THE PURIFICATION AND PARTIAL CHARACTERIZATION OF SEVERAL FORMS OF HOG RENIN SUBSTRATE

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The elevation of blood pressure in human essential hypertension is believed by many to be caused by the renin-angiotensin system (1, 2). This pressor system is activated when renin is liberated from the kidneys and acts upon its substrate contained in the plasma to produce angiotensin I, a decapeptide with the structure asp-arg-val-tyr-ileu-his-pro-phe-his-leu (3, 4). This compound is further degraded by the action of a chloride-activated converting enzyme (5) contained in the plasma which has the function of removing the dipeptide his-leu from the C-terminal of angiotensin I (6, 3). The resulting octapeptide, angiotensin II, is the powerful vasoconstrictor substance (5) responsible for the elevation of blood pressure in essential hypertension (7, 8).

Renin substrate is a protein thought to be contained in the α_2 globulin fraction of the plasma (9), and which has been obtained in only moderately purified form by means of partial acid denaturation and ammonium sulfate fractionation (10). It has been degraded by means of the enzyme trypsin to yield a highly active polypeptide fragment (11). This compound was isolated in pure form, and its structure was determined and confirmed by synthesis (12). It was thus conclusively determined that renin hydrolyzes this tetradecapeptide between two leucine residues, producing the decapeptide angiotensin I and the tetrapeptide leu-val-tyr-ser.

Although the tetradecapeptide in aqueous solution and the protein renin substrate in hog plasma are attacked at the same rate by hog renin, when the peptide is added to serum its rate of hydrolysis is severely reduced. This would indicate that the protein portion of the natural renin substrate molecule contains a function which greatly accelerates its reaction with renin in serum, and yet is not directly related to the hydrolytic process. In order to improve our understanding of this problem, an attempt was made to purify the protein substrate from hog plasma. This effort has been successful, and has resulted in the discovery of not one but several different forms of hog renin substrate. The purification and partial characterization of some of these forms is described in this communication.

EXPERIMENTAL

Method of Assay.—Renin for assay purposes was prepared by the partial acid denaturation method of Haas (13), and further purified by ammonium sulfate fractionation and dialysis. The product was diluted in 0.05 M sodium phosphate, 0.1 M sodium chloride solution to a final concentration of 2.0 Goldblatt units (1) per ml. The pH of the final solution was adjusted to 7.5.

Unknown solutions to be assayed were diluted in saline to a concentration estimated to be between 0.1 and 0.3 units per ml. 1 ml aliquots of such solutions were then mixed in siliconized tubes with 1.0 ml portions of renin. The tubes were incubated for 15 minutes at 37°C. At the end of this time, 1 ml of a buffer solution was added which contained sufficient sodium dihydrogen phosphate and hydrochloric acid to bring the final solution to a pH value of about 5.5 ± 0.3. The tubes were then heated on a boiling water bath for 10 minutes, cooled, and centrifuged. The supernatant was assayed in the rat (14), using a stock laboratory solution of angiotensin II, standardized in terms of Goldblatt units.

Methods for the Determination of Protein Concentration.—Analyses for protein were usually performed by means of automatic equipment.[1] The preferred method (Folin) used the phenol reagent of Folin-Ciocaulteu, and employed copper as suggested by Lowry, *et al.* (15). When samples contained either ammonium sulfate in a concentration greater than 0.04 M or polyethylene glycol, an alternate method (Pauly) was used which employed the Pauly diazo reaction for histidine. In either case, the methods employed standards prepared by dilution of a standard bovine serum of known concentration.

In certain cases (D_{280}) when only single determinations were needed or when the amount of samples was extremely limited, the optical density at 280 mμ was used.[2] A factor of 90, derived from results obtained with diluted standard bovine serum, was used to convert optical density readings to mg of protein per 100 ml.

The results obtained by the three different methods usually differed markedly, particularly in the latter stages of the purification. For this reason, at any one point in the process, the same method was used consistently and empirically.

Preparation of Crude Renin Substrate.—Hog blood, in batches of about 50 gallons, was collected with stirring in 0.1 volume of 4 per cent sodium citrate solution containing 0.5 gm of phenylmercuric acetate per liter. The mixture was centrifuged within a few hours of collection by means of a steam-driven, continuous feed centrifuge[3] equipped with a bowl for liquid-liquid separations. The plasma was usually clear and not hemolyzed.

The plasma, with a volume of about 75 liters, was chilled as soon as possible to refrigerator temperature. All fractionation procedures were performed at these temperatures unless otherwise indicated.

The plasma was adjusted to pH 6.0 by the addition of approximately 10 ml of 2.5 N sulfuric acid per liter. Exactly 0.6 volumes of 4 M ammonium sulfate solution was added with constant stirring over a period of several hours. The precipitate (1.5 M ammonium sulfate) was removed by gravity filtration

[1] Auto analyzer, Technicon Instruments Corporation, Chauncey, New York.

[2] Automatic recording spectrophotometer model 505, Bausch & Lomb, Inc., Rochester, New York.

[3] The Sharples Corporation, Philadelphia, super centrifuge type T 41-24 18DY.

and discarded. The molarity of ammonium sulfate in the filtrate was then increased to 2.3 M by a second slow addition of 0.47 volumes of ammonium sulfate. The precipitate was gathered on a large vacuum filter where it was finally squeezed to a hard, dry cake with the aid of a rubber dam.

The protein cake was dissolved in water, and after determination of its protein concentration (Folin), was diluted to yield a 5 per cent solution. This solution was once again fractionated at pH 6.0 between the limits of 1.5 and 2.3 M ammonium sulfate in a manner exactly similar to that just described. The final product, after the last filtration, was collected as a hard, dry cake and stored in the freezer (−20°C) until processed further.

Approximately 450 gm of protein (Folin) were obtained from each 50 gallon batch of hog blood with a purity averaging 50 u/gm. The total yield was about 22,500 units per batch.

Partial Acid Denaturation and Ammonium Sulfate Fractionation.—The crude ammonium sulfate filter cake was dissolved in water and clarified by filtration. The protein content of the resulting clear, blue-green solution was determined (Folin), and enough water was added to lower the concentration to 5 per cent. Eight liters of the solution were taken for the following process, the balance being returned to the freezer for processing at another time.

The ammonium sulfate concentration was estimated by means of the conductivity of suitably diluted aliquots. A sufficient quantity of solid ammonium sulfate was then added to raise the concentration to 0.5 M. The temperature was adjusted to 25°C, and 680 ml of exactly 1.00 N hydrochloric acid was added with constant stirring over a period of 5 minutes. The solution, with a pH of about 2.5, was allowed to stand on the bench for a 1 hour period. About 500 ml of 1 N sodium hydroxide was then added to bring the pH to 4.0, together with sufficient solid ammonium sulfate to give a 1.2 M concentration.

After thorough stirring, the batch was centrifuged for 2 hours in a large refrigerated centrifuge.[4] The supernatant solution was set aside and the gummy, precipitated proteins were suspended by means of a Waring blendor in 7 liters of 1.2 M ammonium sulfate having a pH of 4.0. The mixture was centrifuged as before. The precipitate was discarded, and the two supernatant solutions combined and adjusted to pH 5.0 with 1 N sodium hydroxide. Ammonium sulfate was added to a molarity of 2.5. After thorough stirring, the mixture was centrifuged for 1 hour. The supernatant was discarded and the precipitate dissolved in a minimum volume of water. If not used immediately, the solution was stored in the frozen state.

A large number of batches, prepared by the foregoing scheme, yielded an average of 41 gm of protein (Folin) with a specific activity of 430 units per

[4] International serum centrifuge model No. 13L, International Equipment Company, Boston.

gram. The substrate was purified more than eight-fold with a recovery of about 85 per cent.

Green and Bumpus, (10) were the first to use partial acid denaturation followed by ammonium sulfate fractionation as a means of purifying renin substrate. The acid denaturation procedure used in the present work is taken in almost exact detail from their work. The ammonium sulfate fractionation scheme used by these authors has been greatly modified and made considerably more practical through the use of large scale centrifugation rather than filtration.

Method for Batch DEAE Cellulose Fractionation.—Several batches of substrate, purified by acid denaturation and ammonium sulfate fractionation, were combined to yield a single pool containing 250 gm of protein and about 100,000 units. This large pool was dialyzed in a continuous apparatus—first against cold tap water and later against cold distilled water. The dialysis was continued until the conductivity approached a limiting value.

At the completion of the dialysis, the solution was milky white with precipitate, and usually had a pH value of about 5.8 ± 0.1. It was centrifuged in a large, refrigerated centrifuge for 30 minutes. The supernatant was set aside and the gummy precipitate was dispersed in 4 liters of cold distilled water with the aid of a Waring blendor. The material was centrifuged, and the resulting supernatant added to that first obtained. The precipitate was washed once more in similar fashion and then finally discarded. The three supernatants were combined and diluted to a volume of 60 liters.

8¾ kg of DEAE cellulose[5] was added, and the mixture stirred for 30 minutes. The mixture was then filtered through a muslin filter cloth on a large vacuum funnel. The pad, while still on the funnel, was squeezed as dry as possible with the aid of a rubber dam. The filtrate contained less than 25 mg of inactive protein (Folin) per 100 ml, and was discarded.

The DEAE cellulose pad was suspended in 60 liters of cold distilled water. The pH of the mixture was very carefully adjusted to 5.50-5.60, using approximately 750 ml of 2.5 N acetic acid. During this adjustment, the pH was measured on a small filtered aliquot, which was warmed to room temperature and to which 0.1 volume of 1 M sodium chloride had been added. As soon as the pH adjustment was accomplished, the mixture was filtered by the same method previously used. The filtrate, which was discarded, was nearly inactive, and contained between 125 and 200 mg of protein (Folin) per 100 ml.

The DEAE cellulose pad was suspended once more in 60 liters of cold distilled water. The pH was adjusted to 7.1 ± 0.2 with about 600 ml of 1.0 N sodium hydroxide. In this instance, the pH measurement was performed directly on the cold slurry. The batch was filtered as before, and the inactive and nearly protein-free filtrate discarded.

[5] The adsorbent, in the free base form, was stored under refrigeration as a moist pad.

The active material was eluted from the DEAE cellulose by two separate extractions with 60 liters of cold pH 7.0, 0.04 M phosphate buffer. In each instance, the suspension was stirred for a 1 hour period and then filtered by vacuum. The final DEAE cellulose pad was saved for regeneration and reuse.

The combined eluates were adjusted to pH 5.0-5.2 with 2.5 N acetic acid. The active substance was precipitated from solution by the addition of 400 gm of ammonium sulfate per liter. 3 gm of celite were then added for each gram of protein contained in the batch and the mixture filtered on Whatman No. 52 paper on a large vacuum funnel. The protein in the filter cake thus obtained was dissolved in water, and the celite was removed by filtration, washed, and discarded.

The protein solution, with a volume of about 1500 ml, was placed in cellophane bags and dialyzed against frequent changes of cold distilled water for 3 days. At the end of this time, the solution was removed from the bags and clarified by centrifugation. If necessary, the solution was stored in the frozen state.

The final product of this procedure usually contained 55,000 units with a specific activity of about 2100 units per gram of protein (Folin). The purity was increased about fivefold by this step with an approximate 55 per cent recovery.

Much of the purification accomplished by this procedure was due to a failure of inert protein to elute in the final phosphate buffer washes of the DEAE cellulose. Equally important was the partial elution of impurities performed at pH 5.60. This latter pH value was very critical, and must be redetermined for each lot of DEAE cellulose.

DEAE Cellulose Column Chromatography.—After preliminary purification by means of DEAE cellulose used in batchwise fashion, the substrate was subjected to chromatography in a column of the same adsorbent. An amount of substrate preparation containing 20 gm of protein and about 40,000 units was diluted to 2.0 liters and adjusted to pH 5.50 with 1 N acetic acid. This load was allowed to flow onto the top of a DEAE cellulose column 15 cm in diameter and 24 cm high which had been previously equilibrated with 0.01 M, pH 5.50 sodium acetate buffer. Effluent was pumped[1] from the bottom of the column at the constant rate of 30 ml per minute, and was delivered to a fraction collector capable of measuring aliquots of 900 ml volume at 30 minute intervals. As soon as the sample had been applied, the column head was connected to a supply reservoir containing 38 liters of the 0.01 M, pH 5.50 acetate buffer. Buffer was allowed to flow to the column from this reservoir at the pump speed of 30 ml per minute until a total of 8 liters had been used. At this time, a second pump was started which delivered 0.01 N acetic acid to the supply reservoir at the rate of 15 ml per minute. The slow addition of this acid, accompanied by stirring, produced a gradual decrease in the pH of the buffer in the reservoir. After 50 fractions had been collected, the rate of the acid stream was increased

to 25 ml per minute. Usually about 75 900 ml fractions were collected by which time the pH of the column effluent was about 4.2.

At the conclusion of the chromatogram, the column packing was regenerated by washing *in situ* with 20 liters of 0.1 N acetic acid containing 0.1 M sodium chloride, followed by 20 liters of 0.1 M, pH 5.50 sodium acetate buffer. After a final wash with 20 liters of 0.01 M, pH 5.50 sodium acetate, the column was ready for reuse.

The effluent fractions were analyzed for their substrate and protein concentration (Folin). The pH values were also determined on small aliquots at room temperature, to which 0.1 volume of 1 M sodium chloride had been added. Five different pools, each representing a different form of substrate, were then formed by combination of the appropriate fractions. To each pool was added 50 gm of the free base form of DEAE cellulose for each gram of protein (Folin) which they contained. The pH of the solutions were adjusted to 7.0-7.5 if the addition of the adsorbent itself was not adequate to effect this change. After 1 hour of stirring, the adsorbent was collected from the solutions and the protein-free filtrates discarded. The active materials were eluted from the DEAE cellulose pads with two portions of 0.1 M, pH 7.0 sodium phosphate buffer. These solutions were stored in the frozen state until needed.

The chromatographic process separates hog renin substrate into the three major and two minor forms shown in Fig. 1. These have been identified by capital letters in the order of their elution from the column. The substrates may be more precisely identified in terms of the pH value of the column effluent at the time of their elution. Thus repeated chromatograms of substrate A show that this form is always eluted when the pH value is 5.5. The elution pH value of substrate B is 5.0; that of C is 4.8; while D and E are 4.4 and 4.3 respectively.

The approximate quantitative distribution of the different forms of substrate shown in Fig. 1 was always obtained in chromatograms performed on preparations from the batchwise DEAE cellulose process. The quantitative distribution was the same when batches were chromatographed which had been processed without acid denaturation, although they were treated identically in all other respects. The possibility that the different forms were artifacts caused by the acid treatment was thereby eliminated.

Attempts to chromatograph substrate directly from serum in order to determine the naturally occurring quantitative distribution were unsuccessful due to the very low specific activity.

The average over-all recovery of A, B, and C substrates from thirteen chromatograms was 45 per cent. Contributing to this low figure is loss of activity on the adsorbent itself, the necessity for accepting only the peak tubes from the three major bands, and finally a loss of 10 to 30 per cent sustained in recovering the substrates from the pooled fractions.

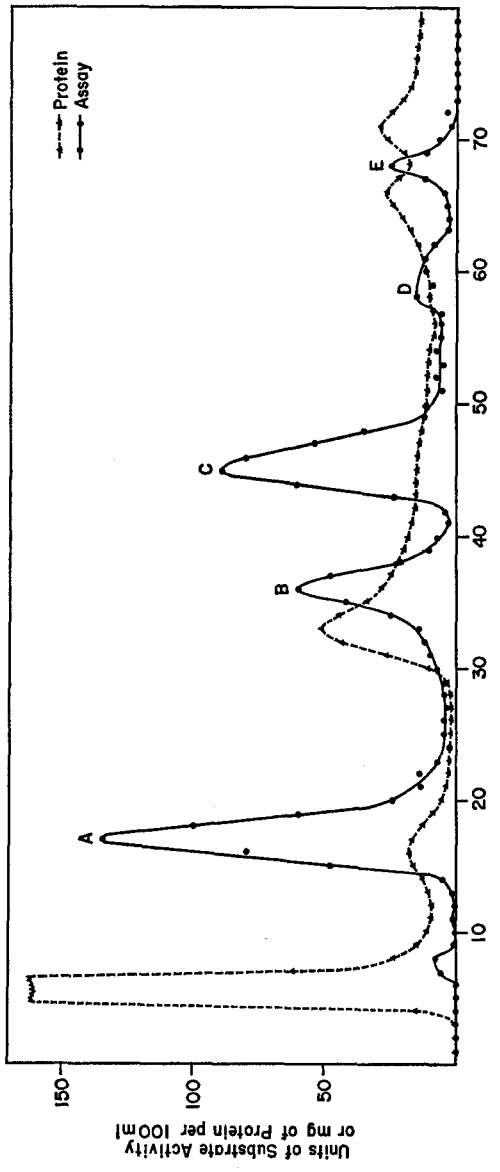

FIG. 1. Separation of several forms of renin substrate by DEAE cellulose chromatography.

Purification by Solvent Extraction.—The preparations of the different forms of substrate derived from the DEAE cellulose column were next purified in a two phase solvent system formed of aqueous solutions of polyethylene glycol and sodium sulfate (16). The procedure was necessarily performed at room temperature due to the low solubility of sodium sulfate decahydrate at reduced temperatures.

Two solvent systems were needed for the procedure and were prepared in advance. Solvent A was composed of 300 ml of a 50 per cent solution of carbowax 1540[6] and 92 gm of anhydrous sodium sulfate dissolved in 540 ml of water. 12 ml of 1 M sodium dihydrogen phosphate was added and, while the preparation was stirred vigorously, the pH was adjusted to 6.5 with 5 N sodium hydroxide. Solvent B was composed of 300 ml of 50 per cent carbowax 1540 and 85 gm of sodium sulfate dissolved in 540 ml of water. Tris (10.9 gm) was dissolved in the mixture, and the pH was adjusted, while the preparation was stirred, to 8.5 with about 10 ml of 4 N sulfuric acid.

A preparation of substrate containing between 2 and 3 gm of protein (Folin) and representing only one of the three major forms of substrate, was diluted to 540 ml with water. 12 ml of 1 M sodium dihydrogen phosphate was added together with sufficient solid sodium sulfate so that the total number of moles of both salts equaled 0.65. The pH was adjusted to 6.5 with sodium hydroxide. 300 ml of 50 per cent carbowax 1540 was added, the mixture stirred for 15 minutes and then centrifuged to facilitate separation of the phases.

The lower salt layer was separated and set aside. The interfacial pad of protein and the upper phase were stirred for 30 minutes with a 200 ml portion of the lower phase of solvent A. The mixture was centrifuged, and the lower phase again set aside. The pad and upper phase were extracted once more in similar fashion with a second 200 ml portion of the lower phase of solvent A. This time the upper phase and protein pad, which contained a significant amount of activity, were stored in the frozen state. The lower layers were combined, adjusted to pH 8.5 with sodium hydroxide and extracted twice with 800 ml of the upper phase of solvent B. The lower phases were discarded. The two upper phases were combined with 400 ml. of the lower phase of solvent B. The pH was adjusted to 5.0 with 1 N acetic acid. After thorough equilibration, the phases were separated and the upper phase extracted twice more in similar fashion at pH 5.0. The upper layer contained less than 5 per cent of the active substance and was discarded. The lower layers were combined, their volume measured, and 165 gm of sodium sulfate added for each liter of solution. After thorough stirring and warming to about 30 to 35°C, the mixture was centrifuged. The upper and lower protein-free phases were removed by aspiration

[6] Polyethylene glycol with an average molecular weight of 1500 obtained from Union Carbide Chemicals Company, Division of Union Carbide Corporation, New York.

and discarded. The interfacial pad of protein was dissolved in 75 ml of water. Solid lithium sulfate monohydrate was added to give a 2.2 M solution. The pH was adjusted to 5.0 and the mixture was centrifuged. Once again the inactive upper and lower phases were discarded. The precipitated proteins contained in the interfacial pad were recovered in a concentrated, salt-free solution by washing with carbowax solutions of decreasing concentrations. The major portion of the sodium sulfate had been removed during the precipitation with lithium sulfate monohydrate. The procedure then could be performed at refrigerator temperatures. The interfacial pad was stirred with 40 ml of a 50 per cent solution of carbowax, the pH of which had been carefully adjusted to 5.2. The mixture was then centrifuged at 18,000 *g* for 30 minutes. The nearly inactive supernatant solution was removed by decantation and discarded. The precipitate was washed twice more in an identical fashion. The supernatant from the third wash was virtually salt-free. The precipitate was washed one time with 40 ml of 25 per cent carbowax, and once with 10 ml of 10 per cent carbowax solution.

At the conclusion of the last washing, the thoroughly drained precipitate was dissolved in a few ml of water. After clarification by centrifugation, the solution was stored in the frozen state until needed for the next step in the purification process.

It was important during the final washing process with carbowax solutions that the pH of the mixture be maintained at about 5.2. Since this is thought to be the approximate average of the isoelectric points of the different forms of substrate, it is not surprising that it is also the point of minimum solubility.

The solubility of a protein in a carbowax solution is a function of ionic strength. The last washing with 10 per cent carbowax in the foregoing scheme was possible since the salts had been previously removed by washing with higher concentrations of carbowax.

The distribution coefficients of the three major forms of substrate were sufficiently similar so that they could all be processed by the same procedure. The per cent of active material recovered by the solvent extraction scheme was disappointingly low, averaging about 33 per cent. A major portion of the loss occurred during the initial extraction of the active substance into the solvent system. Apparently 20 to 25 per cent of the active material remained adsorbed to the interfacial pad of inactive protein. A further loss of about 15 to 20 per cent occurred due to failure of the active substance to extract into the upper phase at pH 8.5. In both instances, the losses to the main fraction were accompanied by very substantial gains in specific activity. The over-all increase in specific activity was three- to four-fold.

Countercurrent Distribution.—The five large scale purification steps which have been described were used to purify a total of 3750 liters of plasma estimated to contain 2,250,000 units with a specific activity of 10 u/gm. (Fig. 2).

As a result of this effort, a total of 50,300 units of substrate A with an average purity of 15,900 u/gm was obtained, as well as 9550 units of substrate B at 16,600 u/gm., and 15,300 units of substrate C at 27,500 u/gm. Unfortunately, it was learned from starch gel electrophoresis as well as other tests, that none of these products was pure. Final purification was, therefore, undertaken by means of countercurrent distribution.

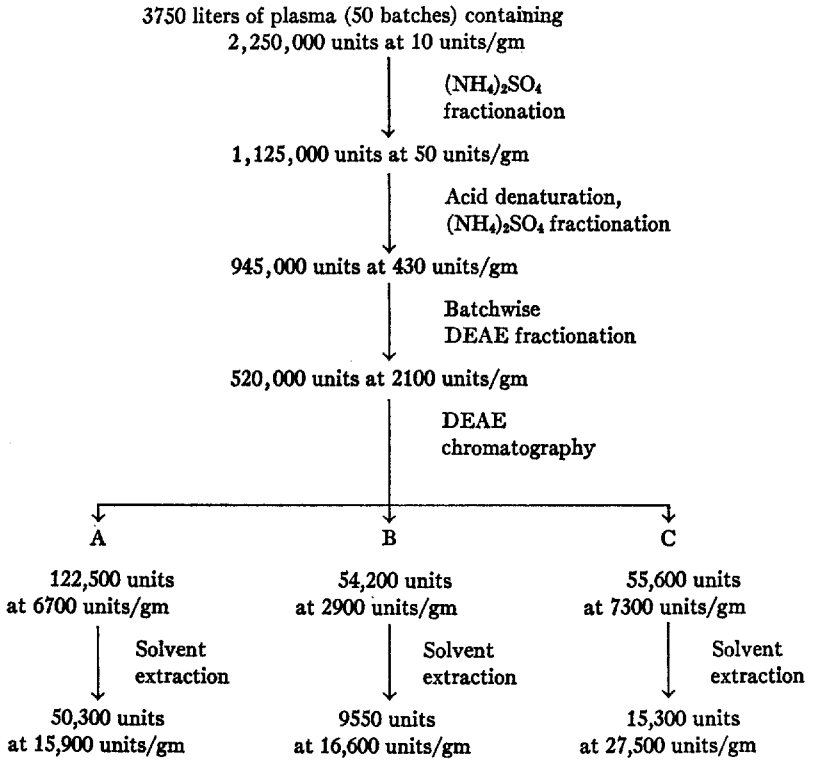

FIG. 2. Large scale steps in the purification of hog renin substrates.

A 200 tube, 10 ml phase, robot-operated Craig-Post apparatus was used for this procedure. The equipment was operated in a refrigerator maintained at 10° ± 0.5°C.

One solvent system was used for all of the distributions. For this purpose, a solution was first prepared by dissolving 1150 gm of lithium sulfate monohydrate in 3930 ml of water. 75 ml of 1 M, pH 6.5 sodium succinate buffer was added and the solution clarified by filtration through Whatman No. 52 paper on a vacuum funnel; and 3275 ml of a 50 per cent solution of carbowax 1540 was then added to the filtrate. The pH of the mixture was precisely adjusted to

6.5 with 1 N sodium hydroxide or sulfuric acid while at room temperature. After pH adjustment had been accomplished, the temperature was lowered to 10°C and, after re-equilibration, the phases were separated.

Several preparations of substrate A, resulting from the solvent extraction procedure, were pooled, giving a total of 985 mg of protein (D_{280}) containing 19,150 units. The volume was adjusted to 100 ml by addition of water and 29.3 gm of lithium sulfate monohydrate, followed by 83.3 ml of 50 per cent carbowax 1540. The pH of the mixture was adjusted to 6.5 and 1.9 ml of 1 M, pH 6.5 sodium succinate buffer was added. After thorough stirring it was apparent that a significant portion of the protein was not in solution in the system. Stirring was therefore continued while small increments of water were added. When a total of 20 ml of water had been added, the sample was nearly all in solution. The mixture was then centrifuged, and the small interfacial pad containing 92 mg of nearly inactive protein was discarded.

The upper and lower phases were loaded into tubes 0 to 9 of the distribution apparatus. A total of 400 transfers were accomplished using the method of single withdrawal. The average time allowed for separation of the phases was 30 minutes.

An analysis of the distribution is illustrated in Fig. 3. One major active band ($K = 0.36$) is shown to be virtually separated from an inactive band of much lower K value. The active band exhibits spreading beyond that which would be theoretically predicted for a single substance. However, assay and protein (Pauly) values are parallel throughout the major portion of the curve.

The material contained in tubes 95 to 135 was removed from the machine in a single pool. 10 gm of solid lithium sulfate monohydrate was added for each 100 ml of volume and, after thorough stirring, the pH was adjusted to 5.2. The mixture was centrifuged, and the inactive upper and lower phases discarded. The small interfacial pad was then washed with carbowax solutions of decreasing concentration as described under solvent extraction. The final aqueous solution, with a volume of 10.5 ml, contained 164 mg of protein (D_{280}) and 7500 units. The specific activity was therefore 45,700, which represents a 2.4-fold purification.

The preparation was then subjected to ultracentrifugation for the purpose of removing the small amount of remaining carbowax, and to permit recovery of the protein in a concentrated form. For this reason, the entire preparation was centrifuged at $175{,}000 \times g$ for 40 hours in a model L Spinco apparatus. The supernatant, which contained only 11.4 mg and very little activity, was removed by aspiration. The very slightly yellow pellet was dissolved in water to give a clear, colorless solution with a total volume of 5 ml.

The solid content of the solution, as determined by drying small samples to a constant weight at 105°C, was 292 mg. A total of 7500 units were again found by assay. The specific activity, as calculated on the basis of solids, was 25,700

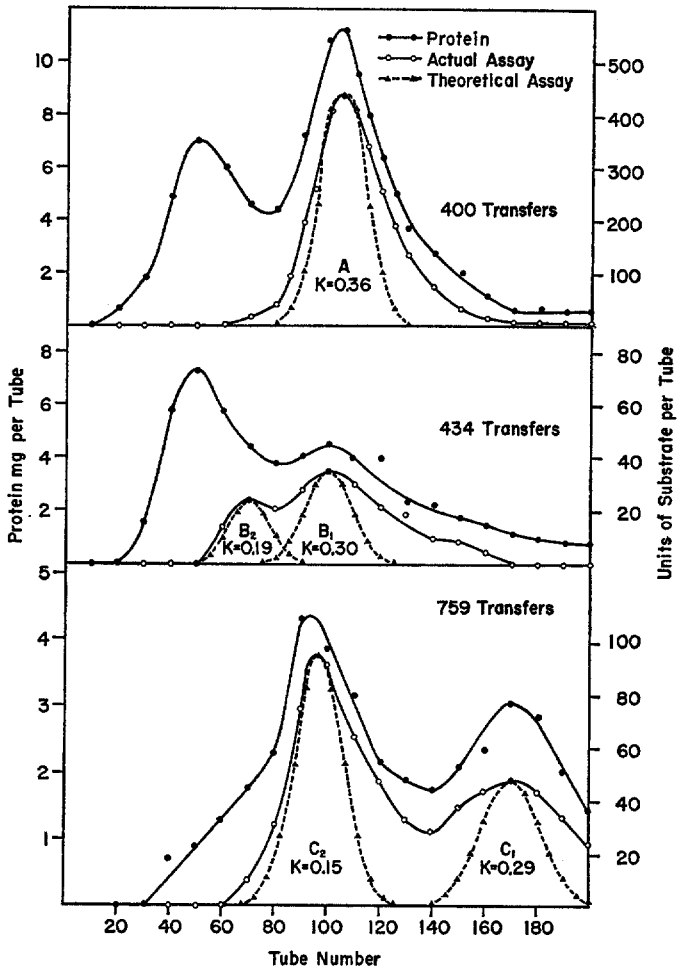

FIG. 3. Countercurrent distributions of renin substrates A, B, and C.

units per gm. (47,700 u/gm by D_{280}). Since the optical density of a solution containing 10 mg of protein per 100 ml as measured at 280 mμ was found to be 0.059 instead of 0.110 as had been assumed, specific activities previously calculated by this means were grossly erroneous, although still useful for comparative purposes.

A second countercurrent distribution of substrate A was also performed. In this instance the sample for distribution, which resulted from the solvent extraction process, contained 1035 mg and had an assay value of 11,400 units, indicating a specific activity of 11,000 u/gm. The material was dissolved in the

solvents and distributed for a total of 216 transfers. Analysis at this time revealed a single active peak nearly separated from a band of slower moving impurities. The solutions in the inactive tubes were removed and replaced with fresh solvents. The apparatus was then recycled for an additional 270 transfers. At this point, analysis revealed a single active band with a K value of 0.34. The plotted curves of assay and protein values were very similar to each other and to the predicted theoretical assay curve.

The peak tubes were pooled, and the material recovered as in the case of the first preparation. A total of 3980 units, or 35 per cent of the starting load, was obtained with a purity of 47,000 units per gm. (D_{280}). The material was purified 4.1 times. An additional 30 per cent of the starting load could be accounted for in various side fractions, but the remaining 35 per cent represented unaccountable loss.

The purified fraction was recovered in pellet form after ultracentrifugation. The pellet was dissolved in water, yielding 112.1 mg of a final preparation containing 2700 units. The specific activity was 24,200 u/gm of solid (46,800 u/gm by D_{280}), which compares favorably with the 25,700 units obtained in the first preparation. The nitrogen content was 14.63 per cent.

A total of 575 mg (D_{280}) of substrate B, which contained 9550 units with a specific activity of 16,600 u/gm. (D_{280}), were available for distribution. The load was prepared in the solvents and placed in tubes 0 to 8 of the distribution apparatus as described for substrate A. The apparatus was operated for 434 transfers using the method of single withdrawal.

Analysis revealed the pattern illustrated in Fig. 3. In addition to a slowly moving inactive band, there was a principal B_1 active band ($K = 0.30$). In addition, the assay curve reveals a second active peak intermediate ($K = 0.19$) between these two bands, B_2. The assay curve suggests the possible presence of still a third, more rapidly moving active band.

The peak tubes representing the B_1 and B_2 fractions were collected and the materials recovered from the solvents. A total of 82.2 mg of the B_1 preparation were obtained with an assay value of 1320 units. The specific activity of the material was 16,100 u/gm (D_{280}). The second fraction, B_2, yielded 70 mg of protein assaying 845 units. The specific activity was 12,000 u/gm (D_{280}). A large amount of inactive protein was removed during countercurrent distribution. However, the specific activity of the products actually decreased. Thus a very serious degree of inactivation occurred for which no explanation is available.

A portion of each of the two preparations was subjected to ultracentrifugation. The pellet resulting from the B_1 fraction yielded 46.3 mg of solid containing 458 units. The specific activity was, therefore, 9900 u/gm. of solid (13,900 u/gm. by D_{280}). Similarly the B_2 fraction yielded 46 mg and 300 units with a specific activity of 6500 u/gm. (7700 u/gm by D_{280}).

The amount of substrate C available for countercurrent distribution totalled 15,300 units contained in 558 mg of protein (D_{280}). The specific activity was 27,500 units per gm. The entire amount was prepared in the solvent system and loaded into tubes 0 to 10 of the distribution apparatus. The equipment was operated for 758 transfers using the method of single withdrawal. A total of 21 days were required for the process.

An analysis of the distribution is presented in Fig. 3. Two major active bands (C_1 and C_2) were obtained with K values of 0.29 and 0.15 respectively. The assay and protein values are approximately parallel. However, the C_2 band is only partly separated from some more slowly moving inactive material. Assay of the effluent fractions showed two small active bands. These were both found, on small scale DEAE cellulose chromatography, to be of the D form of substrate, which is sometimes poorly separated from the C form on large scale chromatography.

The solutions from the appropriate peak tubes only from the C_1 and C_2 fractions were pooled and the proteins recovered as previously described. Thus 82 mg of protein, representing the C_1 fraction, was obtained which contained 1610 units with a specific activity of 19,700 u/gm (D_{280}). In addition, 57 mg of C_2 protein was isolated containing 1490 units with a specific activity of 26,200 u/gm. (D_{280}).

The specific activity of the C_1 preparation was decreased somewhat during the distribution, while C_2 remained about the same. Since 21 days were required for the distribution, the severe loss of total activity is understandable. It must be pointed out, however, that only the peak tubes were used. More important is the clear-cut separation of the C form into two different fractions.

Both the C_1 and C_2 preparations were subjected to ultracentrifugation. The pellets were dissolved in water and yielded, on the basis of their solid content, 69.3 mg of the C_1 form containing 847 units with a specific activity of 12,200 u/gm. (22,400 u/gm by D_{280}), and 60.4 mg of the C_2 form containing 872 units with a specific activity of 14,500 u/gm (28,000 u/gm. by D_{280}). Although no significant loss of specific activity occurred as a result of this procedure, a considerable loss of total activity did occur with the unexplained formation of an inactive and insoluble precipitate.

Neither the protein nor the assay curves in the three countercurrent distributions illustrated in Fig. 3 agree with those which would be theoretically predicted. The actual patterns are broader and are skewed to the left. These departures from the theoretical curves need not be accepted as certain evidence that the preparations are impure. Such behavior can result, at least in part, from a non-linear partition isotherm (7). In this instance, a decrease of distribution coefficient with increased solute concentration is indicated. Since the solubility of protein in the lithium sulfate–carbowax system is low, this explanation seems possible.

In early work on this problem a system formed of sodium sulfate and carbowax was used at room temperature. In this case the curves for substrate activity made much closer approaches to the theoretical curves. The system was also capable of dissolving more protein, and emulsions separated much sooner. However, the rate of inactivation of substrate was intolerably high. For this reason, lithium sulfate was used since it forms systems with carbowax which are usable at reduced temperatures where the rate of inactivation of substrate is much less.

Electrophoresis of the Purified Substrates.—The several substrates were examined by means of paper electrophoresis.[7] Small samples of each preparation were studied alone and after being mixed with fresh hog serum. The electrophoresis was performed in veronal buffer, pH 8.6, having an ionic strength of 0.075. After being stained with bromphenol blue, the strips were evaluated by direct inspection and by photometric scanning. The two type C substrates migrated as α_2-globulins. The type B preparations, displaying a lower mobility, traveled near the trailing edge of the α_2-globulin zone. Type A substrate, showing the lowest mobility, migrated to a position intermediate between the α_2- and β-globulin zones. The relative mobilities of the three main substrate types were the same, whether compared singly or in mixtures with hog serum.

The five different purified protein preparations were also subjected to starch gel electrophoresis. The method of Smithies (18) was used. The samples were applied to small pieces of filter paper which were inserted vertically in the gel. By this method, it was possible to apply 1.71 mg of A, 1.03 mg of B_1, 0.96 mg. of B_2, 0.45 mg of C_1, and 0.58 mg of C_2. Electrophoresis was conducted in sodium borate buffer having a pH of 8.48. A potential gradient of 6 volts per cm was applied for a 22 hour period in order to allow the maximum possible time for separation of possible impurities.

At the conclusion of the run, the gel was removed from its tray, and 37 small vertical indentations were cut in the slab to serve as alignment marks. The gel was then sliced horizontally into two similar sections. The first section was stained immediately with amido black. The second section was cut transversely at the alignment marks into 37 small gel segments. The segments were macerated and extracted with 2 ml of cold saline and finally centrifuged. The supernatant solutions were assayed for their substrate activity. The protein concentrations were determined by the Folin method, using a 1.5 cm flow cuvette and a tenfold electrical range expansion. Under these conditions, the sensitivity of the analytical system is very greatly increased, a solution containing 5 mg of protein per 100 ml, yielding an optical density of 0.7.

The electrophoretic mobility of the five different substrates parallels that

[7] A Spinco model R, series D paper electrophoresis apparatus with Durrum type cell and analytrol scanner was used. The reagents and methods used were those recommended by the manufacturer.

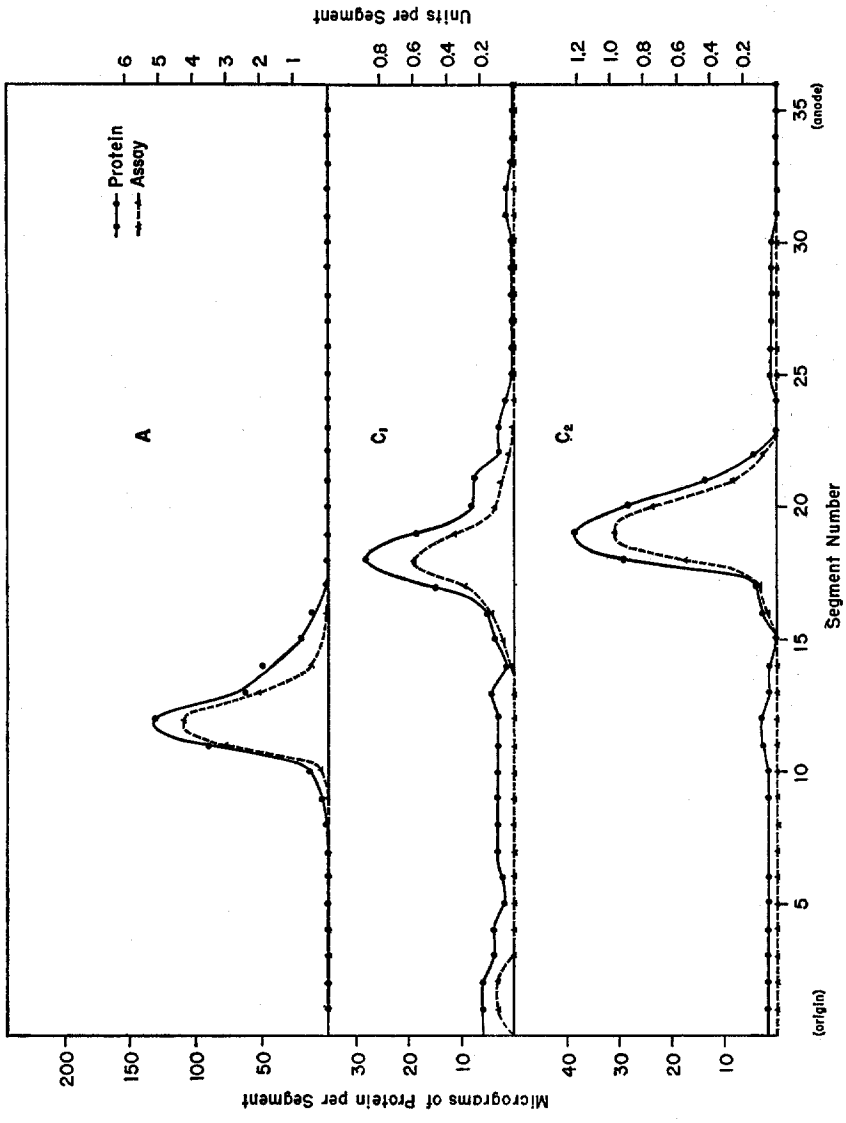

FIG. 4. Electrophoresis of renin substrates A, C_1, and C_2 in starch gel.

observed by paper electrophoresis. As shown in Fig. 4, the two C substrates migrate the farthest, and the type A the shortest distance toward the anode.

In the case of the A substrate, a single protein band was found either by amido black staining or by direct protein determinations. The assay values paralleled the protein concentration throughout the major portion of the curve. A small amount of inactive material appeared on the leading edge.

The two C substrates yielded single bands of protein. Traces of protein impurities appeared along the base line. These traces of impurities were not visible on the sections stained with amido black. In both cases, the assay and protein curves paralleled each other.

In contrast, the two B substrate preparations showed gross evidence of contamination by impurities, and are not illustrated in Fig. 4; nor were they considered suitable for further analysis. Such a result had not been unexpected, since the countercurrent distribution pattern also clearly indicated the relative impurity of these preparations. In addition, further inactivation occurred during ultracentrifugation.

Ultracentrifugation[8].—The method of Archibald, as described by Schachman (19) was used for the direct determination of molecular weights in a Spinco model E ultracentrifuge. The same synthetic boundary cell was employed for both the approach to equilibrium and the synthetic boundary runs for each sample to assure uniform cell optics. All determinations were made at 20° with a schlieren bar angle of 70°. The synthetic boundary runs were all performed at 14,290 RPM. The Kodak metallographic plates were enlarged 18 to 19 times before printing. Areas in synthetic boundary runs were measured with a planimeter, while the areas of the meniscus in the Archibald runs were integrated from measurements made with the aid of a millimeter grid.

All protein solutions were dialyzed 4 times at 6° for 23 to 24 hours against 20 volume portions of 0.1 M sodium phosphate at pH 7.2; the same buffer was used to prepare the synthetic boundaries. Concentrations were based on the absorption at 280 mμ.

The partial specific volume for each type of substrate was calculated from the amino acid composition. The partial specific volume of glucosamine, as determined by the method of Schachman (19), was 0.713.

Each Archibald run was made at two speeds by increasing the speed of the centrifuge during operation from 1.5- to 1.7-fold after the first seven pictures had been taken. When the pattern had been re-established (minimum of 25 minutes), three more frames were exposed. By this procedure, samples contain-

[8] We are greatly indebted to Dr. Helmut Mueller, Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, for instruction and guidance in the use of the Archibald method, and to Dr. Fred Tsuji, Department of Biophysics, Veterans Administration Hospital, Pittsburgh (Oakland), for making the ultracentrifuge in his department available to us.

ing high molecular weight components were expected to show a decrease in meniscus molecular weight with increased centrifugal force at the higher speed. As shown in Table I, the molecular weight of substrate A and substrate C_2 did not change significantly when the speed was increased. The molecular weights of these two substrates were also nearly identical. The meniscus weight for C_1 substrate decreased at 16,200 RPM to the same value as found for A and C_2 at this speed, showing the separation of a heavy component.

Chemical Analysis.—Samples of substrate A containing an estimated 0.17 μmole or 11.7 mg of protein were subjected to the FDNB method as outlined by Fraenkel-Conrat, Harris, and Levy (20). Hydrolysis, solvent extraction and

TABLE I

Molecular Weights by Ultracentrifugal Analysis

Substrate	mg/ml	Partial specific volume $\bar{V}$	First stage			Second stage			Probable molecular weight
			RPM	Frames*	Molecular weight	RPM	Frames*	Molecular weight	
A	10.25	0.741	10,589	6	56,120 ± 1020	16,200	3	58,230 ± 1580	57,180‡
C_1	9.20	0.741	9,341	5	74,510 ± 2470	16,200	3	57,660 ± 1150	57,660
C_2	10.75	0.741	9,341	5	57,040 ± 1080	16,200	3	54,130 ± 2020	55,590‡

* Indicates the number of separate molecular weight determinations used to obtain the averages shown.

‡ Averages of values from first and second stages.

chromatography of the extract of the substituted protein yielded only DNP aspartic acid in approximately a 23 per cent yield.

Treatment of a similar size sample of substrate A by the PTC method, as outlined by the same authors, also yielded only PTH aspartic acid. In this case, the apparent yield of amino acid derivative was 125 per cent of the protein sample.

Still another sample of substrate A of the same size (300 units) was first treated with 3 units of highly purified hog renin. The mixture, with a volume of 3 ml and a pH of 7.4, was incubated for 3 hours at 37°C. At the end of this time, by direct assay, it was found that angiotensin I had been completely released from the substrate. The enzymatic reaction was then stopped by heating to 100°C for 10 minutes. The entire mixture was treated by the FDNB method. Again DNP aspartic acid was obtained. In addition, a second N terminal was found which was probably leucine or isoleucine. Valine could not be completely excluded as a possibility.

Substrates C_1 and C_2 were examined by the PTC method. The sample sizes were 4.07 and 3.48 mg respectively. In both cases, the only N terminal which could be found was aspartic acid.

Hydrolysates of the three substrate preparations were prepared from accurately measured aliquots containing approximately 0.5 mg of protein. The samples were sealed under vacuum in a tube with 2 ml of glass-distilled, constant boiling hydrochloric acid. One set of samples prepared in this fashion was

TABLE II

Composition of Substrate A

Constituent	Residue weight per 100 gm of protein	N as per cent of total N*	Calculated No. of residues per mole‡
	gm		
Aspartic acid	7.39	6.16	36.8
Threonine	6.28	5.97	35.6
Serine	5.47	6.03	36.0
Glutamic acid	10.22	7.60	45.3
Proline	5.08	5.03	30.0
Glycine	3.36	5.65	33.7
Alanine	5.17	6.97	41.6
Cystine	0.57	0.53	1.6
Valine	5.86	5.68	33.9
Methionine	1.55	1.13	6.8
Isoleucine	1.84	1.56	9.3
Leucine	13.28	11.27	67.2
Tyrosine	2.31	1.36	8.1
Phenylalanine	6.06	3.95	23.6
Ammonia	—	6.71	40.0
Lysine	4.07	6.10	18.2
Histidine	2.40	5.03	10.0
Arginine	5.65	13.88	20.7
Glucosamine	1.18	0.71	4.2
Sialic acid	1.07	0.36	2.1
Neutral hexose	2.20	—	7.8
Total	91.01	101.68	

* The total nitrogen found was 14.6 per cent.

‡ Based upon a molecular weight of 57,180 obtained by ultracentrifugal analysis (see Table I).

heated at 105°C for 20 hours; a second set for 40 hours. Extrapolation of the data obtained at these two times allowed corrections to be made for the loss of serine, threonine, and methionine on hydrolysis.

Separate aliquots for the chromatographic determination of glucosamine were hydrolyzed in 2 N hydrochloric acid in sealed evacuated tubes at 105°C. Additional samples for the determination of cystine were oxidized with performic acid and hydrolyzed by the procedure recommended by Moore (21).

In all cases, the acid used for hydrolysis was removed by low temperature

evaporation under reduced pressure. The residues were dissolved in buffer and subjected to automatic amino acid chromatography.[9]

The chromatographic procedure was standardized by the addition of small exact quantities of the unnatural amino acid norleucine to the unknowns

TABLE III

Composition of Substrate C_1

Constituent	Residue weight per 100 gm of protein	N as per cent of total N*	Calculated No. of residues per mole‡
	gm		
Aspartic acid	7.13	5.86	35.8
Threonine	6.35	5.95	36.3
Serine	5.90	6.41	39.1
Glutamic acid	10.33	7.57	46.2
Proline	4.77	4.66	28.4
Glycine	3.36	5.57	34.0
Alanine	5.12	6.81	41.6
Cystine	0.70	0.66	2.0
Valine	5.79	5.53	33.7
Methionine	1.49	1.08	6.6
Isoleucine	1.83	1.53	9.3
Leucine	13.07	10.94	66.7
Tyrosine	2.17	1.26	7.7
Phenylalanine	5.91	3.80	23.2
Ammonia	—	7.16	43.7
Lysine	3.93	5.81	17.7
Histidine	2.34	4.85	9.9
Arginine	5.48	13.28	20.2
Glucosamine	1.40	0.82	5.0
Sialic acid	1.69	0.55	3.3
Neutral hexose	2.45	—	8.7
Total	91.21	100.10	

* The total nitrogen found was 14.8 per cent.

‡ Based upon a molecular weight of 57,660 obtained by ultracentrifugal analysis (See Table I).

before hydrolysis as well as to standard mixtures of amino acids. The final verification of the analysis was accomplished by preparation and chromatography of a mixture of amino acids approximating as closely as possible the preliminary analysis of substrate A.

[9] A Technicon amino acid analyzer was used which employs continuously variable gradient chromatography, and a high pressure column of sulfonated polystyrene resin. Technicon Chromatography Corporation, Chauncey, New York.

The solid content of the final preparations was determined by drying several accurately measured aliquots which contained between 1 and 3 mg of protein to constant weight at 105°C.

The small amount of residual carbowax not removed by the ultracentrifuga-

TABLE IV

Composition of Substrate C_2

Constituent	Residue weight per 100 gm of protein	N as per cent of total N*	Calculated No. of residues per mole‡
	gm		
Aspartic acid	7.41	6.05	35.8
Threonine	6.57	6.11	36.1
Serine	5.62	6.07	35.9
Glutamic acid	10.32	7.51	44.5
Proline	4.88	4.72	28.0
Glycine	3.35	5.52	32.7
Alanine	5.43	7.18	42.5
Cystine	0.57	0.52	1.5
Valine	5.81	5.52	32.6
Methionine	1.56	1.12	6.6
Isoleucine	1.77	1.48	8.7
Leucine	13.51	11.23	66.5
Tyrosine	2.04	1.17	7.0
Phenylalanine	5.62	3.59	21.2
Ammonia	—	7.89	46.8
Lysine	3.90	5.73	17.0
Histidine	2.18	4.48	8.8
Arginine	5.62	13.53	20.0
Glucosamine	2.00	1.17	6.9
Sialic acid	1.82	0.59	3.5
Neutral hexose	3.43	—	11.8
Total	93.45	101.18	

* The total nitrogen found was 14.9 per cent.

‡ Based upon a molecular weight of 55,590 obtained by ultracentrifugal analysis (See Table I).

tion procedure was estimated by use of Dragendorff's reagent as described by Ginn *et al.* (22). Approximately 2 per cent of the solid content of the A substrate preparation, and 1 per cent of the two C preparations was found to be carbowax. These estimates were used to correct the solid content of the solutions.

The nitrogen content of the substrate preparations was determined by use of the microdigestion procedure of Wong. Wong's procedure is described by Peters and Van Slyke, (23). Determination of nitrogen on the digests was

made by use of the alkaline phenol-hypochlorite color reaction as adapted to the autoanalyzer. The entire procedure was verified by means of trial analysis of protein samples of known nitrogen content.

The validity of the method for determination of solid was checked by coagulating small samples of the A substrate with heat. The coagulum was washed with hot water, methyl alcohol, 2:1 chloroform and methyl alcohol, and finally

TABLE V

Comparison of the Chemical Composition of Substrates A, C_1, and C_2

Constituent	Assumed No. of residues per mole		
	A	C_1	C_2
Aspartic acid	37	36	36
Threonine	36	36	36
Serine	36	39	36
Glutamic acid	45	46	45
Proline	30	28	28
Glycine	34	34	33
Alanine	42	42	43
Cystine	2	2	2
Valine	34	34	33
Methionine	7	7	7
Isoleucine	9	9	9
Leucine	67	67	67
Tyrosine	8	8	7
Phenylalanine	24	23	21
Ammonia	40	44	47
Lysine	18	18	17
Histidine	10	10	9
Arginine	21	20	20
Glucosamine	4	5	7
Sialic acid	2	3	3
Neutral hexose	8	9	12

with peroxide-free diethyl ether. The small sample of dried protein thus obtained was then desiccated to constant weight at 105°C in a high vacuum. The dried samples were hydrolyzed *in vacuo* with constant boiling hydrochloric acid for 24 hours at 105°C. Aliquots of the hydrolysates were analyzed for their nitrogen content and leucine equivalents by the ninhydrin method. The results were not significantly different from those obtained on 24 hour hydrolysates prepared from aliquots of the same type A substrate which had not been coagulated, washed, and dried, but had been corrected for their carbowax content.

Small aliquots containing approximately 0.5 mg of the three forms of substrate were made 5 N with sodium hydroxide, sealed in a small capillary and

hydrolyzed for 20 hours at 100°C. The hydrolysates were diluted with water, adjusted to pH 2.5 with 5.7 N hydrochloric acid and subjected to amino acid chromatography. No tryptophan was found by this procedure.

Sialic acid was determined by the thiobarbituric acid method of Warren (24).

Total protein-bound hexose was determined by the orcinol–sulfuric acid method as described by Winzler (25). A standard containing equal amounts of galactose and mannose was used.

The result of the chemical analysis of substrates A, C_1 and C_2 are presented in Tables II through VI. The nitrogen contents of the preparations are closely accounted for in terms of their amino acid, sialic acid, and glucosamine con-

TABLE VI

Comparative Data for Several Forms of Hog Renin Substrate

Forms of substrate	Weight of final product	Specific activity	pH of DEAE elution	Distribution coefficient	N terminal	Molecular weight by ultracentrifugation
	mg	*units per mg*				
A	292*	25.7	5.5	0.36	Asp	57,180
B_1	—	—	5.0	0.30	—	—
B_2	—	—	5.0	0.19	—	—
C_1	69.3	12.2	4.8	0.29	Asp	57,660
C_2	60.4	14.5	4.8	0.15	Asp	55,590

* An additional 112.1 mg with a specific activity of 24.2 units per mg were obtained in a second preparation.

tents. The analysis failed to account for the total weight of the preparations. The sum of the residue weights of the constituents totals 91.01 gm per 100 gm of substrate A; 91.21 gm. in the case of substrate C_1; and 93.45 gm for substrate C_2. These findings were confirmed by a direct comparison of the ninhydrin value of substrate hydrolysates with that of a synthetic mixture of amino acids of substrate A combined in the same proportions. It was found that the substrate hydrolysates had a total ninhydrin value which was about 91 per cent of the synthetic amino acid mixture. Thus it is probable that the chemical analysis does not fully account for the total solids, and that the preparations may contain one or more unknown constituents which do not contain nitrogen.

DISCUSSION

Angiotensin I has been isolated from hog as well as from horse blood. In both cases, only single compounds were obtained which had identical structures (2). For this reason, it seems most likely that all of the different forms of substrate would yield the same angiotensin I. Additional evidence in support of this viewpoint was obtained in experiments in which angiotensin I was prepared from each of the major forms of substrate. After preliminary purification, the

products were subjected to countercurrent distribution. Only single active bands were obtained whose distribution coefficients were similar and which agreed with those previously obtained with angiotensin I (26).

Studies of the rate of action of renin on the different substrates showed that all types are attacked at similar rates. These rates were, in all cases, actually somewhat lower than the action of renin on polypeptide substrate. No explanation has been obtained for the great decrease in rate of action of renin on the latter compound when it is added to serum. It can only be suggested that the function of the protein carrier in the natural substrate is to hold the substrate moiety in a position where it can be attacked easily by renin. In contrast, the polypeptide substrate is perhaps adsorbed to serum proteins, and is less accessible to the action of the enzyme.

The chemical composition of the three preparations is remarkably similar (Table V). The amino acid composition, in particular, appears to be nearly the same, and it has been assumed that the peptide portion of the substrates is probably identical.

There are small but significant differences in the carbohydrate section of the three proteins. For example, there are three residues of sialic acid in the two C substrates, and only two in the A preparation. The presence of this one additional highly acid residue may be sufficient to increase the electrical charge of the C substrates and allow their separation from substrate A by electrophoresis and chromatography. The two B and the D and E substrates may also be separable on the basis of their electrical charge.

The three proteins differ significantly in their content of glucosamine and neutral hexose. It is possible that these differences in carbohydrate content may account for differing solubilities and distribution coefficients and permit separation of the two C and the two B fractions by means of countercurrent distribution.

SUMMARY

Hog renin substrate has been separated into three major (A, B, and C) and two minor forms (D and E) by DEAE cellulose chromatography. Two of the major forms (B and C) have been further fractionated into two additional types (1 and 2) by countercurrent distribution.

The purification of substrates A, C_1, and C_2 has been completed. Analysis shows that all three are glycoproteins with molecular weights of about 57,000, and have similar amino acid compositions. Differences exist in the sialic acid, glucosamine, and neutral hexose content, which may account for different physical properties.

All the forms of the substrates are attacked by renin at similar rates, and appear to yield the same angiotensin I.

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