (gemiddelde en 95%-betrouwbaarheidsinterval) tonen dat, naarmate de spiegel van actief renine hoger is, prorenine ook hoger is. De procentuele stijging van actief renine is echter groter dan die van prorenine. Patienten met prekallikreinedeficientie (Fletcher trait) en zwangere vrouwen vormen uitzonderingen hierop.

geheel afkomstig van de nieren. Hoewel het gehalte aan prorenine bij de meeste patienten zonder nieren verlaagd was, was het bij sommigen binnen het normale bereik. Gemiddeld is het proreninegehalte bij deze patienten ongeveer eenderde van de gemiddelde waarde bij normale personen. Dus ongeveer eenderde van het prorenine in plasma van normale personen is van extrarenale oorsprong. Hoewel het onderzoek bij genefrectomeerde patienten er duidelijk opwijst dat prorenine, althans voor een deel, door de nieren aan het plasma wordt afgegeven, is het onder normale omstandigheden moeilijk het directe bewijs hiervoor te leveren d.m.v. bepalingen van prorenine in het plasma van nierarterie en niervene, vanwege de grote bloeddoorstroming van de nieren en de relatief lange plasma-halfwaardetijd van prorenine (2-3 uur)[11]. Anders ligt het, wanneer slechts een van beide nieren prorenine produceert, de doorbloeding van deze nier verminderd is en de bijdrage van deze nier tot de totale productie van circulerend prorenine groot is. Dit is het geval bij eenzijdige nierarteriestenose. Bij patienten met deze aandoening kon dan ook worden aangetoond dat de prorenineconcentratie in de niervene significant hoger was dan in de nierarterie.

Terwijl de concentratie van prorenine is verlaagd bij genefrectomeerde patienten, is deze bij patienten met een toegenomen renale afgifte van actief renine verhoogd. Over het algemeen geldt bovendien hoe hoger de totale concentratie van renine (prorenine plus actief renine) in plasma, hoe groter het percentage aan actief renine (Fig.2). Waarschijnlijk is de procentuele bijdrage van het door de nier geproduceerde prorenine aan de totale produktie van circulerend prorenine onder deze omstandigheden ook groter.

Er zijn echter twee belangrijke uitzonderingen op deze regel nl. patienten met erfelijke prekallikreinedeficiëntie (Fletcher trait) en zwangere vrouwen. Bij patienten met Fletcher trait is prorenine duidelijk verhoogd, terwijl actief renine normaal of verlaagd is. De oorzaak hiervan kan gelegen zijn in het feit dat de omzetting van prorenine in renine geblokkeerd is. Dit zou dan beteken dat onze bevindingen *in vitro*, waaruit bleek dat plasmakallikreine in staat is prorenine om te zetten in het enzymatisch actieve renine, wel degelijk van fysiologisch belang zijn.

Bij zwangeren zijn in het plasma zowel prorenine als actief renine verhoogd, maar prorenine is verhoudingsgewijs veel sterker gestegen dan actief renine. Dit is reeds het geval in het eerste trimester. Het ligt voor de hand om aan te nemen dat in dit geval het lage percentage aan actief renine verband houdt met een grotere extrarenale productie van prorenine. Een van de mogelijke bronnen is de placenta. In menselijk vruchtwater (derde trimester) is de prorenineconcentratie ongeveer 6-10 maal hoger dan in plasma. Na de geboorte van de placenta daalt de concentratie van prorenine in plasma in de eerst 48 uur echter nauwelijks, dit in tegenstelling tot actief renine.

Een andere bron van prorenine zou het ovarium kunnen zijn. Het is bekend dat de prorenineconcentratie van plasma in de luteale fase van de normale menstruele cyclus ongeveer tweemaal zo hoog is als in de folliculaire fase[12]. Bij vrouwen, die worden gestimuleerd met injecties van gonadotroop hormoon

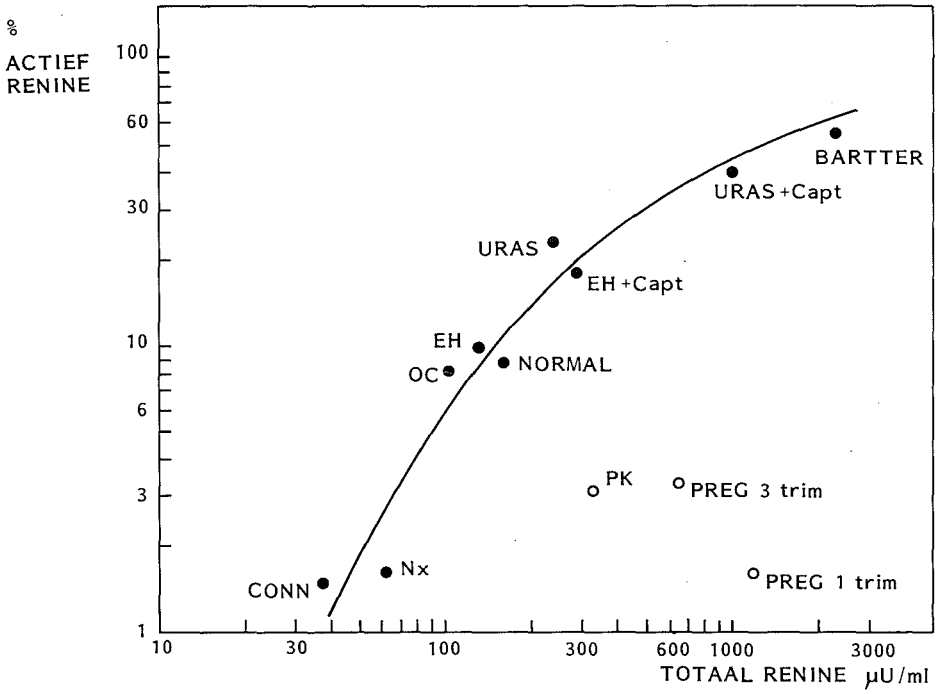

Fig.6.2. De relatie tussen het percentage enzymatisch actief renine en de totale renine concentratie (prorenine plus actief renine) in plasma. Zie ook Tabel 6.1. Capt = captopril behandeling 3 dd 50 mg. EH = patienten met essentiele hypertensie. Nx = genefrectomeerde patienten, OC = vrouwen die orale anticonceptiva gebruiken. PK = patienten met prekallikreine deficientie. Preg 1 trim = vrouwen in eerste trimester van de zwangerschap, Preg 3 trim = vrouwen in derde trimester van de zwangerschap, RAS = patienten met eenzijdige nierarteriestenose.

ter voorbereiding van *in vitro* fertilisatie, treedt tijdens of vlak na de ovulatie een stijging van het plasmaprorenine op tot waarden die ook gedurende zwangerschap worden gezien[13]. Wij hebben kunnen vaststellen dat de ovariële follikelvloeistof van deze vrouwen, ten tijde van het oogsten van het ovum, vlak voor de te verwachten ovulatie een zeer hoog gehalte aan prorenine heeft, ongeveer 30-40 maal hoger dan het gehalte in plasma. Bovendien is er een positieve correlatie tussen de prorenineconcentratie in de follikelvloeistof en het plasma (ongepubliceerde eigen waarneming). Een dergelijke correlatie is er niet voor wat betreft actief renine. Bij een vrouw zonder functionerende ovaria, die zwanger werd na het inbrengen van een 48 uur oud embryo, steeg het prorenine in plasma vrijwel niet tijdens het gehele beloop van de zwangerschap, terwijl bij normale zwangeren het prorenine reeds gedurende het eerste trimester stijgt met een factor 5-6. Het gehalte aan actief renine in plasma liet bij deze patiente het normale beloop zien, namelijk een langzame stijging tot een niveau in het derde trimester tweemaal de uitgangswaarde voor de zwangerschap. Deze bevindingen maken

het aannemelijk dat in het ovarium prorenine wordt geproduceerd. De stijging van de concentratie van prorenine in plasma omstreeks de ovulatie bij de normale menstruele cyclus en de hogere concentratie van plasmaprorenine bij vrouwen die behandeld worden met gonadotroop hormoon maken het aannemelijk dat de productie en afgifte van prorenine door het ovarium door gonadotroop hormoon worden gestimuleerd.

6.4. CONCLUSIES

Wat is de fysiologische betekenis van prorenine? Is er een regelmechanisme voor de activatie van prorenine binnen de juxtaglomerulaire cellen? Is er activatie van prorenine nadat het door de juxtaglomerulaire cellen is afgescheiden en in het circulerende bloed is terechtgekomen? Wat is de functie van prorenine dat niet in de juxtaglomerulaire cellen maar elders, zoals in het ovarium, is gevormd?

Ons onderzoek geeft geen rechtstreeks antwoord op deze vragen, maar onze resultaten laten wel enkele conclusies toe die het antwoord dichterbij kunnen brengen.

1. Onze resultaten wijzen erop dat er inderdaad een mechanisme bestaat dat de verhouding regelt tussen de hoeveelheden actief renine en prorenine die door de juxtaglomerulaire cellen worden gesecerneerd in het circulerende bloed; bij stimulatie van de reninesecretie stijgt deze verhouding. Bij acute stimulatie neemt selectief de afgifte van actief renine toe. Bij chronische stimulatie neemt de afgifte van zowel actief renine als prorenine toe, terwijl de afgifte van actief renine relatief sterker toeneemt dan die van prorenine. De aard van dit mechanisme, dat na de translatie van prorenine-mRNA ingrijpt in de processen van opslag en secretie in de juxtaglomerulaire cellen, is onbekend.

2. Onze resultaten tonen verder dat zowel het factor XII – kallikreine – contactactivatiesysteem als plasmine in staat zijn om prorenine te activeren. Deze activatie is *in vitro* alleen mogelijk onder omstandigheden die sterk afwijken van die in het circulerende bloed. Bij onze eerdere bepalingen bij patienten met hypertensie werden na acute stimulatie van de afgifte van renine door de nier soms reciproke veranderingen in de concentraties van actief renine en prorenine in plasma waargenomen; actief renine steeg terwijl prorenine daalde, wat een omzetting van plasmaprorenine in het enzymatisch actieve renine zou kunnen suggereren[14,15]. Ook andere onderzoekers hebben dit beschreven[16–18]. Deze waarneming kon bij recent onderzoek, o.a. met behulp van de directe RIA, echter niet worden bevestigd en berust op onvolledige activatie van prorenine tijdens de bepaling van dit enzym m.b.v. de indirecte RIA, waardoor te lage waarden worden gemeten. Activatie van prorenine in het circulerende bloed is dus erg onwaarschijnlijk.

Terwijl het natieve prorenine slechts langzaam door plasmakallikreine en plasmine wordt geactiveerd, is prorenine, dat behandeld is met zuur, zeer gevoelig voor activatie door deze en andere proteasen. Zoals boven beschreven, is het

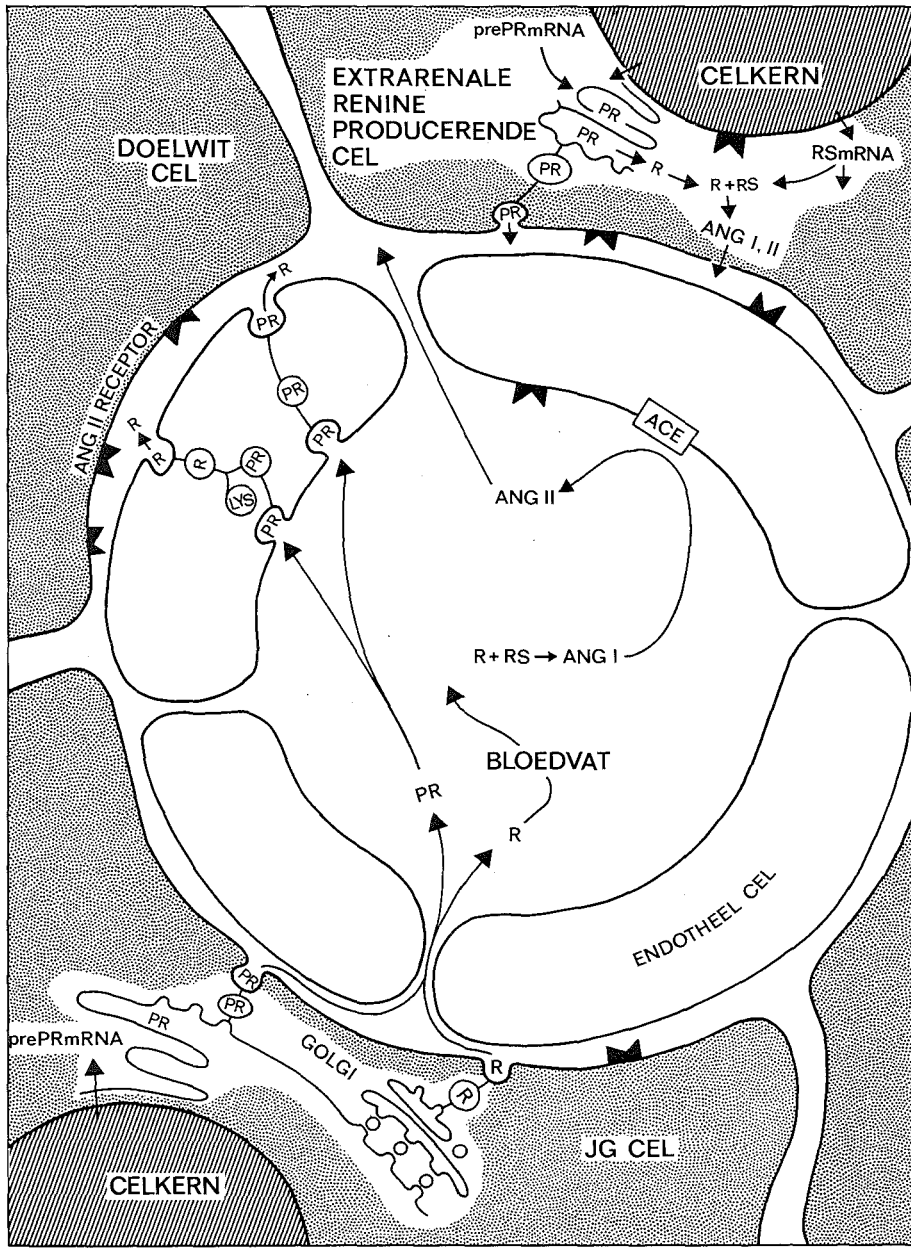

Fig. 6.3. Hypothetische interactie tussen de renale en extrarenale renine-angiotensine systemen. ACE= 'angiotensin-converting enzyme'.ANG I = angiotensine I. ANG II = angiotensine II. JGcel = juxtaglomerulaire cel. Lys = lysosoom. mRNA = messenger RNA. PR = prorenine. R = enzymatisch actief renin. RS = reninesubstraat.

denkbaar dat prorenine door het capillaire endotheel wordt opgenomen via het proces van de (receptor-afhankelijke) endocytose, waarna het wordt blootgesteld aan de lage pH van de receptosomen en aldaar wordt omgezet in de door ons *in vitro* geïdentificeerde intermediaire vorm van geactiveerd prorenine (Fig.3.). Wanneer deze intermediaire vorm van geactiveerd prorenine via het transcellulaire transport van de endocytotische blaasjes de contraluminale zijde van het capillaire endotheel bereikt, kan het door exocytose terecht komen in een milieu met neutrale pH, waarbij het hetzij snel overgaat in inactief renine hetzij door bepaalde proteasen snel kan worden omgezet in de actieve vorm van renine. Het is ook mogelijk dat deze proteolytische activatie binnen de cel plaats vindt, bijvoorbeeld door fusie van de endocytotische blaasjes met (pre-)lysosomen die pepsine-achtige enzymen, zoals cathepsine D, bevatten. Cathepsine D is in staat prorenine *in vitro* bij lage pH te activeren. Het is bekend dat de secretiegranula van de juxtaglomerulaire cellen behalve prorenine en renine ook cathepsine B , Cathepsin D en andere lysosomale enzymen bevatten[19].

De zojuist beschreven hypothese houdt in dat er, naast de vorming van circulerend actief renine door de juxtaglomerulaire cellen, ook lokale vorming van actief renine elders in het lichaam, buiten de nieren plaats vindt en dat deze locale vorming van renine geschiedt door activatie van uit het plasma opgenomen prorenine dat, evenals plasma renine, afkomstig is van de juxtaglomerulaire cellen.

Anderzijds is inmiddels bekend geworden dat, behalve de juxtaglomerulaire cellen, ook andere cellen elders in het lichaam in staat zijn prorenine en renine te synthetiseren. Chorioncellen synthetiseren en secerneren prorenine in het kweekmedium. Neuroblastoma × glioma cellijnen bevatten renine, evenals hersenen, hypophyse (LH en groeihormoon producerende cellen), bijnier, schildklier en testis (Leydigcellen). Bovendien is komen vast te staan dat een aantal van deze cellen behalve renine ook andere componenten van het renine-angiotensine systeem, zoals reninesubstraat, bevatten, waardoor zij in staat zijn tot de intracellulaire vorming van angiotensine I en II (8-10,20).

Het is mogelijk dat het locale (paracriene,autocriene) en intracellulaire (intracriene) renine-angiotensinesysteem en het circulerende (endocriene) renine-angiotensinesysteem onafhankelijk van elkaar opereren, maar het is ook denkbaar dat er een interactie tussen deze systemen bestaat en dat circulerend prorenine via opname en locale activatie door bepaalde cellen, bijvoorbeeld vaatendotheelcellen, bij deze interactie betrokken is[8–10].

3. De hoge concentraties van prorenine in de ovariële follikelvloeistof en in amnionvocht wijzen erop dat renine, via de vorming van angiotensine, niet alleen betrokken is bij de regulatie van de bloeddruk maar ook andere tot heden onbekende functies heeft. In aansluiting aan de ovulatie vindt luteinisatie van de follikel plaats. Dit gaat gepaard met een zeer snelle en uitgebreide vaatnieuwvorming in het zich ontwikkelende corpus luteum. Implantatie van ovariele follikelvloeistof afkomstig van vrouwen en implantatie van angiotensine II in de cornea van konijnen leidt tot vaatnieuwvorming vanuit de limbus corneae[21,22].

Het is dus denkbaar dat locale activatie van prorenine en vorming van angiotensine II betrokken zijn bij de vaatnieuwvorming in het zich ontwikkelende corpus luteum. Een dergelijke functie zou ook het door chorioncellen geproduceerde en in het amnionvocht gesecerneerde prorenine kunnen hebben.

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7. SUMMARY

7.1. AIM OF THE THESIS

Renin hydrolyses the Leu-10-Val-11 bond of human angiotensinogen. Because this reaction is the rate-limiting step in a series of reactions leading to the formation of angiotensin II, a highly potent vasoconstrictor and aldosterone-releasing hormone, renin is considered to have a key role in blood pressure regulation and sodium and water homeostasis (Fig.1). The importance of the renin-angiotensin system is illustrated by the blood pressure lowering effect of antagonists of angiotensin II. Inhibitors of the enzyme that causes conversion of angiotensin I into angiotensin II (angiotensin-converting enzyme) are now widely used in the treatment of patients with hypertension.

On theoretical grounds it seems logical to use the plasma level of angiotensin II as a measure of the activity of the renin- angiotensin system *in vivo*. However, the plasma level of angiotensin II is low, a few picograms per ml, and the antibodies that are used in the radioimmunoassay crossreact with degradation products of angiotensin II. Angiotensin II measurements are therefore not widely used. In clinical practice plasma renin activity (PRA) is generally accepted as a measure of the formation of angiotensin II *in vivo*. In the PRA assay, plasma of the patient is incubated at 37 C. During this incubation *in vitro*, renin is catalysing the generation of angiotensin I from renin substrate. Plasma has angiotensinase activity, by which angiotensin I is hydrolysed. This degradation of angiotensin I can be prevented by the addition of angiotensinase inhibitors. These substances, however, also inhibit 'angiotensin-converting enzyme'. The reaction stops therefore at the generation of angiotensin I. Angiotensin I is measured by radioimmunoassay. Under normal circumstances the plasma concentration of renin substrate (angiotensinogen) equals the Km value, which means that the generation of angiotensin I not only depends on the concentration of renin but also on the concentration of renin substrate (angiotensinogen). The interpretation of PRA measurements is difficult in circumstances where the concentration of substrate is not normal. Techniques were therefore developed, in which the reaction velocity *in vitro* is independent of the substrate concentration. In these assays renin is separated from its substrate or the substrate is selectively destroyed. Subsequently, an excess of more or less purified renin substrate is added. The generation of angiotensin I then only depends on the concentration of renin.

The quantities of angiotensin I generated in this type of assay of the plasma

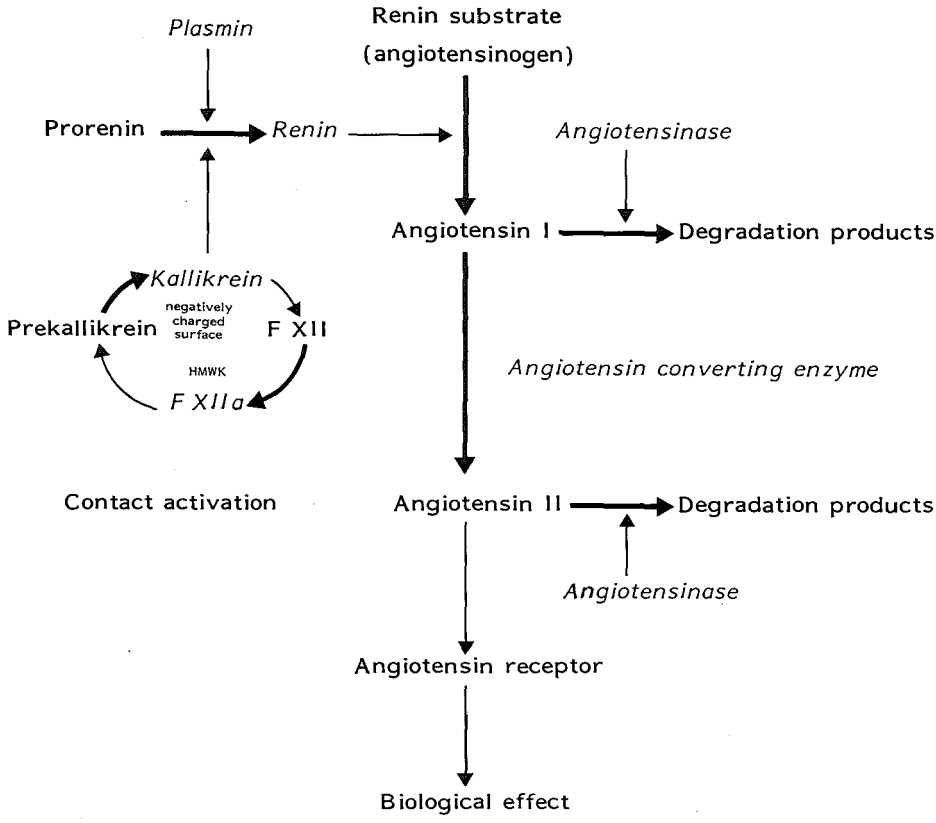

Fig.7.1. Components of the renin-angiotensin system. FXII = coagulation factor XII. FXIIa = activated factor XII. HMWK = High molecular weight kininogen.

renin concentration (PRC) are greater than with the PRA method. This difference is not only caused by the addition of an excess of exogenous substrate. In most of the PRC methods used so far, the plasma is acidified (pH 3-4) in order to destroy the endogenous substrate. After the pH has been restored to neutral, saturating amounts of exogenous substrate are added. Lumbers and Morris[1] demonstrated that the renin activity of human amniotic fluid was much higher after acidification then before acidification. In other words during incubation at 37 C at neutral pH more angiotensin I was generated per unit of time after acidification than before acidification, despite the fact that the concentration of substrate in the incubation mixture was the same. Similar results were obtained with human plasma. The increase in renin activity with acidification (acid-activation) appears to depend on the activation of an enzymatically inactive form of renin. It has been suggested that this so-called inactive renin is a proenzyme (prorenin) and that the activation is caused by an enzyme active at low pH

and causing limited proteolysis of prorenin. Another theory is that inactive renin consists of an enzyme-inhibitor complex that dissociated at low pH[2]. However, activation of inactive renin also occurs at neutral pH, when plasma is stored at 0 – 4 C (cryoactivation).

These observations have important consequences for both the technical procedure and interpretation of plasma renin determinations. We were, however,more intrigued by the observation that about 90 percent of the renin in plasma of healthy volunteers is in the inactive form. In this respect, the renin-angiotensin system is comparable with other proteolytic systems in plasma, such as the coagulation and fibrinolytic systems. Until recently, the renin- angiotensin system was considered to be a circulating hormonal system. In fact, the interpretation of PRA measurements, in which the *in vitro* generation of angiotensin I is thought to be a measure for the *in vivo* generation of angiotensin I (and II), is based on this view. In this thesis we addressed the possibility that the renin-angiotensin system not only acts by the proteolytic activity of circulating plasma renin, through cleavage of angiotensin I from circulating renin substrate, but also that local activation of prorenin may occur and that this activation is caused by enzymes that are also involved in the coagulation and fibrinolytic systems.

This thesis consists of two parts, firstly biochemical *in vitro* experiments and secondly physiological investigations in animals and measurements of prorenin in plasma of healthy volunteers and patients with different diseases and in human amniotic fluid and ovarian follicular fluid

7.2. ACTIVATION OF PRORENIN IN VITRO

7.2.1. Acid-activation and cryoactivation

In the method of Skinner for measuring PRC[3], the plasma is dialyzed for 24 h at 0-4 C against a pH 3.3-buffer and then for another 24 h at 0-4 C against a pH 7.4-buffer, before the plasma is incubated with an excess of exogenous renin substrate. Our kinetic studies revealed that the renin activity immediately after restoration of pH to 7.4, following the first dialysis step was higher than before acidification. The greatest increase in renin activity, however, occurred during the second dialysis step at pH 7.4. This activation at pH 7.4 was blocked by serine protease inhibitors, such as aprotinin, soybean trypsin inhibitor and benzamidine. Experiments with plasma from patients with clotting factor XII (Hageman factor) deficiency or with plasma from patients with prekallikrein (Fletcher factor) deficiency demonstrated that both factors are essential for the activation of prorenin in acidified plasma, after restoration of pH to neutral. Activated factor XII (factor XIIa) and kallikrein are serine proteases, which are generated from factor XII and prekallikrein in acidified plasma after restoration of pH to neutral.

Further investigations with purified β-factor XIIa (light chain with Mr 28,000

and containing the active centre) and plasma kallikrein (the whole molecule Mr 85,000 as well as the light chain Mr 28,000) showed that kallikrein itself or enzymes activated by kallikrein are responsible for the activation of prorenin in acid-pretreated plasma. Factor XII is necessary for the conversion of prekallikrein into kallikrein. High molecular weight kininogen (Fitzgerald factor,HMWK, Mr 110,000), which is required for the rapid activation of factor XII and prekallikrein in the presence of a negatively charged surface (contact activation), is not essential for the increase in renin activity in acid-pretreated plasma; renin activity increased normally in a patient with a hereditary deficiency of HMWK.

Plasma kallikrein is under certain circumstances capable of converting plasminogen into plasmin. Plasmin is also capable of activating prorenin in acid-pretreated plasma. However, the activation of prorenin in acid-pretreated plasma was normal, also after plasminogen had been selectively removed.

Factor XII and plasma kallikrein are activated when plasma is stored at 0-4 C. We expected that this activation was the cause of the aforementioned so-called cryoactivation of prorenin at neutral pH. This was indeed the case, because cryoactivation of prorenin did not take place in plasma of patients with hereditary factor XII or prekallikrein deficiency. Reconstitution of these plasmas with highly purified factor XII or prekallikrein restored normal prorenin activation. Again, HMWK does not appear to be essential for the cryoactivation of prorenin. Cold-promoted activation of prorenin was within the normal range in plasma deficient in either clotting factor V, VII, VIII:C, IX, X or XI, and also in plasma from which prothrombin had been removed by immunoadsorption. Thus, these factors, in contrast to factor XII and prekallikrein, are not essential for the cryoactivation of prorenin.

Cryoactivation of prorenin is a slow process. Cryoactivation was much faster after the most important inhibitors of factor XII and kallikrein , i.e. C_1-esterase inhibitor, α_2-macroglobulin and antithrombin III, had been removed from plasma by immunoadsorption. After addition of the polyanion dextran sulfate (Mr 500,000) to such inhibitor-depleted plasma, prekallikrein is known to be rapidly and completely activated . This so-called contact activation of prekallikrein depends on the presence of factor XII and HMWK, with dextran sulfate acting as a negatively charged surface. When this plasma was subsequently heated to 37 C, there was also a rapid increase in renin activity, which did not occur when the inhibitors had not been previously removed.

We conclude therefore that the increase in renin activity in plasma at neutral pH, both with and without pretreatment with acid, depends on the activation of factor XII and prekallikrein. In both cases the activation of factor XII and prekallikrein is probably facilitated by denaturation or inactivation of serine protease inhibitors. Indeed, when plasma was acidified to pH 3.3 and kept at this pH for 24 h at 0 – 4 C, C_1-esterase inhibitor and antithrombin III were completely inactivated. The activity of α_2-macroglobulin was reduced but not to zero. In addition, the activity of these serine protease inhibitors at 0-4 C is known to be much less than at 37 C.

Our studies of the activation of prorenin *in vitro* were extended with experiments with purified prorenin isolated from human amniotic fluid and plasma. For purification we used ion exchange column chromatography, affinity chromatography on a Blue Sepharose column and a Sepharose column to which a monoclonal renin antibody, reacting with both active renin and prorenin, had been coupled. Traces of active renin were removed by affinity chromatography on a column of pepstatin coupled to Sepharose. Pepstatin is an inhibitor of aspartyl proteases including enzymatically active renin. Prorenin is not bound to pepstatin.

Purified prorenin could be activated by highly purified plasma kallikrein (the intact molecule as well as the light chain), plasmin or trypsin. The activation occurred at 37 C and neutral pH, and pretreatment with acid was not necessary. Purified factor XIIa (α-factor XIIa, the intact molecule), β-factor XIIa (active light chain, Mr 28,000), thrombin and urokinase at concentrations up to 0.1 μM did not activate prorenin.

7.2.2. Activation with and without proteolysis

During the course of our work some investigators showed that inactive renin could be activated by acidification at pH 3.3, also when the aforementioned serine proteases were absent[2,4]. We could confirm these observations and raised the question whether a link might exist between the so-called acid-activation in plasma and amniotic fluid on the one hand and the activation by serine protease on the other. One of the possibilities was that prorenin is not an inactive proenzyme but a complex of active renin and an inhibitor, that this complex dissociates in an acidic environment, and that, after restoration of pH, the inhibitor is inactivated by a serine protease. In agreement with this hypothesis is the finding that acid-activation of prorenin in purified preparations is a reversible process; in a neutral environment the renin activity returns to its original level before acidification. This inactivation is also a reversible process, because after reacidification there is again an increase in renin activity. Also in plasma the acid-activation is reversible, provided the activation of factor XII and prekallikrein can be prevented by the addition of serine protease inhibitors such as aprotinin, soybean trypsin inhibitor or benzamidine.

Enzyme-kinetic experiments with purified renin isolated from human plasma, amniotic fluid or kidney and with purified prorenin isolated from human plasma or amniotic fluid demonstrated that trypsin-activated prorenin from amniotic fluid and plasma had the same enzyme-kinetic properties (Km and Vmax) as naturally occurring active renin from amniotic fluid, plasma and kidney. There was also no difference in the pH optimum. Other investigators found that monoclonal antibodies directed against the pro-segment of renal prorenin, the biosynthetic precursor of active renin, reacted with plasma prorenin but not with naturally occurring active plasma renin[5,6]. Therefore it is very likely that the proteolytic activation of prorenin depends on the cleavage of the pro-segment

from the parent molecule. It is therefore correct to designate inactive renin as prorenin.

In order to gain more insight into the role of the proteases in the acid-activation of prorenin, we studied in more detail the kinetics of acid-activation of prorenin in human amniotic fluid and plasma and in preparations of purified prorenin isolated from amniotic fluid and plasma. Conversion of prorenin (pR) into active renin (R) appeared to be two-step process involving the generation of an intermediary form of activated prorenin (pRa). The pR → pRa step is a unimolecular process and involves an acid-induced reversible change in the conformation of the molecule and is not caused by the dissociation of a renin-inhibitor complex. The pRa → R step is irreversible and proteolytic.

pRa → R conversion occurred in amniotic fluid at low pH by the action of an endogenous aspartyl protease. In plasma pRa → R conversion occurs after restoration of pH to neutral and is caused by the serine protease, plasma kallikrein. PRa → R conversion did not occur in purified preparations of prorenin. Thus, in contrast to pepsinogen, the acid-induced reversible conformational change is not followed by autocatalysis.

pRa of amniotic fluid and plasma could be separated from R by affinity chromatography on Blue Sepharose, and R but not pRa was detected by an immunoassay using monoclonal antibodies reacting with R and not with pR. The first-order rate constant for pR → pRa conversion depends on the protonation of a polar group (or groups) with a pK ~ 3.4, the rate constant being proportional to the fraction of pR molecules that have this group protonated. This is analogous to the reversible acid-induced conformational change of pepsinogen that occurs before its proteolytic conversion into pepsin. Interaction of positively charged amino acid residues in the N-terminal pro-segment (44 amino acids) of hog pepsinogen and negatively charged carboxyl groups of the enzyme-segment keep the pepsinogen molecule in its inactive form. With acidification the electrostatic bonds are broken resulting in a reversible change in the conformation of the molecule, by which the catalytic apparatus becomes exposed. This is then followed by the irreversible proteolytic activation of pepsinogen.

Kcat/Km for pRa → R conversion by plasmin and plasma kallikrein at pH 7.4 and 37 C was 7.8 and 5.2×10^6 M^{-1} min^{-1} respectively, which was about 50 to 70 times greater than for pR → R conversion. The susceptibility of pRa to proteolytic attack is high enough for the intrinsic factor XII-kallikrein pathway to cause rapid pRa → R conversion at 37 C, even in whole blood with its abundance of serine protease inhibitors.

On the basis of these findings we can now describe more clearly the process of activation of prorenin *in vitro*. Cryoactivation of prorenin in plasma depends on limited proteolysis of prorenin leading to the formation of active renin (pR → R). This activation occurs by plasma kallikrein, which in turn is generated from prekallikrein by activated factor XII. These processes do not take place at body temperature, because of the inhibitory action of inhibitors of factor XII and kallikrein in plasma. During acid-activation the prorenin molecule

undergoes a non-proteolytic, reversible, conformational change by which the catalytic apparatus of the molecule is exposed. At low pH however, prorenin, in contrast to pepsinogen, does not possess catalytic activity, because the aspartyl residues (Asp-38 and Asp-226) in the active centre have higher pK-values than the corresponding aspartyl residues (Asp-32 and Asp-215) in pepsin. For the catalytic activity of renin, Asp-38 has to be in the ionized form and Asp-226 in the non-ionized form. Therefore, pRa, which is formed at low pH, has to be brought back to neutral pH before it can hydrolyse renin substrate. Factor XII and prekallikrein are also activated after restoration of pH to neutral, probably because the inhibitors of activated factor XII and prekallikrein are denaturated at low pH. Because pRa is very sensitive for proteolytic conversion into R by kallikrein, the reverse reaction pRa → pR does not take place in normal plasma. Whereas in plasma pRa → R conversion takes place after restoration of pH to neutral, in acidified amniotic fluid the conversion takes place at low pH before pH is restored and this is due to the action of an endogenous aspartyl protease.

The activation of pepsinogen *in vivo* in the stomach occurs at pH 2-3. It is possible that the formation of the intermediary active form of prorenin (pRa) *in vivo* occurs at low pH in intracellular compartments with low pH such as secretory granules and endo- or exocytotic vesicles[7]. The membrane of the endocytotic vesicles (receptosomes), which are involved in the process of receptor-dependent endocytosis, contains an active proton pump, which causes a fall in pH to 5 or perhaps lower. The pH is likely to be even lower at sites near the proton pump. In such an environment pRa can be formed. This intermediary form of activated prorenin is very sensitive to limited proteolysis so that local formation of R may occur. It is possible that part of the locally generated renin has originated from circulating prorenin[8-10].

7.3. REGULATION OF PRORENIN *IN VIVO*

7.3.1. Direct and indirect radioimmunoassays of prorenin

In most of the studies reported in this thesis the renin concentration of plasma and other body fluids was determined with an enzyme-kinetic method. In this assay the velocity of angiotensin I generation *in vitro* under standardized circumstances, is measured by radioimmunoassay of angiotensin I (indirect RIA). For the measurement of prorenin, the proenzyme has to be converted into enzymatically active renin. The difference in renin activity before and after conversion is taken as a measure of prorenin concentration. For this to be valid the conversion has to be complete. It is also necessary that the procedures that are used for the conversion do not cause denaturation of prorenin, activated prorenin and naturally occurring active renin. Finally, it is necessary that the *in vitro* activated prorenin has the same enzyme- kinetic properties (Km and Vmax) as naturally occurring active renin.

The conversion of prorenin into enzymatically active renin *in vitro* can occur in several ways; 1) by storage of plasma or other body fluids at low temperature (cryoactivation), 2) by acid-treatment (acid-activation), and 3) by the addition of proteolytic enzymes.

As discussed in the first part of this chapter, cryoactivation and acid-activation in plasma depend on the presence of factor XII and prekallikrein. When these factors are absent or when their concentration is low, the activation is not complete. Cryoactivation proceeds slowly, also in normal plasma. Therefore, cryoactivation is not suitable for practical purposes. After one week only 15% of the prorenin present in normal plasma is activated at 0 C. Acid-activation appears to be more complete but careful adjustment of pH is of crucial importance. Below pH 3.1 prorenin and active renin are denaturated also at 0 C, and above pH 4.0 there is almost no activation at 0 C. Trypsin is often used for the activation of prorenin. High concentrations of trypsin are required to overcome the activity of the plasma inhibitors but such high concentrations may destroy renin or prorenin. We therefore initially used less aggressive serine proteases such as plasmin and urokinase. The problem with the use of these proteases is that activation of prorenin only occurred after α_2-antiplasmin had been removed. We, therefore, have developed an assay of prorenin, in which the conversion of prorenin into renin occurred under apparently optimal condition, without any loss of prorenin, activated prorenin or naturally occurring active renin. Some characteristics of the assay were 1) prorenin was activated with Sepharose-bound trypsin at 0-4 C, 2) the concentration of the activator was not critical provided the incubation was prolonged until renin activity had reached a plateau, 3) this plateau was stable and had the same height as after maximal activation with acid, plasmin or urokinase, and 4) the activator can easily be removed by centrifugation.

In the last two years monoclonal renin antibodies have become available with high affinity for active renin as well as prorenin. There are now also antibodies available that only recognize the active species. With these antibodies it is possible to measure total immunoreactive renin as well as enzymatically active renin (direct RIA). By comparing the results of the *direct* and *indirect* assays, in plasma as well in preparations of purified prorenin and renin, we could confirm that the *indirect* RIA, in which prorenin is converted into active renin by immobilized trypsin at 0 C, is a good measure of the prorenin concentration in plasma.

During storage and handling of the plasma samples inadvertent cryoactivation of prorenin can occur. This leads to falsely high levels of active renin and low levels of prorenin in the *indirect* RIA. This was especially the case with plasma of women who were on oral contraceptives or with plasma of pregnant women. This might be related to the elevated levels of factor XII and prekallikrein in these women, whereas C_1-esterase inhibitor is decreased in pregnant women. Moreover, pregnant women have elevated plasma prorenin levels, so that more prorenin is available for cryoactivation. The activation of prorenin during handling and storage of the plasma samples could be prevented by taking special

precautions and by adding a serine protease inhibitor, for instance soybean trypsin inhibitor, to the plasma samples.

7.3.2. Concentration of prorenin in plasma and other body fluids under physiological and pathological circumstances

The levels of prorenin and enzymatically active renin in plasma of normal volunteers and patients are summarized in Table 1 and Fig.2. Active renin in

Table 7.1. Plasma levels of active renin and prorenin in controls and in patients with different diseases.

	n	Active renin μU/ml	Prorenin μU/ml	Active renin % of total
Controls	112	17.7 (4.8–65.3)	171 (77.2–377)	9.2 (3.2–26.7)
Anephric patients	37	1.0 (0.2–6.1)	61.0 (13.4–277)	1.6 (0.4–5.9)
Conn's syndrome	14	0.58 (0.11–3.0)	37.3 (17.9–77.4)	1.5 (0.4–6.5)
Normal women using oral contraceptives	47	8.8 (2.3–33.2)	91.8 (32.7–258)	8.6 (3.1–24.4)
Essential hypertension	68	13.8 (3.0–63.3)	115 (40.4–330)	10.0 (2.8–36.7)
Renovascular hypertension	68	52.7 (10.0–277)	165 (50.2–542)	23.1 (8.1–66.1)
Essential hypertension during captopril therapy 50 mg tid	45	65.5 (3.4–1260)	241 (42.9–1350)	19.7 (3.0–68.1)
Renovascular hypertension during captopril therapy 50 mg tid	46	463 (63.6–3360)	593 (200–1750)	41.1 (20.6–81.9)
Bartter's syndrome	9	923 (352–2430)	690 (635–4480)	54.7 (32.7–91.5)
Prekallikrein deficiency	8	11.4 (4.5–29.0)	339 (171–669)	3.3 (1.1–9.4)
Pregnant women first trimester	9	17.5 (10.4–28.9)	1120 (898–1410)	1.6 (0.8–2.0)
Pregnant women third trimester	50	31.7 (10.0–100)	634 (263–1530)	4.7 (1.2–18.6)
Prorenin producing ovary tumor	1	1010	120000	0.8

The results mean and 95% confidence intervals (in parentheses) demonstrate that an increase in the plasma level of active renin is associated with an increase in the plasma level of prorenin. The percentual increase of active renin however is much greater than that of prorenin. Patients with prekallikrein deficiency (Fletcher trait) and pregnant women are an exception.

plasma of nephrectomized subjects is very low. Therefore, circulating active renin mainly originates from the kidney. Prorenin in most patients without kidneys is lower than in controls but some anephrics have plasma levels of prorenin within the normal range. The mean prorenin concentration in anephrics is about one third of that in controls. Therefore, about one third of the plasma prorenin in the normal subjects appears to be of extrarenal origin. Although our

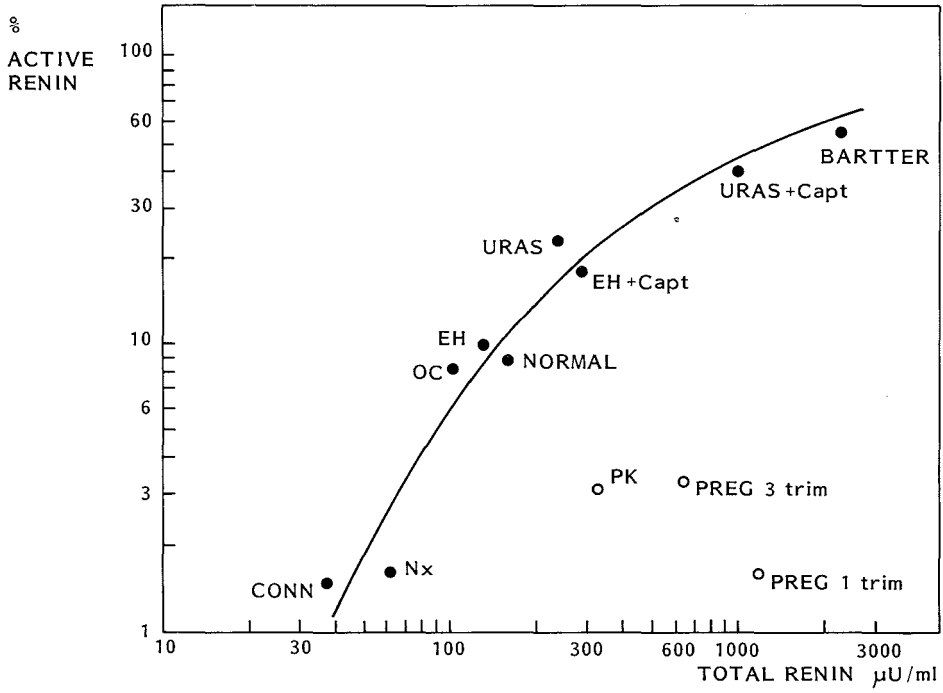

Fig 7.2. The relation between the percentage of enzymatically active renin and the total renin (prorenin plus active renin) concentration in plasma. See also Table 7.1. Capt = Captopril 50 mg tid. EH = patients with essential hypertension. Nx = Nephrectomized patients. OC = Women using oral contraceptives. PK = Patients with hereditary prekallikrein deficiency. Preg 1 trim = women first trimester of pregnancy. Preg 3 trim = women third trimester of pregnancy. URAS = Patients with unilateral renal artery stenosis.

observations in anephrics clearly demonstrate that part of the plasma prorenin is of renal origin, renal production is difficult to prove directly by measurements of prorenin in renal vein and renal artery, because of the high renal plasma flow and the relatively long plasma half-live of prorenin (2-3h)[11]. In patients with unilateral renal artery stenosis, in whom only the affected kidney produces prorenin and the blood flow through this kidney is reduced, we could indeed show that the prorenin concentration in the renal vein was significantly higher than in the renal artery.

Whereas the concentration of prorenin is reduced in anephric subjects, it is

increased in patients with increased renal secretion of enzymatically active renin. Moreover, the higher the concentration of total renin (i.e. prorenin plus active renin), the higher the the percentage of active renin (Fig.2.). The percentual contribution of the kidney to the total production of circulating prorenin is probably also higher under these circumstances.

There are, however, at least two exceptions to this rule. i.e. patients with hereditary plasma prekallikrein deficiency (Fletcher trait) and pregnant women. In patients with Fletcher factor deficiency the plasma concentration of prorenin is increased, whereas active renin is normal or low. The reason for this finding could be a block in the prorenin-renin conversion. This would mean that our *in vitro* finding that plasma kallikrein is capable to convert prorenin into renin is also of physiological significance.

In pregnant women the plasma concentrations of both prorenin and active renin are increased, but the increase in plasma prorenin is much greater. This is already the case in the first trimester of pregnancy. It is possible that in this case the low percentage of active renin is related to the increased extrarenal production of prorenin. One of the possible sources is the placenta. In human amniotic fluid (third trimester) the prorenin concentration is about 6 to 10 times higher than in plasma. In normal pregnant women prorenin did not fall until the second day after parturation. In contrast, the half-live of plasma prorenin after bilateral nephrectomy in patients with renal failure was about two hours. These results suggest that the placenta is not a major source of maternal plasma prorenin.

Another source of prorenin could be the ovary. Plasma prorenin in the luteal phase of the menstrual cycle is about two times higher than in the follicular phase[12]. An increase in plasma prorenin, at the time of the ovulation or shortly thereafter, comparable to the increase in pregnancy, has been reported in women who were on an in *in vivo* fertilization program and received gonadotropin injections[13]. We found that the ovarian follicular fluid of these women, at the time of the collection of the oocyte, contained prorenin in very high concentrations, about 30-40 times higher than the concentration in plasma. Moreover, there was a positive correlation between the prorenin concentration in follicular fluid and plasma (unpublished results). Active renin levels were not correlated. In a woman without functioning ovaries, who became pregnant after transfer of a donated oocyte fertilized with the sperm of her husband, pre-pregnancy plasma levels of active renin and prorenin were normal. Active renin rose two-fold during pregnancy both in the patient and in normal pregnant women. However, in the first eight weeks prorenin rose 156 μU/ml in the patient, whereas it rose by 869$\pm$169 μU/ml (mean$\pm$SD) in normal pregnant women. In the patient prorenin also remained abnormally low throughout the pregnancy. Because of these findings and in the light of recent evidence that the ovary is capable of producing high levels of prorenin in plasma of women with hyperstimulated cycles, it seems likely that the ovary is the main source of the elevated prorenin in pregnancy and that the production and secretion of prorenin by the ovary is under gonadotropic control.

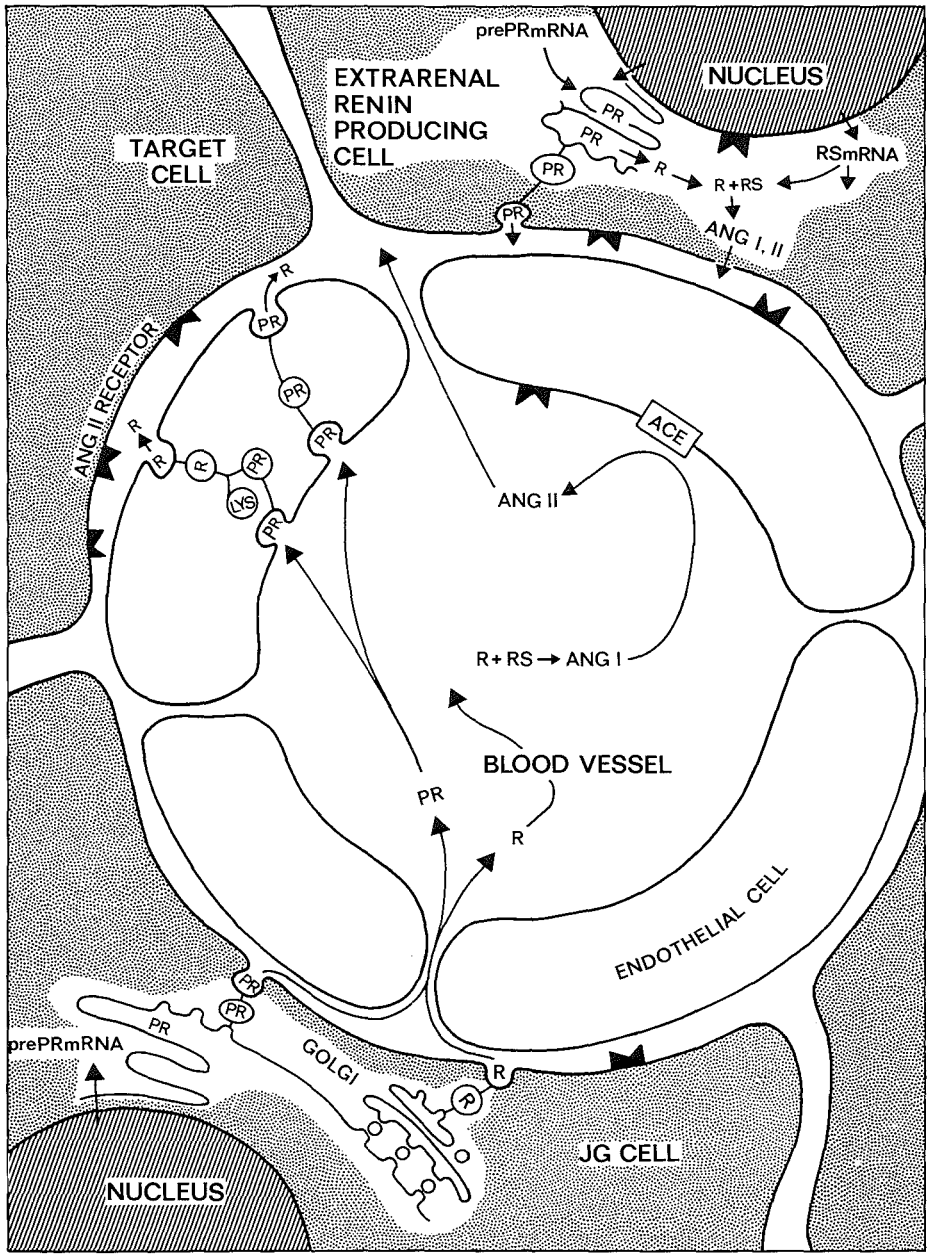

Fig. 7.3. Hypothetical interactions between the renal and extarenal renin-angiotensin system. ACE = Angiotensin-converting enzyme, ANG I = angiotensin I, ANG II = angiotensin II, JGcell = juxtaglomerular cell, mRNA = messenger RNA, Lys = Lysosome, PR = prorenin, R = enzymatically active renin, RS = renin substrate.

7.4. CONCLUSIONS

What is the physiological significance of prorenin? How is the activation of prorenin regulated within the juxtaglomerular cells ? Does activation of prorenin occur after it has been released by the kidney into the circulating blood? What is the function of extrarenally produced prorenin?

Our investigations don't give a straightforward answer to these questions, but some conclusions can be drawn.

1. Our results indicate that there is mechanism regulating the ratio between the amounts of active renin and prorenin secreted by the juxtaglomerular cells into the circulating blood. This ratio increases when the secretion of renin is stimulated. With acute stimulation it is the secretion of active renin that is selectively increased. During chronic stimulation the secretion of active renin as well as the secretion of prorenin are increased, but the increase in active renin is much greater. The nature of this post-translational mechanism, which is regulating the processes of storage and secretion in the juxtaglomerular cells, is unknown.

2. Our results further show that the factor XII-kallikrein-contact activation system as well as plasmin are capable of activating prorenin. This activation *in vitro* is only possible under circumstances that differ from those in circulating blood. In previous experiments in patients with essential hypertension we found that, immediately after acute stimulation of the renal release of renin, the rise of active renin in plasma was associated with a fall of prorenin, suggesting that prorenin-renin conversion also occurred *in vivo*.[14,15]. Such observations were also made by other investigators[16–18]. In recent experiments ,however, using the direct renin RIA, these observations could not be confirmed. Incomplete activation in the *indirect* RIA leading to falsely low values of prorenin is probably the main cause of this discrepancy. Activation of prorenin in circulating blood seems very unlikely.

The activation of prorenin by plasma kallikrein or plasmin is a slow process. Prorenin pretreated with acid is very susceptible to activation by these and other proteases. As described above, it is possible that vascular endothelial cells are capable of taking up prorenin via a process of receptor-mediated endocytosis, after which it is exposed to the low pH of the receptosomes and is converted into the intermediary form of activated prorenin (Fig.3). The activated prorenin may be transported by the vesicles to the luminal or contraluminal side of the endothelial cell. After exocytosis it may enter a milieu with neutral pH, where it refolds to the original inactive form or is rapidly converted into active renin by one or more proteases. It is also possible that the proteolytic conversion takes place within the cell, for example by fusion of endocytotic with (pre-)lysosomes, which contain pepsin-like enzymes, such as cathepsin D. Cathepsin D is capable of activating prorenin *in vitro* at low pH. It is known that the secretory granules of the juxtaglomerular cells contain not only prorenin and renin but also cathepsin B, cathepsin D and other lysosomal enzymes[19].

This hypothesis implies that, besides the formation of active renin by the juxtaglomerular cells, local formation of active renin may occur in other parts of the body, outside the kidney, and that such local formation of renin may occur by the activation of prorenin that originates from the kidney and is taken up from the plasma.

On the other hand, it has now become known that, besides the juxtaglomerular cells, other cells in the body are capable of synthesizing prorenin and renin. Chorionic cells secrete prorenin. Neuroblastoma × glioma cell lines, brain, pituitary gland, adrenals thyroid gland and testis (Leydig cells) contain renin. Moreover, recent studies suggest that some of these cells are capable of synthesizing prorenin, renin and renin substrate, and intracellular production of angiotensin II has been demonstrated[8–10,20].

It is possible that the local (paracrine or autocrine) and intracellular (intracrine) renin-angiotensin systems on the one hand and the circulating (endocrine) renin-angiotensin system on the other are operating independently, but it is also possible that an interaction exists between these systems and that circulating prorenin is involved, via uptake and local activation, for instance by endothelial cells (Fig.3.)[8–10].

3. The high concentrations of prorenin in ovarian follicular fluid and amniotic fluid indicate that renin, via the formation of angiotensin II, is not only involved in blood pressure regulation but also in functions that are unknown as yet. In the ovary luteinization of the follicle takes place following ovulation. This coincides with rapid and extensive neovascularization of the developing corpus luteum. Implantation of human ovarian follicular fluid and implantation of angiotensin II in the cornea of the rabbit leads to neovascularization originating from the limbus cornea[21,22]. It is possible that local generation of prorenin and formation of angiotensin II are involved in the neovascularization in the developing corpus luteum. Prorenin produced by the chorionic cells and secreted into the amniotic fluid could have a similar function.

It is not too far fetched to hope that in the near future further studies on the physiological functions of the extrarenal renin-angiotensin systems will produce some surprising results.

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VERANTWOORDING

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Tenslotte gaat mijn dank uit naar de patienten, in het bijzonder de patiente die in het laatste hoofdstuk is beschreven, studenten en niet genoemde collegae die aan dit onderzoek hebben meegewerkt.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren in 1941 te Venlo. Na het toenmalige Mulo onderwijs was hij tot 1966 analist op het Farmacologisch Research Laboratorium van Organon te Oss. In 1966 werd de medische studie aangevangen aan de Rijks universiteit te Utrecht. Na het kandidaatsexamen werd de studie vervolgd aan de Erasmus universiteit te Rotterdam. Het artsexamen werd in 1972 afgelegd. Tevens werd dit jaar het examen van de Educational Commission for Foreign Medical Graduates (ECFMG) afgelegd. Van 1969 tot 1972 was hij student-assistent op de afdeling Farmacologie (hoofd Prof.Dr. I.L.Bonta) van de Erasmus universiteit. De specialisatie in de Interne Geneeskunde, onder leiding van Prof.dr. J. Gerbrandy, op de Afdeling Inwendige Geneeskunde I in het Academisch Ziekenhuis Rotterdam Dijkzigt te Rotterdam vond plaats in de periode 1972 -1977, waarna inschrijving als internist in het specialistenregister volgde.

In de periode 1977-1981 was hij stafmedewerker op de Hypertensie Unit van de afdeling Inwendige Geneeskunde I (hoofd Prof.Dr. M.A.D.H. Schalekamp). Gedurende deze periode werden enige stages klinische farmacologie gevolgd op de afdeling Clinical Pharmacology (hoofd Prof.Dr.C Dollery) van het Hammersmith Hospital te Londen, Van 1981 tot 1982 was hij research fellow op de afdeling Materia Medica (hoofd Prof.dr. J.L. Reid) van de University of Glasgow. Op deze afdeling werd de opleiding tot klinisch farmacoloog gevolgd. Sinds zijn terugkomst is hij als zodanig werkzaam in het Academisch Ziekenhuis Rotterdam Dijkzigt aanvankelijk op de afdeling Inwendige Geneeskunde I. In 1984 werd hij als klinisch farmacoloog aangesteld en sindsdien is hij verbonden aan de afdeling farmacie (hoofd Dr. J.W. Meilink).

List of abbreviations and definitions

α_2AP	α_2-Antiplasmin
α_1AT	α_1-Antitrypsin
ANGI	Angiotensin I
ANGII	Angiotensin II
ATIII	Anti trombin III
BSA	Bovine serum albumine
C_1-INH	C_1-Esterase inhibitor
CNBr	Cyanogen bromide
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid disodium salt
FXII	Factor XII (zymogen, Mr. 80,000)
α-Factor XIIa	the 80,000 Mr. form of activated factor FXII
β-Factor XIIa	the 28,000 Mr. form of activated factor FXII
HMWK	High molecular weight kininogen
HSA	Human serum albumin
α_2M	α_2-Macroglobulin
Mr	Molecular weight
NaN_3	Sodium azide
8OHQ	8-Hydroxyquinoline sulfate
PMSF	Phenylmethylsulfonylfluoride
pNA	Paranitroaniline
PPAN	H-D-Propyl-L-phenylanalyl-L-argine-p-nitroanilide
pR	prorenin
pRa	Intermediary form of activated prorenin
R	Naturally occurring active renin or protease-activated prorenin
RS	Renin substrate (angiotensinogen)
SBTI	Soybean trypsin inhibitor
SDS	Sodium dodecyl sulfate
Tris	Tris (hydroxymethyl)-aminomethane
v/v; w/v	Volume per volume; weight per volume