Labeled Polymeric Substrate for Renin. Synthesis of *N*-Acetyl-poly(L-glutamyl)-[^{125}]Tridecapeptide and Use for Enzyme Assay†

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ABSTRACT: The synthesis of a labeled polymeric substrate for the enzyme renin has been described. The tridecapeptide Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser was coupled by its N-terminal amino group to the carboxyl groups of *N*-acetyl-poly(L-glutamic acid) of mol wt 60,000–80,000. The resulting product was labeled on the tyrosine residues with ^{125}I. This ^{125}I polymeric renin substrate ([^{125}I]PRS) has been characterized chromatographically. The action of renin on the substrate yields the iodinated tetrapeptide, [^{125}I]Leu-Val-Tyr(I)-Ser, which was identified by thin-layer chromatography and electrophoresis and shown to be identical with ^{125}I-labeled and nonlabeled iodotetrapeptide. A simple assay scheme was developed which takes advantage of the marked difference in the partition of the [^{125}I]PRS and the [^{125}I]tetrapeptide cleavage product in a 1-butanol–water system. Human renin attacked the [^{125}I]PRS at a pH optimum of 5.5. Under these conditions the K_m for the reaction is 0.2×10^{-6} M, a value significantly lower than that reported for renin with tetradecapeptide renin substrate and the natural glycoprotein substrate. As little as 10^{-5} Goldblatt unit/ml of human renin is rapidly measured. The system seems especially useful for purification of the enzyme and studies of its inhibitors. Coupling of substrate to a soluble polymer in order to facilitate the separation of the enzyme product from the substrate would seem to be a useful approach of wider applicability in labeled substrate and other enzyme assays.

Renin, a highly specific endopeptidase from the kidney, splits the decapeptide, angiotensin I, from a plasma globulin substrate. Renin continues to receive considerable attention because of its role in the genesis of certain forms of hypertension and in the control of sodium balance. The enzyme is present in plasma and other biological materials at very low concentrations. Renin assays have, therefore, depended on bioassay of nanogram amounts of generated angiotensin or, more recently, on immunoassay or double-isotope derivative assay of the peptide (Stockigt *et al.*, 1971; Cohen *et al.*, 1971; Gregerman and Kowatch, 1971). While the recent methods offer considerable improvement over bioassay, efforts continue toward development of a technique which will combine simplicity with sensitivity and speed (Levine *et al.*, 1970). This report presents a new approach to the assay of renin which utilizes a labeled polymeric substrate fashioned specifically for this purpose.

Materials and Methods

Chemicals. Compounds and their sources were as follows: Boc-*O*-benzyl-L-serine-resin ester (0.248 mmole of serine/g of resin) and dicyclohexylcarbodiimide, Schwarz BioResearch, Orangeburg, N. Y.; 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC)[1] and the *p*-nitrophenyl esters of Boc-L-leucine, Boc-L-valine, and Boc-*O*-benzyl-L-tyrosine, Cyclo, Los Angeles, Calif.; 1,2,4-triazole, Calbiochem, Los Angeles, Calif.; 3-iodo-L-tyrosine, Aldrich Chemical, Milwaukee, Wis.; lysozyme (three-times recrystallized) and DFP, Sigma, St. Louis, Mo.; aminopeptidase M, Rohm and Haas, Darmstadt, West Germany; papain, Mann, New York, N. Y.; Chloramine-T, Matheson Coleman & Bell, Cincinnati, Ohio; ^{125}I, Union Carbide, Tuxedo, N. Y.; Fast Red TRN, General Aniline and Film Corp., Dyestuff and Chemical Division, New York, N. Y. Purity of Boc-amino acids was checked with thin-layer chromatography (tlc) by methods described below. Other chemicals, including solvents, were high-quality commercial materials from a variety of sources, generally those described by Stewart and Young (1969).

Poly-L-glutamic Acid. Two separate preparations were obtained from Pilot Chemicals Inc., Watertown, Mass.. lot G-120, mol wt 60,000–80,000, and lot G-146, mol wt 120,000. The characteristics of the polymeric renin substrates prepared from each of these lots were identical, both in terms of chromatographic behavior on the thin-layer and column systems used and as substrates for renin.

Thin-layer chromatography of all compounds except the polymeric renin substrate was performed on Eastman Chromagram (6060) silica gel sheets. For both labeled and nonlabeled polymeric renin substrate, silica gel sheets of 250 μ thickness on aluminum backing (Brinkmann Instruments, Inc., Westbury, N. Y.) were most satisfactory. For sample applications 1- to 4-μl volumes were used. Solutions contain-

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[1] Abbreviations used are: EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide; Boc, *tert*-butyloxycarbonyl; TRS, tridecapeptide renin substrate; PGA, poly(L-glutamic acid); N-Ac-PGA, *N*-acetyl-poly(L-glutamic acid); NL-PRS, nonlabeled polymeric renin substrate (*N*-Ac-PGA-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser); [^{125}I]PRS, labeled polymeric renin substrate ([^{125}I]N-Ac-PGA-Arg-Val-Tyr(I)-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr(I)-Ser).

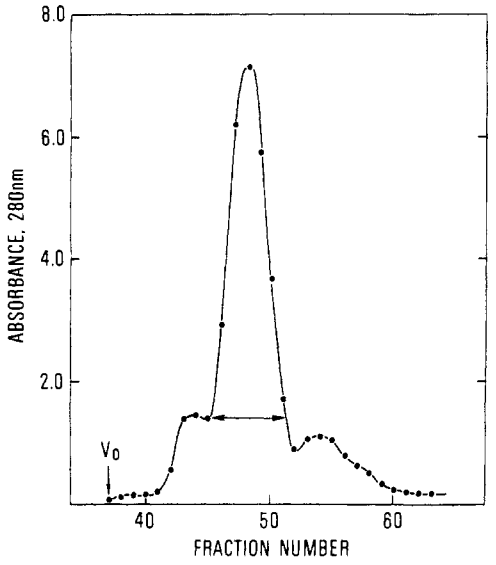

FIGURE 1: Purification of 107 mg of the tetrapeptide, Leu-Val-Tyr-Ser, on Sephadex G-15 in 0.2 N acetic acid. Column, 2.5 × 71 cm; fraction size, 6.2 ml; flow, 28 ml/hr. The fractions indicated by the arrows contained the purified tetrapeptide and were pooled.

ing trace amounts of labeled peptide were spotted over 10 μg of the appropriate carrier compound. The chromatograms were developed for 3–4 hr in ascending fashion following a 30-min equilibration. Nonlabeled peptides and amino acids were localized by spraying the dried chromatogram with ninhydrin, Pauly reagent (Smith, 1969), or in some cases, *t*-butyl hypochlorite (Mazur *et al.*, 1962). Labeled peptides were localized by radioautography, usually with overnight exposure, using Ilford No-Screen X-Ray film. The tlc solvent systems used were as follows: (1) 1-butanol–acetic acid–water (3:1:1); (2) 1-butanol–acetic acid–water (12:3:5); (3) 1-butanol–acetic acid–water (3:1:2); (4) 1-butanol–pyridine–acetic acid–water (30:20:6:24); (5) 1-butanol–pyridine–water (1:1:1); (6) chloroform–acetic acid (99:1); (7) methyl ethyl ketone–pyridine–water–acetic acid (75:15:15:2); (8) ethyl acetate–pyridine–acetic acid–water (5:5:1:3); (9) 1-butanol–isopropyl alcohol–water–acetic acid (65:15:25:3); and (10) 1-butanol–pyridine–acetic acid–water (5:5:1:3).

Amino Acid Determination. HCl (6 N) and enzyme hydrolysates of peptides were quantitated on an automatic ion-exchange-type analyzer (Jeol Model 5AH). Values are not corrected for losses which may have occurred during hydrolysis. Qualitative determinations were made with tlc.

Thin-layer electrophoresis (tle) was performed on cellulose sheets (10 × 20 cm) (Eastman 6065) for 3 hr at 400 V, 5 mA, using a Brinkmann-Desaga apparatus, and a formic acid–acetic acid–water buffer (10:15:75).

Tridecapeptide Renin Substrate (TRS). This compound with the sequence Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser was obtained from Schwarz (lot 7001). It had been prepared by solid-phase peptide synthesis. Its purity was verified by tlc which showed only one component when 10 and 20 μg were chromatographed in systems 1, 3, 5, 8, 9, and 10 and by tle. In addition, the $(DNP)_4$ derivative of the peptide was prepared and examined in tlc by the method of Gregerman and Kowatch (1971). Only a single component derivative was seen when up to 40 μg of the DNP-peptide was chromatographed.

Colorimetric Assay. A diazo coupling reaction using the stabilized diazonium salt, Fast Red TRN, for the determination of imidazole- and tyrosine-containing compounds (Abraham *et al.*, 1962) was used to quantitate the TRS content of the NL-PRS.

Renin. The human renin used in these studies was the generous gift of Dr. Erwin Haas. It was prepared by his procedure A (Haas *et al.*, 1966) and was preparation 13, specific activity 0.13 Goldblatt unit/mg. Although this preparation is said to be free of "angiotensinase" we have regularly used EDTA and DFP to inhibit nonspecific peptidase activity which might be present at high concentrations of the enzyme.

Synthesis of Peptides

Leu-Val-Tyr-Ser. This tetrapeptide was prepared by solid-phase synthesis (Merrifield, 1969). Boc-*O*-benzylserine-resin ester (5 g), containing 1.24 mmoles of serine, was coupled successively with the *p*-nitrophenyl esters of Boc-*O*-benzyltyrosine, Boc-valine, and Boc-leucine in the presence of an equimolar amount of 1,2,4-triazole. Each coupling step proceeded overnight at room temperature using an 8-fold molar excess of active ester. Boc groups were removed with trifluoroacetic acid in methylene chloride (50%, v/v) for 30 min at room temperature. For cleavage of the peptide from the resin HBr gas was passed through a 10% solution of resorcinol in trifluoroacetic acid and then through a suspension of the resin in trifluoroacetic acid for 60 min. Following evaporation of the trifluoroacetic acid under reduced pressure, the syrupy product was dissolved in 2 ml of glacial acetic acid and precipitated with ether, washed with ether, and dried. Purification was performed by chromatography on Sephadex G-15 using 0.2 N acetic acid (Figure 1). Peptide material was localized by measurement of absorbance at 280 nm and by Pauly reaction. Three ultraviolet-absorbing, Pauly-positive peaks were identified. The largest peak was shown to contain a single component on tlc in solvent systems 1, 2, 3, and 4, and on tle. Enzymatic hydrolysis of the purified peptide (Stewart and Young, 1969) gave the four expected amino acids on tlc in solvent systems 1, 2, 5, and no unknown products. Quantitative amino acid analysis after hydrolysis with 6 N HCl gave the following molar ratios: Leu, 1.00, Val, 0.92; Tyr, 1.03; Ser, 0.85.

Leu-Val-Tyr(I)-Ser. The iodotyrosine-containing tetrapeptide was prepared by solid-phase synthesis starting with 4.25 g of Boc-*O*-benzylserine-resin ester containing 1.05 mmoles of serine and using dicyclohexylcarbodiimide for coupling. Boc-3-monoiodotyrosine was used in 3.75 molar excess and Boc-valine and Boc-leucine in 2.5 molar excess. The Boc-3-monoiodotyrosine was synthesized from the iodoamino acid by the method of Schwyzer *et al.* (1959). The product of this synthesis was an oil which dissolved in ethyl acetate and was crystallized with difficulty by addition of hexane. Complete substitution of the moniodotyrosine with the Boc group was demonstrated as follows. The synthetic Boc derivative was chromatographed in tlc system 6. Ninhydrin spray revealed no residual ninhydrin-reactive component. After removal of the Boc group by exposure of the dried chromatogram to HCl fumes for 15 min, a single ninhydrin-positive component appeared with R_F 0.8. The parent compound, iodotyrosine, had an R_F of 0.05 in this system. The crude iodotetrapeptide was cleaved from the resin by the HBr method described above and an approximate yield of 500 mg was obtained.

Purification was carried out on Sephadex G-15 using 0.2 N acetic acid. The peptides were localized by absorbance at 280 nm. The third and largest peak (Figure 2; fraction 88–

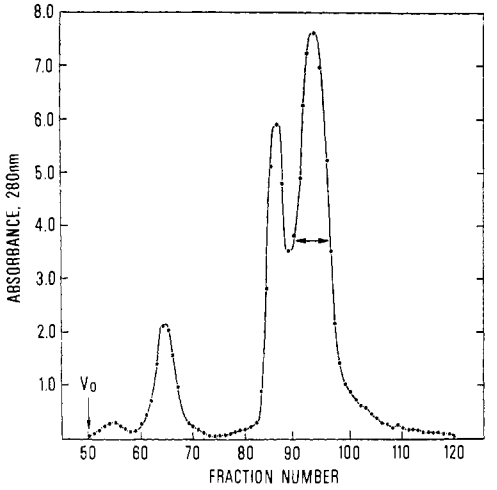

FIGURE 2: Purification of 120 mg of the iodotetrapeptide, Leu-Val-Tyr(I)-Ser, on Sephadex G-15 in 0.2 N acetic acid. Fraction size, 4.2 ml. Other conditions as in Figure 1.

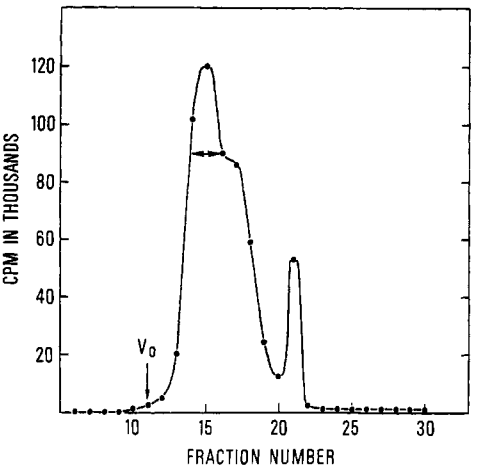

FIGURE 3: Purification of [^{125}I]Leu-Val-Try(I)-Ser on Sephadex G-50 in 1 N NH_4OH. Compound previously purified on Sephadex G-10 following iodination of Leu-Val-Tyr-Ser. Column, 0.9 × 75 cm; fraction size, 2.0 ml; flow, 3.6 ml/hr. The fractions indicated by the arrows contained the purified [^{125}I]tetrapeptide.

96) contained the iodotetrapeptide. On tlc in solvent systems 1, 2, 4, 7, and after tle, one major component and several very minor impurities were seen. The iodotetrapeptide was separable from the tetrapeptide by tlc in solvent system 7 (R_F iodotetrapeptide, 0.6; R_F tetrapeptide, 0.5). The purified iodotetrapeptide contained a minute component that migrated in the same area as the purified tetrapeptide. Enzymatic hydrolysis of the purified iodotetrapeptide gave only the four expected amino acids on tlc. Quantitative amino acid analysis after enzymatic hydrolysis yielded the following molar ratios: Leu, 0.83; Val, 0.83; Tyr(I), 0.98; Ser, 1.00.

[^{125}*I*]*Leu-Val-Tyr(I)-Ser*. The purified tetrapeptide (10 μg) Leu-Val-Tyr-Ser in 25 μl of 0.5 M sodium phosphate buffer (pH 7.4) was added to a solution containing 2.74 mCi of ^{125}I in 15 μl (supplied in 0.1 N NaOH but neutralized with a few microliters of 0.1 N HCl immediately before use). Chloramine-T (25 μl; 4 mg/ml in buffer) was added and after 1 min the reaction was terminated by addition of 50 μl of sodium metabisulfite (8 mg/ml). The reaction mixture was diluted with 1 N NH_4OH to a volume of 1 ml. Purified Leu-Val-Tyr(I)-Ser (100 μg) was added as carrier and the mixture was immediately chromatographed on Sephadex G-10 using 1 N NH_4OH. The labeled tetrapeptide eluted as a symmetrical peak with the void volume and was widely separated from ^{125}I which eluted much later. Further purification was achieved by chromatography on G-50 Sephadex using 1 N NH_4OH (Figure 3). Aliquots (5 μl) of each tube were counted. Two peaks were obtained, the first peak having a shoulder. The material from fractions 12–16 was shown to be 90% pure by tlc in solvent 2 and radioautography. The labeled product corresponded exactly to the nonlabeled iodotetrapeptide during tlc in solvent systems 1–4. Specific activity of the purified material was approximately 5 mCi/mg.

N-Acetyl-poly(L-*glutamic Acid*) (*N-Ac-PGA*). PGA (100 mg), mol wt 60,000–80,000, was suspended in 20 ml of 0.05 M sodium phosphate buffer (pH 7.0), and the pH of the suspension was adjusted with 1 N NaOH to pH 9.0 to effect solution and then readjusted to pH 7.0. Acetic anhydride (0.225 ml) was added in 25-μl aliquots and the pH was maintained at close to 7.0 by intermittent additions of 5 N NaOH. The reaction mixture was allowed to remain at room temperature for 2 hr and was then dialyzed exhaustively against distilled water containing 10^{-4} M sodium azide as a bacteriostat. This solution was evaporated under reduced pressure to yield a stock solution containing 100 mg/ml of N-Ac-PGA.

*N-Acetyl-(poly-*L-*glutamyl)-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser* (*NL-PRS*). To 5 mg (2.7 μmoles) of TRS in 0.5 ml of 0.5 M sodium phosphate buffer (pH 7.0) was added 20 mg (136 μmoles of glutamic acid equivlent) of N-Ac-PGA in 0.2 ml, and 31 mg (162 μmoles) of EDC in 0.3 ml of water. The reaction mixture was allowed to stand at room temperature overnight and was then dialyzed exhaustively against 0.1 N NH_4OH containing 10^{-4} M sodium azide. The TRS content of the product, and hence the extent of the coupling, was determined colorimetrically on an aliquot by Pauly reaction. Control experiments showed that TRS could be used directly as a standard in the colorimetric assay for the unhydrolyzed NL-PRS. The content of coupled TRS in the polymer could also be determined with the Pauly reaction following hydrolysis with 6 N HCl at 100° for 16 hr. In this case a solution containing known equimolar amounts of histidine and tyrosine was used as an internal standard. The use of an internal standard in these determinations was necessary because of the inhibition of color yield due to other components of the hydrolysis mixture. Three separate preparations of NL-PRS were made. The degree of coupling ranged from 30 to 65% of theoretical based on the amount of TRS used.

[^{125}*I*]*N-Acetyl-poly*(L-*glutamyl*)-*Arg-Val-Tyr*(*I*)-*Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr*(*I*)-*Ser*. NL-PRS containing 0.42 μmole of TRS equivalent was dissolved in 0.15 ml of 0.5 M sodium phosphate buffer (pH 7.0). Iodination with ^{125}I was performed as described for the peptide Leu-Val-Tyr-Ser. Following termination of the reaction with sodium metabisulfite, the reaction mixture was diluted to 2.0 ml and chromatographed on Sephadex G-50 in 1 N NH_4OH (1.7 × 70 cm column; 2.0-ml fractions; 10 ml/hr). The [^{125}I]PRS eluted in a symmetrical peak in three tubes near the void volume, while the ^{125}I eluted much later. The fractions from the [^{125}I]PRS peak were combined, evaporated, and reconstituted in 0.01 N NH_4OH in which the material was stored at −20°. Assuming no losses of starting NL-PRS during the procedure, the approximate minimum specific activity was 1 mCi/mg. In the experiments to be described references to molar quantities of

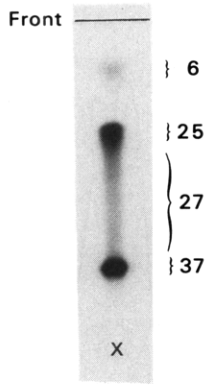

FIGURE 4: Tlc of [^{125}I]PRS on silica gel with 1-butanol–pyridine–water (1:1:1). Localization by radioautography. Per cent distribution of radioactivity is shown at the right. X represents origin.

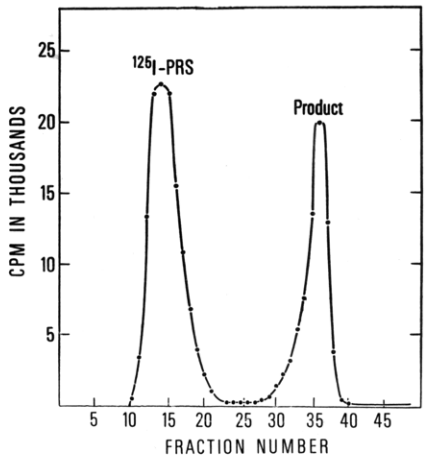

FIGURE 5: Separation of the products of the action of renin on [^{125}I]PRS. 0.01 unit/ml of renin was incubated with 300 mμmoles/ml of [^{125}I]PRS at pH 7.4 for 2 hr. Sephadex G-50 in 1 N NH_4OH; column 2.5 × 70 cm; fraction size, 2.0 ml; flow, 12 ml/hr. The product was identified as [^{125}I]Leu-Val-Tyr(I)-Ser (see text and Figure 6).

substrate refer to molar equivalents of contained tridecapeptide.

Stability of the [^{125}I]PRS. Assays for the presence of impurities were made over a 4-month period by rechromatography on Sephadex G-50 under the same conditions described above. There was no evidence of any significant formation of lower molecular weight ^{125}I products (including iodide) and the total amount of impurities demonstrable ranged from 0.1 to 1%.

Characterization of the [^{125}I]PRS. Chromatography of the [^{125}I]PRS on Sephadex G-10, G-50, and G-100 with 1 N NH_4OH gave a single peak with the void volumes. On tlc in solvent system 5, followed by radioautography, a number of components were demonstrable with two predominating as shown in Figure 4. Chromatography of a mixture of carrier NL-PRS and [^{125}I]PRS under these same conditions gave an identical radioautograph. With the Pauly reagent two diazonium-positive spots were seen in the carrier NL-PRS which corresponded exactly to the two major radioactive spots of the [^{125}I]PRS. PGA, localized with Bromocresol Green (Smith, 1969), did not leave the origin in this system.

A number of experiments were performed in an effort to elucidate the character of the two major radioactive components in the [^{125}I]PRS. Extensive dialysis, first against 2 M NaCl, and then against 1N NH_4OH, produced no change in the tlc pattern. This result tends to exclude the presence of an ionically bound component of molecular weight less than 10,000. Two-dimensional chromatography, using solvent system 5 for both dimensions, gave no evidence for breakdown during chromatography. Thus, the appearance of multiple spots was not likely due to chromatographic artifact. The possibility that any of the [^{125}I]PRS components had formed from self-condensation of TRS during the diimide coupling with N-Ac-PGA was excluded by performing the coupling reaction with tridecapeptide in which the C-terminal carboxyl was protected. The methyl ester of the tridecapeptide was prepared by treatment of 4 mg of TRS in 2 ml of 0.6 N HCl–methanol for 16 hr at room temperature. Tlc in solvent system 1 showed complete esterification with the ester migrating more rapidly than the parent compound. The ester was then coupled to N-Ac-PGA and the TRS content determined as described above. The methyl ester group was removed by saponification with 0.1 N NaOH at 0° for 20 min and the product dialyzed exhaustively against 0.1 N NH_4OH containing 10^{-4} M sodium azide. The product was iodinated with ^{125}I. This material was found to be identical on tlc with the [^{125}I]PRS made from unblocked tridecapeptide. This experiment excludes the possibility that the multiple components are the result of end to end condensation of TRS during the coupling reaction to N-Ac-PGA.

Both major radioactive components of the [^{125}I]PRS were isolated using tlc. Localization was by radioautography (20 min) and elution was performed with 0.1 N NH_4OH. Each component was shown to undergo cleavage by renin to the same extent and each yielded the same [^{125}I]tetrapeptide product (see below). These experiments and the demonstrated high degree of purity of the TRS suggest that the presence of two major components is due neither to the formation of self-condensation products during coupling nor to small molecular weight impurities in the starting TRS. The possibility that the two components are fragments formed during the iodination step is, of course, excluded by the fact that they are demonstrable in the NL-PRS prior to iodination.

Preparation of [^{125}I]PRS for Use in Renin Assay. The stock solution of [^{125}I]PRS in 0.1 N NH_4OH is diluted with 0.05 M sodium phosphate buffer (pH 7.0) to yield a solution containing coupled TRS at a concentration of 5 nmoles/μl. A 5-ml volume of this solution is then extracted four times with 5 ml of water-saturated 1-butanol and the butanol extracts are discarded. This step results in removal of approximately 10% of the total radioactivity. Most of this radioactivity is removed in the first two partitions. Each further extraction removes only small amounts (less than 1%) of additional radioactivity. The preextraction step serves to reduce the assay blank.

Cleavage of [^{125}I]PRS by Renin and Characterization of the Products. To 300 nmoles of [^{125}I]PRS in 1 ml of sodium phosphate buffer (0.05 M, pH 7.4), containing DFP (0.027 M) and EDTA (0.02 M), was added 0.01 unit of renin. After 2 hr at 37° the mixture was diluted to 2 ml with 1 N NH_4OH and chromatographed on Sephadex G-50 in 1 N NH_4OH. The elution pattern produced two peaks (Figure 5), each of which corresponded to that produced in a control experiment in which a mixture of [^{125}I]PRS and [^{125}I]tetrapeptide were co-chromatographed. The product of renin action contained in the second peak was pooled, concentrated by evaporation, and examined by tlc in solvent systems 2 and 5, and by tle.

It proved to be identical with standard [^{125}I]Leu-Val-Tyr(I)-Ser and Leu-Val-Tyr(I)-Ser (Figure 6). A small but significant amount of a labeled compound which was not identified could be seen in addition to the [^{125}I]tetrapeptide.

This experiment demonstrated that specific Leu–Leu bond cleavage occurs by the action of renin on [^{125}I]PRS under these conditions. There was no evidence for the presence of appreciable nonspecific peptidase activity despite the use of a large amount of renin.

If the iodination of NL-PRS gave an incompletely substituted product with ^{125}I randomly distributed between the two tyrosines, or if both tyrosines were completely labeled, the maximal cleavage of the substrate would be 50%. In an experiment testing this point very nearly 50% of the radioactivity was cleaved by action of renin on the [^{125}I]PRS. In this experiment [^{125}I]PRS was subjected to a prolonged incubation (16 hr) with a large amount of renin (0.03 unit) to ensure complete cleavage. As determined by Sephadex G-50 chromatography, the incubation mixture contained 51.5% [^{125}I]PRS and 48.5% [^{125}I] tetrapeptide.

Distribution of [^{125}I]PRS and [^{125}I]Tetrapeptide in an Aqueous 1-Butanol System. Approximately 2000 cpm of [^{125}I]-tetrapeptide was placed into a series of 0.1 M sodium phosphate buffers of 1-ml volume, ranging in pH from 3.5 to 8.5 in increments of 0.5 pH unit. After two extractions with water-saturated 1-butanol (2 ml each), the total radioactivity extracted into the butanol was determined. From pH 3.5 to 6.5, a nearly constant 75% of the counts was recovered in the butanol extracts. At pH 7.0 and 7.5 the figure rose to 90% and then fell to 80% at pH 8.0 and 8.5. In a separate experiment, approximately 2000 cpm was placed into ten tubes containing 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) and the butanol extractions were performed as described. In addition, back-extraction of the combined butanol extracts with a single 4-ml volume of 0.05 M sodium phosphate buffer (pH 7.5) was performed. The overall recovery of the [^{125}I]-tetrapeptide in the butanol phase was 65 ± 2% (std dev).

The effect of pH on the partition of [^{125}I]PRS was studied under the same conditions described above, using 100,000 cpm of preextracted [^{125}I]PRS. From pH 3.5 to 7.5 a constant total (for two extractions) of 2% of the radioactivity was found in the butanol phase, while at pH 8.0 and above the proportion of radioactivity extracted into the butanol rose to 20%.

In a separate experiment, paralleling that above with the [^{125}I]tetrapeptide, 100,000 cpm of [^{125}I]PRS was pipetted into ten tubes containing 2 ml of 0.1 M sodium phosphate buffer (pH 7.0). Two extractions with 2.0 ml each of butanol were followed by back-extraction of the combined butanol extracts with 4 ml of 0.05 M sodium phosphate (pH 7.5). The amount of radioactivity in the entire butanol phase, equivalent in this case to a "polymer blank," was 0.98 ± 0.05% (std dev).

These observations provide the rationale of the assay scheme below. A rapid and simple renin assay was devised which takes advantage of the different partition into 1-butanol from dilute neutral buffer solutions of the [^{125}I]PRS and [^{125}I]tetrapeptide. The most favorable conditions prevail at pH 7.0–7.5 as shown above. After performing an extraction of a mixture of [^{125}I]PRS and [^{125}I]tetrapeptide as outlined above at pH 7.0, the final entire butanol phase contains 65% of the [^{125}I]tetrapeptide, but only 1.0% of the starting [^{125}I]-PRS. Thus the conditions allow measurement of the relatively small amount of iodotetrapeptide renin-cleavage product with acceptable contamination by the labeled substrate.

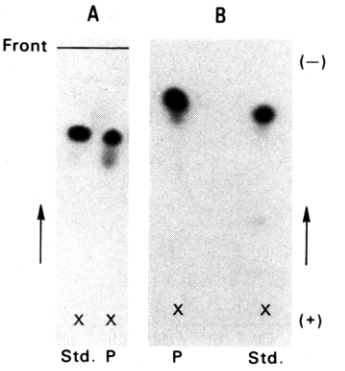

FIGURE 6: Tlc (A) and tle (B) of the product of the reaction of renin on [^{125}I]PRS. Standard [^{125}I]Leu-Val-Tyr(I)-Ser is shown as *Std.* and isolated enzyme product as *P*. Localization shown is by radioautography. Carrier nonlabeled Leu-Val-Try(I)-Ser was used. Localization of this carrier peptide with Pauly reagent (not shown) indicated its exact correspondence with labeled peptide in the radioautographs. Several minor unidentified radioactive impurities are visible.

Routine Enzyme Assay. (1) To a solution of sodium phosphate buffer (0.05–0.2 M), containing lysozyme (1 mg/ml) to prevent adsorptive losses, and DFP (0.027 M) and EDTA (0.02 M) to prevent nonspecific protease activity, are added [^{125}I]PRS and renin. The total volume of the reaction mixture is either 0.5 or 1.0 ml in a 12-ml glass test tube (plastic tubes adsorb both the [^{125}I]PRS and the [^{125}I]tetrapeptide). For incubations longer than 3 hr, the buffer contains phenylmercuric acetate (10^{-4} M) or sodium azide (10^{-4} M) as a bacteriostat. Incubations are at 37°. (2) The reaction is terminated by placing the tubes in a boiling-water bath for 10 min. The tubes are removed and cooled. (3) The pH is adjusted, if necessary, to 7.0. (4) The reaction mixture is extracted twice by mixing on a Vortex test-tube mixer with 2 ml of water-saturated 1-butanol. The butanol phases are transferred to a glass test tube and the 4-ml volume was back-extracted by mixing on the Vortex with 4 ml of 0.05 M sodium phosphate buffer (pH 7.5). A 2-ml aliquot of the butanol phase is then removed and counted in an automatic gamma scintillation spectrometer using plastic counting tubes. The results are expressed as actual counts per minute observed and/or as millimicromoles of iodotetrapeptide product formed as calculated from the known recovery of [^{125}I]tetrapeptide, as determined under identical conditions in a separate experiment, and from the specific activity of the [^{125}I]PRS. All values for [^{125}I]tetrapeptide product have been corrected with a blank value obtained by performing triplicate incubations for each level of [^{125}I]PRS in the absence of renin. This blank has been shown in separate experiments to be identical to values obtained when enzyme is added and the mixture immediately boiled.

Assay Results

Effect of Substrate Concentration. Initial studies were made at relatively high pH in order to simulate conditions under which interference by nonspecific protease activity (contained in the renin preparation, or, potentially, in plasma) would be minimal. Figure 7 shows that for a 16-hr incubation at pH 7.5 with 6×10^{-4} unit/ml of renin, the generation of [^{125}I]tetrapeptide becomes independent of substrate concentration at [^{125}I]PRS levels above 200 nmoles/ml.

Effect of pH. Reaction mixtures were prepared containing

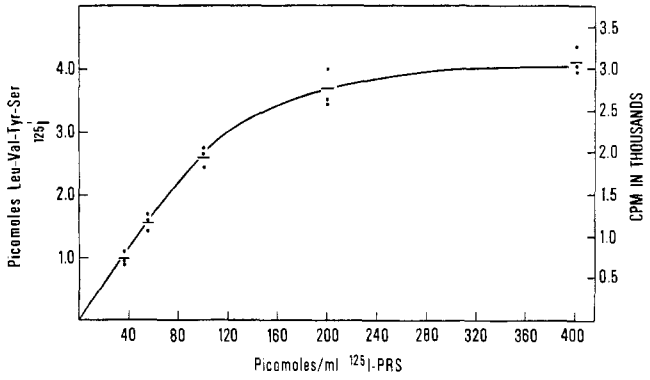

FIGURE 7: Relationship of concentration of [^{125}I]PRS to rate of cleavage by renin. 6×10^{-4} unit/ml of renin was incubated for 16 hr at pH 7.5. Blank values for 100 and 400 pmoles per ml of [^{125}I]PRS were 304 ± 20 cpm (range) and 1194 ± 42 cpm (range).

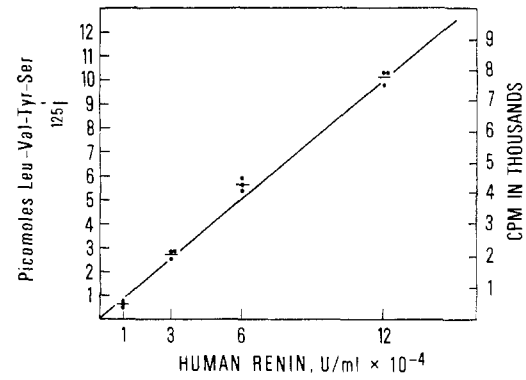

FIGURE 9: Assay of renin at pH 7.5 using [^{125}I]PRS. 16-hr incubation at pH 7.5. The blank for this experiment was 1085 ± 35 cpm (range).

1×10^{-4} unit/ml of renin and 300 nmoles/ml of [^{125}I]PRS in 0.05 M sodium phosphate buffer at the specified pH levels between 3.0 and 8.5. Incubation period was 16 hr. Maximal activity was seen at pH 5.0–5.5 (Figure 8A). In order to determine activity at pH levels above 6.0, a separate experiment was performed. The incubation mixtures were identical, but renin was added in a twelvefold greater concentration (12×10^{-4} unit/ml). This facilitated measurements at the higher pH values where enzyme activities were observed to be much lower (Figure 8B).

Renin Assay at pH 7.5. Renin in concentrations from 1×10^{-4} to 12×10^{-4} unit/ml was assayed as described above using 16-hr incubation and 300 nmoles/ml of substrate. A linear relationship between amount of renin added and the amount of product formed was observed over this range of renin concentrations (Figure 9). Comparison of this experiment with those below demonstrates the greater sensitivity of the assay at lower pH.

Time Course of the Reaction. The action of 1×10^{-4} unit/ml of renin on 300 nmoles/ml of substrate at pH 5.5 was followed as a function of time for a 1-hr period. Generation of [^{125}I]tetrapeptide was linear with time over this interval (Figure 10). These same incubation conditions were used for the K_m determination described below.

K_m of the Reaction of Renin with [^{125}I]PRS. The apparent K_m was determined in the following manner. For each substrate concentration indicated (Figure 11) incubation was with 2.5×10^{-5} and 5.0×10^{-5} unit per ml of renin in a volume of 5 ml of 0.1 M sodium phosphate buffer (pH 5.5). Aliquots of 0.5 ml were withdrawn at 3-min intervals for 21 min. The generation of [^{125}I]tetrapeptide was linear with time for all samples. The K_m for the reaction determined from the plots of the reciprocals of velocity and substrate concentration is 0.23×10^{-6} M and the $V_{max} = 2.1 \times 10^{-6}$ M/min per unit of renin.

Discussion

Although the natural substrate for renin is a plasma protein, the partial degradation of this protein by trypsin yields a tetradecapeptide which is still a satisfactory substrate for renin. The kinetics of the reaction of renin with this substrate as well as with smaller peptide congeners have been described (Skeggs *et al.*, 1968). An automated assay has also been developed which is based on the colorimetric determination of Leu-Val-Tyr-Ser formed as renin cleaves the synthetic nonapeptide His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser (Levine *et al.*, 1970). The use of a [^{14}C]tetradecapeptide has also been described recently (Mendelsohn and Johnston, 1971) in which the decapeptide product, [^{14}C]angiotensin I,

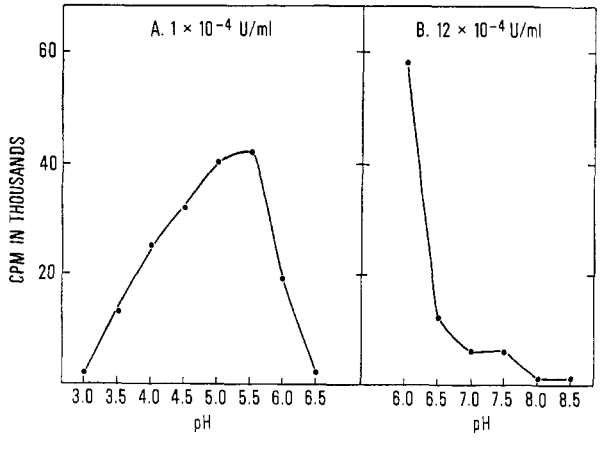

FIGURE 8: Effect of pH on the cleavage by renin of [^{125}I]PRS. Shown in A is the pH optimum at a low concentration of the enzyme. B shows the effect of higher pH using a larger amount of enzyme because of the greatly reduced reaction velocity in this pH range.

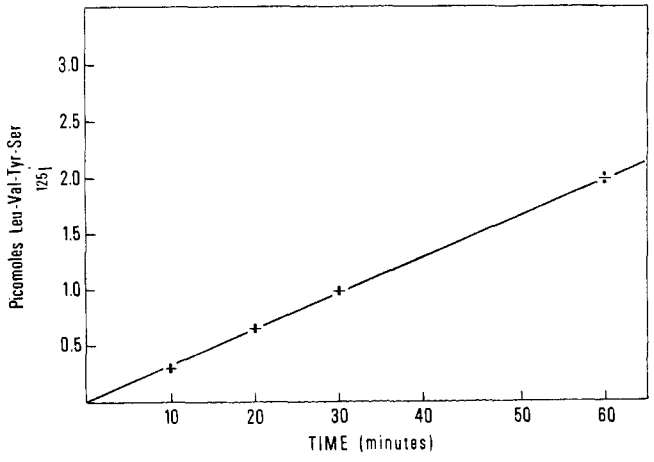

FIGURE 10: Time course of the reaction of renin, 1×10^{-4} unit/ml, with [^{125}I]PRS at pH 5.5.

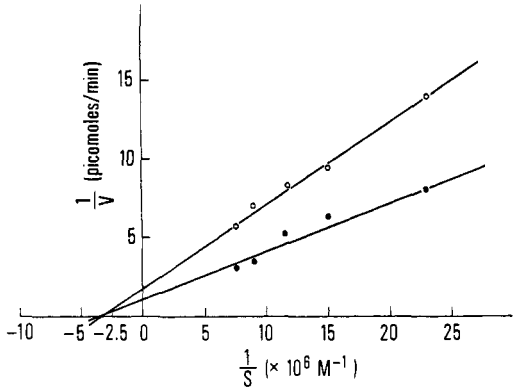

FIGURE 11: Lineweaver–Burk plots for the cleavage of [^{125}I]PRS by renin at pH 5.5. (○) 2.5 × 10^{-5} unit/ml; (●) 5.0 × 10^{-5} unit/ml. Average K_m, 0.23 × 10^{-6} M. Average V_{max}, 2.1 × 10^{-6} mole/min per unit of renin.

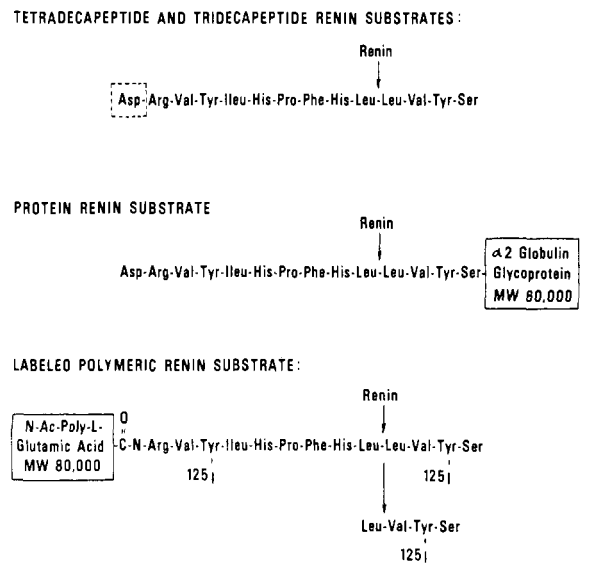

FIGURE 12: Relationships of amino acid sequences in tetradecapeptide, tridecapeptide, protein, and ^{125}I polymeric substrates for renin.

is separated from the labeled substrate by paper chromatography. Both of these recently described techniques are relatively insensitive and require rather elaborate or tedious means for separation and quantitation of the products of the reaction.

The present approach to the problem of development of a sensitive and simple assay was based on our expectation that the tetradecapeptide or one of its synthetic congeners would, when coupled to a high molecular weight polymer and properly labeled, prove to be a nearly ideal substrate for renin. It was clear that cleavage of such a polymeric substrate would produce a small product with physicochemical properties very different from the original substrate. This difference could then be expected to allow a simple separation of product from substrate. The use of a labeled substrate would provide the required sensitivity.

We chose to couple the tridecapeptide Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser to *N*-acetyl-poly(L-glutamic acid) (N-Ac-PGA). In this polymeric substrate the N-terminal amino group of the tridecapeptide is linked to the carboxyl groups of the poly(amino acid). The sequence in this molecule is different from that of the natural protein substrate so that cleavage by renin at the Leu–Leu bond yields a tetrapeptide rather than the decapeptide which is formed when the natural protein substrate is attacked (Figure 12). The impact of this deviation from the normal sequence in the protein on the specificity of the molecule as a substrate for renin is not clear, and we would have prefered a sequence in which that of the natural protein substrate was more closely simulated. However, for a number of reasons our present approach was much simpler. We had to perform only the synthesis of the tetrapeptide, Leu-Val-Tyr-Ser, its labeled iodotetrapeptide congener, and the unlabeled iodotetrapeptide as carrier. Coupling of the tridecapeptide by its C terminus to the amino groups of a large poly(amino acid) would have necessitated the synthesis of a nonapeptide and its labeled congener. Although the tridecapeptide has not itself been previously studied as a renin substrate, published data for other closely related peptides (Skeggs *et al.*, 1968) made it quite likely that this peptide would also be a good substrate for the enzyme. We considered an analogous coupling to N-Ac-PGA of the other available renin substrate, the tetradecapeptide, but at the time our work began the relative availability of the two substrates and the higher purity of the tridecapeptide led us to its use. Had the tetradecapeptide been used, it is quite likely that it would have yielded a very similar polymeric substrate, since after coupling to N-Ac-PGA the tridecapeptide substrate becomes at its N-terminal end Glu-Glu-Arg, a sequence which is not very different from the Glu-Asp-Arg which results from the coupling of tetradecapeptide. Alternative coupling schemes involving tetradecapeptide were considered but were rejected for a variety of reasons. Directed coupling of the C-terminal carboxyl of serine is impossible with the unprotected tetradecapeptide since, unlike the tridecapeptide, it contains another carboxyl group, the β-carboxyl of aspartic acid, at its N terminus. Thus a mixed substrate would result which on cleavage would yield both tetrapeptide and decapeptide. Obviously, for the simplest syntheses of polymeric substrates more closely simulating the natural protein substrate, the tridecapeptide is the substrate of choice.

The preparation of the nonlabeled polymeric renin substrate (NL-PRS) offered no problems in terms of the coupling reaction. This proceeded in reasonable yield (30–65%) in aqueous solution with water soluble carbodiimide. The characterization of the resulting polymeric substrate, however, presented a special problem since analytical methods for the characterization of such polymers are limited in number and resolving power. However, we found that tlc resolved the NL-PRS into two major Pauly-reactive components, both of which migrated well and were clearly distinct from uncoupled poly(L-glutamic acid).

The next point in the strategy of the synthesis of our labeled substrate concerns the method of labeling. In theory, synthesis of labeled tridecapeptide renin substrate in which one or more of the amino acids contains ^{14}C or ^{3}H would be preferable to labeling the peptide moiety after the coupling of the peptide to the polymer. Modification by labeling of functional groups on the tridecapeptide substrate might be expected to result in decreased substrate specificity, or, worse yet, to inhibit the cleavage reaction. The action of renin on peptide substrates has been shown, for example, to require a tyrosine in position 13 of the tetradecapeptide sequence (Skeggs *et al.*, 1968). Nonetheless, we chose for technical ease to label the tridecapeptide substrate after coupling rather

than to attempt a total synthesis of labeled tridecapeptide substrate. Iodination with ^{125}I was a logical first choice which proved successful.

The nonlabeled polymeric renin substrate was iodinated to yield the [^{125}I]polymeric renin substrate ([^{125}I]PRS). Tlc revealed material, localized by radioautography, which was very similar to the original NL-PRS. Two major radioactive components were seen corresponding exactly to the two major Pauly-reactive components (Figure 4). Both major radioactive components yielded [^{125}I]tetrapeptide on cleavage with renin. The possibility that the two components differed as the result of varying degrees of modification of phenolic hydroxyls of tyrosine by carbodiimide was not excluded (Carraway and Koshland, 1968). However, it was demonstrated that neither component represented the product of end-to-end TRS condensation or material uncoupled to N-Ac-PGA. Virtually all of the labeled coupled polymer eluted with the void volume from Sephadex G-100, so it is unlikely that either component represents TRS coupled to a low molecular weight fraction of N-Ac-PGA. Clearly, the iodination itself was not responsible for the formation of two distinct labeled materials, since the NL-PRS also had two Pauly-reactive components which appear to have taken the label equally. At present we have no explanation for these findings, but since both labeled components appeared to be satisfactory substrates for routine assays, the mixture was used without fractionation.

The synthesis of the stable iodotetrapeptide, Leu-Val-Tyr-(I)-Ser, deserves some comment. We are unaware of solid phase or other syntheses in which 3-iodo-L-tyrosine has been incorporated into peptide form. Although the crude solid-phase product was more heterogeneous than the product from the synthesis of the noniodinated tetrapeptide, purification and isolation of the pure iodotetrapeptide were simply accomplished. The use of the stable iodotetrapeptide as carrier proved to be not absolutely essential for the work reported here, but did facilitate tlc of the tracer quantities of iodine-labeled tetrapeptide by prevention of deiodination during tlc.

To our knowledge, the deliberate manipulation of solubility by the coupling of substrate to polymer has not been used in other labeled substrate assays for enzymes which perform cleavage reactions. In our case, the difference in solubility of the labeled polymeric substrate and the iodotetrapeptide reaction product obviates the need for cumbersome means of separating product from labeled substrate. Obviously, the ease with which this is accomplished determines in large measure the usefulness of any labeled substrate assay. Moreover, with our material the effect of coupling of peptide to polymer on the K_m of the reaction is not unfavorable. If anything, the K_m (0.2×10^{-6} M) appears to be lower with the polymeric substrate than with either the tetradecapeptide (4×10^{-6} M) or the protein (0.7×10^{-6} to 1.9×10^{-6} M) (Skeggs *et al.*, 1967, 1968; Gould and Green, 1971). The pH optimum of the reaction at pH 5.5 is similar to that of human renin on human substrate which occurs at pH 5.5–6.0 (Cohen *et al.*, 1971); Skeggs *et al.*, 1969). Since pseudorenin exerts considerable activity on tetradecapeptide substrate at low pH, we suspect that some of the cleavage of the polymeric substrate by our human renin preparation can be attributed, at least at low pH, to contamination of the enzyme preparation with pseudorenin. However, we have also tested pseudorenin-free human renin (Waldhäusl *et al.*, 1970) against our substrate and find that both the pH optimum and K_m are very similar to what we have reported here with the cruder enzyme.

The action of pseudorenin, hog renin, and purified human renin on our substrate will be described elsewhere, as will the cleavage of our substrate by human plasma. While it seems probable that this polymeric substrate assay may not be useful in its present form for the determination of plasma renin, several interesting observations have already emerged. Plasma contains a potent inhibitor of both renin and pseudorenin. Moreover, the pseudorenin activity of plasma against our substrate appears to be significantly correlated with that of the renin content as determined by another technique (Gregerman and Kowatch, 1971). This result is in contrast to the observation of Skeggs, Lentz, and their coworkers (1969) who found no relationship between plasma pseudorenin and renin, although those investigators used synthetic tetradecapeptide as substrate. The relationship between plasma renin and pseudorenin and its possible physiologic significance are presently under investigation.

The synthesis of other labeled substrates is in progress with the hope that one will provide sufficient specificity to allow the simplicity of this approach to be used for direct measurements of renin in plasma. However, the ease and rapidity of the present assay have already made it extremely useful in our current studies of renin inhibitors. For this purpose the polymeric substrate assay seems clearly superior to other available methods, including immunoassays, which depend on the determination of angiotensin I formed from natural protein substrate. The labeled polymeric substrate assay should also prove useful in the purification of renin and pseudorenin.

Acknowledgment

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Activation of the Coagulation Factor VII by Tissue Thromboplastin and Calcium†

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ABSTRACT: Factor VII has been isolated in an activated state after incubation with tissue thromboplastin and calcium and subsequent destruction of all tissue thromboplastin activity by phospholipase C (EC 3.1.4.3). This activated state is dependent upon the presence of phospholipid bound to factor VII, since the activation is reversible by prolonged phospholipase C treatment. Binding of factor VII to tissue thromboplastin membranes is thus not necessary for the activation of factor X by factor VII provided factor VII previously has been exposed to tissue thromboplastin.

In the presence of calcium ions, tissue thromboplastin and factor VII form a complex which activates factor X. Whether thromboplastin activates factor VII to an enzyme, VII_a,[1] which alone can activate factor X, or the complex is necessary for the activation, is not definitely known. Pitlick *et al.* (1971) recently described a peptidase activity in purified tissue thromboplastin preparations. If the activation of factor VII in the extrinsic blood coagulation system were due to a limited proteolysis by this peptidase, the product of this activation, VII_a, might possibly be irreversibly activated and able to act alone as the extrinsic factor X activator.

We report experiments to decide between these alternative mechanisms for the first reaction of the extrinsic coagulation pathway. After interaction between thromboplastin, factor VII, and calcium ions, we tested the product for its ability to activate factor X following destruction of thromboplastin by phospholipase C. We also have separated factor VII_a from thromboplastin.

Materials and Methods

Cephalin from human brain was prepared and stored as described by Hjort *et al.* (1955). The stock was diluted 150-fold in barbital buffer (sodium diethyl barbiturate (11.75 g), sodium chloride (14.67 g), 0.1 N HCl to pH 7.3 (about 430 ml), and distilled water to 2000 ml) (Owren 1947) prior to use.

Tissue thromboplastin was a saline extract of human brain (Hjort, 1957) further purified as described by Hvatum and Prydz (1966, 1969).

Factor VII and factor X were purified from serum (Gladhaug and Prydz, 1970). The preparations were homogeneous in immunoelectrophoresis (Prydz and Gladhaug, 1971), disc electrophoresis (Gladhaug and Prydz, 1970), and analytical ultracentrifugation (to be published) and did not clot fibrinogen (Prydz, 1965; Gladhaug and Prydz, 1970). An antiserum against pure factor X was produced and treated with barium sulfate (Prydz and Gladhaug, 1971).

Coagulation Tests. One stage assays for prothrombin and factors VII, IX, IX_a and X were carried out as described by Østerud and Rapaport (1970), and tissue thromboplastin was measured as described by Hvatum and Prydz (1966) using both normal and factor VII deficient plasma as substrates.

The bentonite-adsorbed plasma used in the factor X assay contained 21% prothrombin. The final system had a buffer time about 120 sec. The test sample was always preincubated separately to avoid the effect of phospholipase C upon the cephalin of the test system.

The extrinsic factor X activator activity (VII_a) was measured in plastic clotting tubes with factor VIII deficient plasma as substrate, using a cephalin reagent without kaolin powder. Substrate (0.1 ml) and cephalin (0.1 ml) were incubated for 3 min at 37°, the test substance (0.1 ml) and calcium (0.1 ml of prewarmed 40 mM $CaCl_2$) were added and the clotting time was noted.

Barium sulfate eluates were prepared according to Hjort (1957), from plasma of patients congenitally deficient in factor VII, factor IX, factor X or factor XI, respectively. The anticoagulants used (one volume per nine volumes of plasma) were a citrate buffer (for factor VII, VIII, IX, and X deficient plasma samples) (Rapaport *et al.*, 1965) and a 2% solution of Na_2EDTA adjusted to pH 7.3 with 2 M NaOH (for factor XI and some samples of factor X deficiency plasma). One volume of the same EDTA solution was added to nine volumes of citrated plasma samples before $BaSO_4$ treatment. The plasma was made factor X deficient before or after the $BaSO_4$

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§ Research Fellow of the Norwegian Council on Cardiovascular Diseases.

[1] The subscript "a" is usually applied to the activated form of various coagulation factors (X_a, IX_a, etc.) which are assumed to be derived from the native form by a change in the primary, secondary or tertiary structure of the molecules. Since the activated form of factor VII described here apparently is a complex of factor VII and phospholipid, we suggest that it should be called VII_{pl}.