Rat angiotensin - converting enzyme : Tissue specific expression during pharmacological inhibition.

by

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Dedication

To Lindy and my parents

for their encouragement and support

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Abstract

The renin - angiotensin system plays a central role in the maintenance of blood pressure. Angiotensin II, the main effector of this system, results from the action of angiotensin - converting enzyme (ACE) on angiotensin I. Angiotensin II, maintains vasomotor tone via its vasoconstrictor action, and also increases salt and water retention by stimulating the release of aldosterone. ACE inhibitors, such as captopril, enalapril and lisinopril, are highly effective in the treatment of hypertension and congestive cardiac failure.

Previous studies have suggested that angiotensin converting enzyme (ACE) production may be enhanced during pharmacological inhibition of the enzyme. Little is known, however about the mechanism of this induction. After demonstrating increases in circulating ACE protein in cardiac failure patients receiving the ACE inhibitor captopril, a rat model was used to study this effect. A sensitive enzyme linked immunosorbent assay for rat ACE was developed and a partial cDNA for rat ACE cloned to enable examination of ACE mRNA and protein expression during enzyme inhibition with enalapril. Rat lung ACE mRNA increased by 156% ($p<0.05$) and ACE protein doubled within 3 hours of administering a single dose of enalapril. Testicular ACE mRNA also increased by 300% ($p<0.05$) within 2 hours and returned to pretreatment levels by 6 hours. The angiotensin II antagonist saralasin similarly caused a significant ($p<0.0001$) 800% enhancement of mRNA expression. Aldosterone pretreatment of rats prior to enalapril administration was found to abolish this mRNA induction. These findings indicate that increased ACE expression during inhibition results from reduced levels of angiotensin II with consequent reduced stimulation of the angiotensin II receptor and its effects, such as aldosterone release. This suggests that ACE levels are regulated by a negative feedback loop involving the distal components of the renin-angiotensin system, namely angiotensin II and aldosterone. *In situ* hybridisation and immunohistochemical techniques were employed to localise the site of this inductive response in rat tissue sections. It was found that lung macrophages were markedly induced to produce ACE, as was ACE in seminiferous tubules. ACE induction was also noted in the expected sites of renal tubular epithelium and glomerular tissue. Interestingly, ACE expression was also enhanced in cardiac valves.

In these studies it has been conclusively demonstrated that new ACE expression is induced by enzyme inhibitor therapy. A variety of techniques have been developed that will allow futher study of ACE in rat tissues.

Chapter 1

LITERATURE REVIEW

1.1. HISTORICAL PERSPECTIVE

The foundation for the discovery of the renin-angiotensin system was the work of Goldblatt and his colleagues in the early 1930's demonstrating that experimental hypertension had a renal basis (Goldblatt, 1947). In attempting to isolate a circulating hypertension factor, attention focused on renin which, as early as 1898 (Tigerstedt and Bergman, 1898), had been shown to be able to cause marked elevations in blood pressure. Confusion, however, arose from the finding of Friedman and his co-workers (Friedman et al., 1938), that renin itself did not cause vasoconstriction in the isolated dog tail.

Clarity was obtained from the work of two groups who independently found that renin acted on a substance in plasma producing a potent vasopressor which the one group called angiotonin (Munoz et al., 1939) and the other termed hypertensin (Braun-Menedez, 1940). Only in 1958 was the hybrid term angiotensin agreed upon (Braun-Menedez and Page, 1958).

Angiotensin-converting enzyme (ACE) was discovered in 1954 (Skeggs et al., 1954). In these early studies, Skeggs and his co-workers demonstrated the existence of two forms of angiotensin which they called hypertensin I and hypertensin II. Their studies also revealed the presence of a "hypertensin-converting enzyme" in equine plasma, capable of converting hypertensin I to hypertensin II. These and further studies by Helmer (Helmer, 1955) suggested that hypertensin II was the true vaso-constrictor and established that angiotensin-converting enzyme (ACE) produces the final effector of the renin-angiotensin system - angiotensin II (Skeggs et al., 1954a & 1954b and 1956a, 1956b & 1956c; Lentz et al., 1956).

These early studies also elucidated some of the most important properties of ACE; its chloride dependence, metalloprotein nature and specificity in the removal of intact dipeptides from the C-termini of peptide substrates.

More than a decade passed before further discoveries were made on ACE. This was largely due to the absence of a simple chemical assay for the enzyme. Ng and Vane (Ng and Vane, 1967 & 1968) found that the lungs, and not plasma, was the major site for the production of angiotensin II. They went on to propose that ACE may also inactivate the vasodilator bradykinin. This proposal was supported by the finding that bradykinin potentiating factor could inhibit ACE. Bradykinin potentiating factor was a mixture of uncharacterised peptides that Ferreira had isolated from the venom of the South American pit viper *Bothrops jararaca* which he showed could inhibit the degradation of bradykinin (Ferreira, 1965 & 1966).

These findings rekindled interest in ACE which had been rediscovered twice during the preceding decade, firstly by Elisseeva and Orekhovich who extracted an enzyme they called "carboxycathepsin" from beef kidney (Elisseeva and Orekhovich, 1963) and later by Erdös and Yang who identified a dipeptidyl carboxypeptidase in human plasma (Erdös and Yang, 1967) which they called "kininase II" to distinguish it from kininase I, a circulating carboxypeptidase which inactivates bradykinin.

The development of simple chemical assays utilising model NH_2-protected substrates (Piquilloud et al, 1970; Cushman and Cheung, 1971; Yang et al, 1970) greatly assisted in the purification of ACE from a variety of animal tissues, including human.

The isolation of pure enzyme resulted in much progress toward the understanding of the biochemistry of ACE as well as its tissue distribution and biological roles. This led to the development of potent orally active inhibitors of the enzyme which are currently widely used in the treatment of hypertension and congestive cardiac failure.

1.2. BIOCHEMISTRY

1.2.1. Physiochemical Properties

ACE appears to be similar in most mammalian species in that it is an acidic glycoprotein consisting of a single large polypeptide chain which initially was thought to contain 1 mol/mol of zinc (Soffer, 1981). Subsequent information obtained from study of cloned ACE has shown this not to be true as will be detailed later. Analysis of the enzymes purified from different tissues of various mammalian species revealed that, within a particular species, the structure of ACE appears to be identical at all anatomical loci except for the testis (Lanzillo et al.,1985) and perhaps the brain (Strittmatter et al.,1985).

In most mammalian tissues ACE has been shown to have a molecular weight of between 140000-160000. ACE has a high carbohydrate content which resulted in spuriously higher molecular weights on gel filtration, thus complicating the determination of its molecular weight (Andrews,1965). This form has been termed the "lung" or somatic form of ACE, reflecting its widespread distribution. A smaller form, of approximately 90000 MW (human) and 100000 (rabbit), is found within the testis.

ACE has a relatively high percentage of acidic amino acid residues, partially explaining its low isoelectric point. The pI lies between 4.3 and 5.2 (Soffer, 1976; Nakajima et al., 1973; Stewart et al., 1981)). Varying degrees of sialylation may explain this range of pI values. Hydrophobic residues account for 44% of the amino acid content and the polar to apolar residue ratio ranges from 1.2 (Das & Soffer, 1975) to 1.4 (Weare et al., 1982) - thus suggesting moderate hydrophobicity.

N-terminal sequence data has been obtained for bovine, rabbit, pig and human ACE (Ehlers & Riordan, 1990). The results demonstrate considerable interspecies homology at the N-terminus. In contrast, limited C-terminal sequence data has been obtained from only two species, rabbit and human. The differences between these are profound and may be due to variations in the purification techniques employed. Although ACE has been isolated from a variety of body fluids, this soluble ACE is thought to result from specific cleavage of membrane bound enzyme. Evidence for this has been obtained from the experience in purification of enzyme from tissues which typically requires solubilisation during purification (Soffer, 1981). This is either accomplished by using non-ionic detergents (Ehlers et al., 1986) or trypsin which is thought to cleave off a short C-terminal membrane embedded anchor peptide (Weare et al., 1982). Comparison of molecular weights between naturally occuring soluble ACE (in blood or seminal plasma) and solubilised ACE from human lung and kidney, either through the use of Triton X-100 or trypsin revealed no difference (Lanzillo et al., 1985). It was noticed however, that detergent solubilised ACE showed an increased tendency to aggregate, suggesting that detergent solubilisation of the enzyme results in the retention of the short hydrophobic membrane anchor sequence. In a study of porcine ACE (Hooper et al.,1987) no difference was found between detergent or trypsin solubilised porcine kidney ACE. When the enzyme was solubilised by Triton X-100 in the presence of EDTA (thought to inhibit endogenous solubilising activity), however, a hydrophobic protein was obtained. These results provided evidence for the anchoring of ACE through its C- terminus.

Study of the rabbit testicular enzyme showed that it differed in both the C- and N- terminal sequences when compared to the lung enzyme (Iwata et al., 1982). This result, taken with those of competitive radioimmunoassays suggesting immunological identity of lung and testicular ACE (Soffer et al., 1987), indicated that testicular ACE possibly consisted of a portion of the somatic enzyme.

Proof of this relationship between lung and testicular ACE was subsequently obtained from the cloning and sequencing of ACE cDNA. These studies will be detailed later in this review.

1.2.2. Carbohydrate content

Rabbit ACE has been found to contain approximately 26-30% cabohydrate (Das & Soffer, 1975 and Das et al., 1977). Up to 188 sugar residues have been found to be present per protein molecule. These consist of fucose, mannose, galactose, N-acetylglucosamine and sialic acid in the ratio 1:7:6.9:6.8:1.75 for lung ACE and for the serum enzyme in the ratio 1:6.3:8.2:8.3:7.5. It is interesting to note the higher content of sialyl residues in the serum enzyme suggesting that sialyl deficient enzyme molecules are selectively removed from the circulation by hepatic asialoglycoprotein receptors recognising sialic acid deficient, galactosyl terminating glycoproteins (Hudgin et al., 1974 & Ashwell , 1974). Human kidney ACE has been found to contain 24-28% carbohydrate by weight with ratios of fucose to mannose to N-acetylglucosamine to galactose being 1.6:3.0:4.5:4.4 with only trace amonts of sialic acid being present (Weare et al., 1982). Human pulmonary ACE contains approximately 20 sialic acid residues per molecule, supporting the endothelial origin of serum ACE. Evidence that ACE may be differentially glycosylated in different organs has been demonstrated in the pig which contains a smaller enzyme in the striatum (170000) compared to that in the kidney (180000). However, on deglycosylation, enzyme from both sources was shown to have identical molecular weights (Hooper et al., 1987). The rabbit testicular enzyme contains 20% carbohydrate and differs from the rabbit lung form in its relatively large content of galactosamine and the absence of fucose (El-Dorry et al., 1982). Recent data (Ehlers et al., 1992) has found that the unique N-terminal sequence of human testicular ACE, not shared by the somatic enzyme, is heavily O-glycosylated.

1.2.3. Metal content

ACE has been shown to be a metalloenzyme containing 1 gram atom of zinc per mole of protein. This has been confirmed in rabbit lung ACE (Das et al., 1975), equine serum ACE (Fernley, 1977) and canine pulmonary ACE (Conroy et al., 1978). With ACE, in contrast to other metallopeptidases, zinc appears to be less tightly bound, dissociating readily below pH 7 (Bunning and Riordan, 1981 and Kleemann et al., 1986). The metal content of testicular ACE has not been determined but testicular ACE appears to be less sensitive to the action of metal chelators (Velletri et al., 1985).

More accurate methods have recently been utilised to re-examine the metal content of ACE and it has been demonstrated that there are two zinc atoms per enzyme molecule allowing both active sites of the somatic enzyme to be functional (Ehlers and Riordan, 1991).

1.2.4. Active site residues

Four functional residues at or near the active site of rabbit pulmonary ACE have been determined by chemical modification (Bunning et al., 1978). These active residues are tyrosine, arginine, glutamine and lysine. In another study, (Fernley, 1977) tyrosine was also implicated as a functional residue at the active site of equine ACE. Similarly, glutamate has been shown to be an active site residue in bovine pulmonary ACE (Harris and Wilson, 1983). These residues have also been shown to be present at the active site of testicular ACE.

Recently, recombinant ACE has been expressed with mutations at the putative active site corresponding to the short consensus sequence of zinc metallopeptidases His-Glu-X-X-His (Wei et al., 1991), confirming that mutations in this region result in a loss of enzymatic activity.

1.2.5. Catalytic Properties

ACE has been found to be able to cleave a wide range of substrates *in vitro*, some as long as the ß chain of insulin to those as short as N- terminal blocked tripeptides. It was originally thought that there were certain rigid substrate requirements for ACE action including a free C-terminal carboxyl group, the absence of a C- terminal dicarboxylic acid and the absence of a penultimate proline residue (because of the inability of ACE to cleave imide bonds). It has now been found that only the last of these appears to be invariable. In fact, ACE has such a wide substrate specificity that it has been used as a tool for C terminal sequence analysis of polypeptides (Krutzsch, 1980 & Beckner and Caprioli, 1980). ACE appears to sequentially cleave dipeptides from the carboxy-teriminal ends of peptide substrates (Ehlers and Riordan, 1990).

ACE has also been shown to be able to cleave peptides having a C-terminal blocking group such as nitrobenzylamine which is released upon cleavage (Hersh et al., 1983).

Another situation in which ACE has been shown to cleave blocked peptides is its action on amidated neuropeptides such as Substance P (Yokosawa et al., 1983) and substance K (Thiele et al., 1985). While substance K degrading activity could not be shown for porcine striatal or kidney ACE, striatal ACE from rat brain does possess this unique activity and, owing to its slightly smaller size as compared to the lung enzyme (165000 vs.175000), has been described as a brain isozyme of ACE (Strittmatter et al., 1985). However, this has been disputed and the smaller size of this brain "isozyme" has been attributed instead to differential glycosylation rather than differences in polypeptide length (Hooper and Turner, 1987).

While ACE normally cleaves dipeptides from carboxy terminal ends of peptide substrates, tripeptidyl caboxypeptidase activity has been reported for ACE in the cleavage of substance P (Thiele et al., 1985), Arg^9-bradykinin (Inokuchi and Nagamatsu, 1981) and Luteinizing Hormone-releasing hormone (LH-RH) (Skidgel and Erdös, 1985). Even the enzyme specificity for peptide bonds is not absolute as esterase activity has also been shown (Keung et al., 1980).

When considering the wide range of substrates shown *in vitro* to be cleaved by ACE where both non-physiological conditions and prolonged cleavage times may be employed, it must be remembered that true *in vivo* substrate specificity under physiological conditions is regulated by a complex combination of substrate affinities, chloride dependence, hydrolytic rates and the concentration of biologically relevant substrates.

When one observes the kinetic similarity between natural peptides such as angiotensin I and bradykinin, their C-terminal peptide analogues and analogous C-terminal dipeptide competitive inhibitors, the importance of the amino acid residues on either side of the cleaved peptide bond, and their determining role in binding and substrate specificity, becomes evident (Cheung et al., 1980). Thus while the selectivity of ACE appears to depend on the C-terminal sequence of its substrate, it has also been shown that this can be significantly modified by sequence alterations distant from the active site toward the N-terminus of the substrate. For example, it was found that Des^1-Asp angiotensin I is a better ACE substrate than angiotensin I (Gaynes et al., 1978), based on its more favourable V_{max} to K_m ratio (Tsai et al., 1975 and Chiu et al., 1976). Similar findings have also been made with tetradecapeptide renin substrate and its C-terminal nonapeptide homologue (Dorer et al., 1975) and also bradykinin and its higher C-terminal homologues (Dorer et al., 1974). In both cases the shorter peptides were found to be hydrolysed at rates as much as 13 and 22 times faster, respectively.

It must be realised that substrate specificity of ACE is a complex process particularly when its anion activation is considered. Most studies on substrate specificity have depended on structure-activity studies of snake venom peptide inhibitors and of more recently developed metal coordinating ACE inhibitors rather than on substrates themselves.

Moreover, studies have utilised fixed time sampling assays to detail the kinetic parameters for natural substrates such as angiotensin I and bradykinin and numerous synthetic N- blocked tripeptides. These assays are not generally well suited to precise kinetic analyses and thus only when a continuous spectrophotometric ACE assay was developed by Holmquist et al. (1979), was it possible to rigorously study ACE catalysed peptide hydrolysis. This assay depended on Furanacryloyl blocked tripeptides in which a decrease in absorbance accompanies hydrolysis, and the first such tripeptide to be used was Fa-Phe-Gly-Gly (Bunning et al., 1983).

1.2.6. Anion activation and the importance of pH

It has long been known that the catalytic mechanism of ACE is dependent on anion activation (Skeggs et al., 1954). It was subsequently found that the anion requirements vary between substrates and are affected by pH. For example the comparative activating potencies of anions for Z-Phe-His-Leu hydrolysis (Piquilloud et al., 1970) are $Cl^- > NO_3^- > Br^- > F^- > I^-$ and for Fa-Phe-Gly-Gly (Bunning and Riordan, 1983) was $Cl^- > Br^- > F^- > NO_3^- > CH_3COO^-$. It appears that there are stringent anion size requirements governing the degree of activation, because despite bromide, nitrite, iodide and acetate all appearing to bind ACE at least as well as chloride by kinetic criteria, these are only 52%, 14%, 10% and 2% as effective as chloride, respectively.

There is also substrate dependence of anion activation since angiotensin I and some tripeptide substrates such as Hip-His-Leu have a strong requirement for chloride whereas bradykinin can be hydrolysed in the absence of chloride at approximately 37% of the optimum rate (Dorer et al., 1974). It has also been found that where the substrate is tightly bound to ACE there is less dependence on chloride for hydrolysis and in fact supraoptimal concentrations of chloride have been shown to inhibit hydrolysis (Cheung et al., 1980). Thus it appears that, where a substrate binds tightly to ACE, activation by small amounts of chloride could be due to enhanced binding of substrate.

Shapiro et al. (Shapiro et al., 1983) have classified substrates according to their dependence on anion activation by studying hydrolysis of a group 26 Furanacryloyl tripeptides. Three classes of substrate have been described; Class I substrates for which Fa-Phe-Gly-Gly is typical, are relatively weakly binding (K_m values 0.16-4.1 mM) but have high turnover numbers (K_{cat} 32-617 sec^{-1}) with hydrolysis showing an absolute requirement for chloride, demonstrating an essential activator mechanism at neutral pH. Apparent activation constants (K_a') are in the range of 75-150 mM for chloride for Class I substrate hydrolysis. Class II substrates are tight binding (K_m 6.5-110 μM) having much lower K_a' values (2.9-5 mM) and all appear to be hydrolysed by a non essential activator mechanism. The kinetic mechanisms for Class I and Class II substrates are diagramatically shown in the figure below. Class III substrates demonstrate intermediate values of K_m (49-520 μM) with activation constants of between 18 and 30 mM and are hydrolysed by a more complex non essential activating mechanism.

pH dependence of the activation constants for chloride differ markedly between each of the three classes, varying 60 fold over the pH range 6-9 for Class I, 3-fold for Class II, and 19-fold for Class III substrates. While for Class II substrates these findings can be related to the amino acids found at the C-terminus of the peptide, no distinguishing features for Classes I and III can be clearly discerned, except that none contain charged residues. In contrast to Class II substrates, all Class III substrates posess an alanyl residue as the penultimate amino acid. These findings have been demonstrated for both rabbit lung (Shapiro et al., 1983) and human kidney ACE (M.R.W.Ehlers, personal communication).

Bradykinin, with its positively charged ultimate residue, appears to be a Class II substrate, which as mentioned previously, undergoes significant ACE hydrolysis even in the absence of chloride. In contrast, angiotensin I, may be both a Class I and Class II substrate, depending on the ionisation state of the penultimate histidine residue (Shapiro et al., 1983). With rabbit ACE it has been shown that angiotensin I behaves as a Class I substrate, having an absolute requirement for chloride (Bunning et al., 1983). Angiotensin I hydrolysis by human kidney ACE follows a non-essential activation mechanism in which it has been shown that the requirement for chloride is markedly pH dependent, increasing with increasing pH (Ehlers and Kirsch, 1988).

Sulphate has been shown to potentiate the chloride activation of ACE. Initially it was thought that this effect was simply due to the change in ionic strength and only recently has this phenomenon been kinetically studied using rabbit lung ACE and the substrate Fa-Phe-Gly-Gly (Bunning and Riordan, 1987). While sulphate decreases the apparent binding constant for chloride (lowering apparent K_m value) it does not change k_{cat}. Sulphate, therefore, potentiates the effect of chloride which appears to activate the enzyme by enhancing substrate binding. Sulphate on its own however, is unable to activate the enzyme.

1.2.7. Effects of Metal Substitution

ACE, being a zinc metalloprotease can be inhibited by metal chelators such as EDTA (Cushman and Cheung, 1971) and the removal of zinc abolishes activity completely (Bunning and Riordan, 1981). Experiments in which enzyme is stripped of zinc and replaced by various divalent cations revealed that the resulting activity is markedly ion and substrate dependent. For example while Co^{2+}-containing ACE demonstrates 120% activity compared with Zn^{2+} enzyme for the substrate Fa-Gly-Leu-Ala it only shows 55% of the zinc enzyme activity for the substrate Fa-Phe-Gly-Gly (Bicknell et al., 1987 & Bunning and Riordan, 1981 & 1985). Zinc dissociates from the enzyme at low pH and therefore, in acidic conditions, zinc must be added in order to maintain activity (Bunning et al., 1983). Supraoptimal zinc concentrations have been shown to inhibit ACE. This effect has been seen for rabbit ACE particularly in alkaline conditions and for human kidney ACE throughout the pH range (Ehlers and Kirsch, 1988).

1.2.8. Catalytic Mechanism

On the basis of structure-activity studies on ACE using numerous snake venom peptide inhibitors, ACE was originally considered to be a dipeptide-releasing carboxypeptidase similar in many respects to carboxypeptidase A (CPA) (Cushman et al., 1977 & Ondetti et al., 1977). Subsequently, when it was found that zinc was required for activity and that arginine, tyrosine and glutamate are important active site residues, ACE became defined as a zinc metallocarboxypeptidase closely related to CPA but also part of a family of zinc metallopeptidases including CPA, thermolysin, bacterial neutral proteases, aminopeptidases, collagenases and others (Bunning et al., 1978).

A catalytic mechanism for ACE appears to include the necessity for a positively charged arginine residue which binds to the C-terminal carboxyl group of the substrate followed by cleavage of an amide bond which is catalysed by both the electrophilic effect of the zinc ion and nucleophilic attack of the gammacarboxylate group of either the glutamate and/or a zinc hydroxide. Thus it would appear that for substrate binding and correct alignment of enzyme to substrate to be achieved there is a necessity for; 1.) binding of the substrate C terminal carboxyl with the enzyme arginine residue; 2.) coordination of the scissile amide carbonyl with the zinc atom; 3.) the formation of a hydrogen bond between the tyrosine at the active site and the terminal peptide carbonyl; and finally,4.) interaction of the side chain of the C-terminal three substrate amino acid residues with as yet undefined binding subsites.

Radiationless energy transfer studies with a fluorescent substrate (Bunning and Riordan, 1981) have demonstrated that zinc is not required for substrate binding and appears to function solely in the hydrolytic step of catalysis. This is further supported by the knowledge that metal replacement mainly affects k_{cat} causing little alteration in K_m (Bunning and Riordan, 1985).

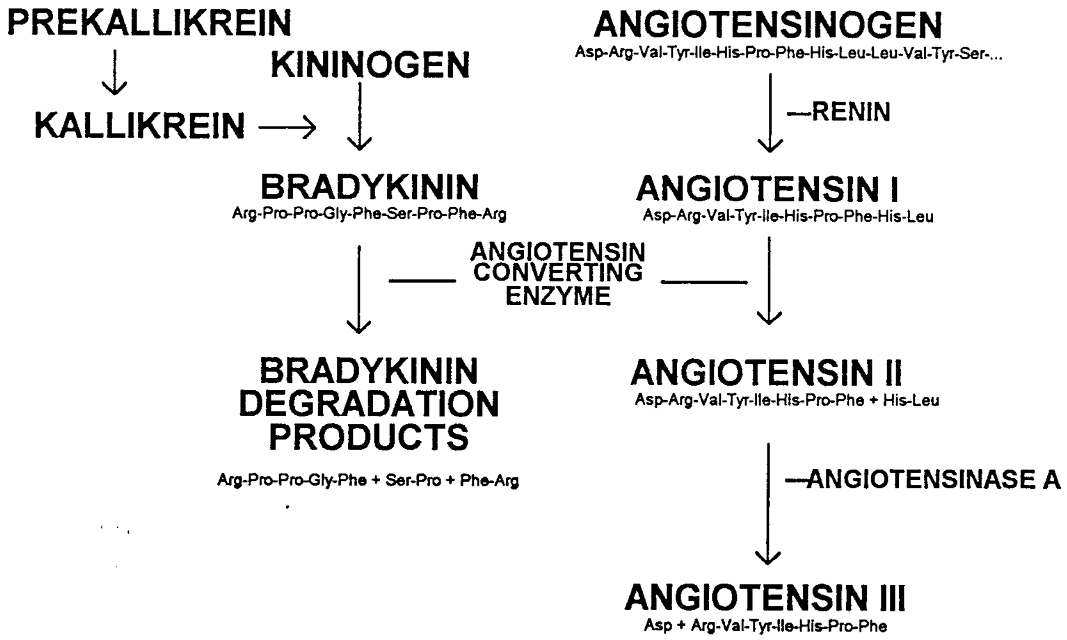
Despite similarities between ACE and other zinc metallopeptidases, a unique feature of ACE not explained by the proposed catalytic mechanism above, is the marked activating effect of chloride and other monovalent anions. It has recently been noted however, that CPA is inhibited by chloride and other anions by a partially competitive mechanism, arginine being implicated as the most likely anion binding site (Williams and Auld, 1986). CPA does not have an active site lysine which has been demonstrated to be the anion binding site of ACE (Bunning et al., 1978). It appears that anion activation is the result of enhanced enzyme substrate binding (Shapiro et al., 1983). Similarly it has been shown that inhibitor binding is enhanced by chloride (Shapiro and Riordan, 1984). It has been suggested (Shapiro, 1983) that the active site lysine of ACE in conjunction with chloride promotes binding. This may be achieved by an improvement, in the presence of the anion, in the alignment of the other active site residues enhancing substrate binding. Some evidence for this effect of chloride causing an active site conformational change does exist as it has been found that chloride results in a small alteration of the near and far ultraviolet absorbance (Oshima et al., 1974) and also enhances the efficientcy of radiationless energy transfer between ACE tryptophan residues and an active site bound danzyl inhibitor (Martin et al., 1987).

A potential biological role for anion activation has been suggested (Ehlers and Riordan, 1990), where apart from promoting a more favourable conformational change in the active site it may also play a regulatory role depending where the enzyme is acting. Whereas perhaps not true for ACE on endothelium throughout the body, ACE in the renal tubule brush borders and on intestinal microvilli is certainly exposed to significant ion fluxes and this may have the effect of regulating ACE activity.

1.3. THE RENIN ANGIOTENSIN SYSTEM.

The renin angiotensin system (RAS) was initially thought to be merely a circulating endocrine system. The role of ACE was considered to be limited to the conversion of angiotensin I to angiotensin II which took place in the pulmonary vasculature. Although the RAS does act as a circulating endocrine system, involved in the production of the vasoconstrictor angiotensin II, there is now firm evidence that it also has important local autocrine and paracrine roles in a variety of tissues. The local tissue RAS provides tonic control of vascular resistance and influences local tissue function while the circulating RAS serves in short term cardiorenal homeostasis (Dzau, 1988). It has even been suggested that the RAS acts on intracellular events, ie. an intracrine system (Re, 1984). Other diverse roles for the RAS, unrelated to cardiorenal homeostasis, are reproduction and immunity (Weinstock, 1986; reviewed in Ehlers and Riordan, 1989). As has been mentioned previously in the section on substrate specificity, ACE is also thought to hydrolyse non angiotensin peptides such as substance P *in vivo* (Strittmatter et al., 1985). These functions will all be covered in the following section.

In considering the role of ACE in the RAS, brief mention should be made of the RAS cascade and its components. The biochemical cascade forming the renin angiotensin system (RAS) is diagramatically detailed in the accompanying figure.



1.3.1. Renin

Renin (EC 3.4.23.15) is an aspartyl (or acid) protease closely related to pepsin, cathepsin and chymosin. The mouse, rat, and human (Rougeon et al., 1981;Imai et al., 1983;Soubrier et al., 1983) renin genes have been cloned. The renin gene consists of nine exons in the mouse and rat, differing from the human gene which has ten exons . Renin is synthesised as 55kDa precursor molecule which is then converted to 44kDa active renin either during packaging in the Golgi apparatus or in renin granules. Renin is synthesised and stored in granules contained in the juxtaglomerular apparatus of the kidney (Barajas, 1979), but it has become increasingly apparent that renin is also synthesised in extra-renal sites such as the brain (Dzau et al., 1986) and the adrenal glands (Deschepper et al., 1986). Renin only acts on its specific substrate angiotensinogen, to form angiotensin I (the substrate for ACE to form angiotensin II) and a large residual peptide of unknown function, Des-angiotensin-I angiotensinogen.

1.3.2. Angiotensinogen

Angiotensinogen is an alpha 2 globulin synthesised by the liver. The gene for angiotensinogen has been cloned (Okhubo et al., 1983). Heterogeneity has been demonstrated in the purified protein which has a molecular weight of between 61 and 65 kDa and is glycosylated (Menard et al., 1983; Tewksbury, 1983). As with renin, there is evidence that angiotensinogen is synthesised at extrahepatic sites such as the brain and kidney (Campbell and Habener, 1986). Angiotensinogen levels have been shown to be increased by oestrogens, oral contraceptives, glucocorticoids, thyroid hormones and post nephrectomy - and appears to be decreased during ACE inhibitor therapy (Rasmussen et al., 1981).

1.3.3. Angiotensin converting enzyme

The biochemistry of ACE has been extensively covered above and its tissue distribution and functions are detailed in section 1.4.

1.3.4. Angiotensin I, II and III

Angiotensin I is a decapeptide that results from the action of renin on angiotensinogen. Having no apparent biological activity, angiotensin I is rapidly converted by ACE to angiotensin II, the biologically active octapeptide effector of the RAS. It has been shown that administration of ACE inhibitors causes an accumulation of angiotensin I (Johnston et al., 1979). While it is likely that most conversion of angiotensin I to angiotensin II takes place in the pulmonary vasculature, there may also be significant local formation across peripheral vascular beds. Angiotensin II has a short half life of between 15 and 30 seconds in the circulation before being destroyed by a series of aminopeptidases or angiotensinases. Action of aminopeptidase A on angiotensin II results in angiotensin III which is formed in very low concentrations in humans but in greater concentrations in the rat (Semple et al., 1976). Angiotensin III may have some biological role in the brain and adrenal. It does possess some biological activity on vasculature, although less potent than angiotensin II, and in stimulating the production of aldosterone.

Angiotensin II can also be formed by the action of enzymes other than ACE on angiotensin I. Although in the rat angiotensin II appears to be formed by ACE, this is not so in the human heart where a major angiotensin II forming serine protease, belonging to the chymase family of leukocyte proteinases, has been isolated (Urata et al., 1990). It has also been shown that kallikrein or kallikreinlike enzymatic activities can result in the formation of angiotensin II directly from angiotensinogen (Gondo et al., 1989).

1.4. TISSUE DISTRIBUTION AND BIOLOGICAL ROLE OF ACE AND THE RAS.

ACE is widely distributed in virtually all mammalian organs and body fluids. The tissue distribution has, however been shown to differ between species. In the rat, highest ACE specific activity is in the epididymis, testis and lung, with the lowest levels in the kidney, heart and liver (Cushman and Cheung, 1971). Quantitatively different values were obtained in a survey of 24 post-mortem human tissues (Lieberman and Sastre, 1983). Highest levels were found in the kidney which has subsequently proven to be a most convenient source in the purification of ACE (Ehlers et al., 1986). Lower levels were found in the lung, prostate, colon, testis and adrenal. ACE is found primarily in three types of cells; endothelial, epithelial and neuroepithelial (Defendini et al., 1983). This grouping provides a convenient system for classifying ACE distribution. Endocrine cells and the ACE of other sources will be termed "Miscellaneous" below. As described earlier, ACE is mostly membrane bound acting outside the cell, although ACE has been located in intracelluler cytoplasmic membranes as in sperm cells (Brentjens et al., 1986) and possibly neuroblastoma cells (Okamura et al., 1981).

1.4.1. Endothelial ACE

ACE is widely found in the vasculature of virtually all organs consistent with its plasma membrane localisation on endothelial cells (Caldwell et al., 1976). In this location ACE is well positioned to convert angiotensin I to angiotensin II and to inactivate bradykinin. Owing to the vast capilliary surface found in the lung it has long been considered that the lungs are the major site for the formation of angiotensin II (Ryan, 1982). This view has been more recently revised following findings suggesting that the peripheral tissues are the major source of angiotensin II (Campbell, 1985).

1.4.2. Epithelial ACE

In humans, and probably all mammals, epithelial ACE located in the kidney, choroid plexus, prostate and epididymis is bathed in an effluent and is well placed to act on soluble substrates in flux.

1.4.2.1. Kidney

ACE in the human kidney has been localised to the brush border of proximal tubular cells (Danilov et al., 1987), glomerular endothelium (Danilov et al., 1987) and juxtaglomerular cells (Celio and Inagami, 1981). Other components of the RAS have also been located in the kidneys of rats: renin mRNA and protein have been located by *in situ* hybridisation and immunohistochemistry in the juxtaglomerular apparatus (Deschepper et al., 1986), and angiotensinogen mRNA has been located in the rat proximal tubule (Ingelfinger et al., 1990). A variety of roles have been suggested for renal ACE: it may a) cleave dipeptides from absorbed oligopeptides and transport them into the cytoplasm (Defendini et al., 1983); b) it could be involved in sodium and fluid reabsorbtion (Harris et al., 1984); or c) its action could regulate the glomerular filtration rate by causing vasoconstriction of afferent and efferent vessels (Celio and Inagami, 1981).

1.4.2.2. Intestine

All the components of the RAS have been shown to be present in the intestine; angiotensinogen and renin have been located in the walls of the splanchnic arteries (Desjardins - Giasson et al., 1981) and ACE has been found on the endothelium of mesenteric vessels (Erdos and Skidgel, 1987) and intestinal brush border (Bruneval et al., 1986). ACE has been localised in the rat intestine in the mucosae of the duodenum and jejunum (Duggan et al., 1989). A more restricted distribution of the enzyme was found in the ileum and colon.

ACE was also detected in the muscle layers of all regions especially at the mucosa muscularis-junction. In the same study, angiotensin II receptors were detected only in the muscularis layer of particularly the colon and to a lesser extent the ileum and duodenum. The authors concluded that ACE in the intestinal mucosa could play a role in the hydrolysis of absorbed peptides while ACE and the angiotensin II receptors in the muscularis suggest a role in intestinal motility. It is well established that angiotensin II stimulates the contraction of intestinal smooth muscle.

1.4.2.3. Choroid Plexus

The apical membranes of the epithelial cells of the choroid plexus are particularly rich in ACE (Igic et al., 1977). It is bound to the microvillus luminal plasma membranes and comes into contact with the cerebrospinal fluid (CSF) which bathes the plexus (Defendini et al., 1983; Strittmatter et al., 1984; Rix et al., 1981). Apart from the large amounts of ACE on the surface of the choroid plexus, angiotensinogen is present in significant quantities in the CSF (Printz et al., 1982) and renin is located in the cytoplasm of the choroid epithelium (Slater et al., 1980). When components of the RAS are administered into the ventricles, dipsogenic and hypertensive effects are seen (Fitzsimmons, 1979). Intracerebroventricular application of various ACE inhibitors lowers blood pressure in stroke prone spontaneously hypertensive rats (Phillips and Kimura, 1986). Controversy exists, however, as to whether ACE inhibitors gain accesss to the CNS during systemic administration. While initially it was thought that captopril does not cross the blood-brain barrier (Hutchinson et al., 1982), some studies have shown inhibition of ACE in brain tissue homogenates following systemic ACE inhibitor treatment (Nakata et al., 1987). Furthermore, when angiotensin I and renin were injected into the ventricles of rats treated with oral captopril, decreased dipsogenic and hypertensive effects were noted (Unger et al., 1983), suggesting that ACE inhibitors may cross into the CSF and inhibit CSF ACE.

1.4.2.4. Prostate and epididymis

The human prostate contains high levels of ACE activity which are increased in benign prostatic hypertrophy (BPH)(Yokoyama et al., 1980). ACE is secreted into the prostatic duct from the tubular epithelium and so is found in the seminal plasma (Yokoyama et al., 1982). High levels of ACE are also present in the rat epididymis (Cushman and Cheung, 1971) where ACE from the luminal surface of epithelial cells is released into the lumen in particulate form (Strittmatter et al., 1985). The role of ACE in the male genital tract is entirely unknown. Evidence suggests that the ACE derived from the prostate and epididymis is of the somatic form: a) failure to purify the smaller testicular form of ACE from seminal plasma (El-Dorry et al., 1983); b) immunoprecipitation of epididymal ACE with a monoclonal antibody to somatic ACE that does not recognise the testicular form (Strittmatter and Snyder, 1984); and c) co-migration by polyacrylamide gel electrophoresis of epididymal ACE and the somatic lung enzyme (Strittmatter and Snyder, 1984).

1.4.2.5. Placenta

Human placental syncytiotrophoblastic microvilli contain ACE (Johnson et al., 1984) which is thought to regulate the passage of angiotensin I and other peptides at the maternal-fetal interface (Johnson et al., 1984).

1.4.2.6. Heart

Strong evidence exists for the presence of a local renin angiotensin system in the heart. Cardiac angiotensinogen gene expression has been demonstrated in rats (Campbell and Habener, 1986), and also in mice (Dzau et al., 1987). It appears that angiotensinogen is expressed at far higher levels in the atria compared to the ventricles. Similarly, renin gene expression has been documented in both mice (Dzau et al., 1987) and in rats (Suzuki et al., 1988). In contrast to angiotensinogen, renin expression appears to be higher in ventricles than in the atria. Although there are, as yet, no published reports of *in situ* cardiac ACE mRNA expression, localisation of ACE in cardiac tissues is well documented using a variety of techniques. Most recently, using a radio inhibitor ligand binding technique to localise ACE in the rat heart (Yamada et al.,1991), it was determined that higher levels of ACE are present in the atria as compared to the ventricles with higher levels being noted in the right atrium compared with left. ACE was localised in the myocardium with only low levels being detected in the endocardium. Interestingly, extremely dense ACE labelling occurred throughout the full thickness of all cardiac valves, suggesting that this may be an important site for the conversion of circulating angiotensin I to angiotensin II and the metabolism of other peptides.

Various functions of an intracardiac RAS include the control of coronary vasoconstriction, positive inotropic and chronotropic actions (Lindpainter and Ganten, 1991), and the stimulatory effect of an activated RAS on the sympathetic nervous system of the heart. A further role for angiotensin II is it's potential action as a growth factor. It has been noted that angiotensin II stimulates increased DNA and RNA turnover and new protein synthesis *in vitro* (Baker and Aceto, 1990). Two possible mechanisms for this effect have been suggested; the first is direct interaction of angiotensin II with nuclear chromatin, (Robertson and Khairallah, 1971), and secondly, activation of the nuclear proto-oncogene *c-fos* which plays an important role in the regulation of cell proliferation and protein synthesis in cardiac muscle (Taubman et al., 1989).

1.4.3. Neuroepithelial ACE

Certain regions of the brain have been found to contain ACE, with highest levels being found in the choroid plexus and subfornical organ, lower levels in the caudate putamen, globus pallidus, substantia nigra, and pituitary and the lowest levels in the hypothalamus (Defendini et al., 1983).

Angiotensin II receptors have been found in the subfornical organ and also in the magnocellular nuclei of the hypothalamus, suggesting that ACE in these sites is responsible for the local generation of angiotensin II. Angiotensin II has been shown to stimulate the release of vasopressin by the hypothalamus (Lang et al., 1983). In the pituitary, where it appears that ACE may produce angiotensin II from circulating angiotensin I (Strittmatter and Snyder, 1987), angiotensin II causes the release of both ACTH and prolactin (Steele et al., 1981).

Basal ganglia ACE does not appear to be involved in angiotensin II production as no angiotensin II receptors are present in this region (Mendelsohn et al., 1984). There is evidence that a striatonigral ACE is associated with tachykinin metabolism as it is uniquely able to cleave substance K (Thiele et al., 1985).

1.4.4. Endocrine ACE

1.4.4.1. The testis

The testes contain the unique 90 000 MW enzyme which has been localised to the cytoplasmic droplets of sperm (Yotsumoto et al., 1984). ACE, along with other components of the RAS, has also been demonstrated in the Leydig cells (Pandey et al., 1984). It is thought that ACE may be a factor in male fertility by affecting sperm motility and capacitation through bradykinin inactivation and may also modulate steroidogenesis and/or regulate cell growth and differentiation.

1.4.4.2. The ovary

All components of the RAS have been localised in mammalian ovaries (Bumpus et al., 1988). Angiotensin II receptors have been shown to be localised on the granulosa and theca cells of developing follicles and thus appear to be involved in the control of follicular function and steroidogenesis.

1.4.4.3. Adrenal

Renin (Deschepper et al., 1983) and angiotensinogen (Campbell and Habener 1986) messenger RNA's have been demonstrated in the adrenal. Angiotensin II receptors are present in all zones of the adrenal gland and angiotensin II has been shown to stimulate aldosterone, corticosteroids and other steroids as well as the release of catecholamines from the adrenal medulla (Peach et al., 1971 and Nakamura et al, 1985). ACE is found in association with blood vessels in the adrenal cortex and also large quantities of the enzyme have been demonstrated in the adrenal medulla (Strittmatter et al., 1986) which, by producing angiotensin II which interacts with medullary angiotensin II receptors, stimulates the release of catecholamines (Peach et al., 1971).

1.4.5. Miscellaneous ACE

1.4.5.1. Serum

ACE in serum is thought to be derived from endothelial ACE either through non specific sloughing or perhaps by specific cleavage by an, as yet, undefined enzyme. Details of it's carbohydrate content and physicochemical properties have been discussed above. Circulating levels of ACE may be elevated in a variety of diseases including leprosy, primary biliary cirrhosis, diabetes mellitus, silicosis and hyperthyroidism (Studdy et al., 1983). The disease with which serum ACE is most commonly associated however, is sarcoidosis, in which measurements of elevations in the activity of the circulating enzyme have proven useful in the diagnosis and follow-up of patients with this disease (Ainslie and Benatar, 1985).

1.4.5.2. Mononuclear phagocytic cells

Studies in patients with sarcoidosis have revealed that ACE activities are markedly elevated in serum and in lymph nodes. More detailed localisation has revealed that ACE is to be found in the epithelioid and giant cells of sarcoid granulomata (Silverstein et al., 1979), where angiotensin II has also been found (Silverstein et al., 1978).

The involvement of ACE in immunity has been extensively studied in the model of murine schistosomiasis where it has been shown that granulomas contain all components of the RAS (Weinstock and Blum, 1984). Angiotensin II receptors are expressed on granuloma macrophages (Weinstock and Kassab, 1984), and angiotensin II stimulates phagocytosis (Weinstock and Kassab, 1984) and recruits splenic mononuclear cells to the developing granulomas by a chemotactic mechanism (Weinstock and Kassab, 1986). These studies have demonstrated that ACE plays an immunoregulatory role in granulomatous inflamation by a mechanism involving angiotensin II production and/or inactivation of bradykinin and various neuropeptides (Weinstock, et al.,1986).

1.5. ACE REGULATION

Little is known about the regulation of ACE both in terms of its biosynthesis, control of existing enzyme and it's metabolism. Several studies have demonstrated hormonal control of the enzyme and some studies have suggested the existence of endogenous ACE inhibitors which will be discussed below.

1.5.1. Hormonal control

1.5.1.1. Sex Hormones.

Cushman and Cheung (1971) in their survey of ACE activity among various tissues in the rat, found that high levels of ACE in the epididymis and testis were only attained at maturity, being low in immature rats, and that hypophysectomy prevented the maturity related increase in ACE activity. Subsequently it was shown (Velletri et al., 1985), that even mature rats which were hypophysectomised lost their testicular ACE activity over a period of two to three weeks after the operation, suggesting that continued stimulation by the pituitary is required for the maintenance of testicular ACE activity. This conclusion was further strengthened in the same study where it was shown that hormonal replacement with FSH/LH (follicle stimulating hormone/leutinising hormone), HCG (human chorionic gonadotrophin) or testosterone immediately following surgery, maintained ACE at near normal levels. In the rat testis, ACE biosynthesis is under hormonal control but it is not known whether androgens directly induce ACE in existing germinal cells or whether they stimulate germinal cell maturation and thus non specifically increase ACE synthesis (Velletri et al., 1985).

1.5.1.2. Corticosteroids.

ACE induction by corticosteroids has been demonstrated in cultured alveolar macrophages (Friedland et al., 1977), cultured human monocytes (Friedland et al., 1978), cultured endothelial cells and rat lung in vivo (Mendelsohn et al., 1982). In alveolar macrophages and human monocytes, enzyme induction appears to be due to new messenger RNA and enzyme biosynthesis as it was inhibited by actinomycin D and cycloheximide. *In-vitro* it has been shown that peripheral blood monocytes can be induced to produce ACE in the presence of autologous lymphocytes and the existence of a soluble ACE inducing factor produced by lymphocytes has been determined (Conrad and Rohrbach, 1987). These findings may be of relevance in an understanding of the development of sarcoid granulomata.

1.5.1.3. Thyroid Hormones.

Patients with hyperthyroidism have elevated levels of serum ACE that return to normal following therapy (Yotsumoto et al., 1982; Smallridge et al., 1983).

ACE levels were found to be reduced in hypothyroidism and it appears that thyroxin (T4) and ACE levels in serum fluctuate in parallel during the evolution of sub-acute and silent thyroiditis (Smallridge et al., 1983). The source of the increased ACE activity in serum may be due to induction of ACE production by thyroid hormones of vascular endothelial cells. A recent study (Dasarathy et al., 1990) showed that bovine endothelial cells could be stimulated to produce increased quantities of ACE in culture in the presence of tri-iodothyronine.

1.5.2. ACE induction by specific ACE inhibitors

In a study assessing the effect of the ACE inhibitor captopril on blood pressure, plasma renin activity, and ACE activity in hypertensives (Larochelle et al., 1979), it was noted that while therapy resulted in a decrease in blood pressure, papadoxically plasma ACE activity after seven days of treatment remained unchanged and in some cases even appeared to rise. Captopril has an opposite effect on ACE activity versus concentration when captopril is allowed to degrade either through storage of the sample or by chemical destruction using N-ethylmaleimide (Boomsma et al., 1981).

Studies in rats demonstrated ACE induction using both captopril and enalapril and the inductive response depends on an intact adrenal gland. Also, in endothelial cell culture captopril induces ACE production. These studies will be discussed in more detail in the introduction to the experimental studies on ACE induction.

1.5.3. Endogenous ACE inhibitors

Little is known about how ACE, once produced, is controlled physiologically. While, as has been discussed in the section on the anion dependence of ACE, that ACE could be regulated by ionic fluxes, it is interesting to consider that specific endogenous inhibitors of the enzyme could exist. The first evidence suggesting the possible presence of such inhibitors was in a study (Lieberman and Sastre, 1983) where it was noticed that ACE activity in a variety of tissues could be increased by dilution of the homogenate. It was found that maximal increase in activity between 80 and 200% over the undiluted sample was achieved at a 1 in 8 dilution. Similarly, an ACE inhibitor was found in human serum (Lieberman and Sastre, 1986) with a prevalence of 25% of 280 serum samples tested. The serum inhibitor appears to be heat labile, have a molecular weight of over 50000 and also possesses the rather unique property of producing irreversible inhibition after the loss of a low molecular weight component, possibly an ion, since loss of inhibition by further dilution no longer occured after dialysis of the sample against saline. However, to date there have been no further reports supporting these findings.

Recently endogenous peptide inhibitors of ACE have been purified from a number of sources. The first of these to be isolated was an ACE inhibitor from porcine plasma (Hazato and Kase, 1986). This inhibitory peptide has the amino acid sequence leucyl-valyl-leucine and demonstrated an IC 50 for rabbit lung ACE of approximately 3×10^{-6} M. Another potential endogenous inhibitor for ACE is des-leu 10 - angiotensin I which is generated from angiotensin I by a releasable platelet peptidase (Snyder and Wintroub, 1985), and suggests that potential pathways exist by which cells such as platelets can generate an endogenous inhibitor of ACE at local vascular sites thus controlling angiotensin II generation in vascular tissue (Snyder and Wintroub, 1986).

An endogenous inhibitor was also inadvertently found in rat heart tissue (Ikemoto et al., 1989) when it was noted that endogenous inhibition of ACE took place during tissue oxidation. The exact isolation of this inhibitor was elusive and awaits further study.

1.6. MOLECULAR BIOLOGY OF ACE

The first study on the molecular biology of ACE (El-Dorry et al., 1982) was an attempt to study the differences between pulmonary and testicular ACE. When reticulocyte lysates were primed with poly A containing RNA from mature rabbit testes the newly synthesised peptide was 35% smaller than that programmed by pulmonary RNA. Furthermore, testicular ACE was not produced if the reticulocyte lysates were primed with RNA from immature testes, in contrast to the pulmonary dependent product which was promoted by RNA from both immature and mature lungs. This suggests that the distinguishing structural and regulatory properties of testicular ACE are determined pretranslationally. These differences were thought to be due either to tissue-specific differential processing of primary messenger RNA transcripts from a single gene, or tissue-specific isozymes arising from different genes.

Recently, great strides have been made in the molecular characterisation of ACE with the isolation of cDNA clones for somatic and/or testicular ACE from a variety of species; rabbit (Roy et al., 1988), mouse (Bernstein et al., 1988), and human (Soubrier et al., 1988; Ehlers et al., 1989). It was soon realised that somatic ACE in all species studied consists of two homologous domains each containing a putative metal binding site and that testicular ACE is identical to the C-terminal half of somatic ACE except for a unique 35 amino acid residue at its N-terminus (Ehlers et al, 1989; Lattion et al., 1989). Similar conclusions were also drawn from a study of rabbit ACE cDNA (Kumar et al., 1989) where various oligonucleotide probes were synthesised and used to probe the structure of pulmonary and testicular ACE. It was found that the two isozymes possesed a segmental mosaic structure which suggested that a single gene may be the source of both forms of ACE. This conclusion had already been proposed following work on the human ACE cDNA (Soubrier et al., 1988). It was considered that the somatic ACE gene was the result of ancestral duplication of a smaller gene corresponding to the testicular form.

Northern blots of mRNA from cultured human umbilical vein endothelial cells revealed a 4.3 kb hybridisation product and similar studies of testicular mRNA detected a 3 kb product (Soubrier et al., 1988). In the rabbit a 5-kb ACE mRNA was found in lung tissue and a 2.6-kb mRNA species was detected in testicular tissue (Roy et al., 1988). In northern analysis of mouse testicular ACE, a single hybridisation signal was obtained of 2.7 kb (Howard et al., 1990), in the same study mRNA hybridisation signals of kidney ACE were obtained at both 4.2 and 5.1 kb. In this study, techniques such as polymerase chain reaction (PCR) revealed multiple RNAs suggesting incomplete splicing - the relevance of this finding is unknown.

The first genomic study was the analysis of DNA just 5' to the cDNA sequence for both human and mouse ACE (Shai S-Y et al., 1990). This revealed potential corticosteroid binding site consensus sequences and other potential regulatory motifs. Mouse testicular ACE cDNA starts with an exon not present in somatic ACE cDNA (Howard et al., 1990). Transcription for the testis isozyme begins within the 12th intron of the gene, 7206 base pairs (bp) 3' to the translation start site for somatic ACE. A search for a potential unique testis specific promoter revealed a TA-rich region 25 bp upstream of the transcription start site of testis ACE.

A rabbit (Kumar et al., 1991) 18.3-kb genomic DNA clone was found to hybridise to oligonucleotide probes specific for both somatic and testicular ACE. As has been reported for mouse ACE, rabbit somatic and testicular ACE are transcribed from two different initiation sites of the same gene. The genomic sequence revealed a unique pulmonary coding sequence followed by a unique testicular sequence which is then followed by a sequence common to both isozymes. Generation of the somatic ACE mRNA involved the splicing out of the unique testicular sequence as an intron. Transcription of the testicular isozyme starts within this intron. Using a variety of techniques, including DNA sequencing, RNA protection assays and primer extention assays, the exact transcription initiation start sites were determined and the sequence odtained for the region upstream of these sites. Analysis of the sequence upstream of the transcription start site for somatic ACE, revealed a TATA box 26 bases upstream of the start site. Transcription factor IID is known to bind to this motif; also a SP-1 binding site was found in this region (Mitchell and Tijan 1989).

Further upstream three more SP-1 sites and an AP-1 binding site were found. In contrast, the only binding motif found upstream of the initiation site for testicular ACE was a TTATT sequence 27 bp 5' to this site. Genomic sequence analysis of the human ACE gene has shown (Hubert et al., 1991) that this gene spans 21 kb and comprises 26 exons. The two homologous domains of the enzyme are coded for by exons 4-11 and 17-24. Each contains the metalloprotease consensus sequence H-E-X-X-H in exon 8 and 21. Ribonuclease protection assays revealed the transcription initiation site with a typical TATA box at -37. The hexanucleotide sequence of a glucocorticoid-responsive element was found at the following four positions far upstream of the transcription initiation site; -1213,-935,-794 and -686. The transcription start site for the testicular isozyme is within intron 12, similar to the findings in the mouse gene as detailed above. A TATA like sequence (TATT) is also present 15 bp upstream of the start site. Other potential regulatory sequences include a sequence similar to the cAMP responsive element consensus sequence at position -44 and a steroid responsive sequence was found at position -190 but this may not be functional (Testut et al., 1993). The increased ACE mRNA and activity of cultured bovine endothelial cells following ACE inhibitor administration must still be explained (Dasarathy et al., 1992).

The potential age of the duplication of the ancestral shorter gene was studied by comparing the approximate rate of divergence between human, mouse and rabbit exons 7 and 20 and concluded that this event occurred between 118 and 194 million years ago.

A recent study (Langford et al., 1991) has shown that testicular ACE in fact does utilise its own unique promoter rather than the somatic ACE promoter, with subsequent splicing out of the non-testicular sequence. Transgenic mice carrying a 698 bp fragment of the mouse testicular ACE promoter linked to the ß-Galactosidase reporter gene was found to express ß-galactosidase protein and RNA only in the testes. Histological examination for ß-galactosidase in sections of testicular tissue revealed staining mostly in the elongating spermatids and later stages of spermatogenesis. This is consistent with immunohistochemistry for ACE in similar sections. Thus clear evidence for a testis specific promoter within the region immediately upstream of the transcriptional start site was presented.

Recombinant native and mutated ACE have recently allowed greater insight into the C-terminal anchor peptide sequence (Ehlers et al., 1991; Wei et al., 1991a) and the enzyme's active sites (Wei et al.,1991b).

In a study utilising recombinant human testicular ACE, Ehlers et al. (Ehlers et al., 1991) transfected Chinese Hamster Ovary cells (CHO cells) with either full length human testis cDNA or a deletion mutant in which a stop codon was inserted preventing the formation of the putative anchor peptide. Cells transfected with the full length cDNA produced both membrane bound and secreted ACE (isolated from the medium). In contrast, cells transfected with the mutant produced virtually only secreted ACE. Both forms of the enzyme had similar enzymatic activity, were indistinguishable on SDS-PAGE, and phase separation studies confirmed that membrane bound ACE is hydrophobic and could be converted to a hydrophilic form, similar to the anchor-minus mutant ACE, by trypsinisation. Amino acid analysis also demonstrated the similarity between soluble hydrophylic ACE and mutant anchor-minus ACE. Similar studies on the somatic enzyme (Wei et al., 1991a) showed that naturally produced soluble ACE was catalytically and immunologically indistinguishable from recombinant mutant ACE lacking a C-terminal anchor sequence. The conclusion of both these studies was that soluble ACE is derived by specific proteolytic cleavage of membrane ACE by an as yet unidentified enzyme.

Investigation of the catalytic domains of the somatic enzyme has also been made possible by the production of recombinant ACE (Wei et al., 1991b). In order to study each of the catalytic domains of the enzyme, deletions or point mutations were introduced in these domains and transfected into CHO cells. It was found that both domains are catalytically active and can cleave Hip-His-Leu and angiotensin I. Also, both domains have an absolute requirement for zinc, are activated by chloride, are inhibited by specific inhibitors and can function independently. There are differences in the catalytic activity of the two domains as evidenced by differing catalytic constants and differing patterns of chloride activation. For example, at high chloride concentrations the C domain hydrolyses substrates faster than the N domain. The most likely residues for zinc binding are His-361,365 and His-959,963 and Glu-362 in the N domain and Glu-960 in the C domain. A rigorous re-analysis of the Zn^{2+} stoichiometries of somatic and testis ACE revealed that, contrary to earlier data, somatic ACE indeed contains 2 mol Zn^{2+} per mol enzyme, whereas testis ACE contains 1 mol/mol Zn^{2+} (Ehlers and Riordan, 1991). Furthermore, an extensive survey of diverse conventional and unconventional peptide substrates, indicated that with the notable exception of luteinising hormone-releasing hormone (LH-RH), virtually all peptides are hydrolysed identically by the somatic and testis isozymes. LH-RH, however, is hydrolysed significantly faster by somatic ACE, particularly by an unusual, N-terminal, tripeptidyl endopeptidase cleavage, which is 300-fold faster than for testis ACE (Ehlers and Riordan, 1991). These studies strongly suggest that somatic ACE contains two active sites, of which the C-terminal site - identical to the testis ACE site - hydrolyses "conventional" substrates, whereas the N-terminal site may confer an additional, as yet unidentified substrate specificity (Ehlers and Riordan, 1991; Wei et al., 1991b).

It appears that circulating ACE levels may be under genetic control (Rigat et al., 1990 and Tiret et al., 1992). Southern blot analysis of DNA from a group of subjects revealed a polymorphism which correlated well with serum ACE levels, highest levels of ACE being seen in subjects homozygous for the deletion of approximately 250bp. Intermediate ACE levels were noted in those heterozygous for the polymorphism and lowest levels corresponded to those subjects in which both alleles contained the 250bp fragment. It seems therefore that this 250bp sequence in some way either affects the proteolytic cleavage of the enzyme from endothelial surfaces or affects its stability in the circulation. This polymorphism has been shown to be a risk factor for myocardial infarction (Cambien et al., 1992). More recently, this polymorphism has been found to be associated with left ventricular hypertrophy (Schunkert et al., 1994), idiopathic or dilated cardiomyopathy (Raynolds et al., 1993) and to determine the expression of ACE in T-lymphocytes (Costerousse et al., 1993). It has also recently been determined that ACE DNA polymorphisms in spontaneously hypertensive rats are related to blood pressure and that ACE is a candidate gene for primary hypertension (Hilbert et al., 1991 and Jacob et al., 1991).

1.7. ACE INHIBITORS

From the earliest studies it was noted that ACE could be inhibited by metal chelators such as EDTA. This allowed the metalloenzyme properties of ACE to be defined as has been detailed (Section 1.2.3.).

The ability of an extractable factor from the venom of the South American Pit Viper (*Bothrops jararaca*) to enhance the action of bradykinin was discovered by Ferreria (1965) and was named Bradykinin Potentiating Factor (BPF). Other snake venoms from the *Bothrops* species were also found to contain this activity. BPF also inhibits ACE in a dog lung homogenate (Bakhle, 1968) thus unifying the fields of ACE and bradykinin research.

1.7.1. Miscellaneous peptide inhibitors

A number of molecules including albumin and its fragments (Klauser et al., 1979), fibrinolytic fragments (Saldeen et at., 1981), insulin and the beta-chain of insulin (Igic et al., 1972) can act as non-specific inhibitors of ACE *in vitro*. Also, any one substrate or its split products can competitively inhibit an enzyme's action on another substrate. Hence, angiotensin I, bradykinin and Hip-Phe-Arg are to varing degrees inhibitory of the action of ACE on Hip-His-Leu (Cheung et al., 1980). Angiotensin homologues, such as angiotensin II and III, and [des-Asp^1] angiotensin I and angiotensin analogues are all inhibitory toward ACE (Tsai and Peach, 1975 & 1977; Chiu et al., 1976).

1.7.2. Multibinding active site-directed reversible inhibitors.

Although the snake venom peptides are potent and specific inhibitors of ACE, they are not ideal inhibitors of the enzyme due to their large size and limited bioavailability. Since no knowledge was available on the catalytic mechanism of ACE, a more complete active site model had to be found in order to design and synthesise specific and potent active site-directed inhibitors.

Carboxypeptidase A, whose active site and catalytic mechanism are well understood (Quicho and Lipscomb, 1971) is, like ACE, is a zinc metalloenzyme with similarities in substrate specificities in that it requires a free C-terminal carboxyl group. Thus a hypothetical model was proposed in which ACE was considered to be a kind of dipeptide-releasing carboxypeptidase posessing an active site and catalytic mechanism similar to that of carboxypeptidase A (Cushman et al., 1977 & 1978; Ondetti et al., 1977a & 1977b). Important components of this model were a site for the binding of the charged C-terminal carboxyl of the substrate, most likely the cationically charged side chain of an arginine residue, a binding site for the C-terminal dipeptide of the substrate, recognition sites for substrate amino acid side chains and a catalytic zinc atom located opposite the penultimate peptide bond of the substrate. Susequently the validity of this model was confirmed by the finding of arginine, glutamic acid and tyrosine at the active site of ACE and also the presence of a zinc atom. A molecule capable of interacting with all these binding sites would be a potent inhibitor. Support for this idea was provided by the finding that carboxypeptidase A can be inhibited by a potent competitive inhibitor D-2-benzylsuccinic acid (Byers and Wolfenden, 1972 & 1973). Thus a similar succinyl containing molecule was synthesised specifically to inhibit ACE. It was realised at this stage that the succinyl group specifically interacts with the zinc atom of the enzyme, a feature fundamental to the action of the active site directed inhibitors. The first such molecule to be produced was succinyl-L-proline, a weak but specific ACE inhibitor.

Amongst other compounds studied is one which contains terminal dipeptide sequences of the snake venom inhibitor BPP_{5a} (a bradykinin potentiating peptide) (Ondetti et al., 1971, 1972). The knowledge that the carboxyl group of the carboxyalkanoyl chain interacts with the zinc atom gave rise to compounds incorporating a sulphydryl group, known to interact stongly with zinc. This resulted in a group of inhibitory compounds the most potent of which was designated SQ 14,225 or captopril. Captopril is a specific inhibitor of ACE with a K_i = 1.7nM for rabbit pulmonary ACE. It does not inhibit either carboxypeptidase A or B (Ondetti et al., 1979), carbonic anhydrase or liver alcohol dehydrogenase (Ondetti and Cushman, 1981). Leucine aminopeptidase, a zinc containing enzyme, is inhibited by captopril with an IC_{50} of 5.4 µM (Cushman et al., 1977). When smooth muscle preparations were exposed to concentrations of captopril 20000 times higher than that required to inhibit ACE, there was no alteration in the response to angiotensin II, acetylcholine, norepinephrine, isoproterenol, dopamine, serotonin, histamine, prostaglandins, nicotine or barium ion (Rubin et al., 1978).

Although clearly effective in enhancing the inhibitory action, the presence of a sulphydryl group such as found in captopril is possibly not ideal in the *in vivo* setting in which the sulphydryl group could contribute to undesirable side effects. The sulphydryl group is also unstable in that it can undergo facile oxidation and disuphide exchange reactions (Patchett and Cordes, 1985). Patchett et al. (1980) re-examined the alkanoyl amino acid 2-methylglutaryl proline, first described by Cushman et al. (1977), for ways to improve its inhibitory potency. Captopril does not make use of two of its potential binding sites: the S_1 binding site and a hydrogen bonding site for the amide nitrogen of the normally scissile peptide bond. A range of synthetic N-carboxyalkyl dipeptides were examined in an attempt to utilise these extra sites and two of the most potent inhibitors were, N-[(S)-1-carboxy-3-phenylpropyl]-L-alanyl-L-proline (enalaprilat) and N-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline (lisinopril), both with an IC_{50} of 1.2×10^{-9} M. It is interesting that enalaprilat closely resembles the C-terminal tripeptide sequence of the snake venom peptide BPP_{5a} which, with an $IC_{50} = 3.6\text{x}10^{-6}$ M, is 1000 fold weaker than the IC_{50} of enalaprilat or lisinopril. If the metal binding carboxyl group is removed from enalaprilat or lisinopril, the IC_{50} approximates that described for BPP_{5a} (Patchett et al., 1980).

Rigorous kinetic methods were utilised to define some of the fundamental properties of the enzyme inhibitor complex by examining the interaction between purified rabbit ACE and enalaprilat, lisinopril, captopril and certain other analogues (Bull et al., 1985; Riordan and Shapiro, 1984a and 1984b). In summary their findings were;

1. These inhibitors are slow binding but tight binding. (Slow binding is observed at low substrate concentrations and inhibition can still be meansured in the absence of substrate and is still slow. Tight binding implies that substantial inhibition is obtained at concentrations of inhibitor similar to those of enzyme).

2. As it could not be shown that initial reaction rates were either independent of inhibitor concentration or decrease with increasing inhibitor concentration, two possible mechanisms are suggested. Either there is slow formation of an enzyme inhibitor complex or a two stage process exists in which competitive inhibition results in the fast formation of an initial enzyme inhibitor complex followed by a rate determining rearrangement of this complex to form a second more stable complex.

3. The mechanism of inhibition is defined as competitive as the rate constants for the formation of the enzyme inhibitor complex decrease with increasing substrate concentration.

4. The enzyme inhibitor complexes posess extremely low dissociation constants resulting in long half lives.

5. In support of the already described function of the metal-binding carboxylate in the overall binding affinity of enalaprilat it is not suprising that the affinity of enalaprilat for ACE in which the zinc has been removed by dialysis against EDTA, is 20000 times reduced as compared to the holoenzyme. The zinc deficient apoenzyme still binds substrate confirming that zinc is not involved in substrate binding but in the hydrolytic step of catalysis.

6. Chloride affects the binding affinity of enalaprilat to ACE: in the absence of chloride binding is 0.5% of that found at saturating levels of chloride ion.

7. Binding is pH dependent and is maximal at pH 6.0, suggesting that either the protonation state of the secondary nitrogen atom of the inhibitor or that ionisations of the enzyme alone determine binding affinity.

Subsequently, many other ACE inhibitors have been synthesised which, although posessing different groups, probably all interact with ACE in a manner similar to that originally proposed by Cushman, Ondetti and their colleagues.

Chapter 2

PURIFICATION OF ANGIOTENSIN CONVERTING ENZYME FROM TISSUE

2.1. OBJECTIVE:

To establish a quick, effective method to purify ACE from human and rat tissues.

2.2. INTRODUCTION

Historically, the purification of ACE from tissue has always been a difficult task (Soffer, 1981) despite its abundance in certain tissues. In real terms ACE is present in very small amounts in relation to other tissue proteins, for example, in rabbit lung, ACE constitutes only 0.1% of total protein (Soffer, 1981) while in rabbit serum ACE constitutes only 0.0017% of protein (Soffer, 1981). Therefore, in order to purify the enzyme to homogeneity, purifications of at least 1000 fold must be achieved for the lung enzyme and 60000 to 100000 fold for serum ACE.

Despite these difficulties, ACE has been successfully purified from many different species and tissue sources. Since this study is primarily concerned with rat ACE, and to a lesser degree human ACE, I shall concentrate my dicussion on purification of ACE from these species.

For most species, the lung has been the most favoured source of ACE where it is relatively abundant due to its endothelial localisation in the extensive pulmonary vasculature. This has been documented in the rat in which ACE specific activity comparisons between various tissues (Cushman and Cheung, 1971) support the lung as being one of the best tissue sources of the somatic enzyme. In contrast, in humans, despite the high ACE levels in lung tissue, a similar homogenate of the kidney cortex contains approximately 6 times more enzyme than the lung (Stewart et al., 1981).

ACE is a membrane bound enzyme, necessitating some form of solubilisation in order to release it from the plasma membrane. Although in some cases this has not found to be required eg. in rat lungs as much ACE is released by allowing the homogenate to stand for 20 days at 2°C as after treatment with deoxycholate (Lanzillo and Fanburg, 1974). Most purifications have, however, relied on the use of some solubilising agent such as the ionic detergent deoxycholate or non-ionic detergents such as Triton X-100 and Nonidet P-40. Enzyme released through the use of detergents retains the hydrophobic anchor peptide and thus has a tendency to aggregate, which can be problematic during gel filtration. An alternative is to trypsinise the homogenate (Nishimura et al., 1976), releasing soluble, hydrophyllic enzyme devoid of the anchor peptide. Human enzyme purified by both these methods were indistinguishable physicochemically, including molecular weight, isoelectric point, catalytic and immunological properties (Lanzillo et al., 1985).

Initially ACE purification relied on homogenisation, solubilisation and conventional chromatography. These included one or more ion exchange steps on resins such as DEAE-cellulose which retains ACE as it is an acidic protein. Further purification has relied on hydroxyapatite or calcium phosphate to which ACE does not adsorb. Gel filtration on resins such as Sephadex G-200 has also been employed. Conventional chromatographic methods, however, are laborious and do not result in good yields of active ACE.

The development and use of various affinity chromatographic methods resulted in improved yields and specific activities of the purified enzyme.

The first of these methods to be employed was immunoaffinity chromatography in which antibody against ACE (previously purified by conventional means) was coupled to a solid phase. In this manner ACE was purified 60000 fold from rabbit serum (Das et al., 1977). Problems of this method include: 1.) the necessity to first purify the antigen by conventional chromatographic means; 2.) antibodies coupled to a solid phase can act as ion exchange ligands resulting in a high degree of non-specific binding; and finally, 3.) the strong binding between antigen and antibody neccessitates harsh elution conditions, resulting in some denaturation of the purified enzyme. In an attempt to avoid this problem of denaturation, baboon ACE was purified by conventional means, used to raise antibodies which were utilised as an immunosorbent for the purification of human lung ACE (Lanzillo et al., 1980). Because of the reduced affinity of anti-baboon ACE antibodies for human ACE, less harsh elution conditions were required, resulting in maintenance of specific activity.

Another ingenious method was that of reverse immunoadsorbtion (Weare et al., 1982). After conventional methods had been used to partially purify human lung ACE, it was subjected to chromatography on an immunoadsorbent of anti-human lung ACE antibodies - the contaminant proteins not bound to the column were collected and antibodies against this fraction were raised. An immunoaffinity column was then made with these anti-contaminant antibodies. When partially purified human kidney ACE was applied to this column, gentle elution conditions resulted in ACE of high specific activity in high yields.

These laborious methods could only be replaced with the advent of specific competitive inhibitors of ACE which, when used as affinity ligands, greatly simplified ACE purification.

The development of potent site-directed inhibitors such as captopril, prompted various workers to employ these and related analogues as affinity ligands.

Dipeptide analogues of captopril such as L,D-cysteinyl - L - proline were (Harris et al., 1981a) coupled to CNBr-activated Sepharose, either directly or via a short spacer arm. In both cases the capacity of the gel for ACE was low, 0.025U/mg gel, which was much improved, to 1.23 U/mg gel, when a longer spacer arm was used (Harris et al., 1981b). This method can be used to purify human serum ACE 130000 fold with a 20% yield. The problem with this method was that pre-affinity columns necessary for optimum results included two polyethyleneglycol precipitation steps and two ion-exchange column steps.

The development of the N-carboxyalkyl dipeptide ACE inhibitors such as enalaprilat and lisinopril heralded a great advance in ACE purification. When lisinopril is coupled to a matrix by a 12-atom spacer arm it was found to have a capacity of 750µg ACE/ml gel (Bull et al., 1985a). This affinity resin was used to purify ACE from human lung and serum but, because free lisinopril was used as the eluant it necessitated 10-14 days of dialysis against EDTA, lowering overall yeilds to 1.15% and 4% respectively.

Captopril as an eluant improved the yields of ACE from a variety of human tissues markedly as the captopril could be chemically destroyed using N-ethylmaleimide (Lanzillo et al., 1985).

The importance of a long spacer arm and the use of a less potent ACE inhibitor as a ligand, thus avoiding elaborate elution and reactivation strategies, was used for the purification of rabbit lung ACE (Pantoliano et al., 1984). These authors synthesised two enalaprilat analogues, N-[1(S)-carboxy-5-aminopentyl]-L-PheGly (CA-PheGly) and N-[1(S)-carboxy-5-aminopentyl]-DL-Ala-L-Pro (CA-AlaPro). In both cases the use of a long spacer arm resulted in capacities of greater than 7mg ACE/ml gel. It was also found that elution could be simply effected by raising the pH from 6 to 8.9 resulting in homogenous enzyme with an overall yield of 34%. Ehlers et al. (1986) synthesised and utilised a related ligand N-[1(S)-carboxy-5-aminopentyl]-GlyGly (CA-GlyGly) resulting also in a facile purification protocol for human kidney ACE with high yields.

It was subsequently found that lisinopril bound via a long spacer arm allowed elution by a change in pH (Bernstein et al., 1988; Ehlers et al., 1989) The more potent inhibitory nature of lisinopril is particularly useful in the purification of ACE from sources with a low ACE concentration. Another advantage is that the leaching of bound enzyme from the column during the pre-elution washing steps is minimal when compared to that found for a ligand such as CA-GlyGly.

2.3. MATERIALS AND METHODS

HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), hippuryl-L-histidyl-L-leucine, o-phthaldialdehyde, L-histidyl-L-leucine, PMSF (phenylmethylsulphonyl flouride) and trypsin were obtained from Sigma. Tris(hydroxymethyl)-aminomethane (TRIS), acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'- methylethylenediamine (TEMED) and bromphenol blue were obtained from BDH. Sodium dodecylsulphate (SDS), sodium azide and Comassie Brilliant Blue R250 were from Merck. Trysylol (Apoprotinin; 100000 K.I.U./10ml) from Beyer, ammonium persulphate from AAL Chemicals, human serum albumin (>99.5% pure) from Miles laboratories. Captopril was a gift from Squibb laboratories, South Africa and enalapril and enalaprilat from Logos pharmaceuticals, South Africa. Lisinopril (Merck, Sharpe & Dohme) attached via a spacer arm to epoxy-activated Sepharose 6B (Pharmacia) was a gift from Dr. Mario Ehlers (Boston, USA).

2.3.1. Tissue

Human ACE was purified from human cadaver kidney tissue which was obtained within six hours of death. The tissue was either immediately processed, as described below, or stored at -70°C until required.

Rat ACE was purified from rat lung tissue. Long-Evans rats (250-400g) were sacrificed by ether anaesthesia, the lungs excised, rinsed in saline and either used immediately or stored at -70°C till required.

2.3.2. Purification

Since purified detergent solubilised ACE is an unstable antigen due to its tendency to aggregate, causing many problems in its use in the immunoassays described below, the tissue was trypsinised to yield soluble ACE.

Fresh or thawed tissue was homogenised with an Ultra-Turrax homogeniser (Model TP 18/2, Janke and Kunkel KG) on ice in 5mM Tris/HCl, pH 7.4, at 4°C to yield a 20% w/v homogenate. This was strained through cheesecloth to remove fibrous tissue and homogenised with glass-teflon homogeniser

(Thomas, USA). The homogenate was centrifuged at 20000g (Sorvall RC-5 Superspeed centrifuge in Sorvall GSA rotor) for 30 minutes at 4°C and the resulting pellet resuspended in 5mM Tris/HCl, pH 7.4, with a glass-teflon homogeniser. This homogenate was again centrifuged at 20000g for 30 minutes at 4°C. The pellet was resuspended in 50mM Tris/HCl, pH 8.5, with a glass-teflon homogeniser to the same volume as the original homogenate.

The protein content was determined by reading the OD (optical density) at 280nm and 260nm and the approximate protein concentration calculated by the formula;

$$[\text{Protein}] = (1.55 \times OD_{280}) - (0.76 \times OD_{260})$$

in mg/ml

Trypsin was added at 10 mg trypsin per gram protein and incubated at 37°C in a shaking water bath for 60 minutes. Digestion was stopped by addition of 1μl 1M PMSF in dimethylsulphoxide (DMSO - Sigma) per ml solution and Trasylol 1ml per 100 ml homogenate, followed by centrifugation at 28000g (Sorvall SS-34 rotor) for 30 minutes at 4°C. The supernatant was placed in dialysis tubing and dialysed at 4°C against 3 x 5 litres 20mM Hepes/NaOH, 0.5M NaCl, pH 7.5 at 21°C. The dialysis buffer was constantly stirred with a magnetic stirrer allowing each dialysis to be completed within four hours.

The lisinopril-Sepharose column was equilibrated with at least 5 column volumes of 20mM Hepes/NaOH, 0.5M NaCl, pH 7.5 at 21°C at a flow rate of 10 ml/hr. The dialysed supernatant was vacuum filtered through a 0.8μm Millipore filter and loaded onto the column at a flow rate of 10 ml/hr. The column was then washed with 10 column volumes of equilibration buffer.

ACE was eluted with 50mM Borate/NaOH pH 9.5, 21°C while monitoring A_{280}. Approximately 3ml fractions were collected and protein measured with the Biorad assay (Bradford, 1976) and ACE activity with the spectroflurometric method (Friedland and Silverstein, 1976). The method is fully detailed in the appendix.

2.3.3. Characterisation of ACE

2.3.3.1. Purity

The purity of the purified enzyme was determined by its specific activity and its electrophoretic homogeneity. Specific activity was determined for the substrate HHL and expressed as Units of activity per mg of protein. Protein concentration was determined by the method of Bradford (1976) using Bio-Rad commercial reagent and human serum albumin as standard.

2.3.3.2. Molecular weght determination

Molecular weight and homogeneity of purified ACE was determined by polyacrylamide gel electrophoresis. All gels contained 0.1% SDS and were run using a discontinuous buffer system (Laemmli, 1970). Protein was analysed on both 7% homogeneous and 3 to 17.5% gradient polyacrylamide gels, at 50-60V for approximately 18 hours. Between 5 and 10 µg ACE was loaded per lane in both a reduced (incubated for 15 minutes in 2% SDS and 2% 2-mercaptoethanol at 60°C prior to loading) and non-reduced states. After electrophoresis, proteins were stained with Comassie Brilliant Blue. Details of the methodology are to be found in the Appendix. A calibration curve was constructed for molecular weight markers (Amersham Rainbow Markers) on 2 cycle semi-logarithmic paper, by plotting molecular weight versus relative mobility.

2.3.3.3. Inhibition by captopril

Utilising the spectrofluorimetric assay of HHL hydrolysis described in the Appendix (section 9.7), the influence of the specific ACE inhibitor captopril on the catalytic activity of the purified enzyme was assessed. Captopril was added to the assay mixtures at a range of concentrations (0.92-55.3 nM).

2.4. RESULTS AND DISCUSSION

2.4.1. Rat Lung ACE

Typically 40 pairs of rat lungs purified by the trypsin/lisinopril-Sepharose protocol yielded approximately 5 mg of pure ACE with a specific activity of 91 U/mg protein. When the pre- and post-column specific activities were compared, it was found that almost 90% (eg.120 U loaded, column eluate contained 14 U therefore 106 U retained) of loaded ACE activity was retained on the column. Very little ACE leached from the column during the washes resulting in the purification of significant quantities of homogeneous enzyme. A table of yields at stages of purification is in appendix 9.10.

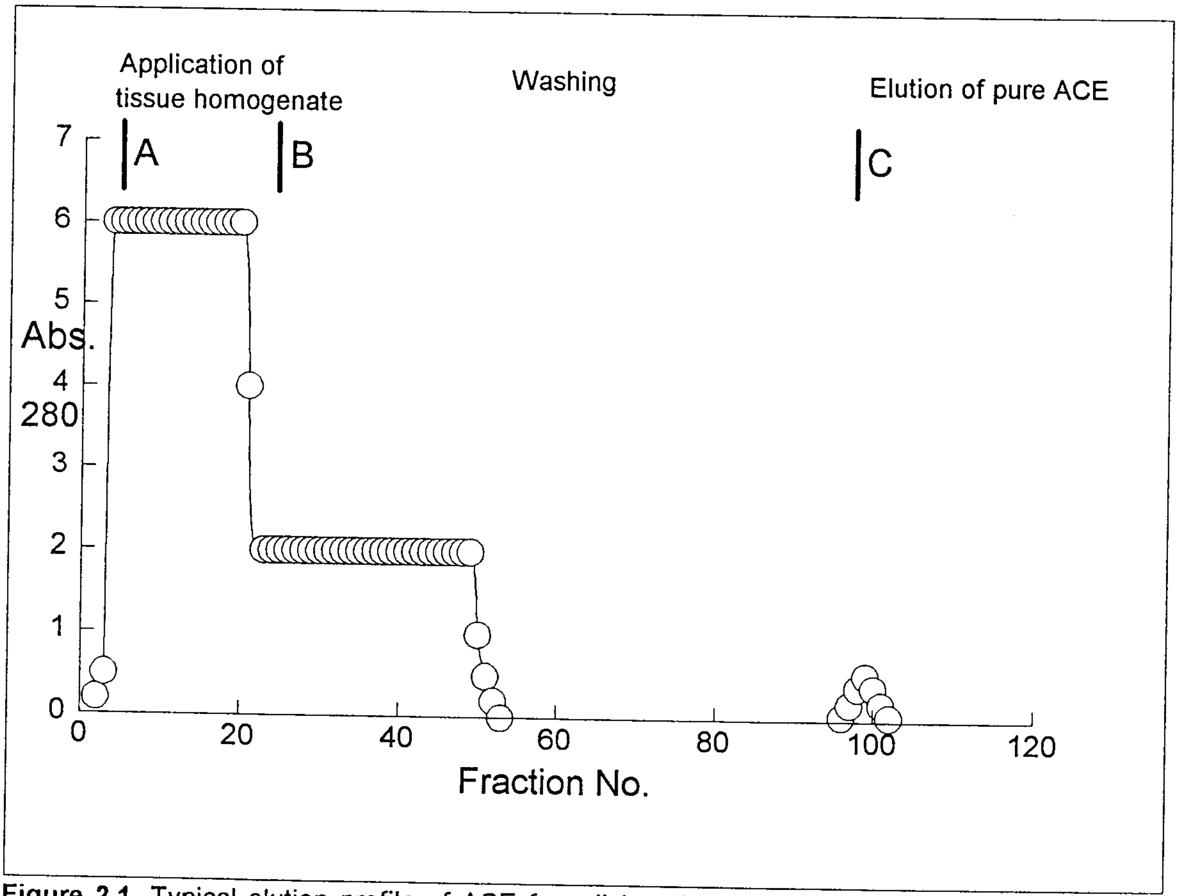


Figure 2.1. Typical elution profile of ACE from lisinopril column. High absorbances (A to B) correspond to the loading of the homogenate to the column in 20mM Hepes/NaOH, 0.5M NaCl, pH 7.5. After washing the column in the same buffer (B to C), pure ACE was eluted by application of 50mM Borate/NaOH pH 9.5 to the column (after C).

2.4.2. Human Kidney ACE

Similar results were obtained for ACE from human cadaver kidneys. Typically 200g of homogenised kidney tissue yielded at least 4mg of homogeneous ACE with a specific activity of 97 U/mg protein comparing favourably with previous reports from the literature.

2.4.3. Molecular weight determination

An example of PAGE analysis of purified soluble rat lung ACE is shown in the accompanying figure. As was also found for human ACE, single bands of homogenous ACE were revealed by Comassie Brilliant Blue staining. It must be realised, however, that some contaminating glycoproteins may exist that do not stain with Comassie Blue (Soffer, 1976). Molecular weights of both human kidney and rat lung ACE were approximately 140000. The glycoprotein nature of ACE does affect the mobility of the enzyme on polyacrylamide gels and thus these molecular weights can only be approximated.

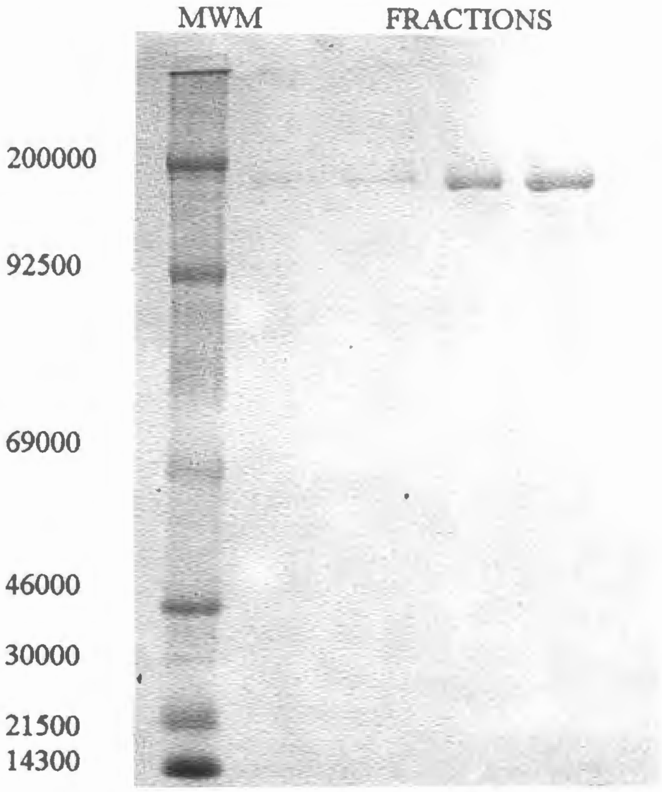


Figure 2.2. Polyacrylamide gel electrophoresis of purified rat lung ACE. The lane marked MWM is the molecular weight marker and those marked fractions correspond to samples of fractions during enzyme elution from the lisinopril - Sepharose column.

2.4.4. Inhibition by captopril

Further proof that the purified protein was indeed ACE was obtained by demonstration of the inhibitory effect of captopril, a specific ACE inhibitor, in the spectrofluorimetric assay. The purified enzyme was sensitive to captopril. Using the method of Dixon (method detailed in appendix 9.11), K_i values of approximately 3nM were obtained which is in good agreement with previously published data for rat lung ACE (Strittmatter et al., 1985).

2.5. CONCLUSION

A rapid and facile method has been presented to allow the purification of high specific activity homogeneous ACE from rat lung or human kidney tissue. Highly purified ACE could be prepared by lisinopril-sepharose chromatography of a partially purified trypsin solubilised tissue extract. ACE was eluted with a high pH buffer as described in Methods and the entire procedure can be completed within two days. This contrasts with earlier multiple stage conventional chromatographic methods and is amongst the fastest of the affinity methods.

This method allows the purification of enzyme from a variety of sources and should prove useful in any experiments studying the enzyme. As will be described in the following chapters, ACE purified in this way was used as an antigen to raise polyclonal and monoclonal antisera and also as standards in radioimmunoassays and ELISAs (enzyme linked immunosorbent assays).

Chapter 3

PRODUCTION OF ANTIBODIES TO HUMAN AND RAT ACE.

3.1. OBJECTIVE

Since no commercial source for ACE antisera was available, our own polyclonal and monoclonal antisera had to be produced. These were required for ACE immunoassays and immunohistochemistry.

3.2. INTRODUCTION

Production of antisera to ACE has been described in the literature. One of the earliest reports was the production of a polyclonal antiserum in goats to rabbit ACE (Caldwell et al., 1976). This antibody was used for the immunohistochemical localisation of ACE in a variety of organs. Goat anti-pig ACE antibodies (Hall et al., 1976) were subsequently utilised in a radioimmunoassay for pig ACE (Odya et al., 1979), and rabbit antiserum to human ACE has been used (Takada et al., 1981) in a radioimmunoassay for human ACE (Alhenc-Gelas et al., 1983).

The production of monoclonal antibodies has also been reported. In 1984, Moore et al. (1984), developed monoclonal antibodies to rat lung ACE and studied their *in vitro* and *in vivo* binding to ACE by immunohistochemistry. Monoclonal antibodies to human lung ACE have also been produced (Danilov et al., 1987a) and utilised for immunohistochemical localisation of ACE (Danilov et al., 1987b), the radioimmunoimaging of lung blood vessels (Danilov et al., 1989) and recently in an ELISA for human ACE (Stevens et al., 1990).

3.3. PRODUCTION OF POLYCLONAL ANTISERA

3.3.1. Materials

ACE was purified to homogeneity as described in the previous chapter. Antisera were raised in New Zealand white rabbits obtained from the Animal Unit at the University of Cape Town Medical School. Protocols for the utilisation of animals were first submitted for the approval of the University Animal Research Review Committee. Freund's complete and incomplete adjuvant was obtained from Difco. Ammonium sulphate was from Sigma and a Hitachi Himac centrifuge was used in the purification of the antisera.

The ELISA utilised 96 well strips and frames from Nunc. NaCl, Tris, Triton X-100, 2,2' azino-di-(3-ethylbenzthiazoline sulphonic acid (ABTS), citric acid monohydrate were all obtained from Sigma. Tween-20 was obtained from Bio-Rad, goat anti-rabbit peroxidase conjugate from Amersham and fetal calf serum (FCS) from Highveld Biologicals. Plates were read in a Titertek Multiscan MCC-340 ELISA plate reader.

3.3.2. Methods

Purified ACE (50µg) in normal saline was thoroughly mixed with complete Freund's adjuvant to form a stable emulsion using the double hubbed needle method (Berlin and McKinney, 1958) and administered at multiple sites intradermally. Three weeks later a booster dose of ACE (25µg) was administered with Freund's incomplete adjuvant in the same manner. Booster injections were repeated at intervals until a sufficient titre of anti-ACE antibodies was detected in samples of blood obtained from ear vein bleeds, utilising a simple ELISA method described below. At this point serum was obtained from the rabbits and stored at -70°C until used.

For most purposes the antiserum obtained required further purification. This was achieved by ammonium sulphate fractionation. A saturated solution of ammonium sulphate was prepared by adding 800g to 1 litre of distilled water at 35°C which was then stirred for 2 hours. The temperature was then lowered to

20°C with continued stirring overnight. The pH of the solution was slowly adjusted to between pH 7 - 8 with ammonium hydroxide after the testing of small aliquots of the solution to avoid errors in pH measurements of the saturated solution.

Precipitation of immunoglobulins in the antiserum was achieved by the addition of the saturated ammonium sulphate solution to a final concentration of 33%. This was stirred for an hour at room temperature and centrifuged at 4000g for 15 minutes. The pellet was redissolved in phosphate buffered saline (pH 7.4) (PBS) to the original volume and reprecipitated and centrifuged as before. The resulting pellet was redissolved in PBS and the excess salt removed by gel filtration on a pre-packed Sephadex G-25 column. Complete removal of the ammonium sulphate was demonstrated by the absence of a precipitate in a small quantity of the purified preparation on the addition of Barium Chloride.

3.3.2.1. Assessment of the antibody titre

In order to assess the titre of the polyclonal antiserum to ACE, an ELISA method was employed. This allowed the test bleed from the rabbits to be tested to determine when sufficient antibody titre had been achieved. Blood was collected in serum separation tubes (SST, Vacutainer), allowed to clot for 30 mins at room temperature and centrifuged at 1000rpm for 5 mins to obtain serum.

The ELISA plate wells were prepared as follows; Purified ACE was diluted in 10mM Na_2HPO_4, 150mM NaCl (pH9) (PBS-9) at a concentration of 5µg/ml. 50 µl was dispensed into each well and the antigen was allowed to adsorb overnight at 4°C. The wells were flicked dry, washed six times with PBS-9, filled with 200µl of blocking buffer (PBS-9 with 1% FCS) and incubated at room temperature for 20 minutes. The wells were emptied and 50µl of doubling dilutions of the rabbit serum diluted in blocking buffer (initial dilution was 1:50 then 1:100, 1:200, 1:400 and so on to 1:6400) were added and incubated at room temperature for an hour. The wells were flicked dry and washed ten times with TST (0.05M Tris/HCl pH8, 0.10M NaCl and 0.05% Tween-20). After drying, the wells were filled with 50µl of 1:100 goat anti-rabbit peroxidase conjugate diluted in ELISA diluent (0.5M NaCl, 0.5% Triton-X100 and 0.01M Na_2HPO_4) and incubated at room temperature for 30 minutes before being washed twice with TST.

The 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate was then added which was prepared as follows; stock solutions of 40mM ABTS in distilled water were stored at -20°C. When required, an aliquot was thawed and diluted 1:100 in citrate buffer (525mg citric acid monohydrate dissolved in 50ml distilled water, pH 4 with NaOH) - to the ABTS dilution was added 0.01/volume of 30% hydrogen peroxide. 100µl of the substrate solution was added to each well and incubated for 30 minutes at room temperature before the absorbances were read in a ELISA plate reader at 414nm.

At each incubation step the plate was wrapped in clingwrap to prevent evaporative losses.

3.3.2.2. Assessment of antibody specificity by Western Blotting

The specificity of the antibody for ACE was assessed by Western blotting (Towbin et al., 1979). This technique allows protein to be electrophoretically transferred to a nitrocellulose membrane which, after blocking non-specific binding, is probed with a specific antibody. Binding of this antibody is detected using a peroxidase conjugate of an antibody directed against the first antibody. The presence of peroxidase is determined by incubation with a peroxidase substrate, 4-chloro-1-naphthol with hydrogen peroxide, to yield a black insoluble precipitate on the membrane.

Duplicate lanes of a SDS-PAGE gradient (3-17.5%) gel were loaded with ACE to provide a seperate lanes for staining and blotting. Purified ACE was denatured in 2-mercaptoethanol and boiled prior to loading.

One gel section was stained and the other was electroblotted onto a piece of nitrocellulose membrane (Immobilon) using an electroblotting apparatus (G.T. Series Gel Electrophoresis unit with a Transphor Power-Lid model TE 50 from Hoefer Scientific Instruments, San Fransisco) placed in an ice-filled polystyrene cool box. The unit was filled with protein transfer buffer (0.025M Tris-HCl, pH

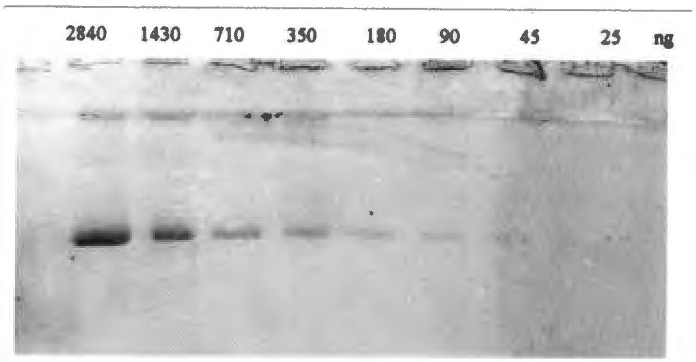
8.8, 0.20M Glycine, 0.1% SDS and 20% [v/v] Methanol). Blotting was performed at 400mA for 2 hours in a cold room at 4°C.

The membrane was removed and blocked with 1% foetal calf serum, 0.3% Tween-20 in 0.2M NaCl, 0.05M Tris/HCl pH 7.4 (blocking buffer) for one hour. Polyclonal antibody was diluted in the blocking buffer at a dilution of 1:500 and incubated with the membrane for 1,5 hours at room temperature. After the membrane was washed six times with 0.05% Tween-20 in 0.2M NaCl, 0.05M Tris/HCl pH 7.4 (Tris buffered saline), it was incubated for one hour with a 1:500 dilution (in blocking buffer) of goat anti-rabbit peroxidase conjugate (Amersham). The substrate, 4-chloro-1-naphthol 24mg was dissolved in 8ml methanol, to which was added 40ml of Tris buffered saline and 5µl hydrogen peroxide. The membrane was transferred into this solution and once the colour had developed sufficiently, the membrane was rinsed in water and dried onto graphic arts film in the dark.

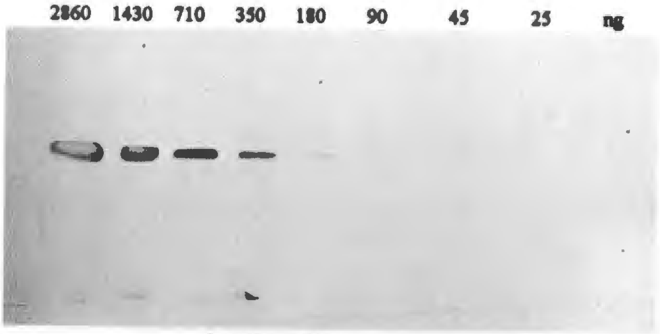
3.3.3. Results

An adequate titre of polyclonal antibody, as assessed by the ELISA assay, was achieved after four booster innoculations. At this point the animal was bled and the serum separated by centrifugation. The serum was either purified by ammonium sulphate precipitation or stored in its crude state at -70°C using 0.02% sodium azide (Merck) as a preservative. Rabbit No.217 was the source for the anti-human ACE antibody and rabbit No.556 the source of the anti-rat ACE antibody.

Antibody specificity was assessed by Western blotting. Doubling dilutions of purified human kidney ACE were loaded into wells of a SDS-PAGE gel and blotted. As little as 180ng ACE could be detected by a 1:500 dilution of the rabbit anti-human ACE antiserum (see figure).



Polyacrylamide gel



Western Blot

Figure 3.1. Upper panel. Polyacrylamide gel electrophoresis of doubling dilutions of purified human kidney ACE. Lane 1 = 2860ng ACE, lane 2 = 1430ng, lane 3 = 710ng, lane 4 = 350ng, lane 5 = 180ng and so on to lane 8.

Figure 3.2. Lower panel. Corresponding western blot of the above gel. The lowest amount of ACE detected by the polyclonal antiserum is to be found in lane 5 ie. 180ng.

3.3.4. Conclusion

Utilising the above methods, polyclonal antisera to rat and human ACE were produced. These allowed for the development of immunoassays for rat and human ACE as will be described in the following chapters.

3.4. PRODUCTION OF MONOCLONAL ANTIBODIES TO HUMAN ACE.

This section describes the development of monoclonal antibodies to human ACE, as well as the isolation of an antibody that cross-reacts with rat ACE. This allows for a continuing supply of specific antibody without the usual problems of cross-reactivity demonstrated by many polyclonal antisera. The main purpose of obtaining these monoclonal antibodies was to allow the development of sandwich ELISAs using these as second antibodies.

3.4.1. Materials and Methods

All reagents were of the highest grade obtainable. Sterile procedures were performed in a laminar flow hood (Laminair) and cell cultures were grown in a CO_2 incubator. BALB/c mice were obtained from Animal Unit at the University of Cape Town. Experimental protocols were submitted for the approval of the University Animal Ethics Committee.

3.4.1.1. Antibody production

3.4.1.2. Stock reagents

Penicillin and streptomycin (P and S) 100x was made by mixing 10000 U/ml penicillin and 10000 μg/ml streptomycin in sterile PBS (0.14M NaCl, 2.6mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4) and then frozen in aliquots.

Human umbilical cord serum (HUCS) was heated at 56°C for 30 minutes, filtered and sterilised before use.

"HT" 100x stock: To 0.68g hypoxanthine powder was added 250ml water then up to 5ml of 10N NaOH was added dropwise until dissolved. Then 0.1935g thymidine, 0.011g glycine and 5.503g sodium pyruvate was added and made up to 500ml with water. This was then filter sterilised and stored frozen at -20°C.

Aminopterin 100x stock: 25ml water was added to 1.76mg aminopterin, followed by 0.5ml of 5N NaOH. This was then neutralised with 5N HCl and made up to 100ml with water. This solution was filtered and stored, protected from light at -20°C.

3.4.1.3. *Immunisation of mice*

Mice were injected intraperitoneally with 30 µg of purified human ACE emulsified in complete Freund's adjuvant. Two weeks later the mice were re-immunised with 30 µg ACE, this time in incomplete Freund's adjuvant. After two weeks the presence of antibody was determined utilising the ELISA assay described above. Three days prior to fusion, the mice were injected intraperitoneally with 50 µg ACE in PBS.

3.4.1.4. *Fusion*

Myeloma SP_2/0Ag14 cells were grown in 2-3 100mm petri dishes. The suspension was centrifuged to pellet the cells, and the supernatant filtered through a 0.45 micron filter and stored as "conditioned medium".

10ml Versene solution (0.69mM EDTA in 0.14M NaCl, 2.6mM KCl, 1.5mM KH_2PO_4, 6mM Na_2HPO_4, pH7.4) was added to dishes containing viable myeloma cells. This was incubated at 37°C for 10 minutes before the cells were dislodged by pipetting and transferred to centrifuge tubes. Cells were pelleted at 500g for 15 minutes and resuspended in RPMI 1640 medium. The cells were counted and their viability assessed by trypan blue exclusion.

The mouse was sacrificed and the spleen removed under sterile conditions. It was placed in RPMI medium in a petri dish at 37°C and strained through a sterile stainless steel sieve into the RPMI medium. The clumps were broken up, the resulting suspension transferred to a 50ml centrifuge tube and allowed to stand for 3 minutes before the supernatant was transferred to a clean tube. This was centrifuged at 500g for 5 minutes and the pellet resuspended in 30ml RPMI. The cells were washed in RPMI once before being counted in white cell diluting fluid (2% acetic acid), which lyses red cells.

Spleen cells and SP-2 cells were mixed in a 9:1 ratio in RPMI in a 50 ml centrifuge tube which was centrifuged at 500g for 5 minutes. The supernatant was poured off leaving a little within which to resuspend the pellet. Two grams of polyethylene glycol (PEG 4000) was autoclaved and to this was added 2ml of RPMI at 37°C. This solution was added dropwise (1.5ml over 1.5 minutes) to the cell suspension with gentle mixing which was continued for a further 0.5 minute. RPMI (1.5ml) at 37°C was added slowly over 1 minute to the suspension with gentle mixing. This was repeated before a further 8ml of warm RPMI was added to the suspension over 2-3 minutes. The volume was then made up to 45ml and was centrifuged at 500g for 5 minutes and the supernatant removed.

The pellet was resuspended in HAT-HUCS-Condi medium (1ml HT / 1ml 100x Aminopterin / 5ml HUCS / 1ml 100x Pen. and strep. / 20ml of SP2 conditioned medium / 72ml RPMI) and the volume made up to 50ml. 0.5ml aliquots of this suspension was pipetted into the wells of 24 well plates to which was added 0.5ml of HAT-HUCS-Condi medium. This volume of suspension was appropriate for a yield of +10^8 spleen cells. Plates were incubated at 37°C in 5% CO_2 and 100% humidity.

Clones were allowed to grow for 10 - 14 days. When the culture medium started to turn yellow, the supernatants were tested for antibody using the ELISA technique described above (Section 3.3.2.1.).

Good positives were selected and the contents of the source wells were stored as follows. Cells were suspended in 20% heat inactivated foetal calf serum in RPMI, without antibiotics, and were transferred to cryotubes (Nunc). These were wrapped in paper towel and stored at -70°C overnight prior to being transferred to liquid nitrogen.

3.4.1.5. Cloning of cells.

Those cells selected for cloning were dislodged by pipetting and transferred to sterile tubes. Cells were counted using a counting chamber while the remaining cells were fed with HT-HUCS (1ml 100xHT / 5ml HUCS / 1ml 100x Pen. and strep. / 93ml RPMI) and returned to the incubator. Cells were diluted to give 1-2 cells/50µl.

50µl of the cell suspension was added to wells of a 96 well flat bottomed microtitre tray, followed by 50µl of medium. The presence of one cell in each well was ascertained by microscopy.

The remaining cells were frozen in a cryotube. Harvest fluid was retained for testing by ELISA and once the cells in the 24-well tray had grown these were also frozen.

The 96-well plate was incubated in a CO_2 incubator at 37°C and after 1 week 0.1ml of HT-HUCS medium was added. When this medium was noted to have yellowed it was screened for antiboby production by ELISA. Strong positives were selected and expanded to a 24-well plate. Once again a strong positive was selected and recloned, the rest being frozen as before.

When the final clones had been selected these were expanded in 100mm dishes in 10% FCS for preparation of ascites fluid. Mice were pristane primed by an intraperitoneal injection of 0.5ml pristane. Ten days later these mice received another intraperitoneal injection of 5-10x10^6 cells in 0.5ml PBS. Mice were examined daily for the development of ascites. Once this was assessed as having progressed sufficiently, the mice were sacrificed. The abdominal skin was carefully sterilised with alcohol before the abdomen was opened and the ascites aspirated with a pasteur pipette.

Cells were spun down (500g for 5 minutes), the supernatant filtered through a 0.45 micron filter and stored at -70°C.

3.4.1.6. *Maintenance of myeloma cells.*

Myeloma cells were grown in 10% FCS in RPMI containing antibiotics and passaged 3 times per week. Semi-adherent cells were dislodged by pipetting and the remaining cells nourished with fresh medium. Cells were not passaged for more than 30 days.

3.4.1.7. *Thawing of cells.*

Cells were removed from liquid nitrogen and thawed in a 37°C waterbath. The contents of the tube was then transferred to a 15ml centrifuge tube and slowly diluted by the addition of 10% FCS at room temperature. Cells were then centrifuged at 500g for 5 minutes, resuspended in fresh medium and placed in a CO_2 incubator. Cells maintained in this way were useful as a source of harvest fluid rich in monoclonal antibody.

3.4.1.8. Detection of an anti-human ACE monoclonal antibody that cross reacts with rat ACE.

In order to find a monoclonal antibody from the positive clones already obtained, the ELISA technique was utilised. Purified rat ACE was adsorbed to the wells of ELISA plates at a concentration of 5µg/ml in carbonate buffer. The assay was identical to that described earlier in this chapter except that culture medium was used undiluted in the ELISA (section 3.3.2.1.)

3.4.1.9. Assessment of antibody specificity.

The specificity of the antibodies for ACE was assessed by the technique of Western blotting. The method employed was identical to that described in section 3.3.2.2.

3.4.2. Results

Clone 14/5/16/65 ascites proved to be the most potent of the monoclonals directed against human ACE, reacting well against both kidney and serum ACE on ELISA and by Western blotting.

Clone 187/18 produced a monoclonal antibody directed against human kidney ACE with the highest degree of cross-reactivity for purified rat lung ACE as assessed by ELISA. In an ELISA in which 5µg/ml rat ACE was adsorbed to the wells of an ELISA plate an absorbance of 0.705 at 414nm was recorded versus a negative control absorbance of 0.025.

Unfortunately, although ascites from clone 187/18 was produced it proved to be of a lesser titre than the harvest fluid derived from the growing cells due to a problem later discovered to involve a less than optimal batch of SP-2 cells. This ascitic fluid was therefore retained for use in immunohistochemistry of rat ACE, while the harvest fluid was used in the ELISA assay for rat ACE. Both these methods are to be fully described in later chapters.

3.4.3. Conclusion

A panel of monoclonal antibody secreting clones was produced as a source of antibody directed against human and rat ACE. Thus an abundant supply of these two, and other clones, were available with which to engage in immunoassay and immunohistological studies of human and rat ACE.

In the following chapters the use of these polyclonal and monoclonal antisera in the development of immunoassays for ACE will be described. The anti-human ACE polyclonal antiserum was used as both an antibody in a radioimmunoassay for human ACE as well as a capture antibody in a sandwich ELISA for human ACE, utilising the anti-human ACE monoclonal as a second antibody. Similarly, the antibodies against rat ACE were used in the development of an ELISA to measure rat ACE as well as in immunohistochemistry.

Chapter 4

DEVELOPMENT OF IMMUNOASSAYS FOR HUMAN ACE.

4.1. OBJECTIVE

To develop reliable and sensitive immunoassay techniques to quantitate human ACE protein in body fluids.

4.2. INTRODUCTION

Usual methods for measuring ACE (Kessler, 1987), assess enzyme activity and employ fluorescence, chromophore- or radioactively-labelled synthetic peptide substrates. Although the clinical value of serum ACE activity measurements is well accepted, it should be recognised that in certain circumstances these may not reflect the true concentration of enzyme in the sample. ACE activity assays may provide falsely high values when other serum peptidases are present, or be falsely low in the presence of endogenous or pharmacological enzyme inhibitors, a situation more likely to occur in disease. This possibility has prompted the development of assays of ACE protein concentration, but these have not been widely applied.

Previously published methods of immunoassay for human ACE have included radioimmunoassays for human serum ACE (Alhenc-Gelas et al., 1983; Hiwada et al., 1987). A competitive ELISA or CELIA (competitive enzyme linked immunosorbent assay) has also been described and used to study human ACE (Lanzillo and Fanburg, 1982).

In order for the concentration of ACE to be measured in human sera in our laboratory, immunoassay methods were established. This chapter describes the development of both a radioimmunoassay (RIA) (SECTION A) and a sandwich ELISA for human serum ACE (SECTION B). A comparison between these assays is also made on a group of patient sera (SECTION C).

4.3. SECTION A: RADIOIMMUNOASSAY FOR HUMAN SERUM ACE.

4.3.1. Materials and methods

Early efforts in the establishment of this assay involved attempts to utilise detergent-solubilised, purified human kidney ACE as the standard. This, however proved to be extremely unreliable as this hydrophobic antigen tended to aggregate in solution, providing inconsistent results. For this reason, and because the assay was to be used to assay the soluble form of the enzyme as found in serum, trypsin-solubilised human kidney ACE was found to give reliable and reproducible results.

ACE proved to be susceptible to damage during labelling with ^{125}I. Chloramine-T labelling was attempted according to the method of Greenwood et al. (Greenwood et al., 1963) but this provided an extremely poor tracer, probably due to extensive denaturation of ACE. Lactoperoxidase labelling was also attempted, both as described in the method of Lanzillo and Fanburg (1982) and using Enzymobeads (Biorad) according to the manufacturers directions. Although this was a better tracer than chloramine-T, it still was not optimal.

The lodogen method (Salacinski et al., 1981), which labels proteins extremely gently, thereby minimising the effects of oxidation of the antigen was then employed.

The RIA involved the purification of ACE from human cadaver kidneys to homogeneity on SDS-PAGE, by affinity chromatography (as described in section 2.3.) on a Sepharose-lisinopril resin, ACE elution being achieved using a chloride-free buffer (pH 9.5).

Polyclonal antisera to ACE were raised in rabbits as described in the previous chapter (3.3).

ACE was labelled with ^{125}I (Amersham) using the Iodogen (1,3,4,6-Tetrachloro-3α,6α-diphenyl glycoluril) method (Salacinski et al., 1981) and the tracer purified by gel filtration on a prepacked G-25 Sephadex column (Pharmacia) as follows.

4.3.1.1. Preparation of Iodogen tubes.

Iodogen tubes were prepared by coating 1.5ml Eppendorf microfuge tubes with Iodogen (Sigma) dissolved in dichloromethane (Merck). 1mg Iodogen was dissolved in 1ml dichloromethane and 20µl of this solution dispensed into each Eppendorf vial. The vials were coated by allowing the solvent to gradually evaporate under a constant, gentle stream of nitrogen while turning the vial. This resulted in an even coating of Iodogen on the vial wall. Vials showing uneven coating were discarded. Vials were stored, protected from light, at -70°C until used.

4.3.1.2. Iodination.

19µl 0.05M Phospate buffer was dispensed into an Iodogen coated tube. To this was added 100µCi of ^{125}I (1µl) and 1-2µg ACE in 10µl PBS (purified ACE was concentrated into PBS using Amicon Centricon-30 miniconcentrators in accordance with the manufacturers directions).

The reaction mixture was incubated at room temperature for 10 minutes, agitating the tube by flicking once each minute. The reaction was terminated by addition of 500µl 0.05M phospate buffer, removal of the mixture from tube and transfer to a glass tube for 10 minutes. This prevents the Iodogen from retaining activity on the column which could result in the iodination of the albumin carrier.

The reaction mixture was loaded onto a PD-10 Sephadex G-25 column pre-equilibrated with 0.05M phosphate buffer containing 1% BSA (bovine serum albumin).

Fractions were collected (6 drops per fraction) into 250µl of column equilibration buffer. 5µl of each fraction was counted in a Cobra gamma counter (Packard). Fractions of highest activity were pooled and stored at 4°C. Tracer stored in this manner lasted approximately 3 weeks. Before use, the tracer was centrifuged in a microfuge at 10000g for 5 minutes to remove any aggregates.

4.3.1.3. Assessment of tracer.

The maximal fraction of added tracer bound in the presence of an excess of antiserum was assessed by performing an antibody dilution curve as follows;

4 tubes containing 100µl tracer with 10000cpm/tube (total count or TC tubes).

4 tubes to assess non-specific binding (NSB) containing 10000cpm tracer (100µl), 400µl RIA assay buffer (10mM sodium phosphate-buffered saline, pH 7.4, containing 0.5% w/v BSA and 0.1% sodium azide).

Duplicate tubes containing 10000cpm tracer, 300µl buffer and 100µl of doubling dilutions of antiserum starting at 1:200 and extending to 1:102400. All tubes were briefly vortexed, covered with parafilm and the primary incubation was allowed to proceed for 48hrs at 4°C.

Cellulose-complexed anti-rabbit precipitating antiserum (Sac-Cel - Wellcome) was used to separate bound from free tracer according to the manufacturer's directions. Briefly 100µl of Sac-cel was added to all tubes, except for the TC tubes, vortexed and allowed to incubate at room temperature for 30 minutes before 1ml of deionised water was added to each tube (except TC tubes) and being centrifuged at 3000g for 10 minutes. The supernatants were decanted and the tubes inverted and allowed to drain on paper towel. The tubes were then individually counted for one minute each in a Cobra gamma counter (Packard) and an antibody dilution curve constructed utilising the counter's data-reduction and curve-fitting software. The maximum percentage of tracer bound was noted, as was the dilution of tracer required for approximately 33% of tracer to be bound.

4.3.1.4. Determination of specific activity of tracer.

Tracer specific activity (ie. assessment of ^{125}I incorporation into ACE) was determined by the method of self displacement (Chiang, 1987). Briefly the method involves two sets of tubes. The first set was essentially a standard curve prepared as described above. The second set of tubes contained the same concentration of antibody with different amounts of tracer, no unlabelled ACE was present. The two sets of results were plotted on the same graph: total/bound counts (T/B) vs. concentration of unlabelled ACE, and T/B vs. cpm of tracer. By interpolation from the two curves the specific activity was derived.

4.3.1.5. Assay of human serum.

As in the antibody dilution curve, 4 TC tubes and 4 NSB tubes were prepared. 4 Maximum binding tubes (B_0) were prepared with 10000cpm tracer, 100µl diluted antiserum (1:25600 - determined to result in the binding of approximately 33% of tracer) and made up to 500µl with assay buffer. Twelve standard tubes containing 10000cpm tracer were prepared in duplicate, to which was added 100µl antibody, 100µl diluted ACE in doubling dilutions from 200ng to 0.097ng ACE per tube and 200µl assay buffer. The concentration of ACE was determined by the method of Bradford (1976) using Bio-Rad commercial reagent and human serum albumin as standard.

The assay of serum utilised 25µl sample, incubated with 100µl anti-ACE antibody at a dilution of 1:25600 , and 100µl of tracer diluted in assay buffer (10mM sodium phosphate-buffered saline, pH 7.4, containing 0.5% w/v bovine serum albumin and 0.1% sodium azide) to obtain 10000cpm per tube. Samples were assayed in triplicate. The assay volume of each tube was adjusted to 500µl with assay buffer and the primary incubation allowed to proceed for 48 hrs at 4°C for standard, sample, NSB and B_0 tubes.

Sac-cel (Wellcome) was used to separate bound from free tracer according to the manufacturer's instructions. Following centrifugation at 3000g for 10 minutes at 4°C the supernatant was decanted and the resulting pellet in each tube counted for 1 minute in a Cobra gamma counter (Packard).

Sample ACE concentrations, in ng/ml, were calculated using the Cobra RIA data reduction computer program from a standard curve of the dilutions of purified ACE.

4.3.1.6. Correlation of serum ACE concentration with activity.

As part of a study investigating the serum concentration of ACE in a large group of subjects (n=165), sera of both normal controls (n=32) and those suffering from a variety of respiratory diseases (n=133) were assayed for ACE concentration (using the RIA) and activity (using the spectrofluorimetric assay described in section 9.7).

Blood from each patient was drawn into SST tubes (Serum separation tubes - Vacutainer), was allowed to clot, and the serum separated by centrifugation. Sera were stored at -70°C until assayed.

ACE activity (in U/ml) were plotted against RIA assayed concentration (in ng/ml). The correlation coefficient was determined by the method of least squares.

4.3.1.7. Assessment of within and between assay variation.

For the intra-assay coefficient of variation (CV), the same sample was assayed 6 times and the CV% calculated by conventional methods. The inter-assay CV% was calculated using the results of the same internal standard that had been assayed in 6 successive assay runs.

4.3.1.8. Assessment of the assay in the presence of pharmacological ACE inhibitors.

As this assay was to be used to assay samples of patients being treated with ACE inhibitors, it was vital to ensure that these inhibitors would not affect assay results. This was determined by assaying the same samples both without and with added captopril or enalaprilat (the active metabolite of enalapril) at a final concentration of 10^{-6} M in the assay volume of 500 µl.

4.3.1.9. Assessment of immunological identity between sample serum ACE and purified ACE standard.

A logit-log transformation of the RIA standard assay binding data was made by calculating the logit of the standard curve binding data using the formula;

> logit b = $\log_e(b/100\text{-}b)$ where b is the proportion of the tracer bound expressed as a percentage of that in the zero standard (B_0). The logits for each of the standards was then plotted on the *y* axis against the log ACE concentrations on the *x* axis.

Likewise four different amounts of each of the samples was assayed and the results plotted in the same way on the same graph.

Thus a logit-log plot was made of the standard curve and various dilutions of the serum of a normal control and sarcoid patient and the slopes of the curves compared. This was to assess the parallelism of the displacement curves, which suggests immunological identity between sample and standard.

4.3.2. Results

4.3.2.1. Iodination of ACE.

ACE was iodinated using the Iodogen vial method described above. Typically, well over 95% of the 100μCi used in the labelling reaction was incorporated into the ACE. Tentative evidence for this high degree of incorporation could firstly be noted from the cpm/fraction eluted from the PD-10 column with virtually all counts being associated with the protein peak (see accompanying figure). Proof of this high degree of ^{125}I incorporation was noted from the results of the self-displacement assay which demonstrated an average of 1 atom ^{125}I incorporated per molecule ACE (method detailed in appendix 9.12).

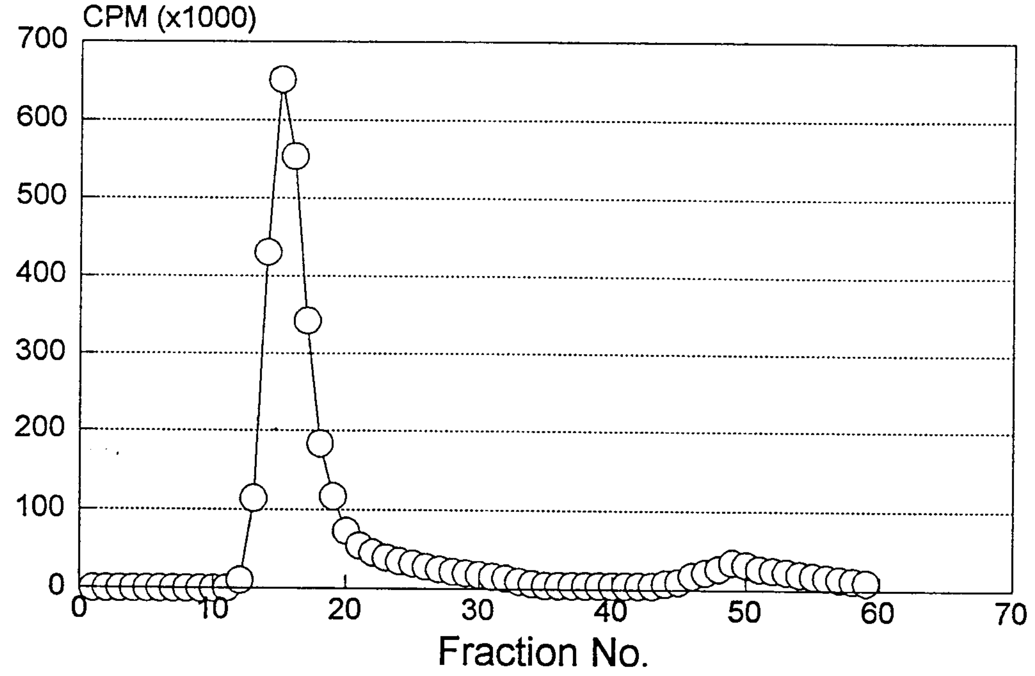


Figure 4.1. Elution profile of ^{125}I labelleled ACE from a PD-10 column. Each fraction of 6 drops was collected into 250µl of 0.05M phosphate buffer, 1%BSA. 5µl of each fraction was collected and counted. CPM for each fraction were plotted as shown. The first peak (fractions 11-20) contained the labelled ACE, while the small peak at fraction 50 contained unbound tracer.

4.3.2.2. Antibody dilution curve

The antiserum raised to purified ACE routinely bound more than 85% of labelled ACE when incubated in excess, in the absence of competing antigen.

The dilution of tracer giving approximately 33% tracer binding was 1:25600 in 100µl and thus this concentration, representing a final dilution of 1:128000 in the 500µl assay volume, was chosen for all assays.

4.3.2.3. RIA of serum samples.

A typical standard curve is shown in the accompanying figure. Non specific binding was commonly less than 3% of B_0. Intra-assay and interassay reproducibility coefficients were normally approximately 5% and 8%

respectively. The RIA detected 0.25 ng ACE/25 μl serum and results were unaffected by the presence in the assay medium of either captopril or enalaprilat (the active metabolite of enalapril) at a concentration of 10^{-6} M.

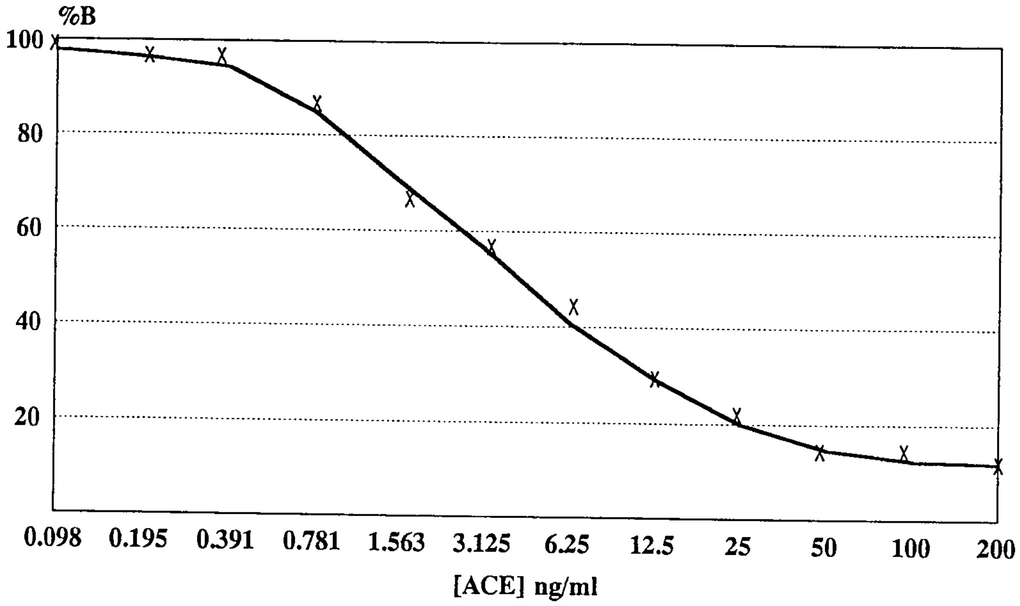


Figure 4.2. Typical standard curve for RIA of human serum ACE. Percentage of tracer bound on the y-axis is plotted against known concentrations of purified human kidney ACE on the x-axis.Crosses denote actual values obtained and the fitted line is shown.

4.3.2.4. *Correlation of serum ACE concentration with activity*

The assay results obtained for each sample group are detailed in the accompanying table.

Group	n	Activity (U/ml)	[ACE] (ng/ml)	Sp.Act. (U/mg)
Normal	32	37.2 ± 2.1	453.7 ± 28.3	83.9 ± 2.0
Sarcoid	51	78.7 ± 5.8^1	979.3 ± 77.4^1	83.6 ± 2.4
Silicosis	21	51.9 ± 4.6^2	646.5 ± 52.2^2	81.2 ± 3.2
MTB	20	50.0 ± 2.8^2	627.0 ± 48.5^2	82.8 ± 3.0
CTB	12	32.5 ± 3.5	387.4 ± 46.2	87.2 ± 6.9
COPD	26	40.2 ± 2.2^3	396.2 ± 53.1^3	113 ± 4.0^1
IPF	7	39.7 ± 2.7	437.2 ± 52.7	96.4 ± 9.1^3

Table 4.1. ACE activity, Concentration ([ACE]), and Specific Activity (Sp.Act.) by patient group. n represents the number of subjects in each group; COPD, Chronic Obstructive Pulmonary Disease; CTB, Cavitating Pulmonary Tuberculosis; MTB, Miliary Tuberculosis; IPF, Interstitial Pulmonary Fibrosis. ^{1} $p < 0.0001$, ^{2} $p < 0,01$ and ^{3} p = N.S. compared with normal control group. Expressed as mean±SEM.

The ACE concentration (mean (± 1 S.D.)) of normal subjects was 453.7(±159.8) ng/ml. Significant elevations in serum ACE concentration were found in the M.T.B., silicosis and sarcoidosis groups.

A plot of ACE activity vs. concentration is shown in the figure below. Least mean squares analysis demonstrated an excellent correlation ($r=0.93$; $p<0.0001$) between these two measurements.

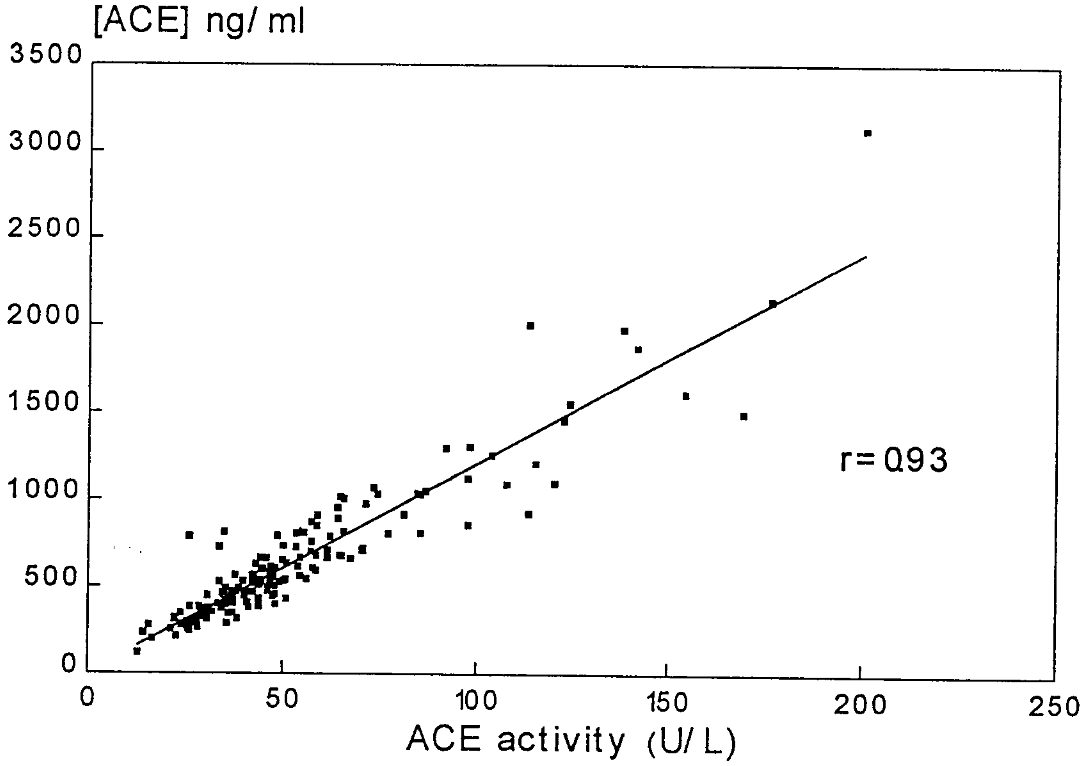


Figure 4.3. Plot of Serum ACE concentration (ng/ml) vs. serum ACE activity (U/L). The line represents the least mean squares correlation.

4.3.2.5. Assessment of parallelism

A logit-log plot of dilutions of purified ACE and samples demonstrated parallelism, suggesting immunological identity between standard and samples from both a control and a sarcoid subject.

4.3.3. Conclusions

The labelling of ACE by the Iodogen technique has not previously been described and it is interesting to note that the degree of ^{125}I incorporation and retention of immunoreactivity is superior to previously reported methods for which such data are given (Alhenc-Gelas et al., 1983).

A RIA assay for human serum ACE has been established which is capable of measuring the quantity of ACE protein in human sera. It is unaffected by the presence of ACE inhibitors and is therefore of great value in the assay of patient sera in the presence of pharmacological ACE inhibitors or in the detection of endogenous ACE inhibitors, unlike assays measuring ACE activity.

The assay is very sensitive, being able to detect as little as 250 pg ACE per 25 µl serum. Multiple runs of this assay incorporating internal standards have attested to the reliability and reproducibility of the assay.

The close correlation between ACE activity and concentration found in the study of a large group of patients (r=0.93) is higher than that reported in other RIA studies (Alhenc-Gelas et al., 1983; Hiwada et al., 1987). The ACE concentration in normal human serum was found to be 453.7(±159.8) ng/ml which is in close agreement with previously reported values of 400(±131) ng/ml (Alhenc-Gelas et al., 1983) and a little higher than the value of 320.9(±105.2) ng/ml reported by Hiwada et al. (1987).

The ACE RIA has the disadvantage of requiring the use of a radio-isotope, but is easier to perform, allows processing of samples in large batches, is more specific, and has a lower level of detection for ACE than the spectrofluorometric assay.

4.4. SECTION B: ELISA FOR HUMAN ACE

An ELISA for human ACE has also been developed, avoiding the need for isotopically labelled tracer. There are many potential disadvantages in radioimmunoassays: apart from the obvious saftey and disposal problems attached to the use of radioisotopes, there is also the need for specialised, expensive radioactivity measuring apparatus and the need to repeatedly relabel tracer due to its short half-life.

As polyclonal and monoclonal antisera to human ACE had already been developed (see section 3.3. & 3.4.), their use in a sandwich ELISA was considered.

ELISA methods for the measurement of human ACE have been described. In their paper detailing four different configurations of a competitive ELISA or CELIA, Lanzillo and Fanburg (1982) used a primary incubation of sample or standard ACE with a polyclonal antibody, before a second incubation in another well allowed any remaining antibody to bind a fixed amount of purified ACE immobilised on the solid phase. The authors found that the most sensitive configuration employed was that using the avidin biotin-horseradish peroxidase conjugate. This resulted in an assay which required approximately 24 hours to complete. The assay had a useful range of between 39 and 700 ng/ml. This assay was suitable for the measurement of ACE in human serum.

More recently, the first method of ACE ELISA utilising monoclonal antibodies has been reported (Stevens et al., 1990). In this assay one monoclonal Ab acts as a capture antibody, extracting the ACE from the sample or standard. This ACE is then detected with a second monoclonal directed against a different epitope as compared to the first antibody. The second antibody was conjugated to alkaline phosphatase. Detection involved the NADP/NAD cycling reaction method (Johannson et al., 1985). The reported sensitivity of this assay was 94 pg/ml making this assay suitable not only for the assay of ACE in serum or tissue but also in cell culture experiments. It is the most sensitive immunoassay for human ACE reported to date.

It was decided that an ELISA would be developed using a polyclonal capture antibody immobilised on a solid phase and detecting bound ACE with a monoclonal antibody.

4.4.1. Materials and methods.

ACE was trypsin solubilised and purified to homogeneity from human cadaver kidney tissue as described in chapter 2.

As the capture antibody required purification prior to use in the ELISA, polyclonal antiserum No. 217 was subjected to DEAE Affigel-Blue (Biorad) chromatography. This method removes all contaminating proteins from the immunoglobulin except for transferrin. 2 ml of antiserum was dialysed overnight against 0.02M potassium phosphate buffer, pH8. A column of DEAE Affigel Blue (bed volume approximately 10 ml) was equilibrated with the same buffer. After loading the column with the dialysed antiserum, the IgG was eluted with the same potassium phosphate buffer (contaminants are retained on the column). The absorbance was monitored continuously at 280 nm and fractions collected. The column was regenerated by washing with 6M KSCN.

The appropriate fractions were pooled after analysis on SDS-PAGE. The approximate IgG concentration was assayed using the method of Bradford (Bradford, 1976) using the Biorad reagent.

Monoclonal antibody 14/5/16/65 ascites was used unpurified.

Initially the assay was set up using the avidin-biotin method. This entailed detection of the second (monoclonal) antibody with a biotinylated anti-mouse antibody (Amersham) and subsequent incubation with Avidin-horseradish peroxidase before reaction with the substrate, ABTS. Although this method provided sensitivity, it was found that use of a goat anti-mouse peroxidase conjugate reduced the number of steps and time required without sacrifice of sensitivity.

Optimum concentrations for all reagents were determined by running multiple checkerboard titration assays. This method allows for a range of concentrations of two reagents to be simultaneously tested in the same assay.

The final assay configuration was as follows;

Nunc round-bottomed U-96 microtitre plates were filled with 100µl of purified antiserum 217 diluted 1:20 in 0.1M carbonate buffer pH 9.6. Incubation was allowed to continue overnight at 4°C.

The wells were then emptied and flicked dry on paper towels before being filled with 200 µl of 2% casien in the above carbonate buffer. This blocking step was for 30 minutes at room temperature.

The blocking buffer was then drained and the samples and standards diluted in ELISA diluent (0.5M NaCl, 0.01M $Na_2HPO_4 \cdot 12H_20$, 0.5% Triton X100). All samples and standards were in duplicate with samples being diluted in 1:2, 1:4 and 1:8. These were incubated for 1.5 hours at room temperature. The wells were then drained and washed 5 times with Tris-saline-Tween (TST, 0.1M NaCl, 0.05M Tris, 0.5% Tween-20).

The monoclonal ascitic fluid was diluted 1:2500 with ELISA diluent and 100 µl dispensed into each well. This was incubated at room temperature for 1.5 hours before the wells were drained and washed 5 times with TST. Goat anti-mouse peroxidase conjugate (Amersham) was diluted 1:30 in ELISA diluent and 100 µl dispensed into each well and incubated at room temperature for 1 hour. This was drained and the wells washed 5 times with TST. 150 µl of the substrate ABTS (made up as described previously) was added to each well, incubated at room temperature for 10 minutes before the absorbances were read at 414 nm on a Titertek Multiscan MCC-340 ELISA plate reader.

Samples were also assayed in the presence of the ACE inhibitors captopril and enalaprilat at a concentration of 10^{-6} M to assess the effect of these inhibitors on the assay.

4.4.2. Results

Polyclonal antibodies from antiserum 217 purified on DEAE Affigel Blue is shown in figure 4.4. The fractions eluted were electrophoresed on SDS-PAGE (also shown in figure 4.5) demonstrating that most contaminating proteins were removed from the serum leaving IgG and transferrin. The final purified IgG was at a concentration of 120µg/ml.

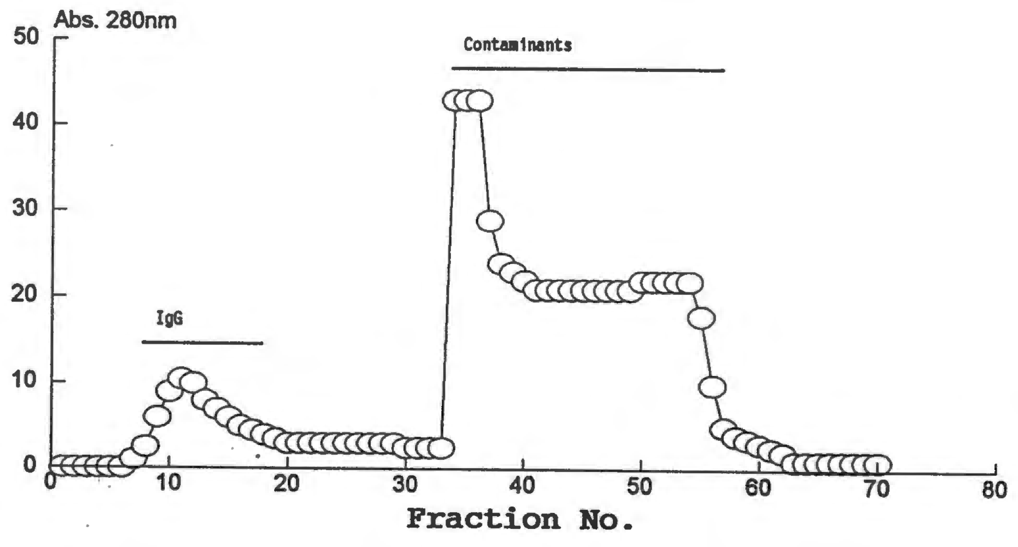


Figure 4.4. Polyclonal antiserum No. 217 was subjected to DEAE Affigel-Blue (Biorad) chromatography removing all contaminating proteins from the immunoglobulin except for transferrin. 2 ml of antiserum was dialysed overnight against 0.02M potassium phosphate buffer, pH 8. A column of DEAE Affigel Blue (bed volume approximately 10 ml) was equilibrated with the same buffer. After loading the column with the dialysed antiserum, the IgG was eluted with the same potassium phosphate buffer in fractions 8 - 16 (contaminants are retained on the column). The absorbance was monitored continuously at 280 nm and fractions collected. The column was regenerated by washing with 6M KSCN.

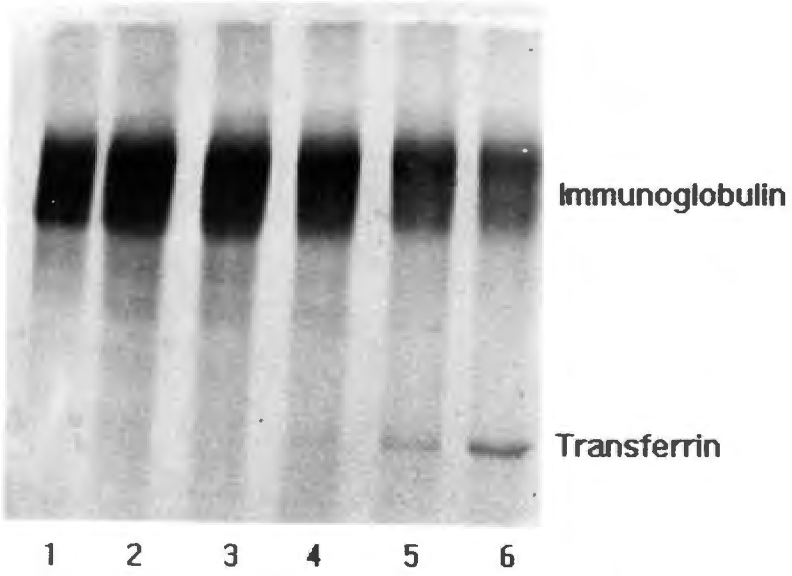


Figure 4.5. Polyacrylamide gel electrophoresis of fractions eluted from DEAE Affigel-Blue column. Lanes 1 - 6 correspond to fractions 8, 10, 12, 14, 16 and 18 respectively. The bold band in each lane is the IgG, minor contaminants are noted in the latter two fractions.

The assay performed as described demonstrated high sensitivity with as little as 5 ng/ml being reliably assayed. There was no interference from added captopril or enalaprilat in assay results. A typical standard curve is shown in the accompanying figure.

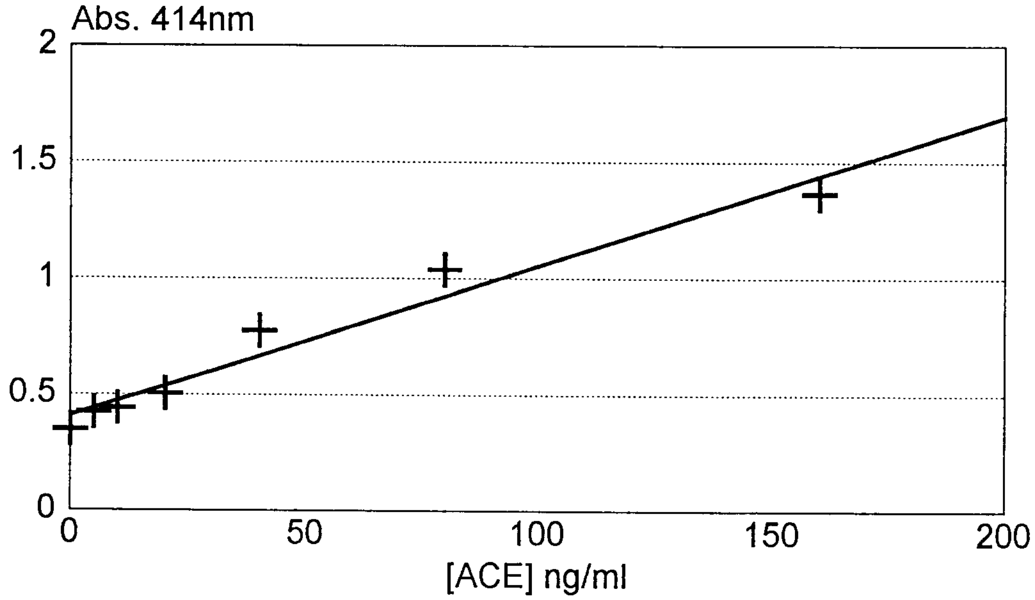


Figure 4.6. A typical ELISA standard curve for human serum ACE. Known concentrations of purified human kidney ACE is plotted on the x - axis against optical absorbance at 414nm on the y - axis. The regression line from which sample values were read, is shown.

4.4.3. Conclusion

An ELISA for human serum ACE was developed utilising a polyclonal antiserum as a capture antibody and a monoclonal second antibody. The assay demonstrated a sensitivity roughly equivalent to that of the RIA described earlier. Unlike the RIA which requires a sample-to-result time of at least 55 hours, the eqivalent time for the ELISA is 16 hours. Most of this time is the overnight (10 hour) coating of the wells with the capture antibody which can be reduced to 2 hours without loss of sensitivity (data not shown).

Although this did not have the same degree of sensitivity as the recently published monoclonal sandwich ELISA for human ACE (Stevens et al., 1990), it is sensitive enough for most purposes, excluding perhaps cell culture experiments. Its assay characteristics compared favourably with the results of the only other published ELISA assay for human ACE (Lanzillo and Fanburg, 1982).

Like the RIA, this assay is unaffected by the presence of ACE inhibitors in the sample, making it useful for studies of patients sera for ACE in the presence of these drugs.

4.5. SECTION C: CORRELATION OF ACE RIA vs. ELISA.

In order to assess the correlation between RIA and ELISA results a random group of patients sera were assayed by both methods and the results compared.

4.5.1. Methods

Sera were obtained from samples (n = 13) submitted to our laboratory for routine ACE activity assay. The samples were processed as described above

The samples were assayed in accordance with the described methods and only once the assays were completed were the results correlated.

The concentrations obtained from each sample, assayed in duplicate, were meaned and the correlation between both methods was determined by the method of least squares analysis.

4.5.2. Results

The results obtained are tabulated below.

Patient	[ACE] by RIA ng/ml	[ACE] by ELISA ng/ml
V.J.	707.8	706.1
B.R.	410.6	419.2
E.F.	510.9	478.6
L.S.	469.6	392.9
S.S.	214.1	259.1
D.F.	1024	1081.2
B.R.	119.2	110.5
E.A.	202.3	184.8
N.J.	337.2	370
J.Y.	395.4	402
K.S.	284.8	245.4
S.J.	266.7	291.1
L.M.	504.9	437.5

Table 4.2. Table showing values for ACE concentration by RIA and ELISA for each of 13 subjects.

A graph demonstrating the correlation between RIA results and those of the ELISA was also constructed and is shown in the figure below. A correlation of r = 0.99 was found between the results of both assays.

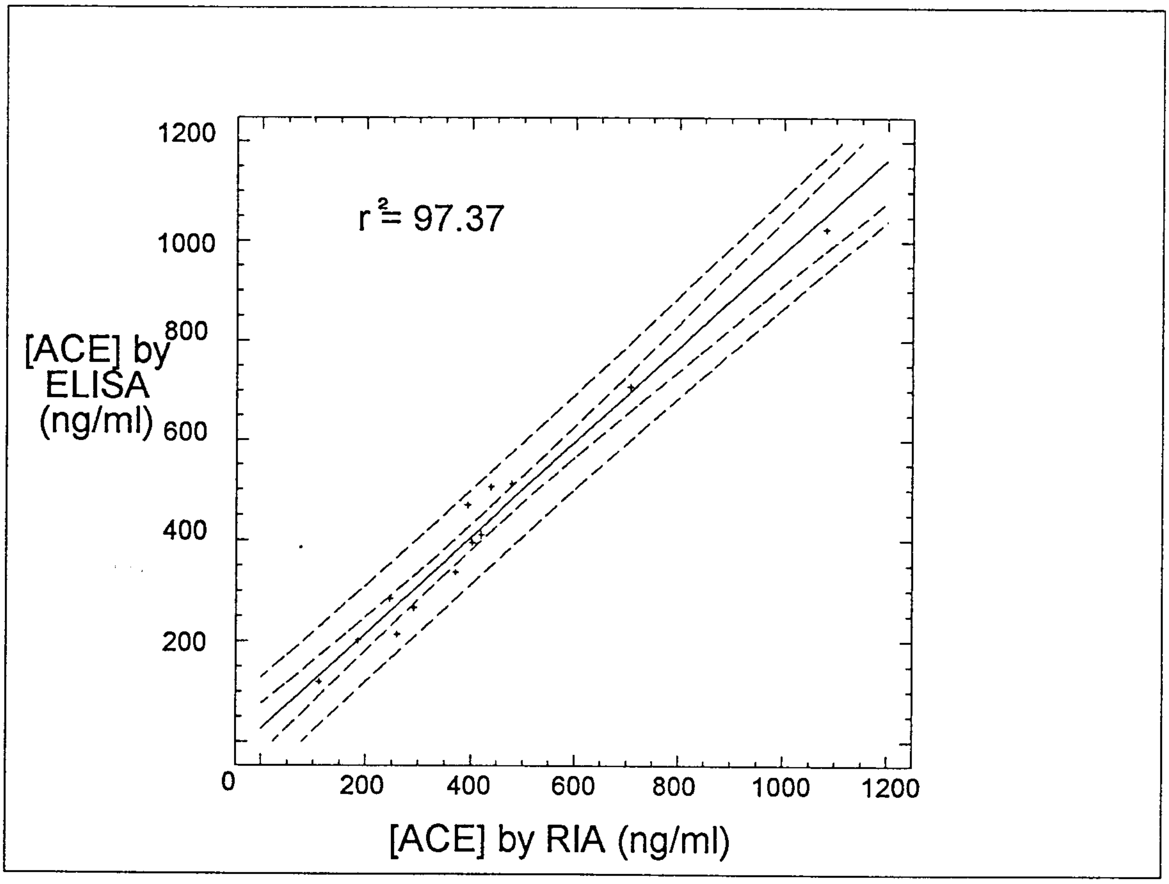


Figure 4.7. Graphical representation of the correlation between ACE concentration measured in patients by both RIA and ELISA. Excellent correlation is evidenced by the r^2 value of 97.4. The pairs of dotted lines represent the 95% confidence and prediction limits.

4.5.3. Conclusion

An excellent correlation was shown between both assays and thus they are valuable in studies of ACE in human serum. As the ELISA is both quicker and has equivalent sensitivity as compared to the RIA, and avoids the hazards of radioisotope usage, ELISA appears to be the assay of choice for future studies. The only potential advantage of RIA is in the simultaneaous processing of many specimens. As one generally tends to only process one ELISA plate at a time, this only allows for a maximum of about 25 samples to be assayed per run. The RIA, in contrast, has been used in runs of 250 samples in duplicate. Thus, the greater throughput possibilities with the RIA may be of advantage in some studies.

Niether of these assays have been applied to tissue extracts, although RIAs are often more susceptible to interference from tissue proteins as compared to ELISAs. As it is often necessary to detergent solubilise ACE from tissue, and ELISA diluent already contains detergent, it is likely that the ELISA will be useful in this setting. The RIA, however, fails to produce a standard curve in the presence of Triton X100 (data not shown). The exact reason for this has not been determined but may involve a conformational change of either antigen or antibody abolishing any mutual interaction.

These assays should prove valuable in future studies of ACE, and the application of the RIA to the study of patients receiving ACE inhibitors will be described in chapter 7.

Chapter 5

DEVELOPMENT OF ELISA FOR RAT ACE

5.1. OBJECTIVE

To develop an ELISA assay to measure ACE in the serum and tissues of rats.

5.2. INTRODUCTION

As the study encompassed by this thesis was, in the main, to investigate serum and tissue ACE responses to pharmacological inhibition, it was necessary to develop a sensitive immunoassay capable of accurately quantitating ACE in rat serum and tissues. A rat model for our studies was chosen because the animals are readily available, easily handled, and the rat is a frequent model employed in cardiovascular, and more particularly, ACE research.

The only other ELISA assay described to measure non-human ACE was for bovine ACE (Dasarathy et al., 1990), and has been employed to measure ACE from cultured bovine endothelial cells under the influence of various pharmacological agents.

The ELISA technique was chosen, since in the human assays it had been shown to be as sensitive as the RIA and potentially more useful in assay of tissue samples (see previous chapter).

Having raised a polyclonal antiserum to rat ACE and having found an anti-human ACE monoclonal antibody that cross-reacted with rat ACE, it was possible to develop a sandwich ELISA, similar to that developed to measure human ACE. The development of this assay is described in this chapter, while its application to the measurement of serum and tissue ACE will be covered in a later chapter.

5.3. MATERIALS AND METHODS

Rat lung ACE was purified to electrophoretic homogeneity as described in Chapter 2 on ACE purification.

Polyclonal antiserum no. 556, directed against rat lung ACE, was raised as described in Chapter 3. This antibody required purification on DEAE Affigel Blue before use as a capture antibody in the ELISA. The isolation of monoclonal antiserum 187/18, raised against human kidney ACE but cross-reacting with rat lung ACE, is also described in section 3.4.1.8. Monoclonal 187/18 was used in the form of harvest fluid collected from antibody-secreting cells.

Optimum concentrations for all reagents were determined by running multiple checkerboard titration assays. This method allows for a range of concentrations of two reagents to be simultaneously tested in the same assay.

The final assay configuration was as follows;

Nunc round-bottomed U-96 microtitre plates were filled with 100 µl of purified antiserum 556 diluted 1:20 in 0.1 M carbonate buffer pH 9.6 (final concentration 9µg/ml). Adsorbtion was allowed to continue overnight at 4°C.

The wells were emptied and flicked dry on paper towels before being filled with 200 µl of 2% casien in the above carbonate buffer. This blocking step was for 30 minutes at room temperature.

The blocking buffer was drained and the samples and standards diluted in ELISA diluent (0.5M NaCl, 0.01M $Na_2HPO_4 \cdot 12H_20$, 0.5% Triton X100). All samples and standards were in duplicate with serum samples being diluted in 1:5 and 1:10. The standard curve ranged from 5000 ng/ml to 4.9 ng/ml in doubling dilutions. Two wells were filled with ELISA diluent to provide the zero standard. These were incubated for 1,5 hours at room temperature. The wells were then drained and washed 5 times with Tris-saline-Tween.

The monoclonal harvest fluid was used undiluted and 100 µl dispensed into each well. This was incubated at room temperature for 1.5 hours before the wells were drained and washed 5 times with TST. Goat anti-mouse peroxidase conjugate (Amersham) was diluted 1:30 in ELISA diluent and 100 µl dispensed into each well. Incubation was at room temperature for 1 hour. This was drained and the wells washed 5 times with TST.

150 µl of the substrate ABTS (made up as described previously) was added to each well. Incubation was allowed to proceed at room temperature for 10 minutes before the absorbances were read at 414 nm on a Titertek Multiscan MCC-340 ELISA plate reader.

To assess the utility of this assay in the measurement of rat ACE and to obtain a normal rat serum ACE concentration, normal serum was obtained from a group of eight female Long-Evans rats of similar mass (approximately 350 g). ACE was assayed by ELISA at two dilutions, each being in duplicate.

Samples were also assayed in the presence of the ACE inhibitors captopril and enalaprilat at a concentration of 10^{-6} M to assess the effect of these inhibitors on the assay.

5.4. RESULTS

Polyclonal antiserum 556 was purified on DEAE Affigel Blue and the fractions eluted were electrophoresed on SDS-PAGE to ensure that most contaminating proteins were removed from the serum leaving IgG and transferrin. The final purified IgG was at a concentration of 170 µg/ml.

The assay could detect as little as 5ng/ml ACE and there was no interference of assay results when captopril or enalaprilat were added to the samples. A typical standard curve is shown in the accompanying figure. An internal standard of pooled rat serum ([ACE]=1980 ng/ml) was included in all assays and showed a between assay CV% of 8%.

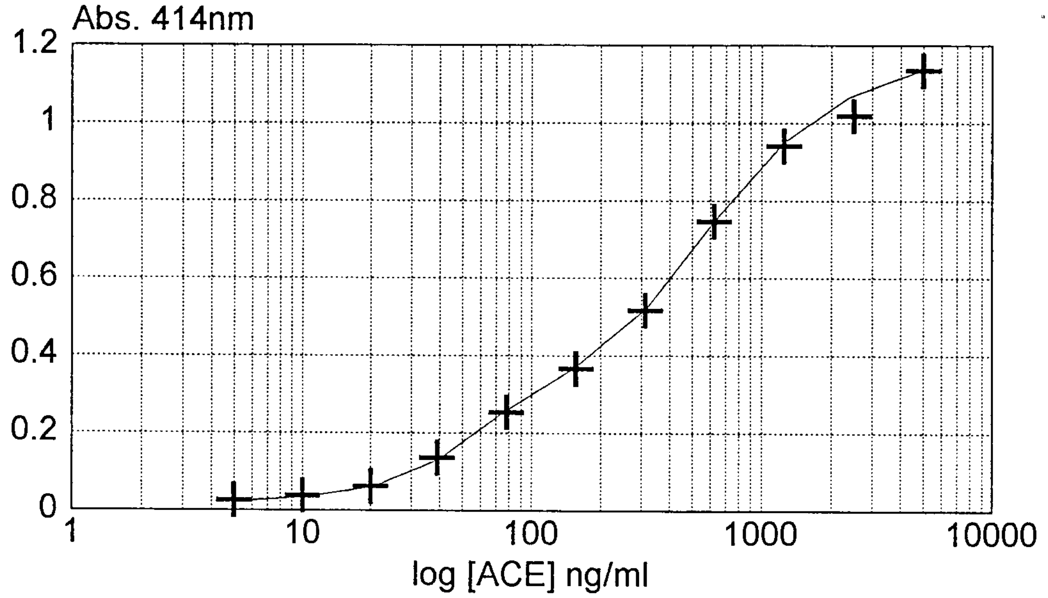


Figure 5.1. A typical standard curve for the rat ACE ELISA. The crosses represent absorbances read for known concentrations of rat lung ACE vs. a log scale of the standard [ACE] up to 5000ng/ml. The line linking the crosses represents a best fit curve from which the concentrations of samples could be read.

Assay of serum ACE from the group of eight rats showed a normal serum ACE concentration of 1189±213 ng/ml (mean±SD).

5.5. CONCLUSION

An ELISA measuring rat ACE was developed utilising a sandwich technique. This assay, the first rat ACE ELISA to be described in the literature, is sensitive enough for use in the study of the effects of pharmacological ACE inhibition on serum and tissue levels of ACE. The ELISA is simple to perform and, following overnight coating of wells with antibody, the assay can be completed within six hours.

The level of a serum ACE concentration in rats is approximately three times that of humans, and this is not surprising as it has previously been noted that rat serum ACE activity is elevated compared to human serum ACE (Kase et al., 1986).

This assay should prove to be a valuable tool in the study of ACE in the rat, an animal frequently utilised in cardiovascular research. Although this assay was used in this study to assess the effect of ACE inhibitors on ACE levels in serum and tissues, it may well be useful for other studies examining the renin-angiotensin system in the rat.

Chapter 6

CLONING AND SEQUENCING OF RAT ACE.

6.1. OBJECTIVE

To obtain a nucleic acid probe that could be used to study the changes in ACE RNA in rats during treatment with ACE inhibitors.

6.2. INTRODUCTION

This study set out to examine ACE expression in rats in response to pharmacological ACE inhibition, not only in terms of ACE protein, but also ACE mRNA. This was vital as any detected increase in ACE protein could either be due to increased synthesis of new ACE or to decreased clearance of pre-existing ACE. If elevations of ACE-specific mRNA could be detected in tissues in response to ACE inhibition, strong evidence for the former possibility would be obtained.

The expression of specific mRNAs in tissues can most easily be determined by the technique of northern blotting where RNA extracted from tissue is separated on an agarose gel, transferred to a solid support, and specific mRNA detected with a suitable labelled DNA or RNA probe (Thomas, 1980). In order to perform such a study, a suitable probe was required. Fortunately, a partial cDNA clone of human testicular ACE (designated 10A see Ehlers et al., 1989) was obtained and, on the assumption that considerable homology existed between rat and human ACE, this human clone was used for northern blots of rat RNA. The clone, however, hybridized weakly to rat ACE mRNA; at least 20 µg total RNA had to be loaded per lane to obtain a barely detectable signal (data not shown). It was therefore decided to isolate a rat clone for ACE. A rat lung cDNA library was screened for ACE sequences using clone 10A as a probe.

This chapter details the isolation of two partial rat ACE cDNA clones and comparison of the sequence obtained with mouse and human ACE sequences already published.

6.3. MATERIALS AND METHODS

A cDNA library of lung mRNA obtained from a male Sprague-Dawley rat of 9 weeks of age in lambda gT11 was purchased from Clontech (Catalogue No. RL1008b).

6.3.1. Labelling of clone 10A

Clone 10A is a partial cDNA clone of human testicular ACE and was supplied subcloned into the pBluescript (Stratagene) vector at an approximate concentration of 1.3 µg/µl. The clone spans 2056 base pairs (bp) and covers most of the testicular ACE sequence. The insert was released by digestion with EcoRI and the purified insert used in the library screening.

Clone 10A was transfected into competent E.coli, grown up in L-broth and DNA purified by the QIAGEN (Diagen) method (protocols detailed in appendix). Clone 10A (50µg) was cut with EcoRI in a volume of 120 µl for 2 hours at 37°C. A small portion of the digest was used to assess whether cutting was complete.

In order to obtain sufficient insert separated from vector, the rest of the digest was loaded into 3 wells of a 1x TAE/1% Agarose gel (TAE = 0.04M Tris-acetate, 0.001M EDTA). The gel was run at 20mA and the gel containing the insert excised from the gel using a scalpel blade. The insert was then isolated by electroelution (section 9.1.8.). As the A_{260} was too low to read, concentration was assessed by running alongside various known concentrations of lambda DNA on a 1% agarose gel.

100ng of linearised insert DNA was labelled with ^{32}P-dCTP by random priming (section 9.1.10.). The probe was purified from unincorporated label by Sephadex G-50 chromatography prior to use.

6.3.2. Isolation of rat ACE clones

The commercially obtained library was titred by plating serial dilutions of the library on a lawn of LE392 (section 9.2.) E.coli cells (Borck et al., 1976) on L-Broth agar / top agarose plates. This was necessary so as to find the optimum ratio of library to cells for plaque screening.

For the primary screening of the library, four 150mm plates were plated with a dilution of the library. After overnight incubation at 37°C, sufficient plaques had developed on the lawn for plaque lifts to be performed. The detail of the plaque lift procedure is to be found in the appendix (9.2.). The filters were probed with ^{32}P labelled clone 10A insert. Following hybridisation and washing, the filters were exposed overnight to X-ray film at -70°C. Positive signals on the developed film represented plaques with potential rat ACE containing sequences.

After the initial screening at higher dilution, the positive plaques were cored from the plates and replated in order to obtain pure growths of positive plaques. Once this was achieved, large scale lambda preparations in NZC medium (containing NZ amine, NaCl, bacto yeast extract, casamino acids and $MgSO_4 \cdot 7H_2O$) were made of the positives and the DNA purified by standard techniques (see appendix).

The release of two putative rat ACE inserts (named RACE1 and RACE2) was achieved by EcoRI digestion of the purified DNA of each of the clones and the digests were analysed on a 1% agarose gel. Sizes of the inserts were assessed by running alongside a marker of HindIII digested lambda DNA.

6.3.3. Subcloning of RACE1 and RACE2 into pUC19

In order to facilitate the sequencing of the two RACE inserts they were subcloned into pUC19. This was achieved by running EcoRI digested lambda gT11/RACE on a TAE agarose gel containing ethidium bromide. The gel was visualised on a UV light box and a slit cut in the gel just ahead of the insert band. Into this slit a small piece of Whatman 3M paper was inserted and electrophoresis resumed to move the insert into the paper. When this was judged to have occurred, the buffer containing the insert DNA was centrifuged from the paper (Errington, 1990).

The inserts collected in this way were ligated into pUC19 using T4 DNA ligase and used to transform competent DK-1 cells. The cells were plated on L-broth ampicillin plates. Colonies were picked, DNA isolated and screened for inserts by EcoRI digestion. Large scale plasmid isolation was on QIAGEN columns according to the manufacturer's recommendations.

6.3.4. Sequencing

Double-stranded plasmid DNA of each of the clones was sequenced by the dideoxynucleotide chain-termination method (Sanger, et. al. 1977) using Sequenase (United States Biochemical). Oligonucleotide primers were synthesised on a Beckman model 200A DNA synthesiser. The full sequencing method is described in the appendix (9.4.)

6.3.5. Sequence analysis

Comparison of the sequence obtained for RACE1 and RACE2 to existing sequences contained in the GenEMBL nucleic acid sequence database was made using a VAX workstation running the program GCG (University of Wisconsin).

6.3.6. Confirmation of probe utility

As it is well known that in many mammalian species, somatic or lung ACE is larger than testicular ACE (discussed fully in the literature review), this feature was used to confirm that RACE1 could be used as a probe for rat ACE.

RACE1 insert was labelled with ^{32}P by the random primer method as described for clone 10A above. 5μg rat lung total RNA and 2μg rat testicular total RNA was electrophoresed on a formaldehyde/agarose gel and blotted onto a nylon membrane. The blot was hybridised with labelled RACE1 insert and, after washing, exposed to X-ray film (full details of method in appendix, 9.5. & 9.6.).

6.4. RESULTS

6.4.1. Clone 10A insert isolation

Clone 10A was successfully transfected into E.coli and grown up in L-broth. EcoRI digestion of the plasmid containing clone 10A revealed complete release of an insert corresponding to approximately 2kb.

10A insert DNA was purified from vector, 100ng of insert was labelled by the random priming method and ^{32}P-dCTP labelled DNA separated from unincorporated label by Sephadex G-50 chromatography.

6.4.2. Isolation of rat ACE clones

Serial dilutions of the library were plated on a lawn of competent LE392 cells and the library titre assessed at approximately 10^9 plaque forming units (pfu) / ml.

To screen the library, it was found that 4 µl of a 10^{-2} dilution of the library plated on a 150mm plate with 500µl of plating bacteria and 7ml top agarose, resulted in the optimum plating density for plaque screening. At this dilution there were approximately 50000 pfu per plate. As four plates were used in the initial screening, approximately 200000 pfu were screened.

Plaque lifts were performed and subsequent screening with 10A insert yielded four strong positive plaques. These plaques were purified by further rounds of screening. During this process two of the positives were lost but the remaining two were purified to homogeneity after 4 rounds of screening.

Once single clones had been obtained, each of the two clones were innoculated into flasks of NZC medium (section 9.3.3.) and the DNA purified from large scale preparation.

These two clones were labelled RACE1 and RACE2, and on agarose gel electrophoresis, after EcoRI excision from vector, RACE1 appeared to be approximately 1.9kb and RACE2 slightly longer at 2.1kb (see figure 6.1).

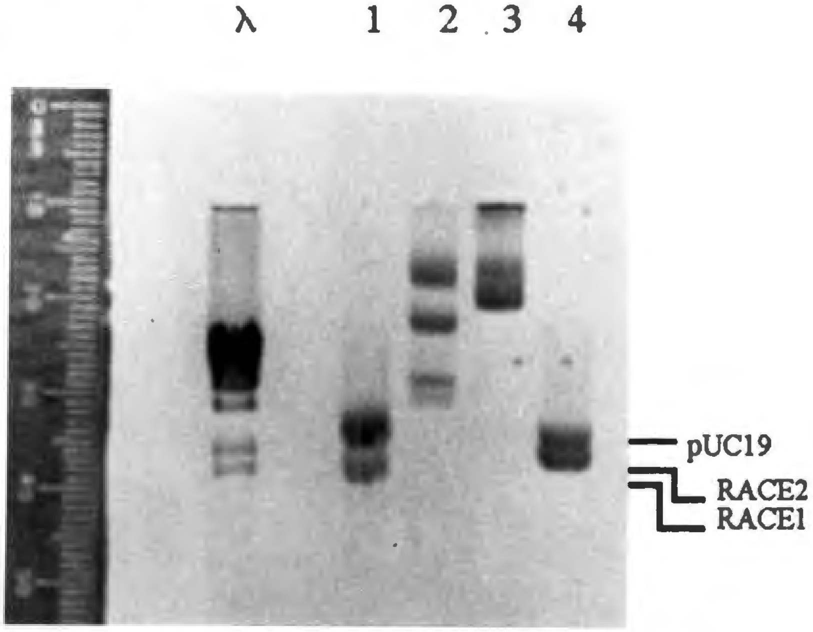


Figure 6.1. 1% agarose gel electrophoresis of RACE 1 and RACE 2 after release from the vector. The lane marked λ contains λ cut with HindIII, lane 1 contains RACE1 in pUC19 cut with EcoRI and lane 4, RACE2 treated similarly.

6.4.3. Subcloning of RACE1 and RACE2 into pUC19

Using the technique of obtaining the cloned inserts in filter paper and subsequently ligating these into pUC19, subcloning was successfully achieved. Out of six random colonies screened for each of RACE1 and RACE2, four of each contained inserts. One of each 1F and 2A were chosen, grown up in a large scale preparation and the DNA isolated using QIAGEN columns.

6.4.4. Sequencing

Sequencing was carried out in both directions using the Sequenase method, initially using M13 universal primers and then other internal oligonucleotides. Usually each sequencing run allowed approximately 200 bases to be read, this sequence was used to determine suitable oligonucleotide sequences to continue the process.

6.4.5. Sequence analysis

Comparison of the RACE1 and RACE2 sequences showed them to be identical over the sequence spanned by RACE1, the shorter clone. Sequence comparison of the RACE1 sequence to that published for mouse ACE show approximately 90% identity for the corresponding section of mouse ACE cDNA. A similar comparison with the published human lung ACE cDNA sequence revealed identity of only 70%.

The sequence of RACE1 corresponded closely to bases 1179 - 3134 of the mouse ACE sequence. This spans 1955 bp of mouse sequence, similar to the length of RACE1 as determined on agarose gel electrophoresis (1.9 kb). RACE2 maps alongside bases 830 - 3134 of mouse ACE (2304 bp).

RACE1 and RACE2 were found to have been subcloned in opposite orientations to each other. Thus the forward sequence of RACE1 corresponded to the complementary reverse sequence of RACE2.

```
                                                                    60
Mouse  GGATCATAAAGAAGCTTCAGAACCTGGACCGGGCAGTGCTGCCTCCCAAGGAATTAGAAG
       ||||||||||||||*||||||||*|||||||||||||||||||||||||*||*|||||||
Rat    GGATCATAAAGAAGGTTCAGAACGTGGACCGGGCAGTGCTGCCTCCCAACGAGTTAGAAG
       ||||||||||||||||||||*||*|*||*|||||||*|||||||*||*|*|||*|*||*|
Human  GGATCATAAAGAAGGTTCAGGACCTAGAACGGGCAGCGCTGCCTGCCCAGGAGCTGGAGG

                                                                   120
Mouse  AGTACAACCAGATCCTGCTAGACATGGAGACAACTTACAGCTTATCCAACATTTGCTACA
       |||||||||||||||||||||||||||||||*||||||||**||*||||**|||||||||
Rat    AGTACAACCAGATCCTGCTAGACATGGAGACGACTTACAGTGTAGCCAATGTTTGCTACA
       ||||||||*|||||||||*|*||*|||||*||*||*|||||*||*||||*|||*|||*||*
Human  AGTACAACAAGATCCTGTTGGATATGGAAACCACCTACAGCGTGGCCACTGTGTGCCACC

                                                                   180
Mouse  CAAATGGCACTTGTATGCCCCTGGAACCTGATCTAACAAACATGATGGCCACATCCCGGA
       |||||*||||||||||*||*|*|||||*||||||||*|||||*||*||||||||*|||||||
Rat    CAAATTGCACTTGTCTGTCACTGGAGCCTGATCTGACAAATATAATGGCCACGTCCCGGA
       |*|||*|||**||*|||***||*|*|||*||||||||*|||*|*||||||||*|||||||
Human  CGAATGGCAGCTGCCTGCAGCTCGCGCCAGATCTGACGAATGTGATGGCCACATCCCGGA
```

```
                                                                  240
Mouse AATATGAAGAATTGCTATGGGCATGGAAGAGCTGGAGAGACAAGGTGGGGAGAGCCATCC
      ||||*|||||||||||*||||**||||||||||||***||||||||||||||||||||||
Rat   AATACGAAGAATTGCTTTGGGTGTGGAAGAGCTGGCAGGACAAGGTGGGGAGAGCCATCC
      ||||*|||||**||*|*||||**|||*||*||||||**|||||||*||||||||||||||
Human AATATGAAGACCTGTTATGGGCATGGGAGGGCTGGCGAGACAAGGCGGGGAGAGCCATCC

                                                                  300
Mouse TTCCTTTTTTCCCAAAGTATGTGGAGTTCTCCAACAAGATTGCCAAGCTCAATGGCTACA
      ||||||||||||||||||*|||||*||||||||||||||*|||||||||||*||||||*|
Rat   TTCCCTTTTTCCCAAAGTACGTGGACTTCTCCAACAAGATCGCCAAGCTCAACGGCTACT
      |*|**||||*|||*||*||||||||**||**||||*||***|||**||||||*|||||**
Human TCCAGTTTTACCCGAAATACGTGGAACTCATCAACCAGGCTGCCCGGCTCAATGGCTATG

                                                                  360
Mouse CGGATGCAGGGGATTCATGGAGATCCATATACGAGTCTGACAACCTGGAGCAAGACCTGG
      *||||||||||||||**|||||||||**|||*||*||*|||*||*|||||||||||||||
Rat   CTGATGCAGGGGATTGGTGGAGATCCTCATATGATTCCGACGACTTGGAGCAAGACCTGG
      **|||||||||||*|*||||||*||****||*||**|******|*|||||||||||||||
Human TAGATGCAGGGGACTCGTGGAGGTCTATGTACGAGACACCATCCCTGGAGCAAGACCTGG

                                                                  420
Mouse AAAAACTGTACCAGGAGCTGCAGCCACTCTACCTGAACCTGCATGCCTATGTGCGTCGTT
      |||||||*|||||||||||*||||||||||*|||*|*||*|||||||||||||||*||*|
Rat   AAAAACTATACCAGGAGCTCCAGCCACTCTCCCTAACCCAGCATGCCTATGTGCGCCGCT
      |****||*|*|||||||||*||||||||||*|||*|*||*|||||||||*||||||||**
Human AGCGGCTCTTCCAGGAGCTGCAGCCACTCTACCTCAACCTGCATGCCTACGTGCGCCGGG

                                                                  480
Mouse CCCTGCACCGCCACTATGGGTCCGAGTACATCAACCTGGATGGCCCCATTCCTGCCCATC
      |||||||||||||*||||||||*||||||||||||||||||||*||||||||||||||*|
Rat   CCCTGCACCGCCATTATGGGTCTGAGTACATCAACCTGGATGGTCCCATTCCTGCCCACC
      ||||||||||*||*||*|||*|**||*|||||||||||||*||*|||||||||||*||||
Human CCCTGCACCGTCACTACGGGGCCCAGCACATCAACCTGGAGGGGCCCATTCCTGCTCACC

                                                                  540
Mouse TGCTAGGGAACATGTGGGCGCAGACCTGGTCCAACATCTATGATTTGGTGGCGCCCTTCC
      ||||||||||||||*||||*|||||*|||||||||||||||||*||||||||*|||||||
Rat   TGCTAGGGAACATGTGGGCACAGACTTGGTCCAACATCTATGACTTGGTGGCACCCTTCC
      ||||*||||||||||||||*|||||*|||||||||||||||||||||||||**|||||||
Human TGCTGGGGAACATGTGGGCGCAGACCTGGTCCAACATCTATGACTTGGTGGTGCCCTTCC

                                                                  600
Mouse CTTCCGCCCCCAATATAGATGCCACGGAAGCCATGATAAAGCAGGGATGGACACCCAGAA
      ||||||||||||*|||||||||||||||*|||||||||||||||||||||||||||||||
Rat   CTTCCGCCCCCAGTATAGATGCCACGGAGGCCATGATAAAGCAGGGATGGACACCCAGAA
      ||||*||||||***||*||**||||*|||||*|||*||||||||||*|||||*||||||*
Human CTTCAGCCCCCTCGATGGACACCACAGAGGCTATGCTAAAGCAGGGCTGGACGCCCAGAG

            608
Mouse GGATATTT
      ||||||||
Rat   GGATATTT
      *|||*|||
Human -GATGTTT
```

Figure 6.3. The 608 base partial sequence obtained from the 5' end of RACE1 showing the nucteotide sequence aligned with those of mouse and human ACE. Bases 1-608 of RACE1 align to bases 1175-1782 of mouse ACE with 86.7% identity and to bases 462-1069 with 74.6% homology to the published sequence for human ACE. The underlined portion of the rat sequence was used as the template for the riboprobes used in the study of rat ACE expression. The partial sequence of RACE1 has been deposited at Genbank, accession number L36664.

6.4.6. Confirmation of probe utility

Northern blot analysis of rat lung and testicular total RNA, probed with labelled RACE1, revealed the expected difference in size between both ACE RNAs. This confirmed that RACE1 was indeed a rat ACE cDNA clone and could be used to probe rat ACE in further studies.

6.5. DISCUSSION

While it would not have been impossible to use the human testicular ACE clone to probe rat ACE, the lesser homology would have required less stringent washing with probably more background in order obtain a readable result on autoradiography. Indeed, a recent paper (Schunkert et al., 1990) described the use of a human ACE cDNA clone as a probe for rat heart ACE. However, each lane had to be loaded with 25μg of poly(A)$^+$ RNA to achieve a useable signal.

Using the methods described above, two partial clones for rat ACE were obtained. These proved to be clones of ACE both from sequence homology with published mouse and human ACE sequences, and by the finding that RACE1 hybridised to both lung and testicular ACE with the expected difference in mRNA size.

The fact that neither of these clones extended 3' to incorporate a polyA tail and instead terminating at EcoRI sites, points to deficiencies in the manufacture of the commercially obtained oligo dT primed cDNA library. The most likely explanation is that rat ACE cDNA contains at least three internal EcoRI sites (because of the three distinct ends of RACE1 and RACE2) and that during library construction, these sites were not protected from EcoRI digestion, due to inadequate methylation. Hence the sequence 3' to the two clones were lost.

As this study only required a portion of the rat sequence for use as a probe, no attempt was made to backscreen the library to obtain a full length clone, if this indeed existed in the library.

6.6. CONCLUSION

Two partial rat ACE cDNA clones were isolated from a rat lung cDNA library. These clones were sequenced and strong homology noted between these and previously published sequences of human and mouse ACE. It was now possible to use these clones, or parts thereof, as probes of rat ACE in a study of the expression of rat ACE mRNA in response to pharmacological inhibition.

Chapter 7

ACE mRNA AND PROTEIN EXPRESSION IN RESPONSE TO PHARMACOLOGICAL ACE INHIBITION.

7.1. OBJECTIVE:

To study the effects of ACE inhibition on ACE mRNA and protein expression in rat tissues.

7.2. INTRODUCTION:

The renin-angiotensin system plays a central role in the maintenance of blood pressure. Angiotensin II, the main effector of this system, results from the action of angiotensin-converting enzyme (ACE) on angiotensin I. Increased production of the vasoconstrictor, angiotensin II, maintains vasomotor tone. Further studies of the renin - angiotensin system have led to the development of ACE inhibitors for the treatment of hypertension and cardiac failure. These inhibitors include captopril, enalapril and lisinopril among others. While it was initially thought that the renin - angiotensin system was purely a circulating system, it has now been realised that local renin-angiotensin systems exist and that inhibition of ACE at the tissue level seems to more closely parallel the blood pressure response (Ferrario, 1990).

Hypertensive patients on treatment with captopril were noted to have no significant change in serum ACE activity (Larochelle et al. 1979). It was initially thought therefore, that inhibition of ACE activity is not the mechanism by which captopril decreases blood pressure. Subsequently it was shown that captopril readily degrades, making it exceedingly difficult to assess the degree of ACE inhibition during therapy with this agent. In another study it was found that plasma ACE activity actually increases in hypertensive patients receiving captopril over a period of several weeks, suggesting that ACE inhibitors actually enhance ACE production (Boomsma et al., 1981).

The above studies prompted other workers to study the effect of ACE inhibitors on cultured endothelial cells, known to express ACE on their plasma membranes. These cells, when treated with captopril, were found to have increased ACE activity in their culture medium compared to control cells (Fhyrquist, et al., 1982a).

Studies on spontaneously hypertensive rats confirmed that ACE activity is elevated in animals treated with captopril. Administration of captopril to rats for a period of 24 weeks resulted in a threefold increase in serum ACE activity and lung plasma membrane ACE activity compared to control animals (Fhyrquist, et al. 1980). Similar results were obtained for normotensive rats treated with enalapril for a period of seven days (Forslund, et al. 1982) where serum ACE activity increases by 280% and by 34% in lung plasma membranes. Spontaneously hypertensive rats treated with captopril show no induction of lung plasma membrane ACE following adrenalectomy suggesting that the adrenals play some role in the membrane incorporation of ACE (Fhyrquist, et al. 1982b). The tissue specificity of the inductive response was suggested by the finding that lung ACE activity increases, whereas kidney ACE activity remains unchanged and testicular activity decreases after six weeks of treatment with captopril (Forslund et al., 1984).

All the above studies utilised measurements of enzyme *activity* to deduce that this observed effect was due to increased ACE protein production. This necessitated the removal of the administered inhibitor either through extensive dialysis, chemical destruction or by allowing time for degradation to occur.

Recently a novel method to assess both ACE inhibition and induction during inhibitor therapy has been described (Kohzuki et al., 1991). This technique involves quantitative *in vitro* autoradiography of serial sections from tissues of interest. The degree of ACE inhibition was determined by direct probing of sections with an I^{125} labelled ACE inhibitor, MK 351A which is a tyrosyl derivative of the ACE inhibitor, lisinopril. After 14 days of treatment with lisinopril, plasma ACE increases 75% while lung ACE as measured by autoradiography of the sections increases by 30% compared to untreated animals. These results showed no significant change in ACE concentration in the kidney, adrenal, aorta or testis.

A greater insight into this induction mechanism is required as it may explain the observation that the degree of ACE inhibition decreases with chronic inhibitor therapy and also the tolerance that patients can develop to previously effective doses of ACE inhibitors (Johnston, et al. 1988). Furthermore, the cost of these agents makes such an investigation all the more relevant.

This study set out, first, to confirm that ACE inhibitor treatment in human subjects induces an increase in the concentration of circulating ACE as detected by radioimmunoassay; and second, to investigate the possible mechanism for this response, by studying the acute effects of ACE inhibition at both the protein and mRNA level in rat tissues. Unlike previous studies of rat ACE mRNA expression that utilised human cDNA probes, all experiments were performed using specific rat ACE cDNA-derived riboprobes. Also, ACE protein was measured by specific immunoassay, avoiding the need to derive data from activity assays. Our findings suggest that the induction mechanism depends on a more distal component of the renin angiotensin system pathway than angiotensin II, possibly aldosterone, the final effector of the system.

7.3. SERUM ACE CONCENTRATION IN HUMAN PATIENTS UNDERGOING TREATMENT WITH CAPTOPRIL.

7.3.1. Methods

7.3.1.1. Patient selection

Ten patients undergoing treatment with captopril for congestive cardiac failure in the Cardiac Intensive Care Unit at Groote Schuur Hospital were included in the study. Blood was drawn for serum ACE concentration assay before the onset of treatment and again after four days of treatment with 6.25 mg captopril

8 hourly. Most patients received concurrent treatment with other standard anti-failure therapy such as digoxin and furosemide (most patients had been on these latter two agents prior to their intensive care unit admission).

7.3.1.2. Radioimmunoassay for human serum ACE

This assay has been fully described in Chapter 4, detailing the development of immunoassays for human ACE. Results obtained were analysed using a paired t-test.

7.3.2. Results

Of the 10 patients studied, 8 demonstrated significant elevation ($p<0.05$) in circulating ACE concentration after 4 days of captopril treatment. These results are shown in table 7.1 and fig.7.1.

Patient	**[ACE] ng/ml**	
	Day 1	**Day4**
I.A.	158.3	189.9
O.A.	379.9	299
M.B.	333.7	289.8
S.D.	209.5	309.7
E.G.	406.7	421.5
E.H.	408.6	504.8
J.H.	453.7	581.6
E.M.	335.4	396
A.O.	287.9	428.9
P.S.	298	356.6

Table 7.1. Tabulation of the ACE concentrations, determined by radioimmunoassay, for each of the congestive cardiac failure patients treated with captopril for four days. Values on day 1 (pre-treatment) and day 4 are listed.

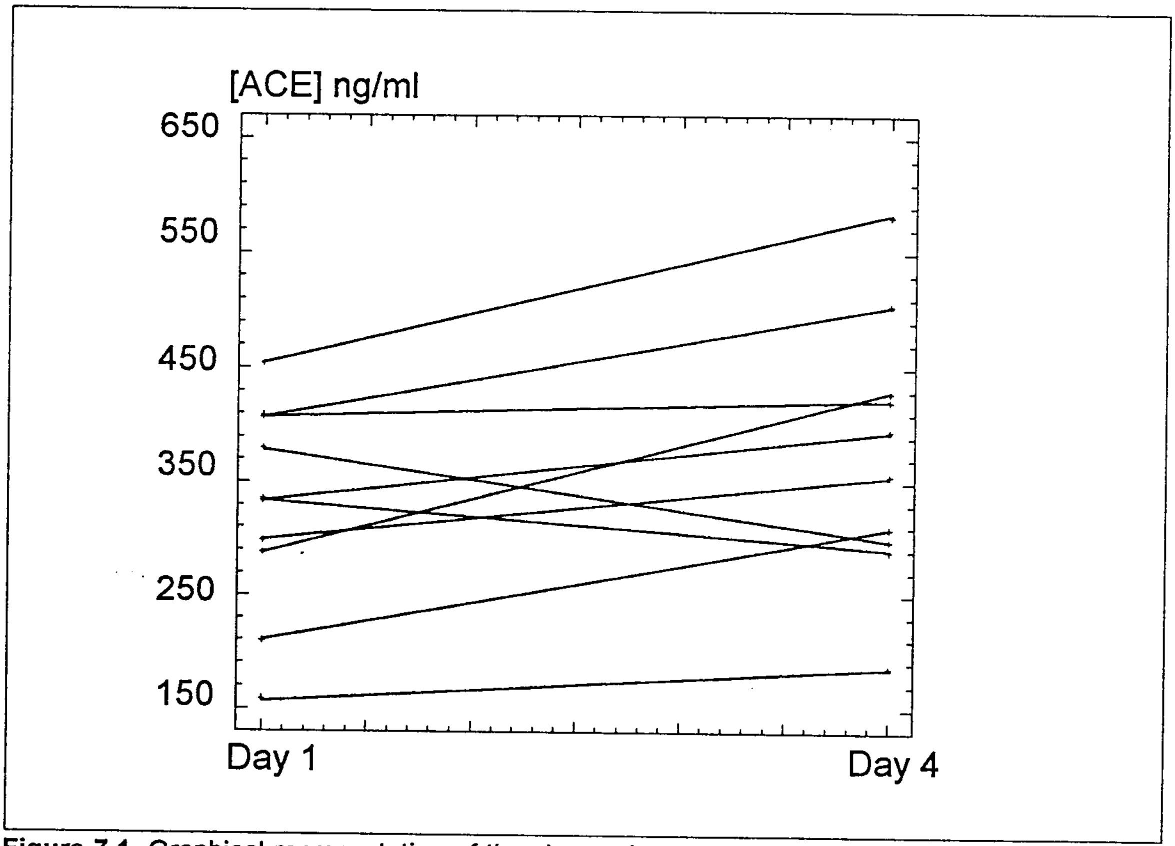


Figure 7.1. Graphical representation of the change in serum ACE concentration, as measured by radioimmunoassay, in patients receiving captopril for congestive cardiac failure for three days. For most patients a significant increase in concentration was observed ($p<0.05$) between days 1 and 4.

7.3.3. Discussion

The results of this pilot study suggest that treatment with captopril elevates circulating ACE concentrations either by an inductive mechanism resulting in increased *de novo* ACE production or increased release of pre-existing ACE from endothelial surfaces.

It must be acknowledged that the effect of the other agents used to treat these patients, most particularly furosemide, could also have resulted in an increased concentration of circulating ACE through diuresis, although the fluid lost during the treatment of congestive cardiac failure is predominantly interstitial and treatment, especially when it includes vasodilators such as nitrates or ACE inhibitors, may in fact initially result in an increase in intravascular volume.

Therapy with ACE inhibitors such as captopril appears to elevate circulating ACE concentrations by an unknown mechanism.

The remainder of this chapter is devoted to an elucidation of this mechanism in a rat model.

7.4. STUDY OF TISSUE AND SERUM ACE RESPONSES IN RATS TREATED WITH THE ACE INHIBITOR ENALAPRIL BY MEASURING BOTH ACE PROTEIN AND ACE SPECIFIC mRNA.

Many animal models of hypertension and studies of the effects of treatment with antihypertensive and other cardiac drugs utilise rats, due to their relative ease of breeding and handling. It was therefore decided to use rats in this study.

The first part of the study set out to examine the acute effect of ACE inhibition on tissue renin-angiotensin systems in rat lung and testicular tissue. In order to study the potential feedback control mechanism of ACE induction, the effects of saralasin (an angiotensin II antagonist) and aldosterone (the final effector of the renin-angiotensin system) on ACE protein and mRNA were examined. Finally, the effect of chronic enalapril administration on circulating and tissue ACE was examined.

In all these studies the ACE inhibitor enalapril (in the form of its maleate salt) was utilised.

Northern blots were probed with a cloned rat riboprobe which has 90% and 70% sequence homology with the mouse and human ACE genes respectively. The digoxigenin (Boehringer) method was used with detection based on the chemiluminescent substrate 3-(2' Spiroadamantane)-4-methoxy-4-3-(3"-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD). Chemiluminescent detection has been reported to provide signals at least as strong as those obtained with isotopes such as ^{32}P. Unlike previous studies of rat ACE mRNA expression which utilised human cDNA probes, all experiments were performed using specific rat ACE cDNA derived riboprobes.

7.4.1. Methods

7.4.1.1. Rats

All rats used in the study were male Long-Evans rats ranging between 250 - 350 grams in mass. All experimental protocols were submitted to the Animal Experimentation Committee of the University of Cape Town for their approval. All animals were fed normal rat chow and supplied with unlimited access to water. Rats were sacrificed by decapitation. Blood was collected into serum separation tubes, allowed to clot and then centrifuged briefly. The serum was aspirated, placed in Eppendorf microfuge tubes and stored at -70°C till assayed. Organs of interest for the tissue studies were rapidly excised, rinsed with physiological saline and snap frozen in liquid nitrogen. Tissue was wrapped in aluminium foil and stored at -70°C until required.

7.4.1.2. Immunoassays for rat ACE

Serum and tissue ACE were measured by ELISA (Chapter 5). In order to be able to assay tissue sample ACE concentrations with the ELISA it was necessary to detergent-solubilise the plasma membrane-bound enzyme. Approximately 100 mg of tissue was chipped from the frozen organs and placed in 1 ml of 25mM HEPES buffer, pH 7.4. The tissue was then homogenised in a glass/glass homogeniser and then a further 1 ml of the same buffer containing 2% CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulphonate) was added. The homogenate was briefly sonicated and incubated at 37°C for thirty minutes. The homogenate was clarified by centrifugation at 10000g for 30 minutes before the supernatant was drawn off and utilised in the ELISA to measure ACE (three dilutions of the sample in duplicate were assayed) and total protein was measured by the method of Bradford (Bradford, 1976) using BioRad commercial reagent. Tissue concentrations are reported in µg ACE/mg protein.

7.4.1.3. Isolation of rat ACE cDNA

The isolation of the rat cDNA clones RACE1 and RACE2 was fully described in chapter 6.

7.4.1.4. Preparation of Digoxigenin-labelled probes

A 400 bp fragment of RACE1 was obtained by restriction enzyme digestion of RACE 1 by the enzymes EcoR1 and Sph1. Complete digestion was ascertained by running the digest on a 2% agarose gel and the required fragment isolated by allowing it to migrate into a piece of Whatman 3M paper that was placed in a slit which was cut in the gel (Errington, 1990). The buffer containing the insert trapped in the filter paper was collected by brief centrifugation in a microfuge tube. This fragment was directionally subcloned (technique detailed in appendix) into the vector pGEM-3 which, after linearisation, allowed for the generation of sense and antisense strands, using the T7 and SP6 promoters of the vector and the corresponding RNA polymerases. Digoxigenin-labelled antisense RNA transcripts were obtained by using SP6 DNA dependent RNA polymerase in the presence of digoxigenin-11-UTP (Holtke & Kessler, 1990) after linearisation with EcoRI (full details of the labelling method are to be found in the appendix).

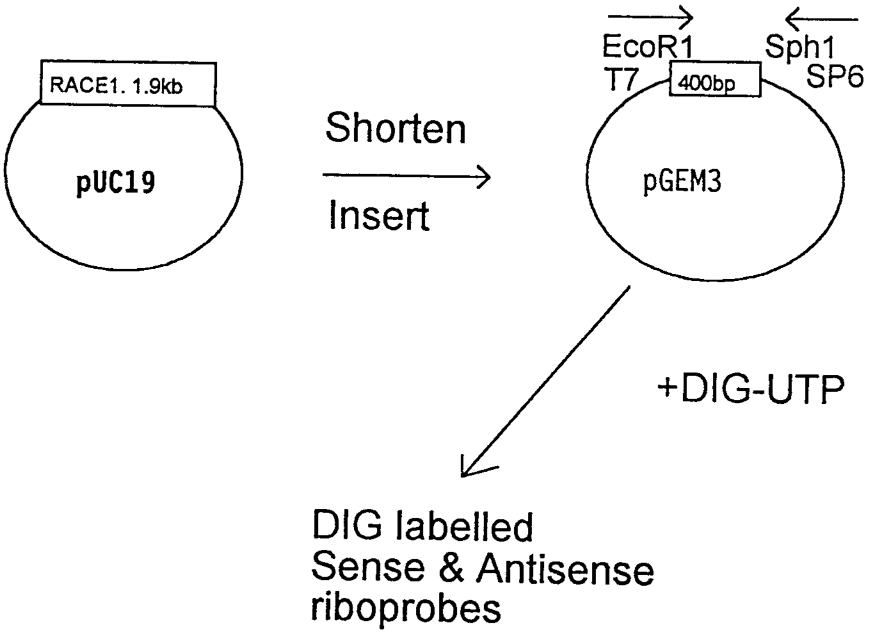


Figure 7.2. Diagrammatic representation of the production of sense and antisense riboprobes from RACE1 in pGEM3.

As an internal standard, human ß-Actin cDNA (Gunning et al., 1983) was cut with XhoII and Sal1 and the resulting ±540 bp restriction fragment directionally subcloned between the SalI and BamHI restriction sites of pGEM-3. Digoxigenin-labelled probe was prepared in an identical manner to that described above. The antisense actin probe was generated by linearising the clone with SalI and using T7 DNA dependent RNA polymerase in the presence of digoxigenin-11-UTP.

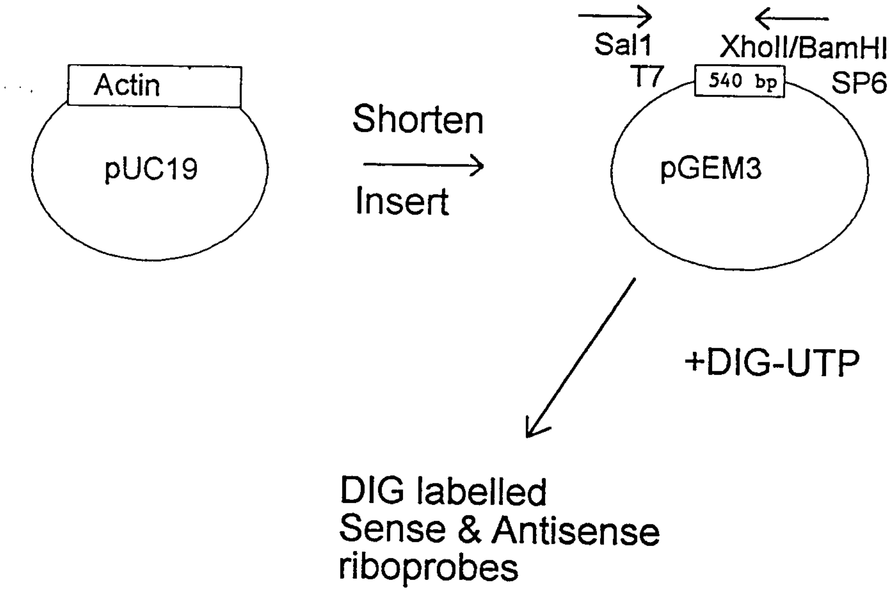


Figure 7.3. Diagrammatic representation of the production of sense and antisense riboprobes from human β Actin in pGEM3.

7.4.1.5. Assessment of probes

Labelled probe was run on a formaldehyde agarose gel and then northern blotted in the standard manner. The membrane was then washed, blocked and probed with an anti-digoxigenin antibody and the labelled probe was visualised directly utilising the colorigenic substrate X-gal/nitroblue tetrazolium. In this way the purity of the labelled transcript could be assessed and the relative titre of different probes compared.

Antisense probes to rat ACE and actin were also tested for their ability to detect their respective mRNA targets in total RNA in northern blots.

7.4.1.6. Northern blotting

For all studies total RNA was isolated by the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987) as described in the appendix from rats immediately after sacrifice. Frozen tissue was retained in liquid nitrogen until required when approximately 100 mg was chipped off with a sterile blade. The tissue was then processed to obtain total RNA. RNA purity was assessed by running on 1% agarose gels in the presence of ethidium bromide and observing the intensity of the 28s and 18s ribosomal bands. Evidence of lack of protein contamination was obtained from the OD 260/280 ratio which was always > 1.8.

To assess the baseline ACE mRNA expression in normal rat tissue, total RNA was isolated from various tissues - lung, liver, kidney, testis, small bowel, spleen, brain and heart. Five µg of total RNA from each source (except for testis where only 2 µg was used) was loaded into wells of a formaldehyde agarose gel and northern blotted as described in the appendix.

For the studies of the effect of ACE inhibitor therapy on ACE mRNA, 5 µg of total lung RNA or 2 µg of testicular RNA (quantitated spectrophotometrically) was run on a formaldehyde agarose gel. After electrophoresis at 30 mA for approximately 3 hours, the RNA was transferred to a nylon membrane (Hybond-N, Amersham) overnight by capilliary blotting in the presence of 10x SSC. After briefly rinsing the membrane in 6x SSC the RNA was fixed to the membrane by UV-crosslinking. The membrane was prehybridised and then probed with a mixture of the digoxigenin-labelled ACE and Actin probes. High stingency washes (final washes 0.1x SSC, 0.1% SDS at 68°C) and subsequent chemiluminescent detection of RNA using the substrate AMPPD was according to the manufacturer's instuctions (Boehringer). Normally an exposure to X-ray film of two hours at room temperature was sufficient.

The relative intensity of the hybridisation signals obtained were quantified with a Hoefer GS300 Transmittance/Reflectance scanning densitometer (Hoefer Scientific Instruments, San Fransisco). Results were expressed in arbitrary transmission units and the ACE mRNA signal strengths controlled for loading by hybridisation to actin. Equivalence of gel loading was also assessed by post transfer staining of the gel with ethidium bromide and post detection staining of the membrane with methylene blue.

To assess the baseline ACE mRNA expression in normal rat tissues, total RNA was isolated from various tissues - lung, liver, kidney, testis, small bowel, spleen, brain and heart. Five µg of total RNA from each source (except for testis where only 2 µg was used) was fractionated on formaldehyde agarose gels and transferred u onto nylon membranes using standard techniques.

7.4.1.7. Rat studies

The effect of enalapril on rat ACE protein and mRNA levels were also assessed. Enalapril maleate, dissolved in physiological saline and filter sterilised (0.22µm filters Millipore), were given by intraperitoneal injection at a dose of 1mg / kg body mass.

Study 1: The effect of a single dose of enalapril maleate on lung ACE mRNA and protein was studied at the following time points, t = 0, 1, 2, 3, 4 and 6 hours (n = 3 per time point). Serum ACE activity was measured simultaneously utilising the spectrofluorimetric assay of Cushman and Cheung (1971). Testicular tissue was studied for ACE mRNA expression at the same time points .

Study 2: The effect of a single dose of the angiotensin II antagonist, saralasin (Sigma) dissolved in physiological saline, administered by bolus intraperitoneal injection at a dose of 100 ng/kg body weight, was studied at t = 0, 2 and 4 hours (n = 3 per time point).

Study 3: The effect of a single dose of aldosterone administered subcutaneously at a dose of 15 µg/kg body weight with rats sacrificed at t = 0, t = 2 and t = 4 hours (n = 3 per group).

Study 4: The effect of pretreatment with aldosterone administered subcutaneously at a dose of 15 µg/kg body weight followed, two hours later, by an intraperitoneal dose of enalapril. Rats were sacrificed at t = 0, t = 2 and t = 4 hours (n = 3 per group).

Study 5: Daily doses of enalapril were administered and animals (8 per group for serum levels and 3 per group for lung ACE mRNA and protein analysis) studied at Day 0, day 7 and day 14.

7.4.2. Results

7.4.2.1. mRNA analysis

The antisense ACE riboprobe sucessfully detected the two distinct forms of rat ACE when lung RNA (5 µg) and testicular total RNA (2 µg) were run on a formaldehyde agarose gel and northern blotted. An identical blot was probed with the actin riboprobe. A marker lane was run with unlabelled mRNA markers of 1.6, 1.9, 2.8, 5.3 and 7.4 kb (Boehringer).

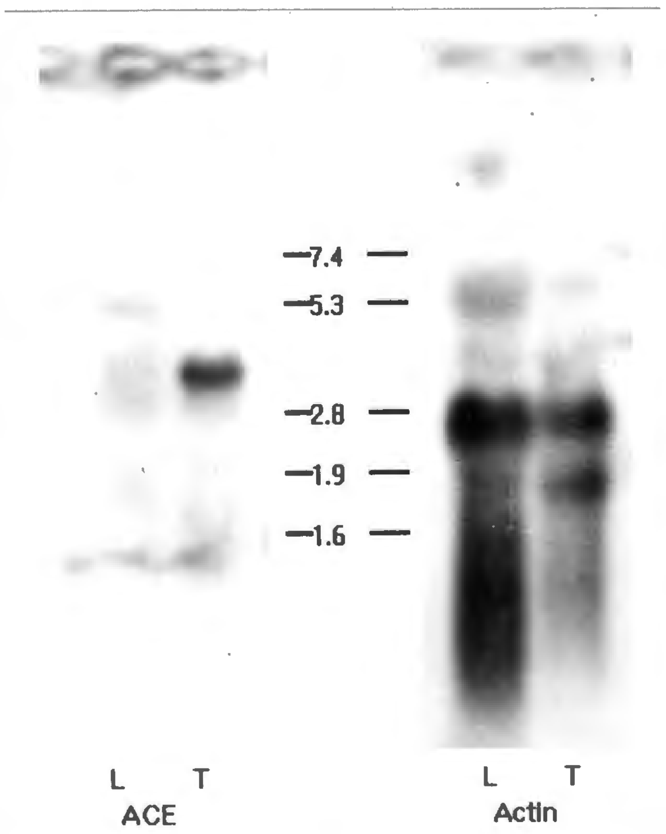


Figure 7.4. Northern blot analysis of lung and testicular ACE. 5 µg total lung RNA and 2 µg total testicular RNA were fractionated on formaldehyde agarose gels. The RNA was blotted onto nylon membranes and the blot on the left probed with the antisense ACE riboprobe while the other blot was probed with the antisense actin riboprobe .

The ACE riboprobe hybridised to a lung mRNA of approximately 5.3 kb and a testis mRNA of approximately 3.5 kb. The actin riboprobe detected mRNA migrating at approximately 2.6 kb in both tissues. This demonstrated that the ACE antisense riboprobe was indeed specific. The human actin riboprobe detected rat actin mRNA with which it is known to share close sequence homology.

Analysis of the labelled antisense riboprobes for ACE and actin showed both of these to migrate as a single band, suggesting that most transcripts were of equal length.

7.4.2.2. Baseline ACE mRNA expression

When 5 µg of total RNA from lung, liver, kidney, small bowel, spleen, brain and heart, and 2 µg total RNA from testis, were northern blotted and hybridized with the ACE riboprobe, only lung, testis and small bowel ACE mRNA were detected, whereas mRNA expression from the other organs appeared to be too low to detect.

Figure 7.5. Northern blot of total mRNAs from the following rat tissues; Lanes 1-8 respectively, lung, liver, kidney, testis, small bowel, spleen, brain and heart. All samples were 5 µg total mRNA except testis which was 2 µg. Hybridisation signal were seen for lung, testis and small bowel.

7.4.2.3. Rat studies

In order to perform a single dose time course, eighteen rats were divided into 6 groups of 3 each and all animals were given an intraperitoneal injection of enalapril, the dose calculated according to their body weight (1mg/kg body weight).

ACE mRNA expression in lung tissue harvested from each group at t=0, 1, 2, 3, 4, 6 hours was assessed by northern blotting. The autoradiographic results are depicted in figure 6 and show a significant elevation in ACE mRNA levels at t = 1 hours with a peak at t = 3 and the t = 6 hour group demonstrating an ACE mRNA level less than that observed at t = 0.

Samples were also processed for lung ACE protein determination and the results are incorporated into figure 6 below. It can be seen that lung ACE concentration more than doubled within 4 hours to a value of 22.8 μg ACE/mg protein.

Within the first hour of treatment, the serum ACE activity had fallen to less than 10% of that observed in the t=0 group (data not shown).

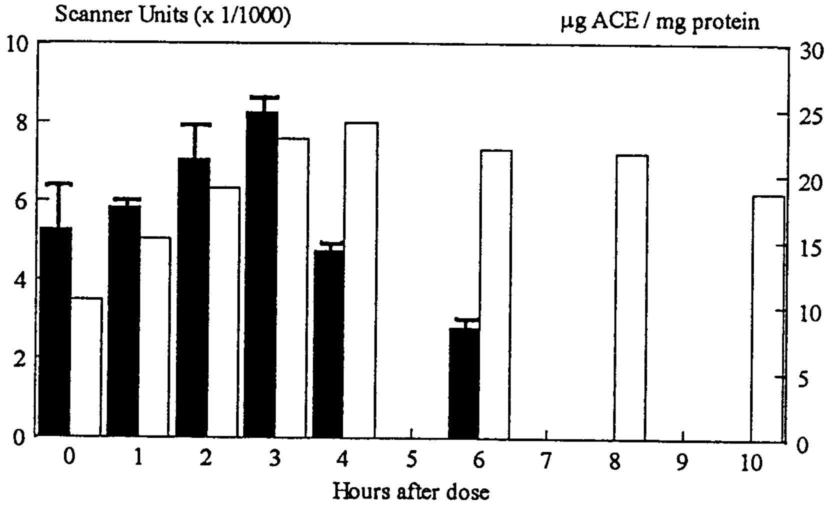


Figure 7.6. Effect of single dose on rat lung ACE mRNA (solid bars) and protein (open bars) at various time points. Eighteen rats were each given a single intraperitoneal injection of enalapril diluted in saline (1mg/kg body weight). Groups of rats (each n = 3) were sacrificed at t = 0, 1, 2, 3, 4, 6 (and 8 and 10 hrs for ACE protein) hours. Lung tissue was obtained and total RNA subjected to formaldehyde agarose gel electrophoresis and northern blotting to a digoxigenin-labelled rat ACE and human actin riboprobe. Results of ACE mRNA levels were obtained by densitometric scanning of the resulting chemilumigrams, and are expressed as means±standard deviation. The actin signal was used for correction of track loading. Lung ACE protein was assayed by ELISA and expressed as mean μg ACE/mg total protein as measured by Bio-Rad assay. A significant peak elevation of ACE mRNA level was observed at t = 3 ($p<0.05$ as compared to t = 0).

Testicular tissue from the same animals was also studied by northern blotting. Results indicate that ACE mRNA expression doubled within two hours and returned to baseline levels within six hours of enalapril administration (figure 7.7). ACE protein was not assayed in these tissues.

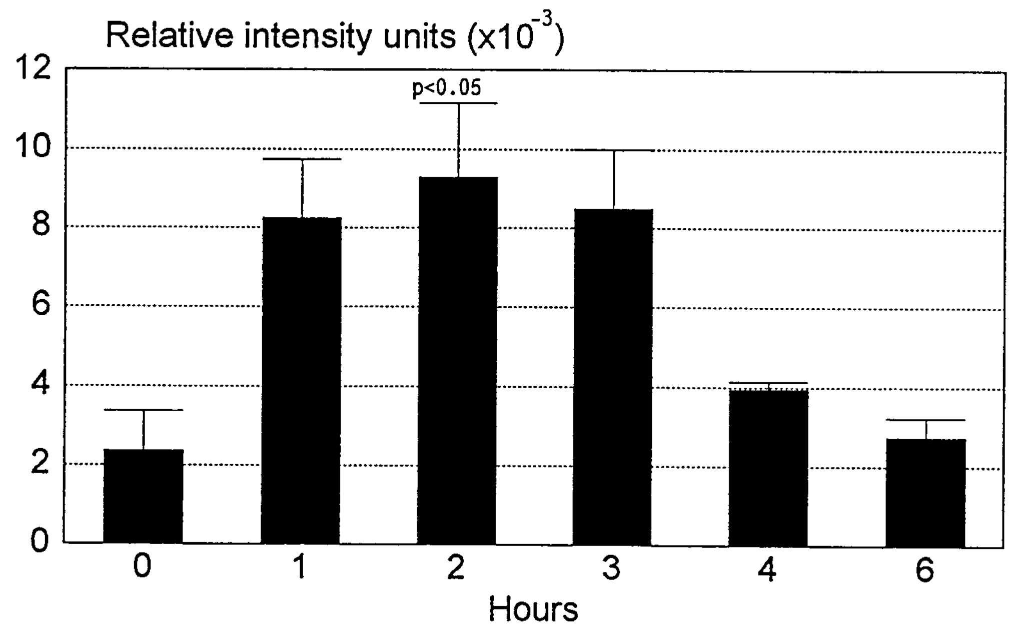


Figure 7.7. Effect of enalapril on testicular ACE mRNA. Eighteen rats given 1mg enalapril/kg body weight by intraperitoneal injection were sacrificed in groups (each n = 3) at t = 0, 1, 2, 3, 4, 6 hours after drug administration. Total RNA was extracted from samples of testicular tissue and subjected to formaldehyde agarose gel electrophoresis and northern blotting. Blots were probed with digoxigenin-labelled rat ACE and human actin riboprobes (to correct for track loading variations). Signals from chemilumigrams were analysed by densitometric scanning and results (in relative intensity units) for each group are depicted in this bar graph. One standard deviation is shown above each bar. A significant elevation of ACE mRNA level was observed at t = 2 ($p<0.05$ as compared to t = 0).

Administration of the angiotensin II antagonist saralasin to rats resulted in a marked increase of lung ACE mRNA expression, with the t = 4 hour level almost eight times that at t = 0. Graphical representation of the results obtained is shown in figure 7.8.

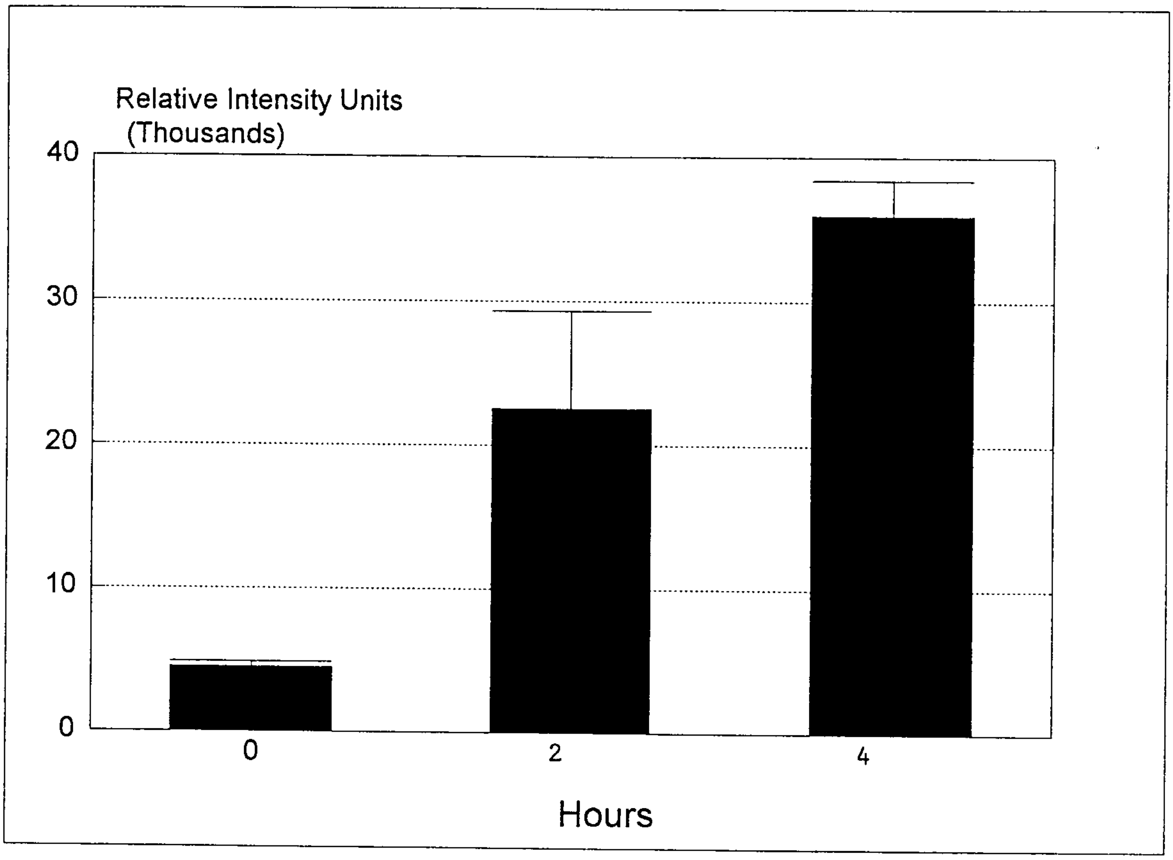


Figure 7.8. Effect of saralasin on lung ACE mRNA expression. Nine rats were each given a single intraperitoneal dose of saralasin (100 ng/kg body weight). Groups (each n = 3) were sacrificed at t = 0, 2 and 4 hours after dosage. Total RNA was extracted from samples of lung tissue and subjected to formaldehyde agarose gel electrophoresis and northern blotted. These were probed with digoxigenin-labelled riboprobes for rat ACE and human actin. Hybridisation signals were detected by chemiluminescence and analysed by densitometric scanning. The results, expressed in relative intensity units (corrected for loading variation by the actin hybridisation) are shown for each of the three groups. In each case the bar represents the mean and the line the standard deviation. A significant elevation of ACE mRNA expression was observed at t = 4 as compared to that at t = 0 ($p<0.0001$).

In order to assess the effect of aldosterone alone, a time course study following a single intraperitoneal dose of aldosterone was pursued. ACE expression was unaffected, as can be seen by the relatively constant ACE expression for all time points (figure 7.9a). This indicated that aldosterone itself has no effect on normal unstimulated ACE mRNA expression.

However, when animals were given aldosterone intraperitoneally followed 2 hours later by a dose of enalapril at the same site, the previously demonstrated enalapril induced increase in ACE-specific lung mRNA was not observed.

Indeed ACE mRNA expression appeared to be greatly diminished compared to that found in untreated rats as evidenced by virtually no hybridisation signal on the northern blot (figure 7.9b). This suggests that the effect of enalapril may result from reduced aldosterone levels caused by ACE inhibition and that when supplementary aldosterone is given no inductive effect of enalapril is observed.

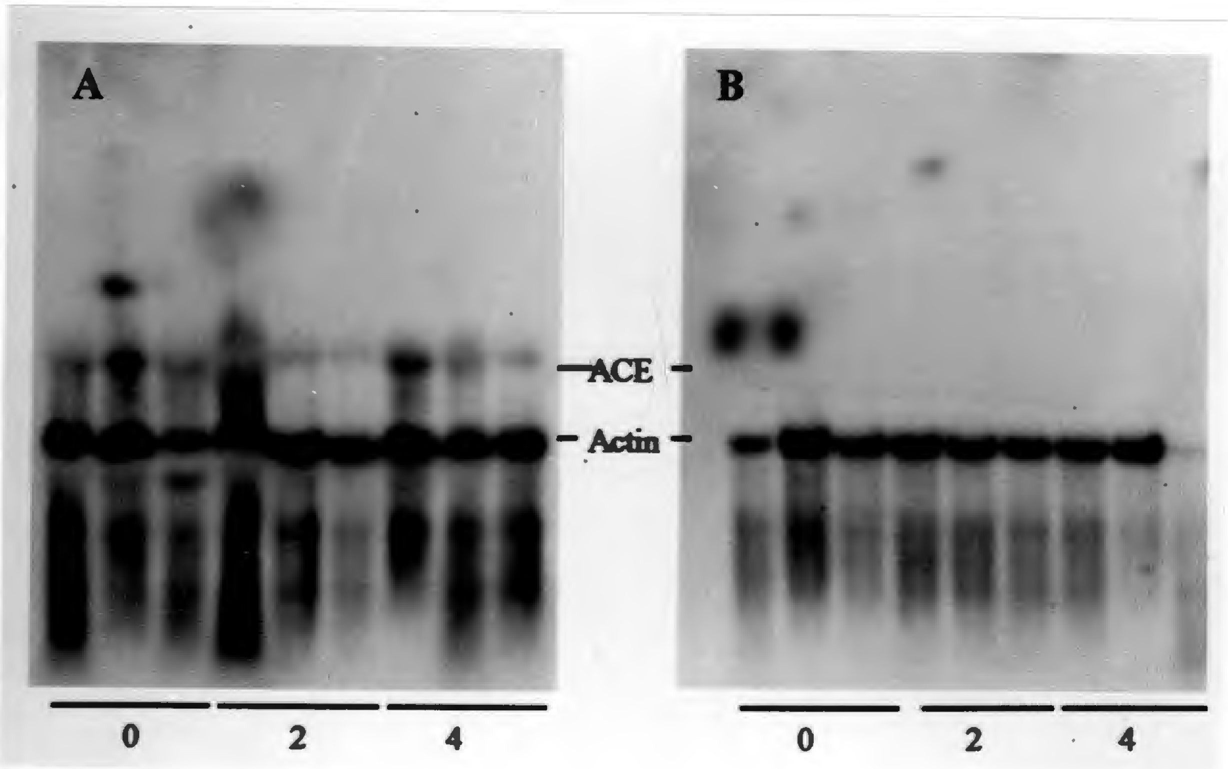


Figure 7.9a. Effect of aldosterone on lung ACE mRNA expression. Nine rats, each given a single subcutaneous injection of 15 μg aldosterone/kg body weight, were sacrificed in groups (n = 3) at t = 0, 2 and 4 hours after dosage. Lung ACE mRNA was studied by northern blotting. The resulting chemilumigram is shown here demonstrating no change in ACE mRNA expression at each of the time points.

Figure 7.9b. Effect of aldosterone pretreatment on response of lung ACE mRNA expression to enalapril. Expression of lung ACE mRNA, where all animals were given a dose of aldosterone followed, two hours later, by a dose of enalapril was studied by northern blotting. Groups of three rats were sacrificed at t = 0, t = 2 and t = 4 after the enalapril dose. No ACE hybridisation signal is seen on the chemilumigram.

To study the effect of chronic enalapril administration, five groups of eight rats were given either enalapril or saline (controls) for varying periods as represented in table 1 and figure 5 (all results expressed as mean±S.D.). At t = 0 the mean serum ACE concentration as assayed by ELISA was 1189±213 ng/ml. After 7 days of enalapril the mean ACE concentration had risen to 2397±511.4 ng/ml (7 day saline control 1201.4±367.6 ng/ml) and by fourteen days of treatment levels had declined to 1765±379 ng/ml (14 day saline control 1257.6±250 ng/ml).

Study Group (each n=8)	**[ACE]±SEM (ng/ml)**
No Rx	1189±213
7 days saline	1201±365
7 days enalapril	2397±511
14 days saline	1257±251
14 days enalapril	1765±379

Table 7.2. Effect of chronic enalapril administration on serum ACE: Groups of rats (each n = 8) were given daily intraperitoneal doses of either enalapril (1mg/kg) or equivalent volumes of normal saline. After the required number of days treatment, and two hours after the last dose, the animals were sacrificed by decapitation. Blood samples were obtained and the serum assayed by ELISA. The results for each group are tabulated showing ACE concentration as mean±S.D.

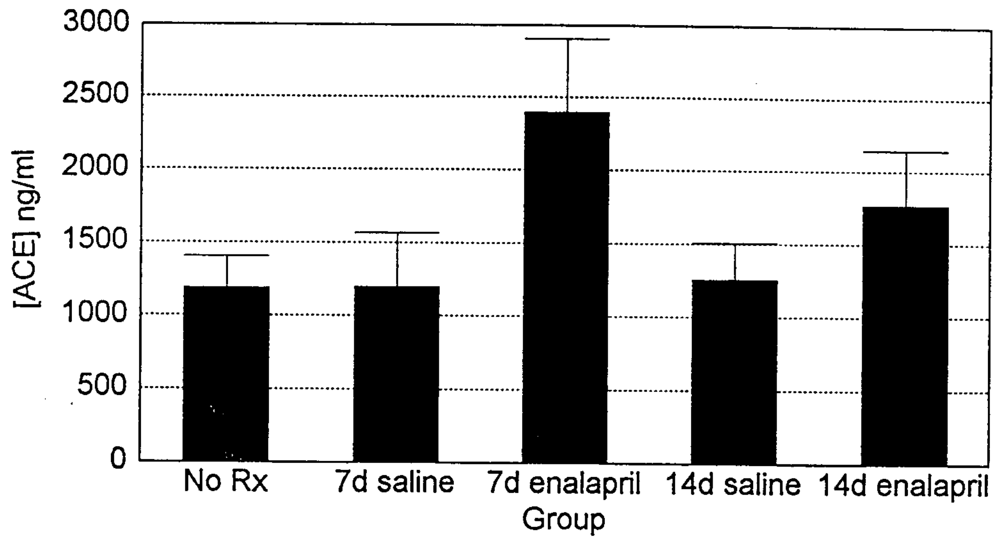


Figure 7.10. Bar chart of ACE concentration for each of the rat treatment groups depicted in table 1 (each n = 8). One standard deviation is represented above each bar.

Similar time points and control groups (all n = 3) were studied for lung ACE mRNA by northern blotting and the results quantified by densitometric scanning. The results showed no marked induction after 7 days of treatment, however it was interesting to note a subnormal ACE mRNA expression in the 14-day treatment group suggesting down-regulated ACE expression (data not shown).

As with lung ACE mRNA, lung ACE protein was also assayed and revealed a mean ACE concentration at t = 0 of 11.6 µg ACE/mg protein, and after 7 days treatment no significant increase was observed (10.9 µg ACE/mg protein). However, after 14 days of treatment, the mean ACE concentration had risen to 14.2 µg ACE/mg protein suggesting that accumulation of ACE protein persists well after ACE mRNA expression has returned to pretreatment levels. This suggests that plasma membrane ACE has a low turnover rate.

7.4.3. Discussion

This study clearly demonstrated that enalapril induced *de novo* synthesis of ACE in the lung and that the previously observed elevations in serum and tissue ACE enzyme activity are due to increased concentrations of ACE protein.

As can be seen from the baseline ACE mRNA expression study in various tissues, it was only possible to accurately quantitate changes in lung and testis mRNA. Small bowel ACE mRNA detection is consistent with previous localisation by *in vitro* autoradiography of ACE within the villi of the duodenum and jejunum (Duggan et al., 1989). In the small bowel ACE is thought to play a role in the hydrolysis of dietary peptides. The structure, formation and secretion of ACE in the small bowel has recently been detailed (Naim, 1992 and 1993)

ACE inhibitors are considered not to affect testicular ACE due to the existence of a blood-testis barrier (Jackson et al., 1988a). It was therefore surprising to find a marked induction of testicular ACE mRNA in our study. A possible explanation is that the earliest post-dose sample in previous studies (6 hours) (Schunkert et al., 1993) was at a time at which ACE mRNA had already returned to baseline in our study. In our study greater sensitivity was obtained by utilising an ACE riboprobe derived from a rat ACE cloned template rather than utilising a human probe to detect rat ACE mRNA which, in a study of rat heart ACE mRNA, required loading of 25µg poly(A) RNA from heart, lung and testis in order to get a hybridisation signal (Schunkert et al., 1990).

The finding that ACE mRNA in untreated tissues appears to be expressed at relatively low levels, despite its widespread endothelial distribution, suggests that the enzyme has a slow turnover rate (its half-life has been previously estimated to be 72 hours (Fhyrquist et al., 1983)). This is further supported by the findings of the single dose time course that demonstrated elevated ACE protein concentration in tissue well after ACE-specific mRNA levels have declined. This indicates a very slow clearance rate of ACE from the body which supports the notion that, in the untreated animal, only a relatively low rate of

ACE production is required to maintain physiological tissue and serum ACE concentrations.

In the single dose time course experiment (figure 5) lung ACE mRNA levels increased by approximately 156% three hours after administration of enalapril. Lung ACE protein concentrations, however reached a peak after 4 hours with an increase of more than 100%, suggesting there is a close temporal relationship between ACE mRNA expression and ACE protein levels. The finding that at 6 hours mRNA expression is near normal (when ACE protein levels are still elevated) suggests that excess ACE may indirectly be involved in feedback inhibition which down regulates further ACE production.

ACE inhibitor administration to cultured porcine endothelial cells also results in a rapid increase of ACE mRNA expression (Oparil and King, 1992). Within 30 minutes, ACE mRNA levels were increased, peaking initially at 2 hours and then again at 48 hours. A delayed increase in ACE activity in the culture medium was also observed.

Although this study examined the acute effects of ACE inhibitor therapy, preliminary chronic studies (data not shown) have demonstrated diminished ACE production following the initial induction; after 14 days of ACE inhibitor therapy, the serum concentration of ACE is decreased compared to the levels attained after only 7 days of therapy. Thus, continuing ACE induction during chronic therapy does not appear to occur and a mechanism exists to limit production of ACE in the presence of excess enzyme. mRNA levels in animals treated for 7 and 14 days, even when harvested 2 hours after their last enalapril dose, did not show the same induction as was observed in the single dose time course. This provides further support for the presence of a negative feedback inhibition on ACE production.

Down regulation of ACE production during chronic inhibitor therapy is further supported by the findings showing that, despite the fact that a single dose of enalapril induces a doubling of lung ACE protein, two weeks of daily enalapril doses produces an elevation of only 40% in the same tissue. This result is in agreement with the finding (utilising the technique of quantitative *in vitro* autoradiography) that a two week administration of lisinopril (Kohzuki et al., 1991), increased total lung ACE by 30%.

Saralasin, an angiotensin II antagonist, produced an eight fold increase in lung ACE mRNA levels within four hours of administration. This suggests that angiotensin II receptor blockade and ACE inhibition provide similar inductive effects. The greater degree of induction observed for saralasin is probably due to more extensive abrogation of angiotensin II effects. A feedback mechanism then operates to overcome this block by stimulating increased ACE production. This finding indicates that the feedback mechanism that operates during ACE inhibition does not depend directly on angiotensin II levels but rather on the extent of angiotensin II receptor stimulation or on a more distal step in the pathway, eg. that of aldosterone production. The binding of angiotensin II and its role in the feedback inhibition of ACE synthesis has recently been suggested from autoregressive modelling of results in a study into the effect of captopril and other enzyme inhibitors on ACE in the rat (Wada et al., 1988). This prompted the study detailed in this chapter on the effect of aldosterone administration on lung ACE gene expression. Pretreatment of animals with aldosterone completely abolished the inductive response to enalapril. This finding, together with the observation that aldosterone administration on its own causes no effect on ACE gene expression, would indicate that the key factor in the enhancement of ACE production during ACE inhibitor therapy is the level of aldosterone present in the tissue. This finding contrasts with the observation that ACE levels are increased threefold when bovine endothelial cells in culture are exposed to aldosterone for 48 hours (Dasarathy et al., 1990). The reason for this difference between *in vivo* and *in vitro* observations is unclear but may point to the existence of other mediators in the feedback process.

Although these studies were performed on lung tissue, it is possible that other tissues in which ACE production is elevated by inhibitor therapy respond in a similar manner, since aldosterone levels are governed by adrenal synthetic activity which could affect diverse tissues. The dependence of the inductive mechanism on adrenal activity was suggested by an earlier finding that adrenalectomy abolished the increase in lung ACE activity during inhibitor therapy (Fhyrquist et al., 1982b). The difference in induction observed by other authors, between lung and kidney tissue for example (Kohzuki et al., 1991), probably relates to a differing degree of inducibility rather than the existence of other mechanisms.

A recent study on the effect of the ACE inhibitor quinapril on tissue ACE activity and ACE mRNA in rats concluded that angiotensin II is the most likely agent responsible for the feedback regulation of ACE gene expression (Schunkert et al., 1993) . Infusion of angiotensin II resulted in decreased lung ACE gene expression and administration of quinapril increased lung ACE mRNA levels. However, this does not appear to be a complete explanation for the feedback process in light of our observations on the effects of saralasin and aldosterone, it is unlikely that angiotensin II is exerting a direct, receptor-independent effect on ACE mRNA and protein expression. Our data suggest that this feedback stimulation is mediated by reduced angiotensin II receptor stimulation, most likely by consequently reduced aldosterone levels. Interestingly, in the same study (Schunkert et al., 1993), no effect on testicular ACE mRNA was observed during ACE inhibitor therapy, but decreased mRNA levels were observed during angiotensin II infusion.

The inductive effect of ACE inhibitors appears to be a general property as it has been reported for captopril (Fhyrquist et al., 1980), enalapril (Forslund et al., 1982) and lisinopril (Kohzuki et al., 1991). The ACE inhibitors vary in their degree of tissue distribution and inhibition, as has been shown in studies of temporal *ex vivo* radioligand binding on various tissues and inhibitors (Johnston et al., 1987). Multiple factors are thought to influence tissue bioavailability of ACE inhibitors (MacFayden et al., 1991); these are: 1. Plasma concentration; 2. Lipophilicity; 3. Relative quantities of plasma to tissue binding sites for the inhibitor; 4. degree and rate of interconversion between the inactive precursor and the active inhibitor (eg. enalapril/enalaprilat); 5. the presence of membrane barriers eg. blood/brain and blood/testis; and finally, 6. the rate of drug elimination from different tissue sites. These could be expected to result in differing degrees of induction between different tissues and inhibitors.

During the treatment of hypertension or cardiac failure, clinical doses of ACE inhibitors normally appear to be sufficient to overcome any degree of ACE induction, especially during chronic treatment (Jackson et al., 1984; Johnston et al., 1987). The results of our studies may explain why variability exists in therapeutic response at the inception of ACE inhibitor administration. This frequently results in ACE inhibitor dosages being increased in the first few days of treatment. It would be interesting to investigate whether, once tissue induction has been down-regulated by feedback mechanisms, patients could be adequately controlled on lower doses of ACE inhibitors than were initially used.

In summary, ACE gene expression appears to be regulated by a feedback mechanism involving angiotensin II binding to its receptor and subsequent events such as aldosterone release. While these studies are among the first to systematically examine, with specific assays, ACE gene expression and ACE protein production following inhibitor treatment, providing greater insights into ACE regulation, further studies are required to gain greater understanding of the mechanisms involved. These may be aided with the sensitive RNA-PCR assays recently described for ACE (Paul and Schunkert, 1992) and the development of specific angiotensin II receptor antagonists (Brunner et al., 1993).

Chapter 8

LOCALISATION OF THE INDUCTIVE RESPONSE IN VARIOUS TISSUES BY *IN SITU* HYBRIDISATION AND IMMUNO-HISTOCHEMISTRY.

8.1. OBJECTIVE

To localise the site of ACE mRNA and protein expression in response to ACE inhibition in sections of rat tissue.

8.2. INTRODUCTION

In the previous chapter, evidence was presented for the induction of ACE in rat lung tissue during treatment with enalapril. It is commonly accepted that the endothelium is the source of ACE in lung tissue and therefore, endothelial cells are most likely to be the site of the observed inductive effect. Endothelial cells in culture have indeed been shown to increase their ACE production during treatment with captopril (Fhyrquist et al., 1982). This has however, never been conclusively demonstrated, as no *in situ* hybridisation studies of ACE mRNA during enzyme inhibition have been reported to date in the literature. Sibony et al. (1993) have reported the use of nucleic acid probes to localise ACE mRNA in normal human and marmoset tissues, but, as for immunohistochemistry, has never been specifically applied during ACE inhibitor therapy.

In situ hybridisation was first used to localise specific DNA sequences on chromosomes (Gall & Pardue, 1971). The technique has since been modified to allow for the detection and localisation of specific RNAs within tissues of many different organisms. Early studies described the localisation of viral or abundant cellular RNAs in cultured cells or tissue sections (Brahic & Haase, 1978; Singer & Ward, 1982). Methods were developed which permitted detection of moderate to low abundance mRNAs and were used to describe the expression and localization of genes regulating the embryonic and larval development of Drosophila. The sensitivity of methods using double stranded (Hafen et al., 1983) or single stranded (Akam, 1983) probes was further increased by the use of single stranded RNA probes (Cox et al., 1984) and ^{35}S labelled probes (Augulewitsch et al., 1986). Non radioactive techniques have also been developed. Most commonly these have used biotin-substituted nucleotides to label probes which are then detected by either a fluorescent or colorigenic enzymatic system (Singer & Ward, 1982; Singer et al., 1986). More recently, greater sensitivity and decreased background signals have been reported for digoxigenin (Boehringer)-labelled probes (Springer et al., 1991). In the northern blot experiments reported in the previous chapter, digoxigenin-labelled riboprobes were utilised and these same probes were used for the *in situ* studies reported here.

While the technique of *in situ* hybridisation is more useful in localising specific mRNAs, it can also be used to provide a semi-quantitative insight into the degree of expression of a particular message. Thus, in the studies reported below, ACE mRNA expression was observed in tissues from rats at different time-points following enalapril administration.

Both polyclonal and monoclonal antisera have been used for immunohistochemical localisation of ACE in a variety of species. From one of the earliest immunohistochemical studies (Caldwell et al., 1976) in the rabbit, it was known that ACE had a widespread tissue distribution in association with vascular endothelial cells. A similar enzyme distribution has been shown in rats (Moore et al., 1984) and human tissues (Danilov et al., 1987) using monoclonal antisera. ACE has also been demonstrated on the epithelial surfaces of renal proximal tubules (Caldwell et al., 1976; Tauc et al., 1988), testicular seminiferous tubules, brain ventricles (Auerbach et al., 1982) and intestinal epithelium (Bruneval et al., 1986). Recently specific polyclonal antibodies capable of differentiating between somatic and testicular ACE have been used to localise ACE in adult human and marmoset tissues (Sibony et al., 1993).

The monoclonal antisera to ACE and a riboprobe to rat ACE mRNA (described in sections 3.4. and 7.2.1.4.) made it possible to combine the techniques of *in situ* hybridisation and immunohistochemistry to detail the induction of rat ACE by enalapril at a cellular level.

8.3. IMMUNOHISTOCHEMISTRY

8.3.1. Detection of rat ACE

As described in Chapter 3, monoclonal 187/18 was developed in mice against human lung ACE, but was found to cross-react with rat ACE. In the immunohistochemical studies this antibody was utilised in the form of ascitic fluid.

8.3.2. Digoxigenin-labelled riboprobes

Riboprobes were produced from the EcoRI/SphI portion of the rat ACE clone RACE1 subcloned into the vector pGem-3. Plamid was linearised by digestion with EcoRI and SP6 RNA polymerase used to synthesize an antisense riboprobe. As a control, a sense riboprobe was derived by SphI digestion and the use of T7 RNA polymerase.

Other controls included in the study of mRNA expression were sections where no probe was used but the subsequent detection steps were still performed, ie. to test for non specificity of the detection system, and pretreatment of the sections with RNAse A prior to hybridisation.

8.3.3. Tissue samples

Male Long-Evans rats (approximately 250 g) were given a single intraperitoneal injection of enalapril maleate dissolved in physiological saline at a final dose of 1mg/kg body weight.

Animals were sacrificed at t = 0 and t = 3 hours for *in situ* hybridisation and t = 0 and t = 4 hours for immunohistochemistry. The organs examined were lung, kidney, testis and heart. After sacrifice these organs were immediately cut into smaller pieces, to maximise fixation, and immersed in Bouin's solution (75 ml saturated aqueous picric acid , 25 ml 40% formaldehyde and 5 ml glacial acetic acid to a final volume of 105 ml). Fixation was allowed to proceed overnight before the tissues were processed and paraffin embedded using standard methodologies.

8.3.4. *In situ* hybridisation

Sections (3-5 μm) of the various samples were cut using a rotary microtome and placed on slides pre-treated with 3- (Triethoxylsilyl)-propylamine (APTES, Merck) and diethylpyrocarbonate (DEPC, Sigma).

The detailed *in situ* hybridisation method is presented in the appendix (9.8.). The sections were dried onto the slides, dewaxed and rehydrated. Proteinase K digestion and fixation with freshly prepared 4% paraformaldehyde was performed before the sections were acetylated with acetic anhydride. After washing, the sections were once again dehydrated, dried and prehybridised for 2 hours at 50°C. Hybridisation was performed overnight at 50°C with probe at 2 ng/μl in hybridisation solution. After washing, digoxigenin-labelled probe was detected with antidigoxigenin/phosphatase conjugate and a colorigenic substrate, X-gal/nitroblue tetrazolium, resulting in a blue-black product.

8.3.5. Immunohistochemistry

ACE protein in the sections was probed with the monoclonal antiserum and binding detected using a peroxidase-conjugated anti-mouse antibody. The presence of ACE protein in the sections is denoted by the brown reaction product. Full details of the method are to be found in the appendix (section 9.9.).

8.4. RESULTS

8.4.1. ACE mRNA and protein localization

In lung tissue little baseline RNA expression could be seen in from cells, probably due to their small size (figure 1). The sense probe control section revealed no staining (not shown). Two hours after treatment with enalapril, it did not appear that there was a change in endothelial ACE mRNA expression, but alveolar macrophages were noted to have significantly increased expression (figure 2). This result was supported by the findings on immunohistochemistry (figure 8).

In the kidney, ACE mRNA was found, as expected, in the glomerular endothelium and in the proximal tubular epithelium (figure 3). Treatment with enalapril significantly increased expression at these sites (figure 4). Immunohistochemistry showed increased ACE protein at these sites four hours after dosage (figure 9 and 10).

Cardiac tissue showed no staining of the myocardium, but the endocardial tissue did appear to have prominent ACE mRNA expression. On treatment with enalapril, ACE mRNA expression was markedly elevated in valvular tissue (figure 5). Immunohistochemistry was not performed on heart tissue.

Cross-sections of seminiferous tubules in sections of testicular tissue revealed ACE mRNA in the germinal epithelium (figure 6). A striking increase in germinal epithelial ACE protein could be seen in developing spermatozoa in the $t = 4$ hour samples (figure 11 and 12).

Figure 1. Section of rat lung tissue (t = 0, x400), probed for ACE mRNA,showing minimal signal detection.

Figure 2. Rat lung tissue (t = 2, x400), probed for ACE mRNA, showing marked alveolar macrophage expression.

Figure 3. Rat kidney tissue (t = 0, x400), probed for ACE mRNA, showing slight glomerular and tubular epithelial expression.

Figure 4. Rat kidney tissue (t = 2, x400), probed for ACE mRNA, showing enhanced glomerular and proximal tubular epithelial expression.

Figure 5. Rat cardiac tissue (t = 2, x400) showing valve with marked endocardial and valvular ACE mRNA expression.

Figure 6. Rat testicular tissue, sections of seminiferous tubule (t = 2, x400), showing germinal epithelial ACE mRNA expression.

Figure 7. Rat lung tissue (t = 0, x400), immunohistochemistry for ACE, showing slight ACE expression in alveolar cells.

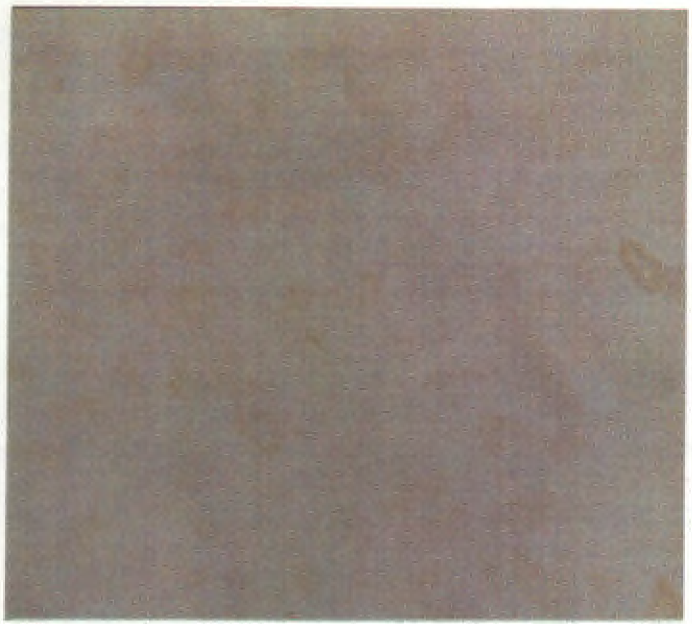
Figure 8. Rat lung tissue (t = 4, x400), immunohistochemistry for ACE, showing enhanced alveolar endothelial ACE expression.

Figure 9. Rat kidney tissue (t = 0, x400), immunohistochemistry for ACE, showing slight glomerular and tubular epithelial expression.

Figure 10. Rat kidney tissue (t = 4, x400), immunohistochemistry for ACE, showing enhanced glomerular and proximal tubular epithelial expression.

Figure 11. Section of seminiferous tubule (t = 0, x400), immunohistochemistry for ACE, showing germinal epithelial ACE expression.

Figure 12. Section of seminiferous tubule (t = 4, x400), immunohistochemistry for ACE, showing marked germinal epithelial ACE expression extending with the developing spermatids into the tubular lumen.



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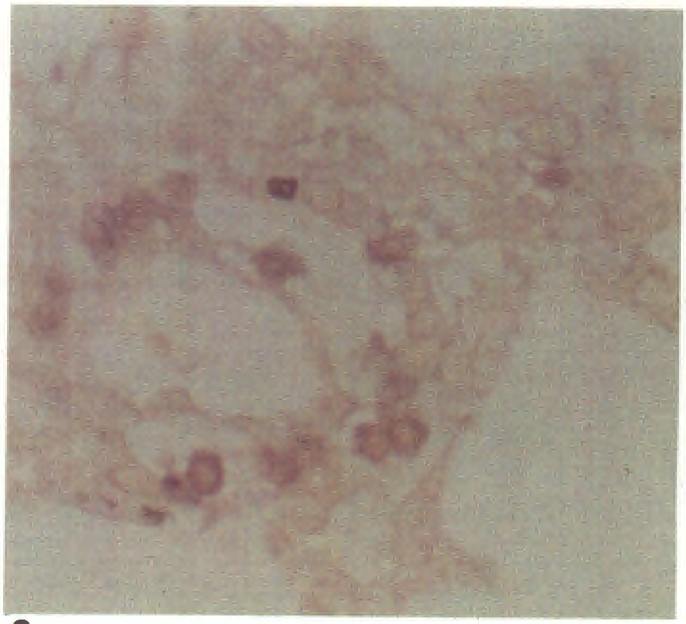

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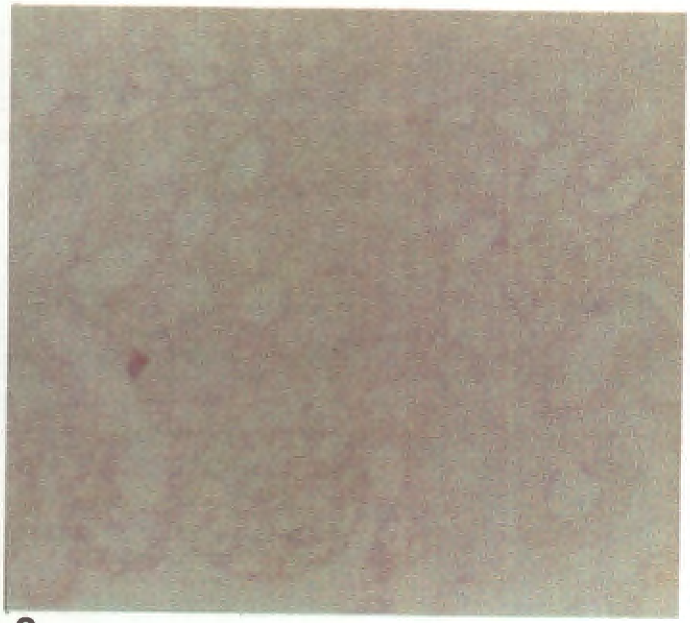

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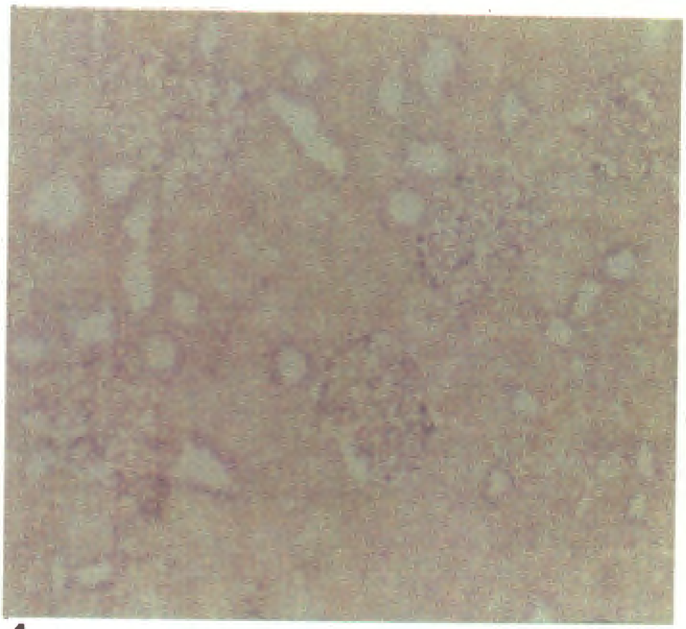

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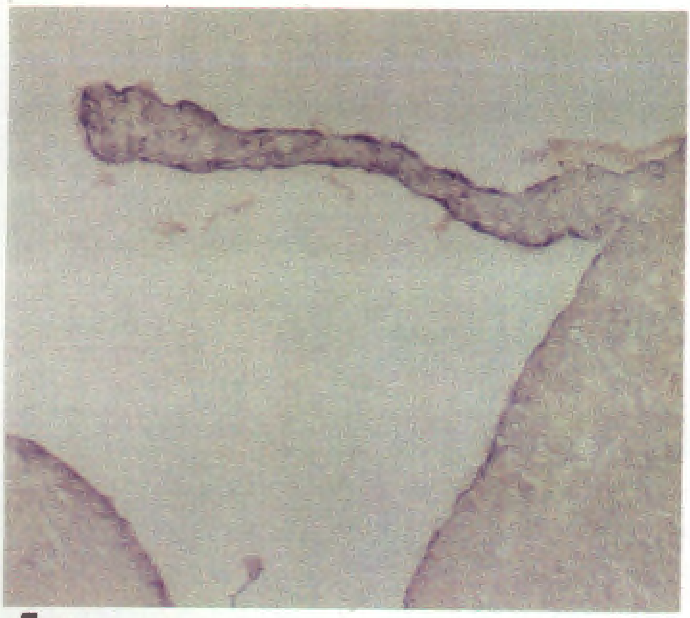

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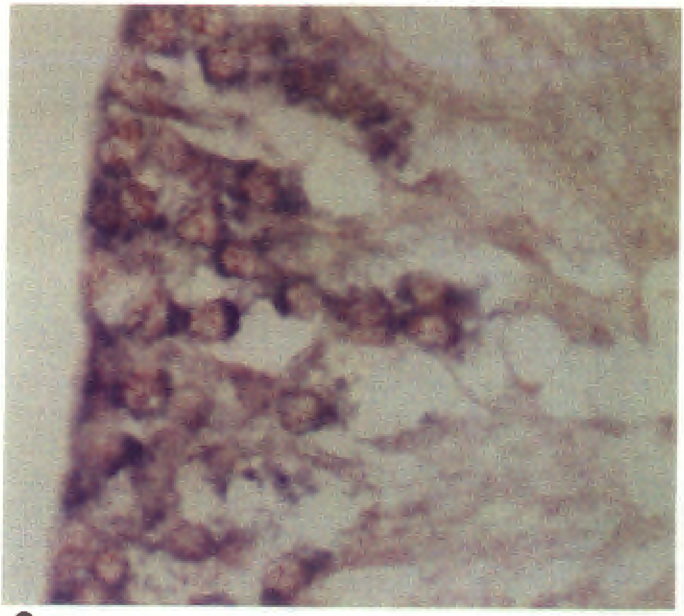

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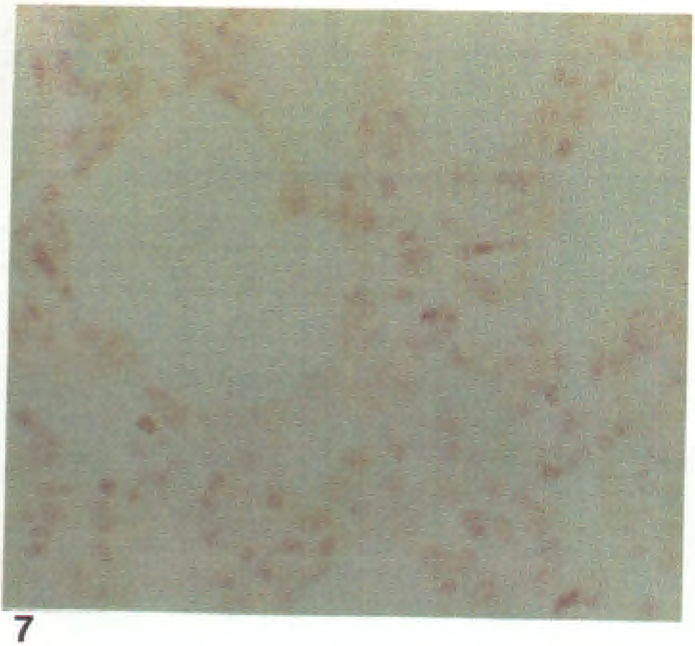
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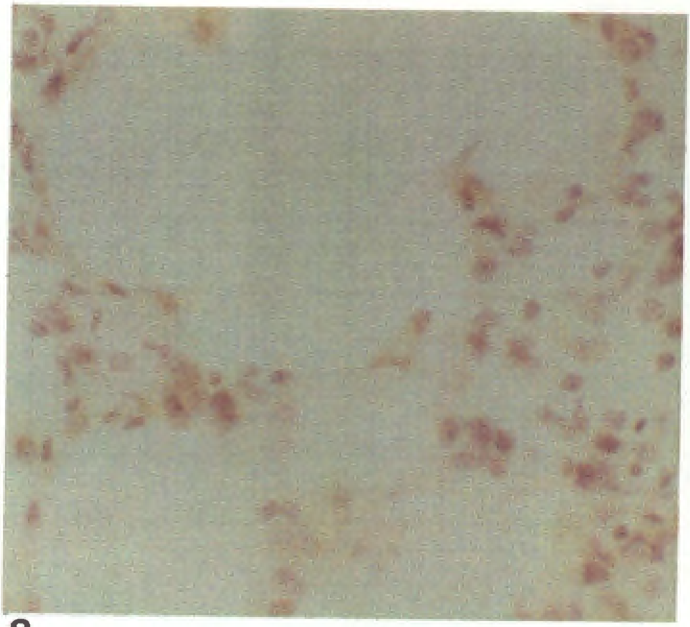
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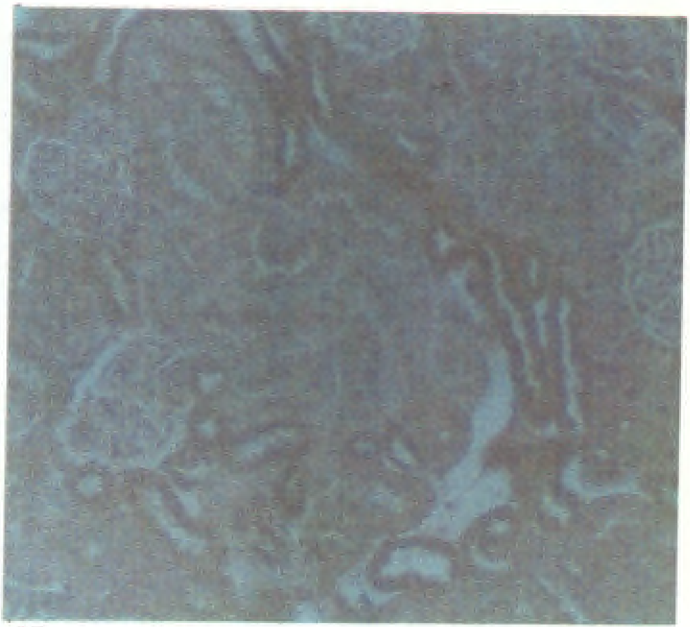
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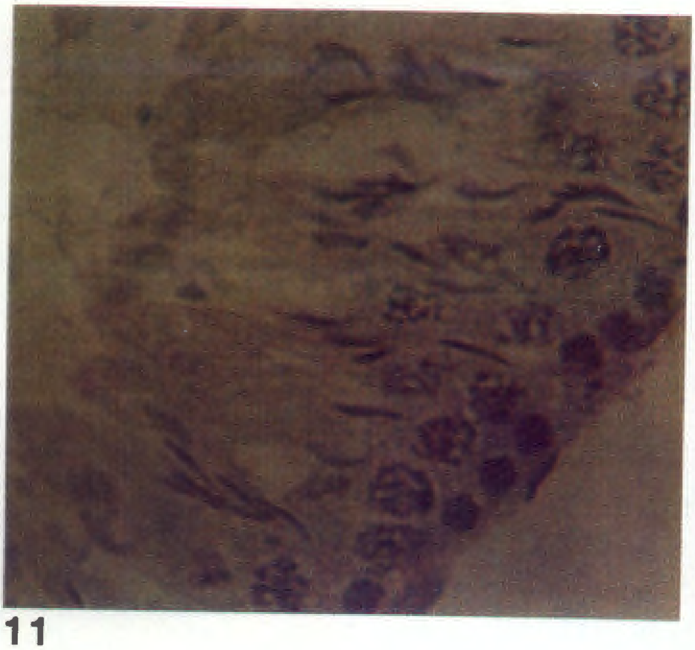
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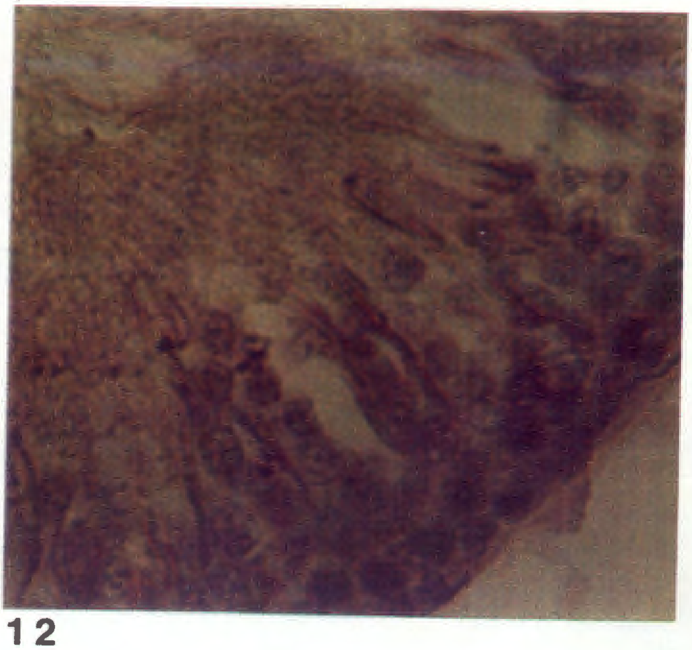
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8.5. DISCUSSION

In these studies the localization of ACE mRNA and protein were examined in rat tissues and the changes in response to ACE inhibition.

While baseline lung ACE mRNA expression located in endothelium was to be expected, a novel observation was the fact that ACE expression was induced in macrophages, rather than in endothelial cells, during enalapril treatment. Macrophages are known to express ACE in sarcoid granulomata (Allen et al., 1986). This effect appears to result from stimulation of ACE producing monocyte-derived granuloma epithelioid cells by T lymphocytes (Vuk-Pavlovic et al., 1988 and Vuk-Pavlovic et al., 1989) and has also been noted in circulating monocytes in sarcoid patients (Okabe et al., 1985). Steroids have also been observed to induce ACE production by rabbit alveolar macrophages in culture (Fiedland et al., 1977), but there are no reports of ACE induction in alveolar macrophages during ACE inhibitor therapy.

Despite having identified alveolar macrophages as being the dominant site of inhibitor induced ACE induction in lung tissue, an important question still remains as to the exact nature of the mediator of feedback induction of ACE in tissues. A response to low levels of aldosterone is supported by decreased ACE mRNA expression in lungs of rats treated with aldosterone and an abrogation of inhibitor-induced induction when aldosterone is administered concurrently. However, adrenalectomy decreased lung and serum ACE activity and, when combined with inhibitor therapy, markedly reduced lung plasma membrane ACE activity, whereas an elevation in serum ACE activity was still observed (Fyhrquist et al., 1982b). This finding suggests that a more complex mechanism involving an adrenal-derived product other than aldosterone is responsible for the feedback mechanism, especially in the lung.

It is interesting to note that bovine endothelial cells in culture (Mendelsohn et al., 1982; Dasarathy et al., 1990), perfused rat lungs (Ialenti et al., 1986) and rat lungs in vivo (Mendelsohn et al., 1982) increase ACE production when exposed to dexamethasone.

Steroid induction of ACE has recently been demonstrated to have a molecular basis with the finding of steroid responsive elements in the human and mouse ACE genes (Shai et al., 1990; Hubert et al., 1991). Mineralocorticoids such as aldosterone may act at these sites. Whether glucocorticoids play any role in the inhibitor mediated ACE induction of alveolar macrophages is unknown, but as yet there appears to be no evidence in the literature of ACE inhibitors inducing endogenous steroid production.

The localisation of ACE mRNA and protein in the glomerular endothelium and the proximal convoluted tubules of renal tissue is consistent with previous immunohistochemical and *in situ* hybridisation studies reported in the literature (Tauc et al.,1988; Sibony et al., 1993). The potential consequences of induction of ACE production at these sites during ACE inhibitor therapy, particularly in the proximal tubules, are unknown. ACE is thought to be involved in sodium transport through the production of angiotensin II (Liu & Cogan, 1987). Recently, angiotensinogen mRNA has been localised to the rat proximal tubule by *in situ* hybridisation (Ingelfinger et al., 1990). Thus angiotensinogen and ACE are produced in the proximal convoluted tubule, implying that angiotensin II (Taugner et al., 1987) is locally generated at this site. As with ACE mRNA, angiotensinogen mRNA (Ingelfinger et al., 1990) has been located in the glomeruli where a local RAS might have a role in glomerular tubular feedback, glomerular contractility and distal tubular function.

The testis is protected from direct access by ACE inhibitors by a blood/testis barrier (Jackson et al., 1988a; 1988b). Finding evidence for ACE induction both in the kidney and in the seminiferous tubules of testicular tissue is in conflict with observations made with the technique of quantitative in vitro autoradiography (Kohzuki et al., 1991), where no induction was observed for either tissue. A possible explanation for this is that the Kohzuki study observed changes after two weeks of inhibitor treatment, by which time down-regulation of induction, observed in the previous chapter, would have occured.

The localisation of ACE mRNA in the germinal tissue of the testis is consistent with the results of Sibony et al. (1993). Marked ACE protein induction after enalapril therapy (seen in figures 11 and 12) shows ACE present in the germinal epithelium and extending into the lumen of the seminiferous tubules.

The induction of testiclar ACE by enalapril points to the existence of an as yet unknown mechanism mediating the inductive process since ACE inhibitors such as perindopril (Jackson et al., 1988b) and quinapril (Fabris et al., 1990) are known not to penetrate the blood/testis barrier to any significant degree. Quinalapril did however, inhibit testicular ACE by up to 30%, which may indicate that certain ACE inhibitors can gain access to testicular tissues. The influence of testicular ACE induction, even if transient, on male fertility is also unknown.

Finally, the localisation of ACE mRNA on endocardium and most markedly in cardiac valve leaflets, is consistent with results of in vitro autoradiography (Yamada et al., 1991). The physiological significance of this observation is unknown, but may suggest a role for ACE in the valve leaflets where it is well positioned for the intracardiac conversion of angiotensin I to angiotensin II, as well as the degradation of bradykinin and other circulating peptides. Little ACE mRNA was found in the myocardium except in association with the occasional coronary vessel.

8.6. CONCLUSIONS

- ACE mRNA and protein have been localised in sections of rat lung, heart, testis and kidney.
- In all these tissues ACE was found to be induced by treatment with enalapril.
- In the lung ACE, induction appears to be predominantly localised in alveolar macrophages rather than endothelium.
- Marked induction was noted in the testis despite the existence of a putative blood/testis barrier.
- In renal tissue, ACE was detected and induced in glomerular endothelium and proximal convoluted tubules.
- Cardiac ACE was markedly induced in the valve leaflets.

SUMMARY AND FUTURE DIRECTIONS

In this thesis methods have been perfected for the purification of rat somatic ACE. Polyclonal and monoclonal antibodies have been raised and these have been used to develop a sensitive ELISA for the measurement of rat ACE in serum and tissue extracts.

A partial rat ACE cDNA clone has been isolated from a rat cDNA library, its identity confirmed, and used as a probe in a study of the expression of ACE mRNA in response to ACE inhibition in various rat tissues. The observed inductive response was localised by the method of *in situ* hybridisation using non-isotopic techniques.

The studies described in the thesis have demonstrated that ACE mRNA and protein are induced in lung and testicular tissue during pharmacological inhibition with enalapril. This response is observed within hours of dosage. The effects of saralasin, an angiotensin II antagonist, and aldosterone have suggested that the induction of ACE by enalapril results either directly from reduced angiotensin II receptor or from a consequent reduction in aldosterone levels. Aldosterone itself does not appear to have any effect on ACE production.

There are examples in the literature of the product of an enzymatic reaction regulating the expression prior enzymatic steps in a pathway. One such example is in the heme biosynthetic pathway in which a mechanism similar to that observed for ACE may be operating. Aminolaevulinic acid (ALA) synthetase is known to be regulated by heme, probably at the transcriptional level, where lack of the repressive effect of heme results in the feedback upregulation of ALA synthetase (Srivastava et al., 1988). Another example of defective binding of a substrate to receptor causing upregulation of precursor enzymatic steps is seen in the syndromes of glucocorticoid resistance (Chrousos et al., 1982). In these syndromes, production of intermediates of steroidogenesis by the adrenal is observed as a result of defects in the interaction of glucocorticoids with target tissues.

In situ hybridisation techniques have not only localised ACE in expected sites of rat tissues, such as renal tubular tissue and testicular germinal epithelium, but have confirmed the induction of ACE at these sites. Of particular interest, is that ACE induction in rat lung tissue, following enalapril dosage, is most marked in lung macrophages, and not solely in the capilliary endothelium.

The various investigative tools established during the course of the studies toward this thesis should allow further detailed study of angiotensin converting enzyme in the rat. Having demonstrated that aldosterone may play an important role in the mechanism of ACE induction by enzyme inhibitors, a search for, and a study of, an aldosterone responsive element in the genomic DNA of rat ACE would conclusively prove this hypothesis.

Appendices

9.1. CLONING AND LABELLING.

9.1.1. Analysis of DNA fragments on 1% agarose gels

1% agarose in 0.5x TBE (0.045M Tris-borate, 0.001M EDTA prepared from 10x solution pH8) was prepared as follows; for 40 ml gel mix add 0.4 g agarose to 40 ml TBE in a conical flask. The flask was weighed and swirled to mix contents before warming on "High" in microwave oven. To prevent from boiling over the heating was stopped regularly and the flask swirled. This was continued until no more "lenses" of undissolved agarose were visible. The flask was reweighed and more buffer added to replace vapour losses during heating. The flask was allowed to cool slightly, 2 µl Ethidium bromide (giving an approximate concentration of 0.5 µg/ml) added and the contents poured into a horizontal gel apparatus. The comb was inserted and the gel allowed to set at room temperature.

The gel was then immersed in a gel tank filled with 0.5x TBE. Samples were prepared by the addition of 1/6th volume sample dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water). A marker consisting of unmethylated lambda DNA digested with either EcoRI of PvuII was added to a well as required. Samples were then denatured at 55°C for 10 minutes, rapidly cooled on ice and loaded into the wells. Gels were run at 40 mA until the tracking dye was considered to have advanced sufficiently. The gel was then removed and viewed on a UV viewing box.

9.1.2. L-Broth.

For 1 liter:

10 g Tryptone / 5 g yeast extract / 5 g NaCl / 2 g glucose made up with deionised water and autoclaved.

9.1.3. L-broth agar plates.

15 g bacto-agar per liter of L-broth. This was sterilised and plates poured.

9.1.4. Ampicillin plates.

The following solution was autoclaved and allowed to cool before adding 2.5 ml of 10 mg/ml ampicillin to a final concentration of 50 µg/ml.

Tryptone 5 g / Yeast extract 2.5 g / NaCl 2.5 g / Glucose 1 g / Bacto-agar 7.5 g in 500 ml water.

20 ml of this solution was poured into each 100mm plate and allowed to set. The plates were either used immediately or stored inverted, protected from light, at 4°C for up to a few weeks.

9.1.5. Transformation of plasmids.

100 µl of competent E.coli (DK-1) cells in suspension were thawed on ice for 30 minutes. 0.5 µl of plasmid ligation mix was added, the tube mixed and placed on ice for 30 minutes. The cells were heat shocked in 42°C waterbath for three minutes before 1 ml L-broth was added, which had been prewarmed to 37°C, and incubated for 1 hour in 37°C waterbath. 100 µl of the solution containing the transformed cells were plated onto a prewarmed L-broth agar/ampicillin plate and spread with flame sterilised shaped glass rod. After a few minutes the plate was inverted and incubated overnight at 37°C. Transformation control was always included using circularised (uncut) pUC19. The next morning, the plates were examined for colonies and some selected for rapid prep. screening.

9.1.6. Rapid prep. screening.

Sterile 10ml centrifuge tubes were filled with 7 ml L-broth under sterile conditions. 70 µl ampicillin stock (to give 50 µg/ml) was added to each tube. Individual colonies were picked with a sterile Gilson pipette tip and expelled into individual tubes.The capped tubes were swirled and placed in an orbital shaker set at 37°C, overnight. The following morning, glycerol stocks of each tube were made by adding 600 µl culture to 400 µl sterile glycerol. This was mixed and allowed to stand at room temperature for a few hours before storing at -70°C. Tubes were then spun for 10 minutes at 2000 rpm. The supernatant was poured off and the pellet resuspended in 200 µl of 25mM Tris pH 8, 10mM EDTA, 50mM glucose and transferred to a microfuge tube. The tubes were left to stand at room temperature for 5 minutes. The 400 µl of a 1% SDS, 0.2N NaOH solution was added, the tubes mixed and stood on ice for 5 minutes. Finally 300 µl of a solution of 3M KAc pH4.8 was added, the tubes mixed gently and placed on ice for 5-10 minutes. Tubes were centrifuged twice in a microfuge at 10000 rpm, each time saving supernatant and transferring to new tube. DNA was precipitated by adding cold isopropanol at a ratio of 0.8 suspension:0.6 isopropanol and incubating at -20°C for 2 hours. Then the tube was centrifuged in microfuge for 10 minutes, the supernatant poured off and 750 µl cold 70% ethanol added. This was repeated. The pellet was vacuum dryed and resuspended in a small volume of TE buffer (10mM Tris/HCl 1mM EDTA, pH 8).

Restriction digest was usually performed to check for inserts on agarose gel electrophoresis.

9.1.7. Large scale plasmid preps. using QIAGEN method.

Plasmids were grown up in in 500 ml L-broth with ampicillin overnight in orbital shaker at 37°C. Pelleting of the bacteria was achieved by centrifugation of the overnight culture at 9000 rpm for 10 minutes at 4°C. The bacterial pellet was then resuspended in buffer P1 (100µg/ml RNAseA, 50mM Tris/HCl, 10mM EDTA, pH 8.0). Once resuspended, 10 ml of buffer P2 (200mM NaOH, 1% SDS) was added and incubated at room temperature for 5 minutes. Then 10 ml of buffer P3 (2.55M KAc, pH 4.8) was added, this was gently mixed and then centrifuged at 16000xg for 30 minutes at 4°C. According to the kit manufacturers, a QIAGEN column was equilibrated with buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100). The supernatant from the preceding centrifugation step was applied to the column and allowed to enter the resin by gravity flow. The column was then washed with 30 ml buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH 7) and the DNA eluted with 15 ml buffer QF (1.25M NaCl, 50mM MOPS, 15% ethanol, pH 8.2). The DNA was precipitated with 0.7 volumes isopropanol at room temperature and pelleted at 10000g for 30 minutes. The DNA was washed with 70% ethanol, air dried and redissolved in a volume of TE buffer to achieve an approximate DNA concentration of 1 µg/µl (as assessed spectrophotometrically).

9.1.8. Electroelution of DNA into dialysis bags

The restriction digest was run on a 1xTAE (0.04M Tris-acetate,0.001M EDTA) / 1% Agarose gel at 20 mA. The gel was viewed on a UV lightbox and working as rapidly as possible, the insert containg gel slices excised with a scalpel blade. Sterile dialysis tubing was prepared by boiling in 1mM EDTA for 5 minutes, rinsing with deionised water and placed in a Schott bottle and autoclaved. The slices were placed in a small piece of dialysis tubing (Spectrapor) which was filled with 1xTAE. The dialysis tubing was immersed in a gel tank filled with 1xTAE and power applied at 25 mA for 2-3 hours. For 1 minute polarity was reversed and run at 100 mA to dislodge DNA from inner wall of tubing. The dialysis tubing was removed from the tank and all the buffer surrounding the gel slices collected. DNA was purified by phenol/chloroform extraction and precipitated with 2.5 volumes 96% ethanol and 1/10 volume 3M sodium acetate. The pellet was washed twice with 70% ethanol, vacuum dryed and resuspended in a small volume of TE buffer.

9.1.9. Chloroform-isoamyl alcohol.

Chloroform : Isoamylalcohol = 24:1

9.1.10. Labelling of DNA by the technique of random priming.

100 ng of linear DNA was denatured by heating at 90°C for 10 minutes then placing on ice. The denatured DNA was incubated with 1x primer mix (containing random primers), 50µCi of ^{32}P-dCTP (3000 Ci/mole - Amersham), 25µM dATP, 25µM dGTP, 25µM dTTP and 2 units of DNA polymerase I in a final volume of 20 µl at 37°C for 30 minutes. The reaction was terminated by the addition of 2 µl 0.2M EDTA pH8 and 78 µl TE buffer. The free ^{32}P-dCTP was separated from that incorporated by chromatography on Sepahadex G-50.

9.2. PLAQUE SCREENING.

9.2.1. Stock of LE392 plating cells.

To 50 ml of L-broth, 1 ml 20% maltose and 20 µl of a glycerol stock of LE392 (derived from a sinle colony by prior plating). The cells were grown overnight in an orbital shaker at 37°C. The next morning 50 ml of sterile L-broth with 0.2% maltose was innoculated with 1 ml of the overnight culture and was incubated in an orbital shaker at 37°C until the OD_{600} had reached between 0.5 and 0.7. The cells were then cooled on ice for a few minutes before being pelleted at 2000rpm for 10 minutes at 4°C. The cell pellet was then resuspended in 15 ml of 10mM $MgSO_4$. The cells were kept at 4°C and were used for up to 3 weeks.

9.2.2. Top Agarose.

0.6g agarose was added to 100 ml L-broth and autoclaved. Just before plating the top agarose was melted in a microwave oven allowed to cool slightly and 1 ml 1M $MgSO_4$ added.

9.3. PLATING OF LIBRARY FOR PLAQUE SCREENING.

9.3.1. Plaque lifts

LE392 cells, an aliquot of the cDNA library and top agarose were mixed and overlaid on a L-broth agar plate, allowed to cool and incubated at 37°C. After sufficient plaque growth had occured, the plates were stored at 4°C for approximately one hour. The plaques were then overlaid with Hybond-N filters and the filter orientation marked by piercing both filter and agar with a sterile needle. The filters were left in contact with the plaques for 10 minutes before being removed and laid, plaque side up, on blotting paper to air dry for 10 minutes. Two glass trays were prepared by placing a piece of Whatman paper at the bottom and soaking that in the first tray with denaturing solution (0.2N NaOH, 1.5M NaCl) and that in the second tray with neutralising solution (0.5M Tris pH 7.4, 1.5M NaCl). The plaque lift filters were then laid firstly in the denaturing tray for 2-3 minutes and then in the neutralising tray for 5 minutes before being allowed to air dry. The filters were then baked in an oven set at 80°C for 2 hours and were then ready for prehybridisation.

9.3.2. Hybridisation of filters

The baked filters were placed in separate glass bottles and each was filled with 5 ml of prehybridisation solution (6x SSC, 5x Denhart's solution, 0.5% SDS and 50µg/ml denatured herring sperm DNA). Prehybridisation was allowed to proceed for 2 hours at 65°C in a Hybaid hybridisation oven. The prehybridisation solution was then replaced with the same volume of hybridisation solution (prehybridisation solution with 2 million cpm/ml of ^{32}P labelled clone 10A. Hybridisation was carried out overnight at 65°C. The filters were then washed twice with 50 ml 2x SSC, 0.1% SDS for 15 minutes each and then twice with 50 ml 0.1x SSC, 0.1% SDS also for 15 minutes each. The filters were sealed in plastic bags and exposed to X-ray film (Cronex) in cassettes which were placed at -70°C overnight. The autoradiographs were developed and the location of positives found.

Areas containing positive plaques that were selected for rescreening were cored out using a sterile pasteur pipette. The cores were expelled into individual Eppendorf microfuge tubes filled with 1 ml SM buffer (5.8g NaCl, 2g $MgSO_4{\cdot}7H_20$, 50ml 1M Tris•Cl pH 7.5, 5 ml 2% gelatin solution and water to 1 liter, autoclave sterilised). The plaques were allowed to elute into the SM on ice with occasional shaking before the buffer was drawn off and placed in a new tube. 20 µl chloroform was added to stop bacterial growth and the plaque extract stored at 4°C till required.

9.3.3. NZC medium with casamino acids.

To 950 ml deionised water, 10 g NZ amine, 5 g NaCl, 2 g $MgSO_4{\cdot}6H_2O$ were added and shaken until dissolved. The pH was adjusted to 7.0 by the addition of 200 µl 5N NaOH. The volume was brought up to 1 liter with deionised H_2O and was autoclaved. Then 250 µl of a sterile solution of 20% casamino acids was added.

9.3.4. Large scale lambda preparation.

In a tube the following were mixed; 1 ml of an overnight culture of LE392 cells, 1 ml of phage lysate with 10^5-10^9 pfu and 1 ml 10mM $MgCl_2$,$CaCl_2$ and this was placed at 37°C for 15 minutes before being added to a flask of 500 ml NZC medium with casamino acids. This culture was allowed to grow at 37°C in an orbital shaker for approximately 6 hours until lysis had been noted and then a further 45 minutes shaking was performed. 1 ml chloroform was added to promote lysis. The flask was placed on ice for 10 minutes before the contents was centrifuged at 9000rpm for 15 minutes at 4°C. The supernatant was collected and was treated with 150 µl 10 mg/ml RNAse and DNAse to destroy host nucleic acids. To dissociate bacteriophage from the debris NaCl was added to a final concentration of 1M and was dissolved by swirling and then placed on ice for 1 hour. Polyethylene glycol (PEG 8000) was added to a final concentration of 10% and the flasks were allowed to stand on ice for at least 1 hour. Bacteriophage was recovered by centrifugation at 9000rpm for 13 minutes at 4°C and the pellet resuspended in 8 ml SM buffer. This was transferred to a glass Corex tubes and 8ml chloroform/isoamyl alcohol added. This was vortexed and then centrifuged at 5000 rpm at 4°C for 15 minutes to pellet the PEG and debris. The phages were collected by centrifugation at 27000 rpm for 2 hours at 15°C and the pellet resuspended in 400 µl 50mM Tris•Cl pH 8. Phage coats were removed by treatments with centrifugation; twice with phenol, twice with phenol/CIAA and twice with CIAA alone. The DNA was then precipitated with ethanol/Na acetate, pelleted by centrifugation, washed twice with 70% ethanol, vacuum dryed and redissolved in approximately 300 µl TE buffer.

9.4. SEQUENCING

Modified T7 DNA polymerase (Sequenase) was used for the sequencing of double stranded, pUC19 derived plasmids. The primer was annealed to the plasmid as follows. 1-2 µg of pUC19 double stranded plasmid in 8 µl water was denatured at 37°C for 30 minutes by the addition of 2 µl of 1N NaOH. The plasmid was then neutralised by the addition of 7 µl of TE and 2 µl of 1N HCl.

The M13 universal primer or the reverse primer (1 µl of 0.5 pg/µl) was then added and left at 37°C for 30 minutes. Precipitation was carried out by the addition of 50 µl of 96% ethanol at -20°C overnight. The annealed plasmid/primer was centrifuged for 10 minutes, washed with 70% ethanol, dried and reconstituted in 8 µl water and 2 µl Sequenase buffer (200mM Tris/HCl, 100mM $MgCl_2$, 250mM NaCl pH 7.5). The annealed DNA mix was incubated at room temperature for 5 minutes with 6.25mM fresh dithiothreitol, 0.2µM dNTPs (dATP, dGTP, dTTP), 10µCi ^{32}P-dCTP and 2 units of Sequenase (modified T7 DNA polymerase) in a final volume of 15.5 µl. The reaction was terminated by adding 3.5 µl of the above to 2.5 µl of prewarmed termination mix at 37°C. There were four termination mixes, each containing a specific dideoxynucleotide. Termination was continued for 5 minutes at 37°C and was then stopped by the addition of 4 µl Sequenase stop buffer (95% formamide, 20mM EDTA, 0.05% Bromophenol blue and 0.05% Xylene cyanol). This mixture was heated for 2 minutes at 75°C and placed on ice. The samples (3 µl) were then loaded onto a 1 meter vertical polyacrylamide gel and electrophoresed for 6-18 hours at a constant voltage of 2400V.

9.4.1. Sequencing Gel

A polyacrylamide/urea gel was prepared as follows:

For a 10% gel the following were mixed and heated until the urea had dissolved:

Bis acrylamide:acrylamide (1:30)	50 ml
10x TBE	20 ml
Urea	84 g
Water	to final vol. of 200 ml

After degassing the solution, 300 µl of ammonium persulphate and 50 µl of TEMED were added to a 50 ml aliquot and poured. Once poured the gel was allowed to set overnight and electophoresed for an hour prior to loading.

9.5. RNA EXTRACTION.

9.5.1. Solution D.

Guanidinium thiocyanate 125 g was added to 146,5 ml water to which was added 8.8 ml 0.75M Na citrate, pH 7 and 13.2 ml 10% Sarkosyl. This stock solution was kept for up to three months at room temperature. To make the solution D working solution, 360 µl of 2-mercaptoethanol was added to 50 ml of the stock solution. This solution had a shelf life of 1 month.

9.5.2. RNA extraction from tissues. (Chomczynski & Sacchi, 1987)

Tissue were stored at in liquid nitrogen until utilised. A pea sized piece of tissue was cut with a scalpel blade and placed immediately in 5 ml solution D. This was then thouroughly homogenised with a small Ultra-turrax till all tissue was disrupted. To this was added 0.5 ml 2M Na acetate pH 4, 5 ml diethylpyrocarbonate (DEPC) water saturated phenol and 1 ml chloroform/isoamylalcohol (49:1) and the tube was mixed and placed on ice for 15 minutes. Samples were then centrifuged at 10000g for 20 minutes at 4°C after which the RNA containing aqueous phase was transferred to a new tube. 1 ml isopropanol was added and the RNA precipitated for 1 hour at -20°C. Centrifugation at 10000xg for 20 minutes was repeated, the supernatant discarded and the pellet resuspended in 300 µl of solution D. An equal volume of isopropanol was added and precipitation allowed to proceed at -20°C overnight. The samples were then centrifuged at 10000xg for 10 minutes at 4°C and the pellet washed with 70% ethanol in DEPC treated water. The pellet was vacuum dried and resuspended either in a small volume of DEPC water or in 0.5% SDS/DEPC water.

Concentration of RNA was assessed by measuring the absorbance at 260nm. RNA concentration (µg/ml) = $A_{260} \times 40$. The A_{260}/A_{280} ratio was taken as a measure of RNA purity, the ratio was always greater than 1.8, 1.9-2.0 being optimal.

9.6. NORTHERN BLOTS

9.6.1. Denhart's solution.

0.02% Ficoll 400 / 0.02% polyvinylpyrolidone / 0.02% BSA

9.6.2. 5x RNA gel running buffer.

0.2M Morpholinopropansulphonic acid (MOPS) / 50mM NaAc / 50mM EDTA pH 7.

9.6.3. 20x SSC.

0.15M NaCl / 0.015M Na citrate pH 7.

9.6.4. Preparation of Formaldehyde/agarose gel for northern blots

0.4 g of agarose was melted in 23.3 ml water. To this solution was added 5 ml 5x RNA running buffer and 8.53 ml formaldehyde. This was thouroughly mixed, poured into a horizontal gel former and allowed to set at room temperature in a fume hood.

5 µl of sample total RNA (in a volume of not more than 10µl) was dispenssed into a DEPC treated Eppendorf microfuge tube. To each tube was added; 3.3 µl DEPC-H_2O, 1.5 µl 10x RNA running buffer, 7.5 µl deionised formamide, 2.7 µl formaldehyde and 1.5 µl RNA loading buffer. Normally the above was premixed and added in a single step to each sample. After mixing each sample was denatured at 55°C for 15 minutes before being rapidly ice cooled and loaded in to the gel wells. After electrophoresis at 30 mA for approximately 3 hours, the RNA was transfered to a nylon membrane (Hybond-N - Amersham) by overnight capilliary blotting in presence of 10x SSC (from 20xSSC stock - 0.15M NaCl / 0.015M Na citrate pH 7) . After briefly rinsing the membrane in 6x SSC the RNA was fixed to the membrane by UV-crosslinking. The membrane was prehybridised in hybridisation solution (5x SSC, 5% blocking reagent, 50% formamide, 0.02% SDS, 0.1% Sarcosyl) and then hybridised in the same

solution containing 50ng/ml digoxigenin-labelled ACE and actin probes. High stingency washes (final washes - 0.1x SSC, 0.1% SDS at 68°C) and subsequent chemiluminescent detection of RNA using the substrate AMPPD was according to the manufacturer's instuctions (Boehringer).

9.7. ACE ENZYME ASSAY

ACE activity was assayed using the spectrofluorimetric method utilising the artificial substrate Hip-His-Leu. This assay relies on the formation of a fluorescent adduct of o-phthaldialdehyde and the histidyl group of the L-histidyl-L-leucine product formed from Hip-His-Leu. The molar quantity of the product formed by the ACE containing sample was estimated by interpolation from a standard curve of flourescent readings determined for five standard dilutions of the product L-histidyl-L-leucine. The standard curve was obtained by subjecting the flourescence data of the standards to least-squares linear regression analysis on a Hewlett-Packard HP97 programmable calculator using a curve fitting program.

9.7.1. Solutions

Buffer: Phosphate-buffered saline (PBS) 0.5M potassium phosphate (pH 8.3), 1.5M sodium chloride.

Substrate: 8.735 mg Hip-His-Leu (HHL) was dissolved in 0.75 ml of 0.025M sodium hydroxide (dissolution effected by agitation and immersion in hot water). The substrate buffer solution was made by adding 0.75 ml PBS and 2.1 ml water to the substrate solution. Thus in the final assay mixture of 120 µl substrate-buffer solution and 5 µl enzyme sample, the concentrations were: 5.4mM substrate, 100mM potassium phosphate and 300mM sodium chloride.

Standard solution: 1.386 mg L-His-L-Leu (HL) dissolved in 10 ml water.

O-phthaldialdehyde solution: 60 mg o-phthaldialdehyde was dissolved in 3 ml methanol (prepared immediately before use).

Preparation of standard dilutions

Standards S1 - S5 were prepared by adding 0.08, 0.17, 0.2, 0.6 and 1 ml of HL solution to 1ml of PBS respectively and making up the volume of each to 4.8 ml with water. A blank was prepared containing no HL. The standards thus contained the following concentrations of HL.

S1 = 1.033 µM
S2 = 2.196 µM
S3 = 2.583 µM
S4 = 7.749 µM
S5 = 12.915 µM

Assay Procedure

0.12 ml of each standard or blank was pipetted into a test tube. 0.12 ml of substrate-buffer solution was pipetted into a test tube for each sample to be assayed, as well as a zero-time blank (all in duplicate). The latter tube was placed on ice.

Reaction was initiated by the addition of 5 µl of the enzyme sample to each sample tube, vortex mixed and immediately placed in a 37°C water bath for 15 minutes. The reaction was terminated by adding 0.725 ml of 0.28M sodium hydroxide to each tube.

0.05 ml of the o-phthaldialdehyde solution was added, the tubes vortexed and incubated at room temperature for 10 minutes. This reaction was terminated by the addition of 3M hydrochloric acid to all test tubes with vortexing.

Flourescence was measured immediately. 1 unit of activity was defined as 1µmol of His-Leu released at 37°C/minute.

9.8. *IN SITU* HYBRIDISATION

9.8.1. Preparation of APTES coated slides

Washed microscope slides were placed in a rack and were immersed in Chromic acid and left to stand overnight. The chromic acid was poured off and slides were washed for 3-4 hours in running tap water. They were then rinsed with deionised H_2O and then left to stand in water overnight. Water was poured off and slides immersed in acetone for 2 hours. This was poured off and slides immersed in a 2% solution of APTES (3-Amino propyl tri-ethoxy silane) for 1 hour and slides then baked in oven at 80 °C overnight.

Slides were then immersed in a 0.1 % solution of Diethyl pyrocarbonate (DEPC) in water for 2 hours. This was poured off and slides autoclaved and then dried at 80 °C in an oven.

9.8.2. Preparation of tissues

Rats were sacrificed and organs harvested on ice, cut into small pieces and placed overnight into Bouin's fixative. The tissue pieces were then processed and wax embedded following standard methodologies.

9.8.3. *In situ* hybridisation

Rehydration of sections

Xylene	2 x 10 minutes
Absolute ethanol	2 x 2 minutes
90% ethanol	1 x 2 minutes
70% ethanol	1 x 2 minutes

Sections were then dipped in DEPC treated water and then in phospate buffered saline (DEPC treated) for 5 minutes. Slides were drained.

Proteinase K digestion

1 µg/ml Proteinase K solution in 10 mM Tris/HCl, 5mM EDTA, pH 7.5 was prepared. 500 µl was laid over each section and incubated in humidified container at 37 °C for 15 minutes. Proteinase K digestion was stopped with 2 mg/ml glycine in PBS for 30 secs. Slides were dipped in PBS twice for 1 minute each.

Optional step for RNAse controls - prepared 40 µg/ml solution of RNAse A in TE buffer and incubated at 37°C for 30 minutes in humidified container. Reaction stopped with paraformaldehyde in following step.

Fixation of sections

500 µl of 4% paraformaldehyde in PBS was placed onto sections and left at room temperature for 5 minutes before being dipped in DEPC water for 30 seconds.

Acetylation of sections.

Prepared 0.1M Triethanolamine as follows;

4 ml Triethanolamine
296 ml DEPC H_2O

Slides were placed in above solution and while stirring solution added 750 µl of acetic anhydride. Continued stirring for 10 minutes then dipped slides in PBS for 5 minutes.

Dehydration of sections

70 % ethanol	1 x 2 minutes
90 % ethanol	1 x 2 minutes
Absolute ethanol	2 x 2 minutes

Slides were air dried.

Prehybridisation

Prehybridisation solution

Dextran Sulphate	0.5 g
1M Tris/HCl pH8	50 µl
100x Denhart's solution	50 µl
0.5M EDTA	10 µl
1M Dithiothryitol	50 µl
dH_2O (DEPC treated)	565 µl
Salmon sperm DNA	250 µl
tRNA 10mg/ml	25 µl

before use diluted 1:1 with deionised formamide

Placed 100 µl for each section on parafilm and inverted slide on this. Incubated at 42 °C in humidified container for 2 hours.

Hybridisation

Hybridisation solution

Dextran Sulphate	0.25 g
1M Tris/HCl pH8	25 µl
100x Denhart's solution	25 µl
0.5M EDTA	5 µl
1M Dithiothryitol	25 µl
dH_2O (DEPC treated)	283 µl
20x SSC	250 µl
Salmon sperm DNA	1 µl
tRNA	1 µl

before use diluted 1:1 with deionised formamide.

Labelled RNA probes were heated for 30 secs. at 80°C then hold at 50°C. Diluted riboprobes to approximately 1 ng/µl in hybridisation solution. Applied 20 µl solution per slide. Inverted on parafilm and incubated overnight at 50°C in a humidified container.

Washing

2x SSC, 2x15 minutes at room temperature

optional: RNAse treatment; 30 min at 37°C with boiled RNAse A in 0.5M NaCl and 10mM Tris.Cl, pH 8.

Then washed twice with 2xSSC as above.

0.1x SSC, 2x15 minutes at 50°C

Digoxigenin detection

a. Slides washed in 100mM Maleic acid, 150mM NaCl pH 7.5 buffer (buffer 1).

b. Blocked for 20 minutes in 100mM Maleic acid, 150mM NaCl pH 7.5 buffer with 2% Normal Sheep Serum (NSS) (buffer 2). Apply 150 µl per slide.

c. Washed with 0.05% Tween-20 in buffer 1 in tank for 5 minutes at room temperature.

d. Diluted anti-digoxigenin antibody 1:200 in buffer 2 . Applied 20 µl/slide inverted on parafilm. Incubated for 30 minutes at 37°C.

e. Washed 2x 15 minutes in buffer 1 at room temperature.

f. Dipped slides for approximately 2 minutes in substrate buffer 100mM Tris/HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ (buffer 3). Then drained slides.

g. To prepare substrate solution - to 1 ml buffer 3 added 4.5 µl NBT solution and 3.5 µl X-phosphate solution.

Added 100 µl to each section and then coverslipped. Incubated in dark at room temperature for approximately 1 hour or until colour reaction was clearly visible.

h. Stopped colour reaction with 10mM Tris/HCl , 1mM EDTA pH8.

i. Dehydrated sections and mounted with Enkitt.

j. Examined under microscope and photographed.

9.9. IMMUNOHISTOCHEMISTRY

Small pieces of tissue were fixed in Bouin's solution, dehydrated in a series of ethanol solutions and then xylene. Embedding in paraffin wax followed standard techniques and 5μm sections were mounted onto slides. The sections were rinsed in phosphate buffered saline (PBS) and incubated in diluted normal rat serum for thirty minutes at room temperature. The slides were washed in PBS before being incubated with monoclonal antibody diluted 1/50 in PBS with normal rat serum for 30 minutes at room temperature. After a further PBS wash the slides were incubated in a solution containing peroxidase conjugated antimouse antibody for 30 minutes. The slides were washed and incubated in a solution containing 3-3' diaminobenzidine and H_2O_2 until staining was considered adequate. Sections were washed, counterstained with haematoxyllin and then mounted.

9.10 Rat lung ACE purification

Purification step	**Volume (ml)**	**Total Protein (mg)**	**Total units (U)**	**Specific activity (U/mg)**	**Purification (fold)**
Tissue homogenisation	400	5424	108	0.02	
Column load	360	5300	477	0.09	4.5
Affinity chromatography	18	5	455	91	1130

N.B. The column load volume of 360 ml was loaded over four purification runs.

9.11 Inhibition of purified ACE by captopril

The purified rat ACE was found to be sensitive to captopril inhibition. In order to derive a value for K_i , standard Dixon plots (Dixon, 1953) of the data were constructed using the values obtained from Hip-His-Leu (1mM) hydrolysis in the presence of concentrations of captopril in the range 0.92-55.3nM. ACE was at a concentration of 0.4µg/ml in 0.1M potassium phosphate (pH 8.3), 0.3M NaCl and 0.12mg/ml albumin.

The values obtained were;

[Captopril] nM	**V nmol/min/ml**
0	12.6
0.92	11.6
9.2	5
18.4	3.2
27.6	2.4
36.8	2
46	1.8
55.3	1.65

As is to be expected with tight binding inhibitors, the resulting plots were non linear (fig 9.1) necessitating the use of the graphical method of Dixon (Fig 9.2) (Dixon, 1972).

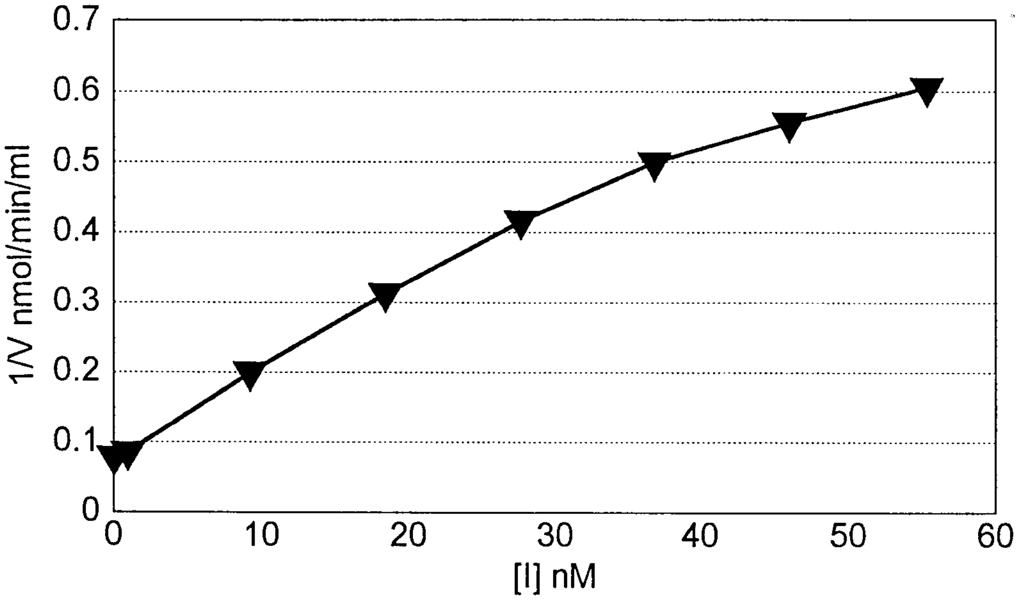

Figure 9.1. Rat ACE inhibition by captopril. Studies were performed with purified rat lung ACE at a concentration of 0.4µg/ml in 0.1M potassium phosphate (pH 8.3), 0.3M NaCl and 0.12mg/ml albumin. Dixon plot of 1/V vs. [I] for Hip-His-Leu (1mM) hydrolysis in the presence of concentrations of captopril in the range 0.92-55.3nM.

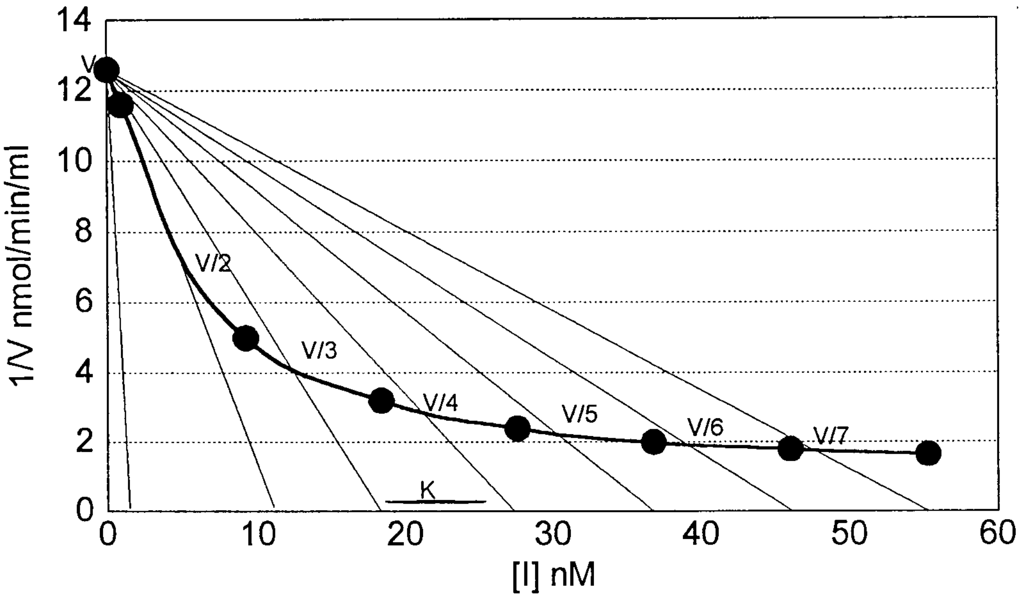

Figure 9.2. Graphical method for the derivation of *K*. 1/V vs. [I] is plotted and a family of lines drawn between V_0 and the various concentrations of captopril. These lines intersect at integral divisions of V_0 giving equally spaced intersection points on the abscissa. The distance between these points is equal to *K*.

Assuming the *K*m for the substrate to be 2mM, the formula for the derivation of K_i was used;

K_i = $K / (1+[S]/K_m)$ where *K* is obtained from the graph.

Captopril was shown to be a competitive inhibitor with K dependent on [S], and a K_i of 2.8nM obtained.

9.12 Analysis of spcific activity of radioiodinated ACE

The specific activity of the radioiodinated human ACE tracer was determined by the self displacement method (Chiang, 1987). A standard RIA assay was prepared and note taken of the Bound/Total (B/T) counts for each of the standards. The results are subjected to linear regression analysis and plotted on a graph vs. the actual standard concentrations on a linear scale. In the same assay, tubes are included with varying excess amounts of radiolabelled tracer. The tubes are assayed in the same way and the resulting B/T values plotted on the same graph vs. total counts in each tube (fig. 9.3). Values for R1,R2,Q1 and Q2 are obtained from the graph by interpolation. The specific activity of the tracer is derived from the formula;

$$\text{Specific activity} = (R1-R2)\ \text{cpm} \,/\, (Q1-Q2)\ \text{ng}$$

The count data and graph obtained are shown below.

	Radioactivity cpm			
Conc. of Std.	Total	Bound	Free	T/B ratio
ng/tube	12449			
0				
0.098		3147	9302	3.96
0.195		3175	9274	3.92
0.391		3201	9248	3.89
0.781		3015	9434	4.13
1.56		2807	9642	4.43
3.125		2210	10239	5.63
6.25		1678	10771	7.42
12.5		1316	11133	9.46
25		1012	11437	12.3
50		752	11697	16.55
Tracer self displacement				
	1952634			4.52
	3743520			7.6
	6132870			9.93
	7824635			12.7
	9536478			15.5

Table 9.1.

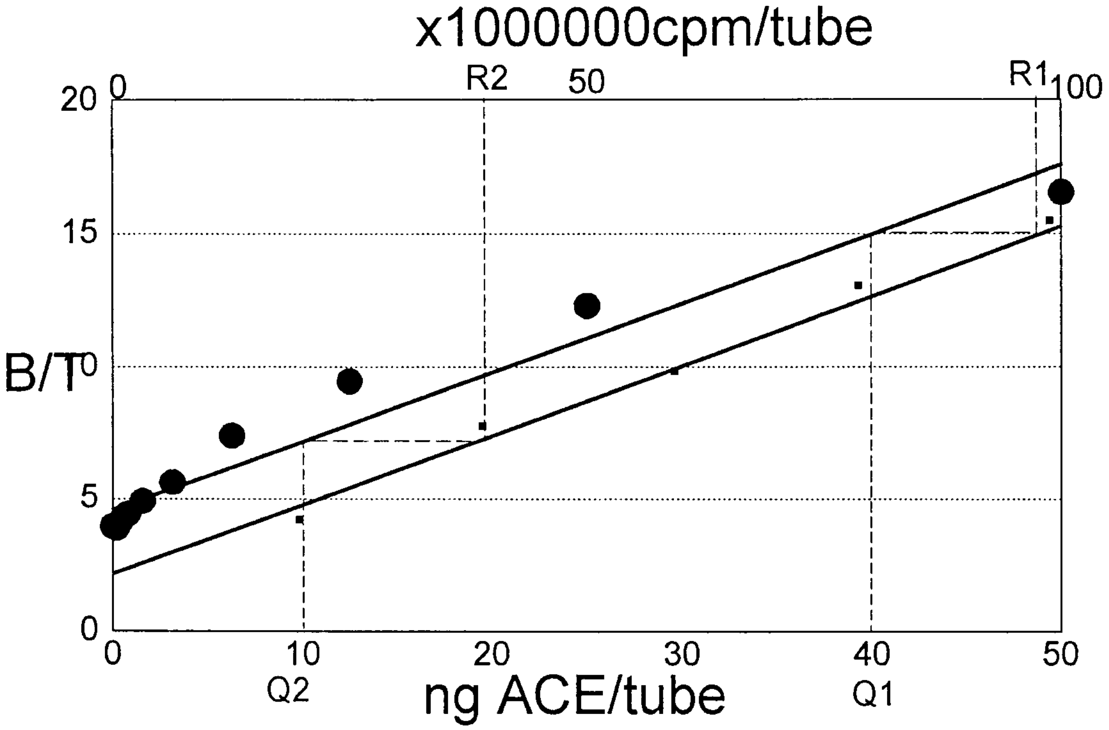

Fig 9.3. Plot of standard curve data from table 9.1. (bold dots), T/B vs. concentration of unlabelled ACE (lower horizontal axis), and tracer self displacement data (small dots), T/B vs. quantity of radioactivity, upper horizontal axis.

Specific Activity = (9800000-3900000)cpm/(40-10)ng = 198000cpm/ng ACE

Counter efficiency = 74%. Therefore tracer specific activity is 267000dpm/ng ACE, which corresponds to approximately 1 atom iodine per molecule ACE.

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