Renin: Inhibition by Proteins and Peptides†

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ABSTRACT: Human renin is inhibited by several proteins. Human, equine and bovine hemoglobins are potent inhibitors (K_i = 2 × 10^{-6} M) while bovine fibrinogen, albumin, and β-lactoglobulin B are much weaker (K_i = 1 × 10^{-4} M). At least one protein (immunoglobulin G) does not inhibit the enzyme. The α- and β-globin chains derived from horse hemoglobin are both about as effective as intact hemoglobin. Portions of the hemoglobin molecule show varying degrees of inhibition, presumably depending on the presence of effective amino acid sequences, but the presence of a Leu-Leu sequence such as is present in renin's plasma protein substrate is not an absolute requirement for inhibitory effectiveness. The 1–55 fragment of the hemoglobin β chain contains such a Leu-Leu sequence and is somewhat more potent than the intact β chain. The 1–32 fragment of the α chain contains no Leu-Leu sequence and is much less potent than the parent chain, but is nonetheless an effective inhibitor at high concentrations. In addition, a number of small synthetic peptides lacking Leu-Leu sequences also prove to be effective inhibitors. Several peptides containing the sequence Leu-Trp are particularly effective (Leu-Trp-Met-Arg-Phe-Ala, K_i = 3 × 10^{-5} M). Kinetic studies with two proteins (hemoglobin; β-lactoglobulin B) and two small peptides (Leu-Trp-Met-Arg-Phe-Ala and Leu-Leu-Val-Tyr-OMe) indicate noncompetitive inhibition. Although most of the studies in this report were performed using a labeled polymeric substrate, the inhibitor effects are not related to use of this particular assay system. Inhibition by protein (hemoglobin) is also demonstrable in assay systems using a renin plasma protein substrate with measurement of angiotensin I generation by both radioimmunoassay and double-isotope derivative assay.

In our recently described labeled polymeric substrate assay for renin the substrate, [^{125}I]*N*-acetyl-poly(L-glutamyl)tridecapeptide, is cleaved by the enzyme to yield a [^{125}I]tetrapeptide which is readily quantitated. The assay is suitable for use with partially purified enzyme preparations from kidney, but attempts to measure renin in plasma led to the discovery that plasma strongly inhibits the enzyme (Bath and Gregerman, 1972). Plasma is known to contain a number of protease inhibitors, but before we could ascribe inhibition to the action of such materials, we undertook the present study in order to examine the possible effects of proteins on the action of renin.

Materials and Methods

Chemicals and their sources are as follows: tetradecapeptide renin substrate[1] and the N-terminal hexapeptide of glucagon, His-Ser-Glu-Gly-Thr-Phe, Schwarz BioResearch, Orangeburg, N. Y.; EDC and CNBr, Pierce, Rockford, Ill.; lysozyme and Dip-F, Sigma, St. Louis, Mo.; Leu-Leu-Gly, Ile-Ile-Ile, Leu-Leu-Val-Tyr, Leu-Leu, Leu-Ile, and Ile-Leu, Fox, Los Angeles, Calif.; Leu-Trp-Leu and Leu-Leu-Leu, Fox and Bachem, Marina del Rey, Calif.; Val-Val-Val, Bachem; Gly-Leu-Gly-Leu and Gly-Leu-Leu-Gly, Cyclo, Los Angeles, Calif.; Leu-Gly-Leu, secretin fragment 21-27 (Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2) and model peptide substrate (Leu-Trp-Met-Arg-Phe-Ala), Research Plus, Denville, N. J.; bovine hemoglobin, bovine albumin, bovine fibrinogen, bovine IgG, β-lactoglobulin B, hog plasma renin substrate and dithiothreitol, Miles, Kankakee, Ill.; methanolic HCl, Supelco, Bellefonte, Pa.; defibrinated horse blood, Bioquest Division of Becton Dickenson, Cockeysville, Md. Other materials including the human renin were from sources previously described (Bath and Gregerman, 1972; Gregerman and Kowatch, 1971).

Radioimmunoassay for angiotensin I was performed with materials from the Angiotensin Immutope Kit from E. R. Squibb, New Brunswick, N. J.

Preparation of Horse Globin Chains. Horse blood was centrifuged and the red cells were washed three times with normal saline at 4°. The washed cells were suspended in two to three volumes of distilled water and lysed by addition of chloroform to a total of 4% by volume. After the solution was stirred for 30 min it was centrifuged for 20 min at 12,000*g* at 4°. The supernatant hemoglobin solution was decanted. Horse globin was prepared with acid acetone (Winterhalter and Huehns, 1964). The globin was split into its component α and β chains by ion-exchange chromatography on a 1.5 × 15 cm column of Whatman CM-32 carboxymethylcellulose using a sodium phosphate gradient from 0.005–0.035 M at pH 6.7 (Clegg *et al.*, 1966; Kilmartin and Clegg, 1967). The buffer contained 8 M urea and 0.32 M dithiothreitol. Elution of the chains was monitored by absorption at 280 nm. The fractions corresponding to the α and β chains were pooled, dialyzed exhaustively against 0.2 N acetic acid, and lyophilized to dryness. Horse globin contains one allelic type of β and two α chains. Only the first or rapidly eluting α chain (α_F) was used in the work described and is referred to for simplicity as the α chain.

Preparation of Horse Globin Fragments α_{1-32} and β_{1-55}. CNBr was used to cleave each globin chain at its single methionine residue using the technique developed for other proteins (Edmundson, 1963; Gross, 1967). The β chain (138 mg) and the α chain (131 mg) of horse globin were dissolved in 3.7 and 3.0 ml of 0.1 N HCl, respectively. CNBr (40 mg in 0.4 ml of water; 50-fold molar excess of CNBr to methionine) was added to each chain solution. The solutions were stirred for 20 hr at room temperature and lyophilized. After redissolving the residues in water and relyophilization several times fluffy products were obtained. The β-chain fragments were dissolved in 0.2 N

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[1] Abbreviations used are: tetradecapeptide renin substrate, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser; CNBr, cyanogen bromide; IgG, immunoglobulin G; [^{125}I]substrate, [^{125}I]polymeric renin substrate; polymeric renin substrate, Ac-poly(Glu)-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser; EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide; Dip-F, diisopropyl fluorophosphate.

TABLE I: Amino Acid Composition of Peptide Fragments α_{1-32} and β_{1-55} Isolated from CNBr Cleavage Mixtures by Sephadex Chromatography.

	α_{1-32}		β_{1-55}	
Amino Acid	Found	Reported[a]	Found	Reported[a]
Asp	2.1	2	5.1	5
Thr	1.1	1	1.0	1
Ser	1.9	2	2.3	3
Pro	0.2	0	1.7	2
Glu	2.6	3	7.4	8
Gly	3.8	4	5.6	6
Ala	5.4	7	5.2	5
Val	3.0	3	7.0	7
Ile	0	0	0	0
Leu	2.45	2	6.7	7
Tyr	0.8	1	1.0	1
Phe	0.3		3.0	3
Lys	2.3	3	1.8	2
His	0.9	1	0.6[b]	0[b]
Arg	0.8	1	1.3[b]	2[b]

[a] Column labeled *reported* contains the number of residues expected from the presently known sequences of horse hemoglobin (see Dayhoff, 1972). [b] See Materials and Methods for comment on this discrepancy.

acetic acid and chromatographed on Sephadex G-50 in the same solvent (2 × 70 cm; flow rate, 22 ml/hr; fraction size, 5 ml). With monitoring at 280 nm, three broad overlapping peaks were obtained. Rechromatography of the third peak area on the same Sephadex G-50 column gave a discrete and dominant single peak which corresponded to the central portion of the original broad third peak. No attempt was made to identify the two earlier eluting peaks which presumably represented unreacted starting material and the 56–146 amino acid fragment of the β chain. The cleavage mixture of the α chain was also chromatographed on Sephadex G-50 yielding two early peaks and a discrete late eluting peak. After rechromatography the late peak appeared as a single component. No attempt was made to identify the materials eluting earlier which presumably represented unreacted α chain and the 33–141 amino acid fragment.

The amino acid analyses of the acid-hydrolyzed CNBr fragments and those expected from the known sequences of horse globin are shown in Table I. A discrepancy was seen in the analysis of the β_{1-55} fragment. This material should have contained 2 residues of Arg and 0 of His. Instead the analysis showed 1.3 of Arg and 0.6 of His consistent with a hemoglobin variant in pooled blood obtained from more than a single animal. The blood used to prepare the globin was known to be pooled since both Tyr and Phe were present in the α fragment (Dayhoff, 1972). The sum of Tyr and Phe was two residues and conformed to expectation. A His substitution for Arg has not been previously reported for horse hemoglobin but would not be remarkable. The analysis below showed the presence in each hydrolysis mixture of homoserine and homoserine lactone, as was expected from the action of CNBr on Met, but no attempt was made to quantitate these components.

Peptic Digestion of Horse Globin β Chain. Globin β chain (14.7 mg) was dissolved in 0.5 ml of 0.1 N acetic acid and divided into equal aliquots. One was diluted with 0.1 M sodium phosphate to a concentration of 1.4 mg/ml at a final pH of 5.0. This solution of intact β chain was saved for later comparison with the second aliquot which was digested with pepsin. The 0.25-ml sample was diluted with 0.6 ml of 0.1 M citrate buffer (final pH 3). Pepsin (10 μg) was added and the mixture was incubated for 150 min at 37°. The digest was then heated for 10 min in a boiling-water bath to inactivate the pepsin and diluted with 0.1 M sodium phosphate to a final pH and concentration equal to the solution of the intact β chain. Both solutions were subsequently assayed simultaneously for their ability to inhibit renin.

Cleavage of β-Lactoglobulin B with CNBr and Dithiothreitol. The procedure followed was that described by Mainferme *et al.* (1971) for this protein.

Preparation of Leu-Leu-Val-Tyr-OMe. The parent tetrapeptide was esterified with anhydrous methanolic HCl (0.6 N in HCl) at a concentration of 10 mg/ml for 20 hr at room temperature. Completeness of esterification was confirmed by thin-layer chromatography (tlc) (Bath and Gregerman, 1972). The ester migrated more rapidly than the parent peptide and was freely soluble in 0.1 M sodium phosphate (pH 5.5); the parent peptide precipitated at a pH lower than 7.2.

Synthesis of [^{125}I]Substrate. This material was synthesized by the method previously described for polymeric tridecapeptide renin substrate (Bath and Gregerman, 1972) using instead the tetradecapeptide renin substrate possessing an additional amino-terminal aspartyl residue. The resulting labeled substrate is indistinguishable from that obtained with the tridecapeptide. In using the tetradecapeptide it is essential that the peptide be homogeneous by tlc in several solvent systems as previously described for the tridecapeptide (Bath and Gregerman, 1972). Those batches of tetradecapeptide which showed considerable contamination by components preceding and trailing the major component were not suitable because they resulted in products with unacceptably high blanks. Stability of [^{125}I]substrate was improved when special care was taken to separate the labeled polymer from the iodination mixture during chromatography on Sephadex G-15. After chromatography the [^{125}I]substrate solution was stored at −70°. Starting with 0.4–0.6 μmol of nonlabeled polymeric renin substrate (tetradecapeptide equivalent), a product was regularly obtained with a specific activity (after extraction with 1-butanol) of about 1 mCi/μmol. For use in the assay 0.5 ml of the stock solution was diluted with 4.5 ml of 0.1 M sodium phosphate (pH 7.0) and extracted eight times with 5-ml portions of water-saturated 1-butanol. The pH of the pre-extracted polymer solution was then adjusted with HCl to the pH desired, usually about 5.5.

Labeled Polymeric Substrate Assay for Human Renin. Enzyme assays were performed using conditions similar to those described earlier: 0.1 M sodium phosphate (pH 5.5); total volume of 1 ml containing lysozyme (1 mg/ml), Dip-F (0.0027 M) EDTA (0.015 M), [^{125}I]substrate, enzyme, and inhibitor. The reaction was started by adding either renin or [^{125}I]substrate. Incubations were at 37°, usually for 1 hr. In occasional instances, owing to insolubility of an inhibitor at pH 5.5, the assay was performed at another pH. For example, horse α and β chains were assayed for inhibitory capacity at pH 5 since at higher pH they precipitated out of solution. The β_{1-55} fragment was assayed at pH 6 since it was insoluble at a lower pH. Reactions were terminated by boiling the tubes for 10 min. Since the [^{125}I]Leu-Val-Tyr(I)-Ser reaction product partitions most favorably into 1-butanol at pH 7–8, 0.5 ml of 0.2 M sodium phosphate (pH 7.6) was added to each tube to adjust the pH prior to extraction of the reaction product. Each tube was extracted as described below for the kinetic studies.

Conditions for Kinetic Studies. The assay was modified slightly for kinetic studies since highly accurate additions of varying concentrations of substrate were essential. Satisfactory reproducibility was attained with a total reaction volume of 5 ml. The reaction solution was prewarmed to 37° and the reaction was initiated by addition of the enzyme. Substrate was added over a range of concentrations so that the ratio [substrate concentration]/K_m varied from 0.1 to 0.6. Reaction rates were shown to be linear with time for at least 20 min by withdrawing 0.5-ml aliquots at 2- or 3-min intervals. Reactions were immediately terminated in these samples by addition of 0.5 ml of 0.2 M sodium phosphate (pH 7.6) and heating in a boiling-water bath for 10 min. The resulting slope of each line at each substrate concentration could be used for kinetic calculations by measuring the rate of generation of product from the graph. However, it was found that the most accurate data could be obtained by incubating the entire 5-ml reaction mixture for precisely 15 min followed by termination of the reaction by addition of 0.15 ml of 3 N NaOH. Each reaction mixture was then heated for 10 min and centrifuged. Two 1-ml aliquots were removed, extracted twice with 2 ml of water-saturated 1-butanol, and back-extracted once with 4 ml of 0.1 M sodium phosphate (pH 7.5); 2 ml of the 1-butanol phase was counted in a gamma scintillation counter. Blanks *for each substrate level* were obtained by adding an equivalent volume of water instead of renin to the initial reaction solution. Since separate experiments showed the reaction to be linear with time the total counts generated per 15-min incubation were used to calculate the counts generated per minute, *i.e.*, the reaction velocity.

The kinetic data were analyzed graphically by three linear transformations of the Michaelis–Menten equation, plotting $1/V$ *vs.* $1/[S]$, $[S]/V$ *vs.* $[S]$, and V *vs.* $V/[S]$ (Webb, 1963; Dowd and Riggs, 1965). The y intercepts and the theoretical lines best fitting the data were calculated with the aid of a computer by the method of least squares. The values of K_m and K_i for human renin acting upon the polymeric substrate in the absence and presence of each inhibitor were calculated from the slopes and appropriate intercepts of each line.

Preparation of Human Hemoglobin for the Renin Assays Using Protein Renin Substrate and Double-Isotope Derivative Assay and Radioimmunoassay for Angiotensin I. Hemoglobin was prepared from human blood collected with EDTA (final concentration of 0.03 M). The red cells were washed three times with normal saline, lysed in distilled water, and centrifuged to remove the erythrocyte membranes. The resultant hemoglobin solution (pH 7.6) was passed through Dowex 50W-X8 in the NH_4^+ form to absorb small peptides which might interfere with the angiotensin I assays (Gregerman and Kowatch, 1971). Since erythrocytes contain high levels of angiotensinase, this enzyme was inactivated by dialyzing the hemoglobin solution overnight against 0.003 M EDTA in 0.01 M sodium phosphate (pH 8.0) followed by heating the dialyzed hemoglobin at 60° for 30 min (Page and McCubbin, 1968). A slight amount of precipitation occurred with heating. After removal of the precipitate the supernatant hemoglobin solution was lyophilized to dryness and dissolved in 0.1 M sodium phosphate (pH 7.6) for addition in the renin assays. Absence of angiotensinase activity in the resultant hemoglobin solution was confirmed by incubating the hemoglobin (final concentration 4 mg/ml) with a known amount of [^{3}H]angiotensin I and measuring the recovery of the intact tritiated peptide following absorption and elution from Dowex 50W-X8 in the NH_4^+ form (Gregerman and Kowatch, unpublished results). Angiotensinase activity of plasma is readily detected by this technique. Compared with an unincubated control there was 108% recovery of [^{3}H]angiotensin I.

The potency of inhibition of renin by such angiotensinase-free heat-treated hemoglobin, when measured in the labeled polymeric substrate assay, was reduced to about one-fourth that of untreated hemoglobin. With the heat treated hemoglobin 50% inhibition (I_{50}) was observed at a concentration of 1.4×10^{-5} M; the I_{50} was 3.2×10^{-6} M for native human hemoglobin.

TABLE II: Inhibition of Renin by Various Proteins and Peptides.

Inhibitor Tested[a]	Concn (M) Producing 50% Inhibn
Horse β_{1-55} fragment[b]	1.5×10^{-6}
Human α chain of hemoglobin[b]	2.3×10^{-6}
Horse β chain of hemoglobin[b]	2.7×10^{-6}
Bovine hemoglobin	2.9×10^{-6}
Human hemoglobin	3.2×10^{-6}
Horse α chain of hemoglobin[b]	3.6×10^{-6}
Bovine fibrinogen	2.3×10^{-5}
Leu-Trp-Met-Arg-Phe-Ala	4.5×10^{-5}
Horse α_{1-32} fragment[b]	1.0×10^{-4}
β-Lactoglobulin B	1.4×10^{-4}
Leu-Leu-Val-Tyr-OMe	3.5×10^{-4}
Bovine albumin	4.4×10^{-4}
Leu-Trp-Leu	5.5×10^{-4}
Leu-Leu-Leu	5.8×10^{-4}
Arg-Leu-Leu-Gln-Gly-Leu-Val·NH_2	1.2×10^{-3}
Ile-Ile-Ile	2.0×10^{-3}

[a] Labeled polymeric substrate concentration varied from 500 to 1000 pmol per ml. Small differences in effectiveness between the various inhibitors may not be significant since a number of different batches of substrate were used and the concentration of substrate varied somewhat. [b] The α and β chains are actually globin chains derived from hemoglobin. Specific fragments were prepared from the α- and the β-globin chains.

Results

Inhibition of Renin by Proteins. To our surprise several proteins inhibited renin in the polymeric substrate assay system. The most potent intact protein tested was human hemoglobin which was inhibitory at 10^{-6} M (Table II). Bovine and horse hemoglobins were effective in the same concentration range. Three unrelated proteins, bovine fibrinogen, albumin, and β-lactoglobulin B, were much weaker inhibitors requiring concentrations approximately 100-fold higher to produce the degree of inhibition shown by the hemoglobins. The single additional protein tested, purified bovine IgG, was completely without effect at 2×10^{-4} M. The wide range of potencies of the several proteins tested and the complete lack of effect by at least one clearly suggests that the inhibition of renin by these molecules has specific structural requirements and is not the result of a completely nonspecific effect of proteins.

Inhibition of Renin by Hemoglobin, Globin Chains, Globin Fragments, and Fragments of β-Lactoglobulin B. Since hemoglobin proved to be an inhibitor of renin and since hemoglobin's structure is well known, we undertook a more detailed study with this protein in the hope of gaining some insight into the amino acid sequence requirement for renin inhibition.

RENIN ↓

RENIN SUBSTRATE: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-
9 10 11 12 13 14

HORSE GLOBIN β CHAIN: Arg-Leu-Leu-Val-Val-Tyr-
30 31 32 33 34 35

FIGURE 1: Relationship of amino acid sequences of renin substrate and of a specific region of the β chain of horse globin. Note similarity of sequences about 9–13 of renin substrate and 30–35 of the β chain. The His-9 of renin substrate and the Arg-30 of the β chain may be considered analogous positively charged residues.

Horse hemoglobin was chosen for study because its unique sequence allowed preparation of fragments with and without Leu-Leu sequences. The likely importance of this particular sequence for inhibition was inferred from the available information on small peptide inhibitors of the enzyme.

Hemoglobin contains four such Leu-Leu sequences. These occur at positions 100–101 and 105–106 in the α chain and 31–32 and 105–106 in the β chain (Dayhoff, 1972). The Leu-Leu at positions 31–32 in the β chain is of special interest since its neighboring amino acids resemble those found in tetradecapeptide renin substrate (Figure 1). When the α and β chains of horse globin were prepared and examined the two chains were about equally potent (Table II and Figure 2). Two specific fragments were then prepared from the α and β chains, respectively. Each chain of horse globin contains a single methionine. Cleavage of the α chain at this residue by CNBr yielded the expected 1–32 amino acid fragment which lacks a Leu-Leu sequence (Table I). The β chain yielded a 1–55 amino acid fragment containing the Leu-Leu with a neighboring sequence similar to that in the natural protein substrate (Figure 1 and Table I). When these fragments were examined for inhibitory capacity, the β_{1-55} fragment proved to be 1.8 times more potent an inhibitor of renin than the intact β chain. However, the α_{1-32} fragment, lacking a Leu-Leu sequence, while less potent than the intact α chain by a factor of about 30, still possesses inhibitory capacity (Table II and Figure 2). These findings demonstrate that a Leu-Leu sequence, especially one resembling that in renin substrate, might still contribute significantly to inhibition but is not essential.

The importance of molecular size for inhibition of renin by proteins was evaluated by digestion of the β chain of hemoglobin with pepsin and of β-lactoglobulin B with CNBr. A pepsin digest retained 60% of the inhibitory potency of intact β chain compared at the latter's I_{50}. A CNBr digest of β-lactoglobulin B was actually more potent than the intact protein. Tested at 2 mg/ml the intact protein inhibited 28% while the digest inhibited 87%. As little as 0.2 mg/ml of the digest was still 44% inhibitory.

These results clearly indicate that large size, *per se*, is not of great importance in the inhibition produced by protein. The results with pepsin, however, are especially noteworthy. Digestion of the β chain of hemoglobin by pepsin has been studied by Konigsberg *et al.* (1963) and is known to disrupt preferentially the Leu-Leu sequences of the molecule. Although we did not prove that our own digest was totally devoid of Leu-Leu sequences, the experiment suggests, as does the comparison of the β_{1-55} and α_{1-32} fragments, that a Leu-Leu sequence is not essential for inhibition of renin by hemoglobin. With these considerations in mind we proceeded to examine a number of small synthetic peptides.

Inhibition by Small Peptides. Despite previous evidence that small peptide inhibitors of renin require a structure closely resembling that of renin substrate (Skeggs *et al.*, 1964; Kokubu *et al.*, 1968) a variety of Leu containing peptides showed a wide range of inhibitory effects (Table II). The hexapeptide,

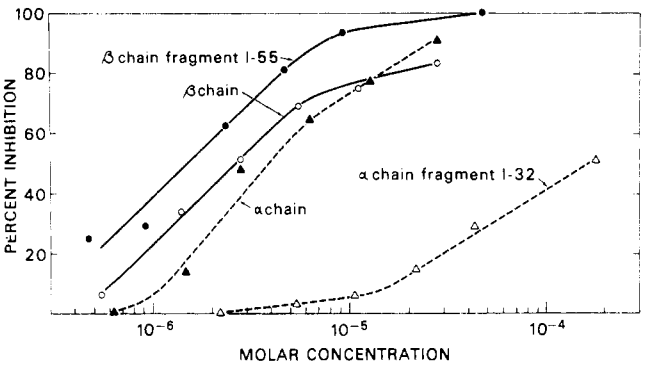

FIGURE 2: Inhibition of renin by horse α- and β-hemoglobin chains and specific chain fragments. The α and β chains both contain two Leu-Leu sequences. The two chains inhibit with approximately equal potency. The β_{1-55} chain fragment which contains a single Leu-Leu inhibits even more strongly than the parent chain. The α_{1-32} chain fragment contains no Leu-Leu sequence; although much weaker than the parent α chain, this fragment is still an effective inhibitor. The Leu-Leu sequence may contribute to the effectiveness of some of the inhibitors but is not an absolute requirement.

Leu-Trp-Met-Arg-Phe-Ala, is a fairly potent inhibitor at 10^{-5} M and was much more potent than Leu-Leu-Val-Tyr-OMe (Table II; Figure 3), the peptide previously studied by Kokubu *et al.* (1968). Several tripeptides are also inhibitory at concentrations in the range of 10^{-4}–10^{-3} M. Specific structural requirements are evident in comparing these compounds. While the tripeptide, Leu-Leu-Leu, produces 50% inhibition at 6×10^{-4} M, the closely related peptides, Leu-Leu-Gly, Leu-Gly-Leu, Gly-Leu-Leu-Gly, Gly-Leu-Gly-Leu, Val-Val-Val (all at 3×10^{-3} M), and Leu-Leu (4×10^{-2} M) are without effect. Even the isomeric tripeptide, Ile-Ile-Ile, is several times less potent than Leu-Leu-Leu. A number of closely related dipeptides are without effect, *e.g.*, Ile-Leu and Leu-Ile (4×10^{-2} M), while some unrelated peptides (His-Ser-Glu-Gly-Thr-Phe, 4×10^{-4} M; oxidized and reduced glutathione, 2×10^{-3} M) are also ineffective. Both peptides containing the sequence Leu-Trp, *e.g.* Leu-Trp-Leu and Leu-Trp-Met-Arg-Phe-Ala, proved to be relatively potent inhibitors, the latter at 10^{-5} M and the former at 10^{-4} M. The results with these small peptides support the conclusion drawn from experiments with the hemoglobin fragments and the peptic digest of the β-globin chain that the Leu-Leu sequence is not an absolute requirement for inhibition of renin.

Kinetics of the Inhibition of Renin by Proteins and Peptides. Detailed kinetic studies were performed on two small peptides and two proteins, four substances which covered a spectrum of inhibitor types and potencies. In each kinetic study the data are presented as plots of $1/V$ *vs.* 1/[S] (Lineweaver–Burk), V *vs.* $V/[S]$ (Eadie; Hofstee), *and* $[S]/V$ *vs.* [S] (Hanes). In no instance did the data conform to that expected from inhibitors of a strictly competitive type (Figure 3). In the case of the protein inhibitors all three plots unequivocally indicate noncompetitive inhibition (Webb, 1963). Laskowski and Sealock (1971) have forcefully presented the view that virtually all protein inhibitors of proteases act by a competitive mechanism. These authors have also pointed out that when the affinity of the enzyme for the inhibitor is very great (K_{assn} much greater than $1/K_m$) or when the rate of dissociation of the inhibitor complex is very slow that noncompetitive kinetics will be observed. The first consideration is not an explanation for our results but our data must be interpreted within the limitations of the second. Nonetheless, even for the small peptides the sum of the evidence also indicates noncompetitive inhibition although a few points should be noted. With Leu-Leu-Val-Tyr-OMe both the

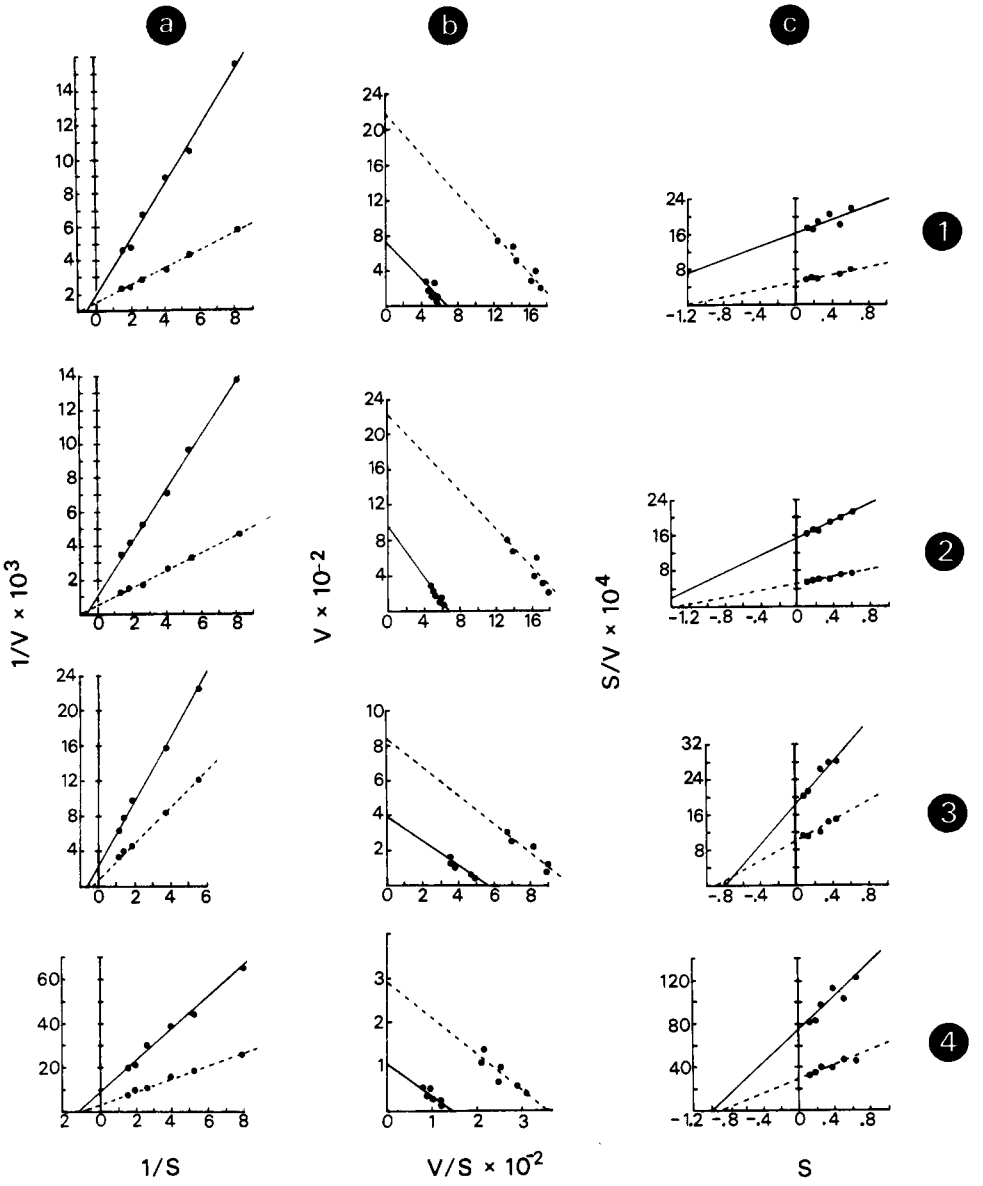

FIGURE 3: Kinetic data for the inhibition of renin by peptides and proteins in the labeled polymeric substrate assay: 1, Leu-Leu-Val-Tyr-OMe; 2, Leu-Trp-Met-Arg-Phe-Ala; 3, bovine hemoglobin; 4, β-lactoglobulin B. Column a, data plotted by method of Lineweaver and Burk ($1/V$ *vs.* $1/[S]$; column b by method of Eadie or Hofstee (V *vs.* $V/[S]$); column c by method of Hanes ($[S]V$ *vs.* $[S]$). Velocities are cpm generated/min of incubation and vary because of differences in the specific activity of substrate used (time elapsed from preparation of the polymer). Substrate concentration, nmol/ml. The data are predominantly of the noncompetitive type. See Results and Discussion.

Lineweaver–Burk and Eadie plots give noncompetitive kinetics. The almost parallel lines in the Hanes plot alone suggest competitive inhibition, but the considerable error of the slope in this instance should be noted. With the Leu-Trp-Met-Arg-Phe-Ala the Lineweaver–Burk plot indicates noncompetitive inhibition. Failure of the lines to intercept on the horizontal axis in the Hanes plot and the deviation from parallelism in the Eadie plot suggest that the inhibition is of a mixed type but is certainly not strictly competitive. Values for K_i are presented in Table III. These results are in contrast to the competitive inhibition reported by Kokubu *et al.* (1968) for the closely related Leu-Leu-Val-Phe-OMe but their data are clearly too imprecise for adequate kinetic analysis (see Dowd and Riggs, 1965).

Inhibition of Renin by Hemoglobin in an Assay System Using Natural Protein Substrate. The possibility was considered that inhibition of renin might result from interaction of a protein inhibitor with the labeled polymeric substrate or was otherwise an artifact related to the use of the synthetic polymer. This was not the case. Inhibition of renin by hemoglobin was also observed in two additional assay systems in which the natural renin substrate, in this instance globulin partially purified from hog plasma, was used instead of the labeled polymeric peptide. In both of these assays the cleavage of substrate is followed by measurement of the generation of angiotensin I. In the first assay system angiotensin I formation was measured by radioimmunoassay. Inhibition by hemoglobin was dependent upon the concentration of hemoglobin and was also affected by substrate concentration (Table IV). Detailed kinetic studies were not attempted with this method. In the second system the angiotensin I was measured by a double-isotope derivative method (Gregerman and Kowatch, 1971). Appropriate control experiments showed no interference by hemoglobin in the double-isotope assay or with recovery of angiotensin I. With similar reaction conditions to those described for the immunoassay experiments of Table IV, the control sample (2×10^{-4} U/ml) generated 25 ng/5 hr with 0.3 nmol of substrate. As little as 1 mg/ml of hemoglobin (1.4×10^{-5} M) produced 8% inhibition while 25 mg/ml produced 49% inhibition. These results were

TABLE III: Inhibitor Constants from the Kinetic Studies Shown in Figure 4.[a]

	Leu-Leu-Val-Tyr-OMe	Leu-Trp-Met-Arg-Phe-Ala	Bovine Hemoglobin	β-Lactoglobulin B
Inhibitor concentration (M)	3.5×10^{-4}	4.5×10^{-5}	2.9×10^{-6}	1.4×10^{-4}
K_m control (M)				
a	1.35×10^{-6}	1.4×10^{-6}	1.1×10^{-6}	0.8×10^{-6}
b	1.1×10^{-6}	1.1×10^{-6}	0.8×10^{-6}	0.8×10^{-6}
c	1.2×10^{-6}	1.2×10^{-6}	0.9×10^{-6}	1.0×10^{-6}
K_m inhibitor (M)				
a	1.95×10^{-6}	1.7×10^{-6}	0.7×10^{-6}	0.8×10^{-6}
b	1.1×10^{-6}	1.45×10^{-6}	0.7×10^{-6}	0.7×10^{-6}
c	2.1×10^{-6}	1.5×10^{-6}	0.8×10^{-6}	0.9×10^{-6}
K_i (M)				
a	3.1×10^{-4}	3.1×10^{-5}	1.6×10^{-6}	0.9×10^{-4}
b	1.8×10^{-4}	3.4×10^{-5}	2.5×10^{-6}	0.8×10^{-4}
c	4.5×10^{-4}	3.2×10^{-5}	2.65×10^{-6}	0.8×10^{-4}

[a] All values for K_i are calculated assuming noncompetitive kinetics: a, Lineweaver–Burk plots; b, Eadie; c, Hanes.

TABLE IV: Inhibition by Human Hemoglobin of Renin Acting on Protein Renin Substrate Derived from Hog Plasma.

A. Inhibition with varying substrate concentration[b]				
Substrate concentration, nmol/ml	0.12	0.24	0.36	0.48
Angiotensin I generated, nanograms[a]				
control	8.0	11.0	14.2	14.2
hemoglobin added	5.0	7.0	10.8	11.3
% inhibition	37.5	36.4	23.9	20.4
B. Inhibition with varying hemoglobin concentration[c]				
Hemoglobin concentration				
mg/ml	0.0	1.0	2.5	5.0
$\times 10^{-6}$ M	0.0	14.7	36.8	73.6
Angiotensin I generated, nanograms[a]	13.0	12.0	8.0	5.6
% inhibition	0.0	7.7	38.5	56.9

[a] Angiotensin I generation measured by radioimmunoassay. Results expressed as nanograms of angiotensin I generated/ml per 5 hr at 37°. [b] Assay mixture of 1 ml contained 2×10^{-4} U of human renin and 24 mg (3.53×10^{-4} M) of human hemoglobin, 0.1 M in sodium phosphate (pH 7.6), and 0.03 M EDTA. An aliquot of 0.1 ml was diluted to 1 ml with 0.9 ml of 0.1 M Tris (pH 9.0), containing 2.5 mg/ml of bovine albumin for determination of angiotensin I by radioimmunoassay. [c] Assay mixture of 1 ml contained 1×10^{-4} U of renin and 0.15 nmol of hog substrate, 0.1 M sodium phosphate (pH 7.6), and 0.03 M EDTA. At the end of incubation an aliquot of 0.1 ml was diluted to 1 ml with 0.9 ml of 0.1 M Tris (pH 9.0), containing 2.5 mg/ml of bovine albumin, 1 mg/ml of human hemoglobin, and 0.024 nmol/ml of protein renin substrate. This addition of hemoglobin and hog substrate to the diluting buffer allowed the assay to be uninfluenced by the small variations of hemoglobin and hog substrate which otherwise produced nonspecific displacement effects in the standard immunoassay curve.

similar to those seen with the immunoassay method (Table IV).

Discussion

The inhibition of renin by human plasma may well be due to its content of a more or less specific inhibitor of the enzyme. Evidence is available from the work of others that plasma may contain a renin inhibitor and does contain inhibitors for a variety of other proteases. However, we have now demonstrated that some purified proteins are highly potent inhibitors of this enzyme and that a variety of proteins exert at least some inhibitory effect. These findings raise the possibility that inhibition of renin by plasma could be due to the summation of the inhibitory effects of a number of proteins in plasma. Although perhaps a model for a putative plasma renin inhibitor, hemoglobin is present in plasma at far too low a concentration to account for any significant portion of plasma's inhibitory effect.

Most of the protease inhibitors of the protein type are themselves cleaved and thus act as alternate substrates (Laskowski and Sealock, 1971). However, our observation of noncompetitive kinetics for the inhibitors hemoglobin and β-lactoglobulin B suggests that cleavage of these proteins does not occur. Inhibitors acting as alternate substrates would ordinarily be expected to produce inhibition of the competitive type. Furthermore, the two small peptides we have studied also give noncompetitive kinetics, suggesting similar mechanisms for the small peptides and the proteins. From these results and from observations on the strict structural requirements for the cleavage of small peptides by renin (Skeggs *et al.*, 1968), one would not expect that cleavage of our small peptides had occurred. Nonetheless, the finding that proteins inhibit renin opens the issue of whether renin may cleave these large molecules and hence of the specificity of the enzyme. This problem is currently under investigation in our laboratory.

Previous studies of structural requirements for inhibition of renin by peptides have considered the Leu-Leu sequence and a close resemblance to renin substrate as essential (Kokubu *et al.*, 1968; Parry *et al.*, 1972). Our results indicate that peptides with sequences quite unrelated to that of renin substrate are potent inhibitors and that the Leu-Leu sequence is not an absolute requirement. A Leu residue in proximity to a hydrophobic

region of sufficient size does appear to be important but it remains to be shown that even a single Leu is absolutely necessary for inhibition of renin by conventional peptides. Pepstatin inhibits a number of acid proteases and is perhaps a special case but is an example of an extremely potent small peptide renin inhibitor which contains no leucine (Aoyagi *et al.*, 1972; Gross *et al.*, 1972; Lazar *et al.*, 1972; Miller *et al.*, 1972). This pentapeptide of microbial origin has the sequence isovaleryl-Val-Val-4-amino-3-hydroxy-6-methylheptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid. Pepstatin's hydrophobic characteristics are thought to be of importance for inhibition (Morishima *et al.*, 1970; Umezawa *et al.*, 1970). Pepstatin also inhibits pepsin, a protease which preferentially hydrolyzes bonds between hydrophobic residues and mimics renin by producing angiotensin I from renin's protein substrate (Franze de Fernandez *et al.*, 1965; Paiva *et al.*, 1972). The importance of hydrophobic interactions of peptides with pepsin has also been recently emphasized (Raju *et al.*, 1972).

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Added in Proof

After our paper was submitted for publication we became aware of a recent additional report on small peptide inhibitors of renin (Kokubu, T., Hiwada, K., Ito, T., Ueda, E., Yamamura, Y., Mizoguchi, T., and Shigezane, K. (1973), *Biochem. Pharmacol. 22,* 3217). This study is an extension of work by Kokubu *et al.* (1968) and includes Leu containing di-, tri-, tetra-, penta-, and octapeptides related to Leu-Leu-Val-Tyr. Inhibition was thought to be of the competitive type for this tetrapeptide and Leu-Leu-Val-Phe, but the conclusion is again based only on Lineweaver–Burk plots of less than optimal precision.

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