Isolation and Amino Acid Composition of Human Angiotensin I

By KIKUO ARAKAWA, MISAKO NAKATANI, ATSUSHI MINOHARA,
AND MOTOOMI NAKAMURA

*Research Institute of Angiocardiology, School of Medicine, Kyushu University,
1276 Katakasu, Fukuoka, Japan*

(Received 13 December 1966)

1. Angiotensin, the most powerful pressor substance known, suspected to be a causal substance in renal hypertension and previously isolated from animal sources, has now been isolated from human sources and the amino acid composition was analysed. 2. The procedures followed in the successful isolation of human angiotensin include: (a) preparation of stable materials to obtain maximum formation of human angiotensin; (b) a relatively selective adsorption of the formed angiotensin on Dowex 50W (X2); (c) gel filtration through Sephadex G-25; (d) cation-exchange chromatography on CM-cellulose; (e) anion-exchange gel filtration on DEAE-Sephadex A-25; (f) molecular-sieve chromatography through Bio-Gel P-2. 3. The homogeneity of the human angiotensin isolated was confirmed by paper and thin-layer chromatography and paper electrophoresis. 4. The biological activity observed indicates the substance isolated to be human angiotensin I. 5. The amino acid analysis suggested the following proportional composition: Asp, 1; Pro, 1; Val, 1; Ile, 1; Leu, 1; Tyr, 1; Phe, 1; His, 2; Arg, 1. This composition is similar to that of horse angiotensin I, i.e. isoleucine⁵-angiotensin I.

The renin-angiotensin system is considered to be one possible mechanism of renal hypertension in man (Braun-Menéndez, Fasciolo, Leloir, Muñoz & Taquini, 1946). The reaction system has been clarified by Skeggs and his collaborators (Skeggs, Marsh, Kahn & Shumway, 1954*a,b*, 1955; Skeggs, Kahn & Shumway, 1956*a*; Skeggs, Lentz, Kahn, Shumway & Woods, 1956*b*; Lentz, Skeggs, Woods, Kahn & Shumway, 1956) *in vitro* with animal materials. Renin, which is an enzyme within the kidney, is liberated by some type of stimulus to the kidney and acts on a plasma protein called angiotensinogen. This action first produces a decapeptide angiotensin I, which is then immediately transformed by a plasma enzyme (converting enzyme) into octapeptide angiotensin II, the most powerful pressor substance yet known. These workers isolated and established the structure of horse angiotensin I and II. Almost simultaneously, Peart (1955, 1956) isolated ox angiotensin I and the structure was determined by Elliott & Peart (1956, 1957) soon thereafter.

Horse and ox angiotensins are identical except in position 5, where the isoleucine residue in the horse peptide is replaced by a valine residue. Pig angiotensin is reported to be identical with that of horse origin (Bumpus, Schwarz & Page, 1957).

Chemical synthesis has been accomplished for isoleucine⁵-angiotensin II (Arakawa & Bumpus,

1961) and valine⁵-angiotensin I and II (Schwyzer *et al.* 1958*a,b*). Isoleucine⁵-angiotensin I has not been synthesized.

Angiotensins from other animal species have not been chemically studied. Nor had human angiotensin been isolated. This fact is somewhat paradoxical in that it was disease of the human kidney (Bright, 1827) which provoked many investigators to study renal pressor substance, and led, by the use of animal materials, to the presently known renin-angiotensin system.

The chemical identity of human angiotensin has been the objective of the present work. The purification procedures have been further improved since our preliminary report (Arakawa, Nakatani & Nakamura, 1967).

EXPERIMENTAL

Materials

Preparation of crude human angiotensinogen. Cohn's fraction IV, which is prepared from human plasma, has been found by Arakawa, Nakatani & Nakamura (1965) to function as a satisfactory substrate for renin. Therefore human plasma was separated from human blood and processed by Cohn's fractionation method (Cohn *et al.* 1946). It was then freeze-dried, pooled in polyethylene bottles and stored at 4° until used. It remained active for at least 2 years.

Preparation of crude human renin. Haas's method (Haas,

Scheme 1. Preparation of crude human renin was carried out by a modification of Haas's method (Haas *et al.*, 1954) for pig renin. The preparation was thoroughly dialysed against distilled water to remove chloride ion, to avoid the action of converting enzyme. It was pooled and stored at -17° .

Lamfrom & Goldblatt, 1954) was modified as follows (Scheme 1). Human kidneys were removed from autopsy specimens within 3-24 hr. of an accidental death, and stored at -17° . One batch of ten kidneys was thawed overnight at a maintained temperature of 4° . They were then chopped into small pieces and homogenized with 2l. of distilled water at 4° . The homogenate was then adjusted to pH 2.8 by adding approx. 60 ml. of 4N- H_2SO_4 . Then NaCl was added to make a final concentration of 0.8M. After vigorous stirring for 1 hr. at 4° , the precipitate was separated by continuous centrifugation and pooled in a polyethylene bag at -17° , to be further extracted later. Then $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant until a 2.5M concentration had been obtained, and this mixture was again centrifuged. The precipitate thus obtained was dialysed overnight in Visking tubing against distilled water. The resultant mixture was adjusted to pH 9.8 with approx. 15 ml. of 2.5N-KOH and then kept for 1 hr. at 4° . Then $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 1.3M and the solution was again acidified to pH 3.5 by adding 4N- H_2SO_4 . The supernatant was again separated by centrifugation, and $(\text{NH}_4)_2\text{SO}_4$ added to increase the concentration to 2.3M. Centrifugation again separated the supernatant from the precipitate, which was then completely dialysed against distilled water. Each batch resulted in a specimen of 100-120 ml., which was

stored in a polyethylene bottle at below -17° . Beginning again with the pooled precipitate obtained from the initial centrifugation, which had been stored in a polyethylene bag for several months at -17° , and by following the procedure outlined above, an additional volume of specimen was recovered that contained 50% of the renin activity of the original. These products were stored separately from the originals. The total renin activity obtained from 280 human kidneys was 0.53 Goldblatt unit per kidney when directly assayed on rat blood pressure and calculated from the correlation ratio (Page & Bumpus, 1961).

Synthetic angiotensins. Isoleucine⁵-angiotensin II was synthesized by the method of Arakawa & Bumpus (1961). All other synthetic angiotensins were obtained from Ciba Ltd. (Basle, Switzerland).

Chromatographic materials. Dowex 50W (H^+ form; X2; 100-200 mesh) (1.7 m-equiv./ml. wet volume) was obtained from Dow Chemicals Ltd. (Midland, Mich., U.S.A.); CM-cellulose of 0.55 m-equiv./g. was from Serva (Heidelberg, Germany); 20-80 μ Sephadex G-25 and 40-120 μ DEAE-Sephadex A-25 (3.5 ± 0.5 m-equiv./g.) were from Pharmacia (Uppsala, Sweden); Bio-Gel P-2 (100-200 mesh) was from Bio-Rad Laboratories (Richmond, Calif., U.S.A.); TLC-cellulose powder was from Serva (Heidelberg, Germany), and Toyo-Roshi no. 51 paper was from Toyokagaku (Tokyo, Japan).

Enzymes. Trypsin was obtained from Sigma (St Louis, Mo., U.S.A.); Pronase P was from Kaken (Tokyo, Japan).

Methods

Bioassay. Female Wistar rats, weighing approx. 100-150g., were anaesthetized by intraperitoneal injection of 10 mg. of sodium amobarbital (Isomytal) per 100g. body wt. followed by the subcutaneous injection of 5mg. of the ganglion-blocker pentolinium tartrate (Ansolsen). Samples dissolved in saline were injected into the femoral vein through a polyethylene tube in 0.1-0.3 ml. volumes, and the blood pressure was measured from the carotid artery. The pressor activity of the sample was expressed in $\mu\text{g.}$ of the equivalent pressor activity of synthetic isoleucine⁵-angiotensin II. The amount of oxytocic activity of the sample was determined by measuring the time required for maximum contraction response of the isolated uteri of rats in oestrus (Schwarz, Masson & Page, 1955).

Specific activity. The ratio of pressor activity of a sample from each purification step to the extinction at 280 m μ was calculated and used as the measure of specific activity.

Protein and peptide determination. Extinction either at 750 m μ , when Lowry's procedure (Lowry, Rosebrough, Farr & Randall, 1951) was applied, or at 280 m μ , when directly read, was determined by the Hitachi Photo Electric Spectrophotometer.

Paper chromatography. The homogeneity of the sample was tested by paper chromatography on Toyo-Roshi no. 51 paper (40 cm. $\times$ 40 cm.) developed for 15 hr. by the ascending technique with the solvent butan-1-ol-acetic acid-water (7:1:2, by vol.). Development was with ninhydrin (0.2%, w/v, in butan-1-ol) and then with Pauly's (1915) reagent or Sakaguchi's (1950) reagent.

Thin-layer chromatography. The homogeneity was also tested by thin-layer chromatography on Serva TLC-cellulose (20 cm. $\times$ 20 cm.) developed for 2 hr. by the ascending technique with the solvent butan-2-ol-aq. 3% NH_3 soln.

(25:11, v/v). Development was as in paper chromatography.

Paper electrophoresis. This was performed on Toyo-Roshi no. 51 paper (2 cm. $\times$ 40 cm.). The sample was applied as a narrow band 1.5 cm. in length. Acidic buffer (pH 2.05) was prepared from 47.5 ml. of acetic acid, 18 ml. of formic acid and distilled water to a volume of 1 l. Alkaline buffer (pH 9.63) was prepared from 39.8 ml. of m-NaHCO_3 , 3.41 ml. of m-NaCO_3 and distilled water to a volume of 1 l. In each case 6.25 v/cm. length of paper was applied. Development was as in paper chromatography.

Amino acid analysis. A portion (1 mg.) of the sample was hydrolysed by 0.3 ml. of 5.5 N-HCl in a sealed ampoule at 105° for 24 hr. The HCl was removed at 40° under reduced pressure and the evaporation was repeated four times with the addition of 0.5 ml. of distilled water each time. The hydrolysed sample was analysed by the Hitachi Amino Acid Analyser.

Preparation of human angiotensin. To find the experimental conditions for the maximum yield of angiotensin several variables were studied: pH, temperature and time of incubation, ratio of renin to angiotensinogen. The best results were obtained as follows. The substrate (200 g.) was added to 8–10 l. of distilled water in a 10 l. Erlenmeyer flask kept in a water bath at 37°, and dissolved by gentle constant motor-driven stirring. Dowex 50W(X2) (1500 ml.), equilibrated with 0.2 M-diethylamine acetate to pH 6.0, was then poured into the flask and mixed. To keep renin activity high, unaffected by the conditions of incubation, 100 ml. of crude human renin (at 0°) was added dropwise to this solution over a period of 6 hr. with continuous stirring, while the mixture was maintained at 37° and at pH 6.0. The stirring was continued for 1.5 hr. after addition of the renin. The Dowex 50W(X2), which absorbs most of the formed angiotensin, was then separated from the remaining protein-containing mixture by filtration with a sintered-glass filter column (8 cm. $\times$ 100 cm.) and washed with 2 l. of distilled water, until the washings had become clear.

Purification of the human angiotensin. The Dowex resin remaining on the filter was eluted with 4 l. of 0.2 M-diethylamine and placed in a 5 l. beaker. The pH was carefully adjusted to 7.0 by adding approx. 45 ml. of acetic acid. A portion was then taken for a pressor bioassay. The total pressor activity, at this point, was calculated to be approximately equivalent to 1200 $\mu\text{g.}$, an average of 30 batches, of synthetic isoleucine⁵-angiotensin II. The specific activity was 0.176.

The neutralized eluate was concentrated to about 200 ml. by a rotary evaporator under reduced pressure. The resulting brownish syrup was centrifuged, and the precipitate (mostly inert protein) was washed four times, each with 15 ml. of distilled water. These washings were combined with the first supernatant. The recovery of pressor activity was 92.5%; the specific activity was 0.504.

The combined supernatant was then dissolved in 4 l. of distilled water by stirring with 200 ml. of Dowex 50W(X2) (equilibrated with 0.2 M-diethylamine, pH 6.0) for 2 hr. The Dowex resin was again collected with a sintered-glass filter column (2.5 cm. $\times$ 50 cm.), followed by washing with 500 ml. of distilled water to remove most of the ammonium acetate. Pressor activity was eluted from the column with 600 ml. of 0.2 M-diethylamine with a recovery rate of 90.2%; the specific activity was 1.24.

The eluate was evaporated under reduced pressure with the temperature kept below 40°. The yellowish solid was

Fig. 1. Purification of human angiotensin on Sephadex G-25. After deproteinization and desalting by readsorption on Dowex 50W(X2), the sample was purified by gel filtration on a Sephadex G-25 column (3.5 cm. $\times$ 50 cm.). Elution was with 0.1 N-acetic acid at a flow rate of 30 ml./hr. The effluent was analysed by measurements of pressor activity (----; $\mu\text{g.}$ of isoleucine⁵-angiotensin II) and extinction at 280 m μ (—). The fractions (5 ml./tube) with pressor activity were all combined and subjected to further purification (see the text).

then dissolved in 10 ml. of 0.1 N-acetic acid and placed on a Sephadex G-25 column (3.5 cm. $\times$ 50 cm.). This was eluted with 0.1 N-acetic acid at a flow rate of 30 ml./hr., and collected in 5 ml. fractions. Three major peaks at 280 m μ were eluted with the pressor activity predominantly superimposed on the descending curve of the second protein peak, i.e. in tubes 66–90 (Fig. 1). The tubes containing the pressor activity were combined, and subsequently evaporated to dryness, with pressor recovery 92%; the specific activity was 4.9.

The evaporated specimen with the total extinction (280 m μ) of 188 was dissolved in 2 ml. of 0.05 M-ammonium acetate, and was next chromatographed on a column (2.5 cm. $\times$ 50 cm.) of CM-cellulose equilibrated with 0.01 M-ammonium acetate buffer, pH 5.6. The elution was carried out at a rate of 20 ml./hr., beginning with 150 ml. of 0.05 M-ammonium acetate solution, switching thereafter to 0.1 M, which, as shown in Fig. 2, in turn was gradually raised to 0.4 M by connecting 250 ml. bottles containing 0.1 M- and 0.4 M-ammonium acetate solution respectively. After this, elution was continued with 150 ml. of 0.4 M-ammonium acetate solution. The eluate was collected in 2.5 ml. fractions and extinction measured at 280 m μ . Three major peaks and one plateau of protein were separated. The pressor activity was in tubes 95–105, which corresponded to a part of the plateau (Fig. 2). Pressor recovery from this column was 79.3%; the specific activity was 27.6.

The fractions with pressor activity were combined, evaporated, and the residue was dissolved in 0.5 ml. of a pyridine-collidine-acetate buffer, pH 8.1 (1 l. of distilled water containing 10 ml. of pyridine and 10 ml. of collidine was adjusted to pH 8.1 by adding approx. 0.3 ml. of acetic acid). This solution was placed on a column (1.5 cm. $\times$ 50 cm.) of DEAE-Sephadex A-25, equilibrated with the same buffer (Fig. 3). It was eluted with 800 ml. of pyridine-collidine-acetate buffer, during which the pH was gradually changed by connecting two bottles: one containing 400 ml.

Fig. 2. Purification of human angiotensin on a CM-cellulose column. After gel filtration by Sephadex G-25, the sample was chromatographed on a CM-cellulose column (2.5 cm. $\times$ 50 cm.) with gradient elution by 0.05–0.4 M-ammonium acetate. It was collected into 5 ml. fractions at a flow rate of 20 ml./hr. The effluent was analysed by measurements of pressor activity (----; μ g. of isoleucine⁵-angiotensin II) and extinction at 280 m μ (—). The fractions with pressor activity were pooled and subjected to further purification (see the text).

Fig. 4. Isolation of human angiotensin on Bio-Gel P-2. The final purification was achieved by molecular-sieve chromatography on a Bio-Gel P-2 column (0.8 cm. $\times$ 100 cm.). It was eluted by 0.1 N-acetic acid and collected into 2.5 ml. fractions at a flow rate of 5 ml./hr. The effluent was analysed by measurements of pressor activity (----; μ g. of isoleucine⁵-angiotensin II) and extinction at 280 m μ (—). The pressor fractions were combined, reduced to a small volume, and recycled on the same column. The second chromatography, as shown on the right, resulted in a perfect association of pressor activity and extinction at 280 m μ . The fractions were combined and freeze-dried.

Fig. 3. Purification of human angiotensin on a DEAE-Sephadex A-25 column (1.5 cm. $\times$ 50 cm.). Gradient elution was from pH 8.1 to 7.0 (pyridine-collidine-acetate buffer) at a flow rate of 20 ml./hr. It was collected into 2.5 ml. fractions. A peak of pressor activity (----; μ g. of isoleucine⁵-angiotensin II) was first associated with a peak of peptide (—), which was determined at 750 m μ with Lowry's colour reaction. Three tubes from the pressor peak were chromatographically homogeneous, but the tubes from the shoulders were not. Therefore pressor fractions were all combined and subjected to further purification (see the text).

the pressor activity, which appeared as one peak, at about pH 7.5, in tubes 247–268. The contour of the pressor-activity peak at first coincided with the colour by Lowry's reagent. Three tubes, containing the highest pressor activity, were shown to be chromatographically homogeneous, but the tubes from the shoulders of the peak subsequently proved to have contaminants. The tubes were therefore all combined and subjected to further, and successful, purification. The pressor recovery was 69.9%; the specific activity was 298.2.

The combined pressor solution was evaporated to dryness under reduced pressure at 40°. The residue was dissolved in 0.3 ml. of 0.1 N-acetic acid, and placed on a Bio-Gel P-2 column (0.8 cm. $\times$ 100 cm.). Elution was carried out with 0.1 N-acetic acid, and 2.5 ml. fractions were collected at a rate of 5 ml./hr. Measurement of the extinction at 280 m μ showed one major peak at tubes 13–18 coinciding with the pressor activity, but this was followed by another peak without any coincident pressor activity. When the fraction producing the first peak was combined, after concentration, and recycled through the same column, the maximum of both extinction and pressor activity coincided exactly in one peak (Fig. 4). The contents of the tubes were then combined and freeze-dried. The recovery of pressor activity through the Bio-Gel P-2 column was 94.1%; the specific activity was 402.7.

RESULTS

of pyridine-collidine-acetate buffer at pH 8.1, and the other containing 400 ml. of the buffer at pH 7.0 (similarly prepared and adjusted to pH 7.0 by adding more acetic acid). The eluate was collected, at a flow rate of 20 ml./hr., in 2.5 ml. fractions; 0.5 ml. of each fraction was used for Lowry's reaction (Lowry *et al.* 1951), and the extinction read at 750 m μ . Most of the inert protein emerged earlier than did

Preparation of human angiotensin. The preparation of the stable materials, i.e. frozen crude renin and freeze-dried fraction IV of plasma protein, were such that storage for long periods (2 years) had no ill effect and allowed for ready use in combined batches of any desired size. Thirty batches were

used in the incubation. The human angiotensin formed was effectively adsorbed on Dowex 50W (X2) as introduced by Boucher, Veyrat, de Champlain & Genest (1964). This was also effective in avoiding the effect of angiotensinase. One batch, on average, yielded pressor activity equivalent to 1200 $\mu\text{g.}$ of synthetic isoleucine⁵-angiotensin II. The specific activity was 0.176.

Purification of human angiotensin. The results of one typical preparation are outlined in Table 1.

The repetition of the initial adsorption procedure on Dowex 50W (X2), and the elution from it, facilitated the removal of most of both the ammonium acetate and protein contaminants (28-fold purification). This procedure accounted for a loss of approx. 16% of the pressor activity.

Gel filtration by Sephadex G-25 eluted with 0.1N-acetic acid (fourfold purification), and cation-exchange column chromatography on CM-cellulose (sixfold purification), led to further purification.

The minute amounts of contaminants still remaining after this step were difficult to remove. Dowex 1, for example, gave relatively good separation, but only poor recovery (30%) of pressor activity. Instead, purification by anion-exchange gel filtration on DEAE-Sephadex A-25 proved to be remarkably efficient and was the method of choice at this step. It also gave the highest degree of purification (11-fold), in graded steps, as shown in Table 1. It was at this step that human angiotensin was first detected chemically by Lowry's colour reaction.

Both slopes, occurring before and after the peaks, however, still contained some impurities, and these were successfully eliminated by molecular-sieve chromatography on Bio-Gel P-2. This replaced the Sephadex G-15 used in our preliminary purification (Arakawa *et al.* 1967), since it was found to afford much better separation.

Chromatographic analysis. The freeze-dried sample was chromatographed on Toyo-Roshi no. 51 paper and on Serva TLC-cellulose thin layers. A single spot was obtained with both systems as shown in Figs. 5 and 6 (R_F 5.1 and 7.1 respectively).

The electrophoresis of the purified human angiotensin in both acidic (pH 2.05, for 2.5 hr.) and alkaline (pH 9.63, for 4 hr.) buffers also revealed a single spot, as shown in Figs. 7 and 8. The mobility relative to the synthetic valine⁵-angiotensin I was 0.98 at both pH values.

Fig. 5. Paper chromatography of isolated human and various synthetic angiotensins. They were chromatographed in butan-1-ol-acetic acid-water (7:1:2, by vol.) on Toyo-Roshi no. 51 paper and developed with ninhydrin. (1) Synthetic isoleucine⁵-angiotensin II; (2) isolated human angiotensin; (3) synthetic valine⁵-angiotensin I; (4) synthetic valine⁵-angiotensin II; (5) synthetic valine⁵-angiotensin II-aspartyl- β -amide.

Table 1. *Recovery and specific activity in each step of the purification of human angiotensin*

Recovery was calculated from the pressor activity. Specific activity was calculated from the ratio of pressor activity to the total extinction determined at 280 m μ .

	Pressor activity ($\mu\text{g.}$ of synthetic Ile ⁵ -angiotensin II)	Recovery of pressor activity (%)	Extinction (at 280 m μ)	Specific activity
1 Elution from Dowex 50W (X2)	1200		6800	0.176
2 Deproteinization by centrifugation	1100	92.5	2200	0.504
3 Recycling on Dowex 50W (X2)	1000	90.2	807	1.24
4 Sephadex G-25	920	92.0	188	4.9
5 CM-cellulose	730	79.3	26.4	27.6
6 DEAE-Sephadex A-25	510	69.9	17.2	298.2
7 Bio-Gel P-2	480	94.1	1.2	402.7

Fig. 6. Thin-layer chromatography of isolated human and various synthetic angiotensins. The isolated human angiotensin along with various synthetic angiotensins (see Fig. 5 for numbering) were chromatographed in butan-2-ol-aq. 3% ammonia (25:11, v/v) on a Serva TLC-cellulose thin layer, and developed with ninhydrin.

Fig. 7. Paper electrophoresis of isolated human and various angiotensins at pH 2.05. The isolated human angiotensin along with synthetic angiotensins (see Fig. 5 for numbering) were subjected to electrophoresis on Toyo-Roshi no. 51 paper at pH 2.05 and 6.25 v/cm., for 2.5 hr. It was developed by Pauly's colour reaction. The relative mobility of the isolated human angiotensin to that of valine⁵-angiotensin I was 0.98.

All the above chromatograms gave a positive colour reaction to ninhydrin, Pauly's (1915) reagent and Sakaguchi's (1950) reagent, and, further, when eluted, gave a strong pressor activity, though none was seen in any other location on the paper strip or on the thin-layer powder.

Biological activity. The isolated human angiotensin gave a specific pressor activity of 402.7

Fig. 8. Paper electrophoresis of isolated human and various angiotensins at pH 9.63. The isolated human angiotensin, along with synthetic angiotensins (see Fig. 5 for numbering), was subjected to electrophoresis at pH 9.63 and 6.25 v/cm., for 4 hr. It was developed by Sakaguchi's colour reaction. The relative mobility of the isolated human angiotensin to that of valine⁵-angiotensin I was 0.98.

(Table 1), in contrast with 700 obtained for the synthetic angiotensin II. The oxytocic activity of the isolated human angiotensin was nil.

The pressor activity was heat-stable, diffusible and readily underwent inactivation with either trypsin or Pronase.

Amino acid analysis. A portion (1.0 mg.) of the purified human angiotensin was hydrolysed with 5.5N-hydrochloric acid, and analysed with an automatic analyser. The result was as follows: Asp, 0.97; Pro, 0.85; Val, 1.00; Ile, 0.95; Leu, 1.07; Tyr, 0.53; Phe, 1.08; His, 2.11; Arg, 1.01.

DISCUSSION

That the purified human angiotensin is homogeneous is supported by: (1) it gave a single peak of pressor activity exactly associated with the extinction at 280 mμ after the final column chromatography on Bio-Gel P-2; (2) it gave a single spot on paper and thin-layer chromatography developed by different systems; (3) it also gave a single spot after electrophoresis at either acidic or alkaline pH; (4) the spot in these chromatograms remained single whether ninhydrin, Pauly's reagent or Sakaguchi's reagent was applied for the detection, and, when eluted, and assayed, only the location of the spot was biologically active.

That the isolated human angiotensin must be a decapeptide angiotensin I is supported by the following: (1) The renin used in the formation of human angiotensin was so prepared, with removal of the chloride ion by dialysis, as to avoid an action of the converting enzyme according to the finding of Skeggs and his collaborators (Skeggs *et al.* 1954a), thus preventing the formation of angiotensin II.

(2) The isolated human angiotensin is not identical, as Figs. 5-8 show, with any synthetic angiotensin II. It only bears close resemblance to valine⁵-angiotensin decapeptide (bovine angiotensin I), having in common additional histidine and leucine residues to the II form. (3) The ratio of the specific pressor activity of the isolated sample to that of synthetic angiotensin II was 4:7, which is not far from the ratio 1:2 reported for that of horse angiotensin I and II (Skeggs *et al.* 1956a). (4) Finally, amino acid analysis of the sample revealed the same composition as that known for horse decapeptide angiotensin I in which the structure also has been established to be isoleucine⁵-angiotensin I, i.e. Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (Lentz *et al.* 1956).

It is unfortunate, however, that isoleucine⁵-angiotensin I has never been synthesized, and no comparison with its behaviour was possible.

The amino acid analysis has revealed only minute amounts of ammonia, indicating that the aspartic acid residue determined was not derived from asparagine; however, this has not yet been confirmed by enzymic degradation.

We thank Dr S. Yoshimura of Tokyo Metropolitan Medical Examiner's office, Tokyo, Japan, for the supply of human kidney, Dr R. Naito of Midori Juji Ltd., Osaka, Japan, for preparing Cohn's fraction IV from human plasma, Dr W. Rittel of Ciba Ltd., Basle, Switzerland, for generous samples of synthetic angiotensins and Dr G. Funatsu, Kyushu University School of Agriculture, Fukuoka, Japan, for amino acid analysis. We are also indebted to Miss J. Yamada and Miss N. Yamashita for their technical assistance. This work was supported by the U.S. Public Health Service (Grant HE 09424-01-02).

REFERENCES

- Arakawa, K. & Bumpus, F. M. (1961). *J. Amer. chem. Soc.* **83**, 728.
- Arakawa, K., Nakatani, M. & Nakamura, M. (1965). *Nature, Lond.*, **207**, 636.
- Arakawa, K., Nakatani, M. & Nakamura, M. (1967). *Nature, Lond.*, **214**, 278.
- Boucher, B., Veyrat, R., de Champlain, J. & Genest, J. (1964). *Canad. med. Ass. J.* **90**, 194.
- Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Muñoz, J. M. & Taquini, A. C. (1946). *Renal Hypertension*, p. 300. Springfield: Charles C. Thomas Inc.
- Bright, R. (1827). *Rep. med. Cases, Guy's Hosp. Lond.*, **1**, 231.
- Bumpus, F. M., Schwarz, H. & Page, I. H. (1957). *Science*, **125**, 886.
- Cohn, E. J., Strong, L. E., Hughers, W. J., jun., Mulford, D. J., Ashworth, J. N. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
- Elliott, D. F. & Peart, W. S. (1956). *Nature, Lond.*, **177**, 527.
- Elliott, D. F. & Peart, W. S. (1957). *Biochem. J.* **65**, 246.
- Haas, E., Lamfrom, H. & Goldblatt, H. (1954). *Arch. Biochem. Biophys.* **48**, 256.
- Lentz, K. E., Skeggs, L. T., jun., Woods, K. R., Kahn, J. R. & Shumway, N. P. (1956). *J. exp. Med.* **104**, 183.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Page, I. H. & Bumpus, F. M. (1961). *Physiol. Rev.* **41**, 331.
- Pauly, H. (1915). *Hoppe-Seyl. Z.* **94**, 284.
- Peart, W. S. (1955). *Biochem. J.* **60**, vi.
- Peart, W. S. (1956). *Biochem. J.* **62**, 520.
- Sakaguchi, S. (1950). *J. Biochem., Tokyo*, **37**, 231.
- Schwarz, H., Masson, G. M. C. & Page, I. H. (1955). *J. Pharmacol.* **114**, 418.
- Schwyzer, R., Iselin, B., Kappeler, H., Riniker, B., Rittel, W. & Zuber, H. (1958a). *Helv. chim. Acta*, **41**, 1273.
- Schwyzer, R., Iselin, B., Kappeler, H., Riniker, B., Rittel, W. & Zuber, H. (1958b). *Helv. chim. Acta*, **41**, 1287.
- Skeggs, L. T., jun., Kahn, J. R. & Shumway, N. P. (1956a). *J. exp. Med.* **103**, 301.
- Skeggs, L. T., jun., Lentz, K. E., Kahn, J. R., Shumway, N. P. & Woods, K. R. (1956b). *J. exp. Med.* **104**, 193.
- Skeggs, L. T., jun., Marsh, J. R., Kahn, J. R. & Shumway, N. P. (1954a). *J. exp. Med.* **99**, 275.
- Skeggs, L. T., jun., Marsh, J. R., Kahn, J. R. & Shumway, N. P. (1954b). *J. exp. Med.* **100**, 363.
- Skeggs, L. T., jun., Marsh, J. R., Kahn, J. R. & Shumway, N. P. (1955). *J. exp. Med.* **102**, 435.