We tested the effect of tris solutions at *p*H values ranging from 7.0 to 9.0. In all experiments tris was increasingly effective as the *p*H was increased.

The results of these experiments suggest that tris is more effective in the uncharged form, but they do not indicate whether it is working at an intracellular or an extracellular site, or, in fact, whether the tris is charged or uncharged at that site.

Tris could be entering the cell and interfering with the processes that occur after transmitter-receptor interaction. On the other hand, the chemical similarity of tris to acetylcholine (see Fig. 2) suggests that tris could be an antagonist of acetylcholine binding to its receptor.

Tris may be a specific antagonist of cholinergic responses. We have performed preliminary experiments with iontophoresed dopamine which show that excitatory responses are minimally altered, if at all, by 10 m*M* tris. In the case of 5-hydroxytryptamine (5-HT), Gerschenfeld and Paupardin-Tritsch (*5*) used tris to substitute for sodium in a study of 5-HT-mediated hyperpolarizations and did not report any difficulty. In contrast, Blankenship *et al.* (*6*) used tris (and occasionally choline chloride and sucrose) as a substitute for sodium in the study of cholinergic excitation in *Aplysia*. Only in a few experiments were they able to demonstrate the expected shift in reversal potential. In the remainder, the response was nearly abolished and estimates of reversal potential could not be made. It is not clear from (*6*) which sodium substitute was used in the various experiments. The abolition of the response may have been caused by direct action of tris on the acetylcholine-mediated excitation.

The results reported here and the confirming results of Parmentier and Zbicz (*3*) were obtained with molluscan systems; further studies should be carried out with cholinergic responses in other systems. Since a number of laboratories have utilized tris-buffered solutions in both biochemical (*7, 8*) and physiological (*9*) studies of cholinergic systems, specific experiments to explore the effect of tris on acetylcholine binding and the associated conductance changes would be most helpful. Until the full extent of the synaptic actions of tris is known, it should be used only after proper control experiments have been performed.

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Design of Specific Inhibitors of Angiotensin-Converting Enzyme: New Class of Orally Active Antihypertensive Agents

Abstract. *A hypothetical model of the active site of angiotensin-converting enzyme, based on known chemical and kinetic properties of the enzyme, has enabled us to design a new class of potent and specific inhibitors. These compounds, carboxyalkanoyl and mercaptoalkanoyl derivatives of proline, inhibit the contractile response of guinea pig ileal strip to angiotensin I and augment its response to bradykinin. When administered orally to rats, these agents inhibit the pressor effect of angiotensin I, augment the vasodepressor effect of bradykinin, and lower blood pressure in a model of renovascular hypertension.*

The nonapeptide SQ 20,881, <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (*1*), has been shown to be a specific and potent inhibitor of angiotensin-converting enzyme in vitro (*2*) and in vivo (*3*). Preliminary clinical studies (*4*) have demonstrated the great potential of SQ 20,881 as a novel antihypertensive drug, limited only by its lack of oral activity. This therapeutic potential led us to test other structural types in our search for new, specific, and orally active inhibitors.

Specific inhibitors of angiotensin-converting enzyme would be expected to show the profile of activities exemplified by SQ 20,881 (Table 1) with respect to inhibition of the isolated enzyme (*5*), inhibition of the contractile response of smooth muscle to angiotensin I (*2*), augmentation of the contractile response to bradykinin (*2, 6*), and no effect on responses to other agonists. Only a small fraction of the nonpeptidic compounds that we have tested were potent inhibitors of angiotensin-converting enzyme, and very few of these showed the desired specificity. However, accumulated knowledge of the chemical and enzymatic properties of angiotensin-converting enzyme suggested the possibility of a new approach, the rational design of specific inhibitors of angiotensin-converting enzyme.

Previous studies with substrates and inhibitors of angiotensin-converting enzyme (*7*) suggested that this peptidase was a carboxypeptidase similar to pancreatic carboxypeptidase A, even though it releases dipeptides rather than single amino acids from the carboxylic acid end

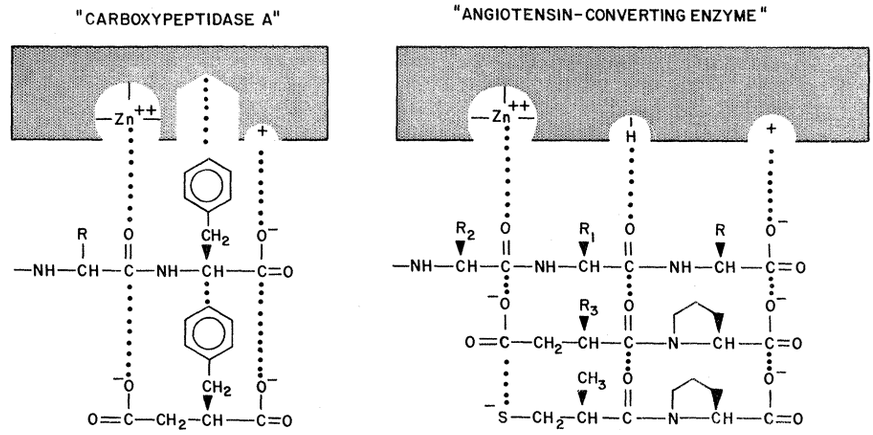

Fig. 1. Schematic representation of the binding of substrates and inhibitors at the active site of pancreatic carboxypeptidase A, and at the hypothetical active site of angiotensin-converting enzyme.

of peptide substrates. These investigations had also indicated that angiotensin-converting enzyme, like carboxypeptidase A, was a zinc-containing metalloprotein, a conclusion that has been verified recently by other investigators (*8*). It thus seemed reasonable to assume that the mechanism of action and, therefore, the active site of angiotensin-converting enzyme would be similar to that of carboxypeptidase A.

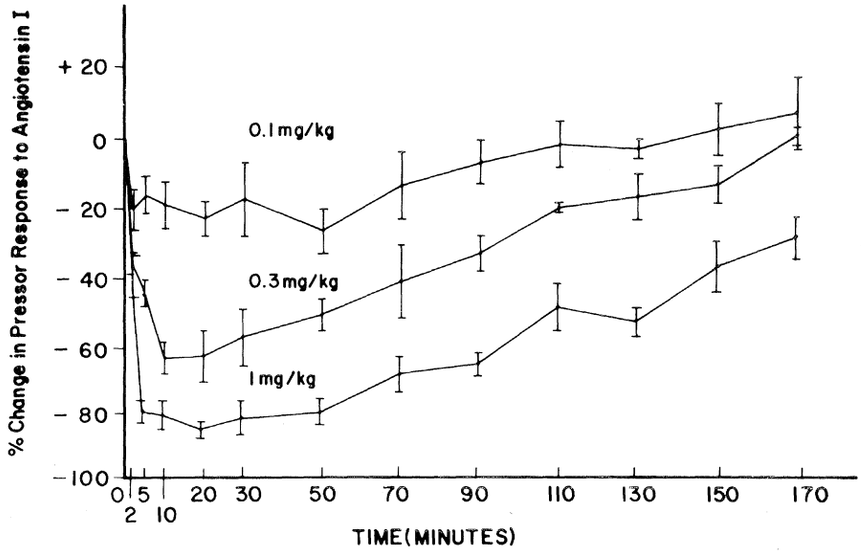

Fig. 2. Mean percent inhibition (± standard error) of pressor responses to angiotensin I after single oral doses of SQ 14,225 in fasted, unanesthetized rats (four animals per dose). The rats were challenged at the indicated times with intravenous injections of angiotensin I at 310 ng/kg.

The important substrate-binding groups at the active site of carboxypeptidase A (*9*), and at the active site of our hypothetical model of angiotensin-converting enzyme are shown in Fig. 1. Positively charged groups at both active sites (Arg^{145}, in the case of carboxypeptidase A) form ionic bonds with the negatively charged terminal carboxyl groups of the substrates. An adjacent "hydrophobic pocket" is responsible for the specificity of carboxypeptidase A toward substrates containing COOH-terminal aromatic amino acids. Angiotensin-converting enzyme, having no such specificity, would not be expected to have a similar hydrophobic pocket. However, the active site of this enzyme might have a group capable of interacting with the COOH-terminal amide bond of the substrate, probably through hydrogen bonding (Fig. 1). The zinc ions must be suitably located at the active site of both enzymes to polarize the carbonyl groups of the scissile amide bonds making them more susceptible to hydrolytic cleavage.

Table 1. Activities in vitro of inhibitors of angiotensin-converting enzyme. Procedures for testing compounds for inhibition of the rabbit lung enzyme and for effects on the smooth-muscle contracting activity of various agonists have been described in (*3*, *5*). Abbreviations: IC_{50}, concentration of compound producing 50 percent inhibition of enzyme activity or agonist effect; AC_{50}, concentration of compound producing 50 percent augmentation of agonist effect; AI, angiotensin I; AII, angiotensin II; BK, bradykinin; Ach, acetylcholine.

	Structure	Designation	Activity (μg/ml)				
			Angiotensin-converting enzyme of rabbit lung (IC_{50})	Excised guinea pig ileum			
				AI (IC_{50})	AII (IC_{50})	Ach IC_{50}	BK (AC_{50})
1	<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	(SQ 20,881)	1.0	0.068	> 32	> 32	0.0017
2	$HO_2C-CH_2-CH_2-CO-N$ ◀ CO_2H		135	94	>100	>100	8.0
3	$HO_2C-CH_2-CH(CH_3)-CO-N$ ◀ CO_2H	(SQ13,297)	12	13	>100	>100	0.2
4	$HO_2C-CH_2-CH(CH_3)-CO-N$ ◀ CO_2H		340	>100	>100	>100	15
5	$HO_2C-CH_2-CH_2-CH(CH_3)-CO-N$ ◀ CO_2H		1.0	4.6	>100	>100	1.0
6	$HO_2C-CH_2-CH_2-CH(CH_3)-CO-N$ ◀ CO_2H		230	>100	>100	>100	4.7
7	$HS-CH_2-CH_2-CO-N$ ◀ CO_2H	(SQ13,863)	0.04	0.06	>100	>100	0.005
8	$HS-CH_2-CH(CH_3)-CO-N$ ◀ CO_2H	(SQ14,225)	0.005	0.005	>100	>100	0.0007
9	$HS-CH_2-CH(CH_3)-CO-N$ ◀ CO_2H		0.50	1.7	>100	>100	3.1

In 1973, Byers and Wolfenden (*10*) reported that D-benzylsuccinic acid was a potent competitive inhibitor of carboxypeptidase A (the inhibition constant K_i $4.5 \times 10^{-7} M$). One of the most plausible explanations (*10, 11*) for this inhibitory potency would be the simultaneous and stereospecific interaction of the inhibitor molecule with three substrate-binding groups at the active site of carboxypeptidase A; namely, the positive charge of Arg^{145}, the hydrophobic pocket, and the zinc atom (Fig. 1). This observation, along with the above-mentioned speculations about the nature of the active site of angiotensin-converting enzyme, led us to pursue the design of specific inhibitors of this peptidase along similar lines.

Since angiotensin-converting enzyme is a "dipeptidyl" carboxypeptidase (peptidyldipeptide hydrolase, E.C. 3.4.15.1), the distance between the cationic carboxyl-binding site and the zinc atom should be greater than in carboxypeptidase A, by approximately the length of one amino acid residue (Fig. 1). Therefore, we postulated that a succinyl derivative of an amino acid, rather than a succinic acid, should be the prototype for inhibitors of angiotensin-converting enzyme. To test this hypothesis, we synthesized succinyl-L-proline (Fig. 1, R_3 = H). Proline was originally chosen as the amino acid moiety because all of the naturally occurring peptidic inhibitors of angiotensin-converting enzyme have this amino acid as the COOH-terminal residue; it was soon demonstrated that proline-containing inhibitors were more potent than those incorporating other naturally occurring amino acids. The activity profile of succinyl-L-proline (structure **2**, Table 1) clearly indicated that this compound, although only slightly active, was a specific inhibitor of angiotensin-converting enzyme.

After exploring the influence of different structural modifications, including length and substitution of the acyl moiety, we were able to increase considerably the inhibitory activity of the prototype compound by synthesis of 2-D-methylsuccinyl-L-proline (SQ 13,297, structure **3**), and 2-D-methylglutaryl-L-proline (structure **5**). These compounds were purely competitive inhibitors of purified angiotensin-converting enzyme of rabbit lung; and, when administered orally or parenterally to rats, they inhibited the pressor effect of angiotensin I. The corresponding 2-L-methyl analogs of these inhibitors were at least 100 times less active (Table 1).

If the interaction of a carboxyl group of compounds such as **3** with the zinc atom of the enzyme (Fig. 1) plays an important role in determining the inhibitory potency, then replacement of the carboxyl function by other groups capable of serving as zinc-ion ligands should yield inhibitors of equal or greater potency. Replacement of the carboxyl group by nitrogen-containing functionalities (amines, amides, or guanidines) did not enhance inhibitory activity, but replacement by a mercapto group (Fig. 1) led to a dramatic improvement of inhibitory potency, without any concomitant loss of specificity. The potency and specificity of 3-mercaptopropanoyl-L-proline (SQ 13,863, structure **7**, Table 1) and 2-D-methyl-3-mercaptopropanoyl-L-proline (SQ 14,225, structure **8**) are, on a molar basis, equal to or better than that of the nonapeptide SQ 20,881. The L-methyl enantiomer of SQ 14,225 is at least 100 times less active.

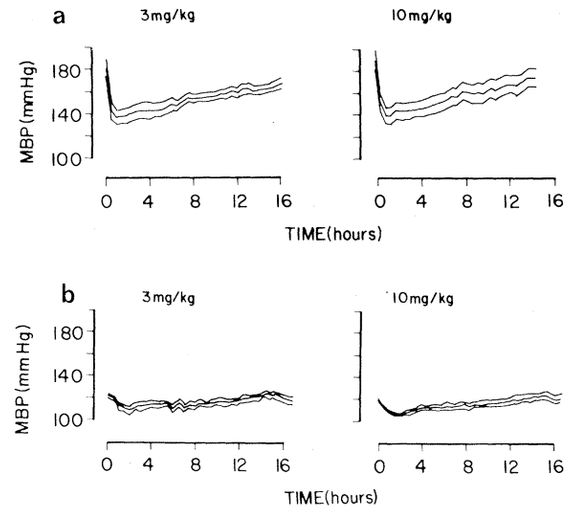

Fig. 3. (a) Effects of single oral doses of SQ 14,225 on mean blood pressure (MBP ± S.E.; ten animals per dose) of unanesthetized Goldblatt two-kidney renal hypertensive rats. (b) Effects of single oral doses of SQ 14,225 on mean blood pressure (MBP ± S.E.; ten animals per dose) of unanesthetized normotensive rats.

The availability of compounds possessing such remarkably potent and specific inhibitory activity prompted the exploration of their effects on the renin-angiotensin system in two animal models. In the first model, aortic blood pressures of unanesthetized normotensive male Sprague-Dawley rats (weighing about 200 g) were recorded directly before and after challenge with angiotensins I and II, bradykinin, and acetylcholine as described (*3, 12*). Single oral doses of SQ 13,297 (300 to 1000 mg/kg), SQ 13,863 (1 to 30 mg/kg), or SQ 14,225 (0.1 to 1 mg/kg), given by gavage to rats that were fasted overnight, produced a dose-related inhibition of the pressor response to angiotensin I (310 ng/kg, given intravenously), but not to that of angiotensin II (100 ng/kg, given intravenously). The degree and time-course of the inhibitory effects produced by SQ 14,225 on the angiotensin I response is plotted in Fig. 2. At 1/3 to 1/100 of the above-cited oral doses, the three compounds also augmented the vasodepressor response to bradykinin (3 to 10 μg/kg, given intravenously), but not that of acetylcholine (0.3 to 1 μg/kg, given intravenously). None of the compounds at any of the doses tested had any appreciable effect on the mean "resting" blood pressure.

The second animal model was the unanesthetized Goldblatt two-kidney renal hypertensive rat, which is renin-angiotensin-dependent for at least several weeks (*13*). Male CFN Wistar rats (weighing about 150 g) were made hypertensive by placing a 0.22-mm silver clip on the left renal artery, with the other kidney being left intact (*3*). Five to six weeks later, blood pressures were recorded from the abdominal aorta of each of these unanesthetized rats. The rats were maintained on commercial (Purina) rat chow and tap water. In these rats moderate to marked antihypertensive effects are produced by oral dosing with SQ 14,225 (3 and 10 mg/kg) administered by gavage (Fig. 3); this drug produced practically no hypotensive effect in normotensive Wistar rats (Fig. 3).

We have thus shown that the rational design of inhibitors of angiotensin-converting enzyme has led to the development of potent oral antihypertensive agents that act by blockade of the renin-angiotensin system. The therapeutic efficacy and the diagnostic utility of these compounds in human hypertension remain to be demonstrated, but the results in animals suggest that these compounds have great potential as a new class of antihypertensive drugs.

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Major Innervation of Newborn Rat Cortex by Monoaminergic Neurons

Abstract. *A major monoaminergic innervation in infant rat neocortex, predominantly in layer IV, has been demonstrated by ultrastructural and biochemical studies after the administration of exogenous catecholamine precursors and congeners. One-third of all cortical synapses have an uptake-storage mechanism for catecholamines. In newborn cortex, the storage capacity for catecholamines is tenfold greater than the endogenous levels, and the uptake-storage mechanism matures earlier than the ability to synthesize neurotransmitter.*

Noradrenergic neurons with cell bodies in the pons give rise to a widespread innervation of the cerebral cortex (*1*). Dopaminergic neurons with cell bodies in the midbrain contribute an additional innervation to restricted areas of the neocortex (*2*). Despite the extensive projection of these catecholaminergic axons, it is estimated that they form less than 1 percent of the total synaptic contacts in the adult cerebral cortex (*3*).

In the neocortex of perinatal rats, the density of aminergic terminals, as revealed by histofluorescence methods, appears to be extremely low (*4*), and the levels of catecholamine synthesizing enzymes and endogenous amines in neocortex remain relatively low throughout the newborn period (*5, 6*). Yet the major catecholaminergic neurons are formed and undergo differentiation at early stages of fetal development (*7*), and their axons reach the neocortex by 1 week before birth (*8*). Either there is a long delay between the arrival of aminergic axons in the cortex and the subsequent formation of synapses, or immature catecholaminergic axons innervate the cortex at birth but are deficient at that age in their ability to synthesize or transport endogenous catecholamines. To address this problem, we have attempted to characterize the monoaminergic innervation of immature cortex by an approach not dependent upon the presence of endogenous neurotransmitters. The in vivo uptake of exogenous catecholamine precursors and congeners by the cortex was studied by electron microscopic and biochemical techniques.

Monoaminergic terminals can be identified at the ultrastructural level by the presence of small (40- to 50-nm) granular vesicles (SGV's), which are the storage sites for the amines (*9*). The demonstration of SGV's in the central nervous system is enhanced by exposure of tissue to the catecholamine congener 5-hydroxydopamine (5-OHDA) (*10*). This "false" neurotransmitter is selectively taken up and concentrated in the synaptic vesicles of monoaminergic nerve terminals, where, after aldehyde fixation, it forms an electron-opaque precipitate. This indirect method permits the ultrastructural analysis of those synaptic terminals that have an uptake-storage mechanism specific for monoamines (*10, 11*). Since the immature blood-brain barrier is permeable (*12*), systemic administration of 5-OHDA was used to study the distribution of monoaminergic terminals in the neocortex of infant rats (*13*).

The morphology and distribution of synapses in the lateral neocortex of infant rats (from birth to 7 days old) were analyzed with the electron microscope [for details see (*13–15*)]. Interneuronal appositions with characteristic vesicles (30 to 50 nm in diameter) adjacent to an area of membrane specialization were designated as synapses. The density of synapses in newborn cortex is extremely low compared with that in the adult. In rats from birth to 7 days old, treated with 5-OHDA, 30 percent of all synaptic terminals in the lateral neocortex contain SGV's; these vesicles are round, 40 to 50 nm in diameter, and contain a dense, eccentric, electron-opaque granule (Fig. 1). In control animals (untreated), all synaptic terminals contain clear (that is, empty) vesicles. In rats that have been treated with reserpine (10 mg per kilogram of body weight) 12 hours before 5-OHDA, no SGV's can be demonstrated. Hence the vesicular accumulation of 5-OHDA is reserpine-sensitive and probably is restricted to monoaminergic terminals.

By 1 week of age, the six cytoarchitectonic layers characteristic of mature cortex can be recognized. The synapses—still relatively sparse—are concentrated in strata that are parallel to the surface (*15*). In 5-OHDA-treated rats, the radial distribution of SGV synapses is characteristic and highly reproducible: in layer IV (the deep third of the cortical plate) more than 70 percent of the synaptic terminals contain SGV's (*13*). In other cortical layers, SGV synapses are sparse, except in the marginal zone where 10 to 30 percent of the boutons contain SGV's. Many of the SGV boutons form synapses de passage as is characteristic of central catecholaminergic neurons, and in layer IV many are on proximal dendrites. The use of 5-OHDA reveals, in immature cortex, a previously unrecognized set of axon terminals deficient in endogenous transmitter but with the capacity to take up and store this catecholamine congener. We attempted to confirm and characterize this uptake-storage capacity by application of biochemical methods.

Rats of various ages were treated with the catecholamine precursor L-dopa. Subsequently, dopamine and norepinephrine levels in neocortex were measured by a sensitive radiometric enzymat-