Synthesis of Renin Substrate by Rat Liver

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The present study attempts to determine if the isolated rat liver is capable of synthesizing renin substrate from ^{14}C -labelled amino acids added in the perfusate. The renin substrate is characterized via reaction with renin, forming a substance that is subsequently identified as proangiotensin. Extensive evaluation of the reaction product is carried out by using molecular-sieve chromatography, countercurrent distribution, reactivity with converting enzyme, radioimmunological technique and bioassay. The results demonstrate that isolated rat liver perfused with artificial salt solution is capable of synthesizing a protein that reacts with renin to form a radioactive substance indistinguishable from proangiotensin.

Over the past 35 years, indirect evidence has accrued to indicate that renin substrate (angiotensinogen), a plasma α_2 -globulin, is synthesized by the liver (Braun-Menendez *et al.*, 1946; Nasjletti & Masson 1971). Nasjletti & Masson (1972) have adduced additional evidence that shows that isolated rat liver perfused with medium 199 synthesizes a substance that reacts with partially purified renin to form a proangiotensin-like compound. Formation of the renin substrate-like substance and protein synthesis in general were inhibited by puromycin.

The compound formed by renin resembled proangiotensin in the following respects. On intravenous injection, it caused a prompt brief rise of mean arterial blood pressure. Its biological activity was inhibited by prior incubation with an antiserum to proangiotensin, but the activity could be recovered by heating the mixture in boiling water. Antiserum to angiotensin was stated to have little or no effect on its biological activity. The proangiotensin-like substance was adsorbed and then eluted from Dowex 50W(X2) as expected for proangiotensin.

We interpret the results of Nasjletti & Masson (1971, 1972) as strong evidence that isolated rat liver is capable of synthesizing a protein or protein fragment capable of reacting with renin to form proangiotensin. The minimum substrate capable of reacting with renin to yield proangiotensin is probably the tetradecapeptide renin substrate (Skeggs *et al.*, 1957). In the present study, we have attempted to obtain further data on the reaction product primarily by examining whether the substrate and the product can be made radioactive by perfusing liver with ^{14}C -labelled amino acids and by comparing the physico-chemical properties of the product to those of authentic [Ile^5]proangiotensin. Because the quantities

of the isolated products were small (100 ng), it was not possible to determine the amino acid composition and sequence of the product.

Materials and Methods

All glassware was coated with silicone (Siliclad; Clay-Adams, Parsippany, NJ, U.S.A.) to decrease surface adsorption of peptides.

Isolated liver preparation

Female Sprague-Dawley rats (0.2–0.3 kg) were injected subcutaneously with 1 ml of diethylstilboestrol (1 mg/ml of peanut oil). The next day the rats were anaesthetized with diethyl ether and subjected to bilateral nephrectomy. After operation, a second injection of diethylstilboestrol was given. The rats received water but no food over the next 24 h. Bilateral nephrectomy and stilboestrol are known to increase blood concentrations of renin substrate and also increase rates of synthesis of renin substrate-like substance by isolated perfused rat liver (Nasjletti & Masson, 1971). One day after nephrectomy, the rats were anaesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg body wt.). Livers were isolated and perfused (portal vein to hepatic vein) as described by Hems *et al.* (1966). After a liver was washed free of blood with aerated Krebs-Henseleit solution (38°C), the perfusion system was connected for recirculation. The volume of the perfusion system was 50 ml. Krebs-Henseleit solution contained the L-amino acids recommended by Jefferson & Korner (1969) plus 1 mCi of [^{14}C]arginine (sp. radioactivity 310 mCi/mmol) and 1 mCi of [^{14}C]proline (sp. radioactivity 157 mCi/mmol). The radioactive amino acids were obtained from Schwarz Bio-Research,

Orangeburg, NY, U.S.A. The solution was maintained at 38°C and was aerated with O₂/CO₂ (19:1, v/v). Circulation proceeded for 60 min, and then the fortified Krebs–Henseleit solution was replaced with fresh solution containing all of the amino acids except for [¹⁴C]arginine and [¹⁴C]proline. Circulation was continued for another 60 min. The solutions collected at the end of each circulation period were processed separately.

Preparation of perfusate protein fractions

Each circulation solution was concentrated by ultrafiltration to about one-fifth of its original volume. The non-diffusible material was then dialysed against 100 vol. of water for 4 h, against 3 mM-disodium EDTA plus 0.9% NaCl for 4 h, and then against water for 16–18 h. Recovery of radioactivity after ultrafiltration and dialysis was about 1.5% for the perfusate collected after 1 h and 3–4% for the perfusate collected after the second hour of circulation. The dialysis membrane (19 mm tubing; Union Carbide Corp., Chicago, IL, U.S.A.) had a nominal retention limit of mol.wt. 10000. After the final dialysis step, each perfusate was freeze-dried and yielded a white powder (200–350 mg/liver perfusate). One such freeze-dried preparation contained ¹⁴C radioactivity at 56000 d.p.m./mg and protein ($A_{280}^{1\%}$ 8.75) at 584 µg/mg of freeze-dried powder. The radioactivity portion of the dry powder (10 mg dissolved in 1.0 ml of 0.9% NaCl) was eluted in the void volume of a Sephadex G-25 column (1 cm × 50 cm) equilibrated and developed with 0.9% NaCl.

Preparation of partially purified renin

Renin from rat kidneys was prepared by using modifications of methods described previously (Ryan & Johnson, 1969; Levine *et al.*, 1972; Skeggs *et al.*, 1972). Rat kidneys were minced in a small meat grinder. The mince (200 g) was mixed with water (400 ml) and was stirred for 10 min and then frozen. The mixture was thawed at room temperature, stirred for 10 min and then centrifuged at 2000g for 30 min at 4°C. The supernatant was saved, and the precipitate was suspended in the original volume of water. After 10 min of stirring, the mixture was again centrifuged. The resulting supernatant was added to the first, and the precipitate was extracted twice as described above. (NH₄)₂SO₄ was added to the combined supernatants at 4°C to give a final concentration of 1.4 M. The precipitate was removed by centrifugation. The supernatant was adjusted to pH 6.0 with 2 M-NaOH, and dry (NH₄)₂SO₄ was added to give a concentration of 2.5 M. The precipitate was collected by vacuum filtration and was dissolved in saline (0.9% NaCl). The filter paper was washed exhaustively with saline because of the strong adsorption of renin to paper (Ryan & Johnson, 1969). The (NH₄)₂SO₄ and NaCl were removed by dialysis

against water. The non-diffusible material was treated batchwise with DEAE-cellulose (free-base form; 10 g of cellulose/g of protein). Acetic acid was added to keep the pH of the mixture between 6 and 7. The mixture was stirred for 30 min and then filtered on a Buchner funnel. The cellulose pad was suspended in the original volume of water and the pH of the mixture was adjusted to 4.7 with 1 M-acetic acid. The mixture was filtered and the cellulose pad was washed once at the same pH. The cellulose pad was mixed with the original volume of 0.1 M-NaCl and the pH was adjusted to 4.0. The mixture was filtered, and the clear faintly yellow filtrate was ultrafiltered and then dialysed against water. The renin solution was passed through a Millipore filter (pore size 0.45 µm) and was stored in vials. The final product contained 28000 renin units/ml (first-order reaction units) and a protein concentration ($A_{280}^{1\%}$ 10.6) of 18 mg/ml. As judged by the results of Skeggs *et al.* (1972), the preparation is probably not free of pseudorenin. However, renin itself was a predominant activity as the enzyme preparation, on intravenous injection, was capable of causing a direct rise of arterial blood pressure (50 µl gave a blood pressure rise of 30 mm Hg), an activity not attributable to pseudorenin or to other enzymes (e.g. pepsin) capable of catalysing the release of proangiotensin.

Assays

Proangiotensin and angiotensin were measured by radioimmunoassays by using anti-proangiotensin and anti-angiotensin. They were also measured by bioassay in terms of effects on mean arterial blood pressure and on isolated rat uterus. The blood pressure assay used anaesthetized rats treated with pentolinium as described by Peart *et al.* (1966). A cannula was inserted in a femoral vein for intravenous injections, and for intra-arterial injections, a cannula was placed via the common carotid artery such that its tip was 2–3 mm above the aortic valves. The rat uterus preparation was that described by Burn (1952). No efforts were made to induce oestrus.

Antibodies against proangiotensin were collected from goats immunized against a proangiotensin-bovine serum albumin conjugate; [Ile⁵]proangiotensin (18 µmol) was dissolved in 0.1 ml of water containing bovine serum albumin at 10% (w/w). The solution was then mixed with freshly prepared 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride (150 mg) in 0.2 ml of water. Over the next 3–5 min a viscous white precipitate formed. The mixture was suspended in 2.0 ml of water and was dialysed against 10 litres of water at 4°C for 48 h. The non-diffusible material was freeze-dried. Freeze-dried conjugate was resuspended in sterile saline (1 mg of conjugate/ml). The suspension was emulsified with an equal volume of complete Freund's adjuvant and 0.25 ml of the emulsion was injected intramuscularly into each leg.

The goats were injected monthly for 3 months and then at intervals of 2 months for the next 8–12 months. The antiserum was fractionated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (33%) saturation, and then was dissolved in 0.5 vol. of saline. The material was dialysed against 5 mM-sodium phosphate buffer, pH 7.5, containing 150 mM-NaCl. Antibodies to angiotensin were prepared in an essentially identical manner by using $[\text{Ile}^5]\text{angiotensin}$ as the hapten. Proangiotensin and angiotensin were synthesized by the Merrifield solid-phase technique and were purified by chromatography on Sephadex G-25 and countercurrent distribution.

Radioimmunoassays for proangiotensin and angiotensin were performed by using dextran-coated charcoal to separate free from bound antigen. ^{125}I -labelled proangiotensin (sp. radioactivity 709.5–1419 Ci/mmol) and ^{125}I -labelled angiotensin (sp. radioactivity 1171–1756.5 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. The iodinated peptides invariably required purification. Chromatography on a column (1.1 cm $\times$ 100 cm) of Bio-Gel P-2 with pyridine/acetic acid, pH 5, usually sufficed to yield a product that behaved like a single substance (R_{avg} 0.49) on paper electrophoresis (for analytical systems, see Chiu *et al.*, 1975) and which was readily bound by specific antibody. The antiserum to proangiotensin bound ^{125}I -labelled proangiotensin (5–10 pg) at >50% at a titre of 1:10000 and did not cross-react with angiotensin over the range of concentrations tested (10–10000 pg/ml). The antiserum to angiotensin II bound ^{125}I -labelled angiotensin (5–10 pg) at >50% at a titre of 1:1000. Its cross-reactivity with proangiotensin did not exceed 3%.

Results

Reaction of liver perfusate renin substrate with renin

Freeze-dried radioactive protein prepared from the liver circulation solution (collected after 2 h) was reacted with partially purified rat renin as follows. Freeze-dried protein (10 mg) was dissolved in 10 ml of 0.1 M-Tris/HCl buffer, pH 6.0, containing 0.1 mM-2,3-dimercaprol and 10.6 mM-8-hydroxyquinoline. At time zero renin (100 μl ; 2800 renin units) was added and mixed, and the final solution was incubated at 37°C. Previous small-scale experiments (see Fig. 1) had shown that the quantity of renin (t_4 15 min) added was sufficient to catalyse the hydrolysis of greater than 98% of renin substrate to yield proangiotensin during a 90 min incubation (cf. Ryan *et al.*, 1968; Ryan & McKenzie, 1968). Samples collected at the subsequent steps were assayed by using the proangiotensin radioimmunoassay. Occasional samples were measured by bioassay (blood-pressure effects). Samples for assay were dissolved or diluted in 0.1 M-

Fig. 1. Release of proangiotensin by renin. Freeze-dried protein (1 mg) was dissolved in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 6.0, containing 0.1 mM-2,3-dimercaprol and 10.6 mM-8-hydroxyquinoline. At time zero, 280 units of renin (10 μl) was added and the final reaction mixture was incubated at 37°C. At the intervals indicated, samples were taken for assay. Proangiotensin was measured by radioimmunoassay. Values for angiotensin release (bars) were corrected to represent the amount of product formed by the entire reaction mixture. Within the limits of the assay, the reaction appeared to obey first-order enzyme kinetics. With the quantity of renin used, approximately one-half of the substrate was hydrolysed every 15 min during the incubation period. For comparison, the line indicates the theoretical rate of substrate utilization for renin with a t_4 of 15 min.

Fig. 2. Chromatography of proangiotensin released from liver perfusate protein.

The proangiotensin-like substance extracted with fuller's earth was applied to a column (see the text) of Bio-Gel P-2 and 60 fractions (2 ml each) were collected. Fractions were assayed for their content of proangiotensin by radioimmunoassay.

Tris/HCl buffer, pH 7.6, containing 1% bovine serum albumin.

Extraction of proangiotensin from the reaction mixture

The proangiotensin-like substance formed during incubation was extracted as described by Boyd *et al.* (1969); 100mg of acid-washed fuller's earth was added to the incubation medium and the mixture was stirred for 10min at 4°C. The fuller's earth was collected by centrifugation (200g × 10min) and then was washed successively with 10ml of water and 10ml of methanol. The angiotensin-like substance was eluted by washing the fuller's earth with 2ml of NH₄OH/methanol (40:60). The fuller's earth/NH₄OH/methanol mixture was stirred at room temperature for 20min and then the eluate was collected by centrifugation. The eluate was air-dried at 40°C.

Molecular-sieve chromatography

The residue was dissolved in 0.5ml of pyridine/0.1M-acetic acid, pH 5.0. The resulting solution was applied to a column (1.1cm × 100cm) of Bio-Gel P-2 equilibrated and developed in the same buffer. Fractions (60) of 2ml were collected and samples of alternate fractions were submitted to radioimmunoassay. The results of one such chromatography are shown in Fig. 2. Fractions 24–45, which correspond to the known elution position of [Ile⁵]proangiotensin, were combined. The solution was evaporated to a small volume (approx. 2ml) in a rotary flash evaporator and then was freeze-dried.

Countercurrent distribution

The freeze-dried residue was dissolved in 1.0ml of 0.1M-sodium phosphate buffer, pH 7.0, previously equilibrated with butanol. The solution was loaded into the first tube of a 9-transfer countercurrent device (Post Instruments, Queens, NY, U.S.A.) and countercurrent distribution was performed by using butanol as the upper phase and 0.1M-sodium phosphate buffer, pH 7.0, as the lower phase (Skeggs *et al.*, 1954). This system provides a wide separation of [Ile⁵]proangiotensin from [Ile⁵]angiotensin and [Val⁵]angiotensin [respective apparent partition coefficients are 1.7–2.2, 0.17–0.21 and 0.11–0.13; Ryan & McKenzie, 1968]. Distribution of the proangiotensin-like immunoreactivity is shown in Fig. 3. No angiotensin-like immunoreactivity was detected. The butanol phases of the indicated samples were combined. The solution was evaporated to dryness; its residue was dissolved in water and then was freeze-dried. Samples of the freeze-dried residue were measured by radioimmunoassay and by effects on mean arterial blood pressure and on isolated rat uterus.

Fig. 3. Countercurrent distribution of proangiotensin from liver perfusate protein

The proangiotensin-like substance obtained by chromatography on Bio-Gel P-2 (Fig. 2) was submitted to countercurrent distribution as described in the text. After the distribution was completed, a sample of each aqueous phase (broken line) and each butanol phase (solid line) was assayed for its content of proangiotensin.

Reactivity of the proangiotensin-like substance with angiotensin-converting enzyme

Material collected by chromatography on Bio-Gel P-2, equivalent to approx. 100ng of proangiotensin, was reacted with pig lung angiotensin-converting enzyme [fraction G (approx. 35% pure) described by Dorer *et al.* (1972)]. The proangiotensin-like material was dissolved in 0.2ml of 0.1M-Tris/HCl buffer, pH 7.6, containing 0.1M-NaCl. The reaction was started by adding 5 µl of converting enzyme (approx. 1 µg of enzyme protein). The solution was incubated at 37°C for 2h, and the reaction was terminated by heating in a boiling-water bath for 5min. At the end of the incubation, samples were assayed by using both the proangiotensin and angiotensin radioimmunoassays. Proangiotensin immunoreactivity was undetectable (<50pg). Angiotensin immunoreactivity, which was undetectable before the addition of converting enzyme, was equivalent to approx. 93ng of [Ile⁵]angiotensin. On subsequent countercurrent distribution, all of the angiotensin-like material partitioned as expected for angiotensin (apparent partition coefficient of 0.11).

As shown in Table 1, recoveries of proangiotensin and angiotensin through the various purification procedures were surprisingly good considering the small gravimetric amounts used. However, it is also evident from Table 1 that most of the radioactivity of the starting renin substrate preparation was not adsorbed on fuller's earth and probably was associated with proteins that were unreactive with renin. If, as appears to be the case (Skeggs *et al.*, 1963), proangiotensin is the N-terminal decapeptide of renin substrate and is released by the cleavage of a

Table 1. *Purification of proangiotensin from liver perfusate protein reacted with renin*

Freeze-dried protein (10mg) was reacted with renin (2800 units) as described in the text. After 90min of incubation at 37°C, the reaction mixture was purified as described in the text.

Purification step	Content of proangiotensin (ng)	Total radioactivity (d.p.m.)
Reaction mixture	280	560000
Adsorption on fuller's earth	250	6400
Chromatography on Bio-Gel P-2	230	2000
Countercurrent distribution	105	835

single peptide bond, then it may well be that part of the radioactivity not adsorbed by fuller's earth is attributable to the protein portion of substrate that remains intact after formation of proangiotensin.

After countercurrent distribution, the apparent specific radioactivity of the proangiotensin-like substance was 4.7mCi/mmol. This value is in fair agreement with the apparent specific radioactivity (11.2mCi/mmol) of the angiotensin-like material obtained after the reaction with converting enzyme and after countercurrent distribution. However, because of the low mass of these substances, we were unable to obtain independent evidence of the purity of the compounds after countercurrent distribution.

Characterization of the reaction products

The proangiotensin-like substance obtained by countercurrent distribution was not reactive with antibodies to angiotensin, but was reactive with antibodies to proangiotensin. Although the substance was capable of raising mean arterial blood pressure by intravenous and by intra-aortic injection, it had relatively weak effects on isolated rat uterus. Its effects on rat uterus were increased (approx. 8-fold) after incubation with a highly purified pig lung angiotensin-converting enzyme preparation. The relative potencies of the material before and after reaction with converting enzyme are identical with those of [Ile⁵]proangiotensin and [Ile⁵]angiotensin. The angiotensin-like substance was reactive with antibodies to angiotensin but not with antibodies to proangiotensin. Its effects on contractility of rat uterus were not modified by further reaction with converting enzyme, and its effects on arterial blood pressure were not diminished by intravenous versus intra-aortic injection (Hodge *et al.*, 1967).

Discussion

The present data confirm and extend the findings of Nasjletti & Masson (1971, 1972). Isolated rat liver

perfused with an amino acid-containing artificial salt solution is capable of synthesizing a protein that reacts with renin to form a substance indistinguishable from proangiotensin. Although we cannot be certain that proangiotensin was obtained in homogeneous form, there is little doubt that the product was, in fact, proangiotensin. The product was adsorbed and eluted from fuller's earth as is authentic proangiotensin (Boyd *et al.*, 1969). Its behaviour on molecular-sieve chromatography was identical with that of proangiotensin. Its apparent partition coefficient on countercurrent distribution and its reactivity with pig lung converting enzyme were the same as those of proangiotensin (Skeggs *et al.*, 1954; Dorer *et al.*, 1972). The pharmacological effects of the product were those expected of proangiotensin. Furthermore it was reactive with antibodies to proangiotensin before reaction with converting enzyme. The product obtained after reaction with converting enzyme was reactive with antibodies to angiotensin and was not reactive with antibodies to proangiotensin. The reactivity of the proangiotensin-like substance with converting enzyme indicates the polypeptide nature of the material. The apparent failure of converting enzyme to degrade the angiotensin-like substance is consistent with lack of reactivity of authentic angiotensin with the enzyme (Dorer *et al.*, 1972).

What remains equivocal is whether the proangiotensin obtained by incubation of the radioactive liver perfusate protein with renin is itself radioactive. Although the vast majority of radioactivity was lost during the purification procedure, the product obtained by countercurrent distribution possessed radioactivity approximately 6-times the background value. The apparent radioactivity of the angiotensin-like product was 11.2mCi/mmol, but since we were unable to prove that the product was pure, we do not know what proportion of the radioactivity was associated with angiotensin.

Indirect evidence suggests that radioactivity was associated with proangiotensin and angiotensin. Although the renin preparation used in these experiments was not pure, all results indicate that the original liver perfusate protein preparation underwent limited proteolysis. Depletion of renin substrate by formation of proangiotensin went to completion, but relatively few peptides were formed as judged by the failure of most radioactivity to adsorb on fuller's earth. Similarly, at the stage of countercurrent distribution, the small amount of radioactivity remaining distributed like proangiotensin. After reaction of the product with converting enzyme and subsequent countercurrent distribution, the radioactivity distributed like angiotensin.

Relatively little is known of the molecular form of renin substrate(s) synthesized by isolated rat liver. The material was concentrated by ultrafiltration and

was not lost on subsequent dialysis. Under these conditions, small polypeptide renin substrates, e.g. the tetradecapeptide renin substrate (Skeggs *et al.*, 1957), would be lost. The radioactive substrate was eluted in the void volume on chromatography with a column of Sephadex G-25. However, further characterization remains to be carried out.

Neither the present results nor those of Nasjletti & Masson (1971) indicate that liver is the only source of renin substrate. However, even taking into account the unphysiological aspects of our perfusion system, it is evident that isolated rat liver is capable of synthesizing relatively large amounts of substrate. As indicated in the Materials and Methods section, each rat liver perfusate system (50ml) yielded a freeze-dried powder of approx. 300mg. Each mg after reaction with renin yielded 25–34ng of pro-angiotensin. Assuming no losses from time of collection through ultrafiltration, dialysis and freeze-drying, the original perfusate contained renin substrate at 150ng of proangiotensin equivalents/ml.

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