Renin Substrate in Granules from Rat Kidney Cortex

By BRIAN J. MORRIS* and COLIN I. JOHNSTON

Monash University Department of Medicine, Prince Henry's Hospital, St. Kilda Road,
Melbourne, Vic. 3004, Australia

(Received 3 June 1975)

1. Subcellular fractions of rat kidney cortex generated angiotensin I continuously over 2 h when incubated at 37°C with rat renin, indicating the presence of renin substrate within cells in the renal cortex. 2. Renin substrate was located in highest specific concentration in particulate fractions. The particles containing renin substrate had a sedimentation velocity slightly lower than mitochondria and renin granules but greater than the microsomal fraction. 3. Isopycnic gradient centrifugation indicated a density of 1.190 g/ml for the particles containing renin substrate, compared with 1.201 for renin granules, 1.177 for mitochondria, and 1.170 and 1.230 for lysosomes in the heavy-granule fraction. 4. In the liver, renin substrate was also found in particles, but these had a lower sedimentation rate than those from the kidney. 5. The molecular weights of renin substrate in kidney and liver granules and rat plasma were similar, namely 61 000–62 000. 6. On the basis of these biochemical findings, a mechanism for the intrarenal production of angiotensin, incorporating a subcellular reaction scheme, is proposed.

The renin–angiotensin system has an important role in the renal regulation of blood pressure and Na⁺ balance. Renin is released from granulated, modified myoepithelial (juxtaglomerular) cells in the wall of the afferent arteriole close to the renal glomerulus in response to changes in sodium concentration in the distal tubule or decreased distension of the afferent arteriole. Renin (EC 3.4.99.19) reacts with a specific α_2 -globulin (renin substrate) in plasma to yield a decapeptide, angiotensin I, which is further hydrolysed to the octapeptide, angiotensin II, in the pulmonary and other peripheral vascular beds. It has been proposed that angiotensin II, a powerful pressor substance, may also be formed locally within the kidney and regulate the calibre of the renal arterioles and therefore glomerular filtration rate and sodium excretion. Evidence has been produced that renin substrate in plasma reacts with newly secreted renin and that angiotensin then constricts the efferent arteriole to increase glomerular filtration rate (Krahé *et al.*, 1970; Hofbauer *et al.*, 1973). However, angiotensin I formed in renal blood may be largely washed out of the kidney before sufficient conversion into angiotensin II can occur to elicit vasoconstriction. The juxtaglomerular apparatus consists of both vascular components (the juxtaglomerular cells) and tubular components (the macula densa cells of the distal tubule) and it has been proposed that the renin–angiotensin system may be involved in the

intrarenal feedback mechanism of maintaining glomerulo-tubular balance proposed by Guyton *et al.* (1964) in a computer model for renal auto-regulation (Thurau, 1964; Thurau *et al.*, 1974). Thurau's group have proposed that an increase in sodium concentration at the macula densa activates a renin–angiotensin system in the juxtaglomerular region and that local formation of angiotensin II results in constriction of the afferent arteriole, with a decrease in glomerular filtration rate and filtered sodium.

For an intrarenal renin–angiotensin system to exist, the requisite components must be present and act within the kidney. Renin substrate, renin, angiotensin I, angiotensin II and the dipeptidyl hydrolase, converting enzyme (EC 3.4.15.1), which converts angiotensin I into angiotensin II, have been measured in renal lymph (Lever & Peart, 1962; Horky *et al.*, 1971) and angiotensin II in lymph has been shown to increase under conditions that release renin (Bailie *et al.*, 1971). Converting enzyme has been measured in cells of the juxtaglomerular apparatus (Granger *et al.*, 1972) and evidence exists for the presence of renin substrate in cells of this region as well (Goldstein *et al.*, 1973). However, the origin and manner of regulation of an intrarenal renin–angiotensin system is not known. The subcellular location of converting enzyme has been established (Oshima *et al.*, 1974) and in the present study the existence, subcellular location and properties of the substrate of renin were investigated in the kidney. The establishment of the location of each component of the renin–angiotensin system in the kidney serves as the baseline for deter-

* Present address (and for correspondence): Department of Physiology, University of Missouri School of Medicine, Columbia, MO 65210, U.S.A.

mining the mechanism and regulation of the production of angiotensin within the renal vascular bed.

Materials and Methods

Tissue fractionation

Ten Sprague-Dawley rats (200–250 g) were killed by cervical dislocation and the kidneys immediately removed and placed on ice. Renal cortical tissue, chopped finely with scissors, was homogenized in 0.3 M-sucrose (1:4, w/w) at 500 rev./min by using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 g for 10 min in the SS-34 rotor of a refrigerated Sorvall RC-2 centrifuge to sediment nuclei and unbroken cells (P_1). Differential centrifugation of the supernatant (cytoplasmic extract, S_1) at 5000 g for 10 min, 20000 g for 10 min and 70000 g for 120 min sedimented a heavy-granule fraction (P_2), light-granule fraction (P_3), and microsomal fraction (P_4) respectively. The final supernatant (S_4) was the soluble fraction. Particulate fractions (P_2 , P_3 and P_4) were gently resuspended in cold 0.3 M-sucrose. The volume of resuspended material was 2.0–2.6 ml for fraction P_2 and 1.0–1.5 ml for fractions P_3 and P_4 . The sample was either stored at -20°C or used in isopycnic gradient centrifugation. Samples of 1.3 ml (50%) of resuspended fraction P_2 and 0.8 ml (60%) of resuspended fraction P_3 were applied to the surfaces of separate 12 ml linear continuous gradients of 1.0–2.0 M-sucrose in 14 ml polycarbonate ultracentrifuge tubes (MSE, cat. no. 34411-125; internal dimensions 9.5 cm $\times$ 1.4 cm) and after gentle mixing with the upper layers of the gradient to lessen any turnover effect (Anderson, 1955) were placed in the buckets of a 6 $\times$ 14 ml Titanium swing-out rotor (MSE, cat. no. 43127-111) and centrifuged in a MSE Super-Speed 65 Mk. II preparative ultracentrifuge at 70000 g (r_{av} 11.1 cm) for 120 min. Approximately equal fractions along the gradient were isolated by using a Beckman tube slicer fitted with 1.5 cm (internal diam.) metal and rubber rings, and were stored at -20°C .

Contribution of renin substrate in renal plasma

In a control experiment the possible contribution of renin substrate in plasma remaining in kidney tissue after excision was tested. Immediately after death the kidneys were perfused *in situ* with 30 ml of cold heparinized 0.9% NaCl, after which fluid leaving the severed renal vein was clear and the kidneys were uniformly blanched. Renal cortical tissue from ten animals treated in this way was fractionated by differential centrifugation, and generation of angiotensin I from renin substrate in subcellular fractions was compared with that of fractions prepared from non-perfused tissue.

Column chromatography

The heavy-granule fraction from 8.0 g of renal cortical tissue was resuspended with 50 mM-sodium phosphate buffer, pH 7.4, to give 2 ml, vortex-mixed vigorously, freeze-thawed twice and vortex-mixed again to rupture granules. The mixture was centrifuged at 10000 g for 10 min at 4°C and 1.0 ml of supernatant applied to the surface of a Sephadex G-100 column (bed height 67 cm, diam. 1.3 cm) equilibrated in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.01% neomycin sulphate. Fractions (2.0 ml) were collected in a LKB Ultrarac 7000 fraction collector and renin substrate, renin and protein determined in each fraction. The procedure was repeated with 1.0 ml of rat plasma and 1.0 ml of an extract of the heavy-granule fraction from 7.8 g of rat liver, prepared as described above. The column was calibrated with the following protein standards: aldolase (mol.wt. 158000), hen's-egg albumin (45000), chymotrypsinogen A (25000) and cytochrome *c* (13000), all purchased from Boehringer Mannheim G.m.b.H., Mannheim, West Germany, and bovine serum albumin (67000) from Commonwealth Serum Laboratories, Melbourne, Vic., Australia. The partition coefficient, $K_{av} = (V_e - V_0)/(V_t - V_0)$ (where V_e = elution volume, V_t = total volume of column, V_0 = void volume), was plotted against the molecular weight of the protein standards on a logarithmic scale [r (correlation coefficient) = 0.987] and the molecular weight of renin substrate in samples was determined from its K_{av} .

Phosphate/saline/casein buffer

Assays of renin substrate and renin used 50 mM-sodium phosphate buffer, pH 7.4, containing 0.1% (w/v) casein (Hammarsten; BDH Chemicals Ltd., Poole, Dorset, U.K.), 140 mM-NaCl, 0.1% (w/v) neomycin sulphate and 10 mM-EDTA (Merck, Darmstadt, West Germany). This standard buffer will hereafter be referred to as 'PSC buffer'.

Measurement of renin substrate

Renin substrate was determined by measuring the amount of angiotensin I enzymically hydrolysed from the protein by renin. Semi-purified rat renin (activity 155 pmol of angiotensin I/h) prepared by a method based on that of Haas *et al.* (1966) (steps 1, 2 and 3) was used. All solutions were kept at 4°C , and each tube contained in addition to the sample and renin, 10 mM-di-isopropyl phosphorofluoridate (Sigma, St. Louis, MO, U.S.A.), 25 mM-2,3-dimercaptopropanol (Merck) and 10 mM-EDTA in 200 μl of PSC buffer. Separate 10 μl portions of fractions isolated by differential centrifugation were incubated at 37°C for 0.5–5.0 h. Fractions isolated by gradient

centrifugation (50 μ l) and fractions from chromatography (1–100 μ l) were incubated for 1.0 h only, since this period was sufficient for complete hydrolysis of renin substrate by renin. A non-incubated control was also included for each sample. The reaction was stopped by the addition of 1.0 ml of cold ethanol and precipitated protein sedimented by centrifugation at 1000g for 15 min at 4°C. The supernatants were decanted into a second set of Wasserman tubes and evaporated at 55°C under an air jet. The residue was reconstituted in 500 μ l of PSC buffer by vortex-mixing and angiotensin I present measured by radioimmunoassay. Angiotensin I standards (0.006–1.54 pmol) initially in 50 μ l of PSC buffer were treated throughout in an identical manner to samples. In addition, sets of standards were mixed with sucrose in amounts similar to those present in the samples and also with 200 μ l of the mixture of renin, di-isopropyl phosphorofluoridate, 2,3-dimercaptopropanol and PSC buffer. This allowed the influence of sucrose and other substances on the angiotensin I antibody-antigen reaction to be monitored. No loss of angiotensin I by ethanol extraction could be detected. Concentrations of sucrose similar to the maximum in samples assayed caused a decrease of bound 125 I-labelled angiotensin I tracer of less than 10%.

Measurement of renin

Renin was determined by the rate of formation of angiotensin I during incubation of 0.001–10 μ l of sample with 43 pmol of renin substrate in 300 μ l of PSC buffer for 1 h at 37°C. Nephrectomized sheep plasma prepared by the method of Skinner (1967) was the source of exogenous renin substrate. A nephrectomized-sheep plasma blank was also included. After incubation the mixture was immediately cooled on ice and angiotensin I measured by radioimmunoassay. The amount of angiotensin I produced was directly proportional to the amount of sample and enzymic activity was expressed as pmol of angiotensin I/h per ml. In control tests the preparation of nephrectomized-sheep plasma was shown to contain endogenous renin activity less than 0.36 pmol of angiotensin I/h per ml, to be free of angiotensinase activity, to generate angiotensin I linearly with increasing amounts of purified Medical Research Council renin, and to generate angiotensin I linearly with time over 4 h when incubated with subcellular fractions. In addition, the preparation was shown to have a K_m , determined by the Lineweaver-Burk method, of 14.6 ± 1.5 pmol/ml (mean $\pm$ S.E.M., $n = 2$) by using semi-purified rat renin, compared with 8.3 ± 0.3 for rat renin with homologous rat renin substrate determined simultaneously. The molar concentration of renin substrate in nephrectomized-sheep plasma was determined directly from the number of mol of angiotensin I produced after complete enzymic

hydrolysis with a large amount of renin, where one molecule of angiotensin I was removed from the N-terminal of each molecule of renin substrate (Skeggs *et al.*, 1963), and was 860 pmol/ml. Angiotensinase activity was undetectable in the very small volumes of differential-centrifugation fractions (0.001–0.1 μ l) and gradient fractions (0.01–1.0 μ l) required for renin assay.

Angiotensin I radioimmunoassay

Angiotensin I was measured by a highly specific radioimmunoassay using [Asp¹,Ile⁵]angiotensin I (Schwarz BioResearch, Orangeburg, NY, U.S.A.) labelled with 125 I, a specific antibody to angiotensin I and doubling dilutions of [Asp¹,Ile⁵]angiotensin I (Research standard A, 70/302; Medical Research Council, Mill Hill, London N.W.7, U.K.) as standard (Johnston *et al.*, 1972). The antibody used showed negligible inhibition by, or cross-reactivity with, angiotensin II (<0.1%), tetradecapeptide renin substrate (<1%) and sheep renin substrate (<0.01%).

Angiotensinase activity

The distribution of activity of enzymes that degrade angiotensin I in the presence of EDTA, di-isopropyl phosphorofluoridate and 2,3-dimercaptopropanol was tested. [Asp¹,Ile⁵]Angiotensin I (0.15 and 1.5 pmol) was mixed with 10 μ l of fractions obtained by differential centrifugation and 200 μ l of PSC buffer containing 10 mM-EDTA, 10 mM-di-isopropyl phosphorofluoridate and 25 mM-2,3-dimercaptopropanol. After incubation at 37°C for 1 h the reaction was stopped with ethanol and angiotensin I measured by radioimmunoassay. Controls were included so that the angiotensin I measurement could be corrected for endogenously generated angiotensin I and the amount of exogenous angiotensin I remaining was expressed as percentage of amount added. The degradation of 0.15 pmol of angiotensin I in the absence of angiotensinase inhibitors was also tested and, in addition, the degradation of 0.15 pmol of angiotensin I by 50 μ l of gradient fractions was determined.

Paper chromatography

The degree of inhibition of angiotensinase activity under the conditions of the resin-substrate assay was tested by paper chromatography. 125 I-labelled [Asp¹,Ile⁵]angiotensin I (280 nCi, 1.0 pmol) was incubated with 10 μ l of heavy-granule fraction in PSC buffer in the presence and absence of EDTA + di-isopropyl phosphorofluoridate + 2,3-dimercaptopropanol. Descending chromatography was performed using butan-2-ol/3% NH₃ (25:11, v/v) in a sealed tank lined with blotting paper saturated with

the same solvent and with the solvent in the bottom of the tank. Portions (5 μ l) of ethanolic extracts of incubates were applied to a 50cm $\times$ 20cm strip of Whatman no. 1 chromatography paper and chromatography was performed until the solvent front had migrated 40cm past the sample zone (about 20h at 22°C), and the paper air-dried in a fume hood. The paper was divided into 2cm strips and radioactivity measured by using a Wallac Decem Series gamma sample counter, model no. GTL 300-500.

Measurement of enzyme markers and protein

Acid phosphatase (EC 3.1.3.2) was measured by the method of Andersch & Szczypinski (1947). Fumarate (EC 4.2.1.2) was measured by the method of Kanarek & Hill (1964). Cytochrome oxidase (EC 1.9.3.1) was measured by the method of Cooperstein & Lazarow (1951). Glucose 6-phosphatase (EC 3.1.3.9) was determined by the method of Nordlie & Arion (1966), except that the technique of Parvin & Smith (1969) was used in the determination of P_i , the reaction product. Hydrolysis of glucose 6-phosphate by acid phosphatase under the conditions of assay was probably quite low (Brightwell & Tappel, 1968; Beaufay, 1972). Lactate dehydrogenase (EC 1.1.1.27) was quantified by a method similar to that of Wróblewski & LaDue (1955). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Cohn fraction V; Commonwealth Serum Laboratories) as standard.

Results

Renin substrate in subcellular fractions of kidney cortex isolated by differential centrifugation

Particulate fractions generated angiotensin I over 2h when incubated with renin (Fig. 1). Since angiotensin I is produced by enzymic hydrolysis of renin substrate by renin, these results indicate that rat kidney cortex contains renin substrate and, further, that the renin substrate is present in subcellular particles which sediment during conventional differential centrifugation. The total amount of angiotensin I produced on complete enzymic hydrolysis of renin substrate by renin is an indication of the amount of renin substrate present in a given sample. Accordingly, since the amount of angiotensin I produced by the heavy-granule fraction was twice that produced by the light-granule fraction and four times that produced by the microsomal fraction, the results indicate that the particles containing renin substrate sediment in greatest amounts at 50000g-min. Renin granules also sediment in greatest amounts at a similar relative centrifugal force. Therefore the faster rate of generation of angiotensin I by the heavy-granule fraction could have been due in part to endogenous renin.

Fig. 1. Generation of angiotensin I from subcellular fractions of rat kidney cortex

Fractions were incubated with semi-purified rat renin, 10mM-EDTA, 10mM-di-isopropyl phosphorofluoridate and 25mM-2,3-dimercaptopropanol in 200 μ l of PSC buffer at 37°C for up to 5h. The means $\pm$ S.E.M. of six experiments are shown. The rapid generation of angiotensin I in the first 2h indicated the presence of renin substrate in the fractions. The progressive decrease in angiotensin I between 2 and 5h was due to metabolism of angiotensin I by uninhibited angiotensinases. The extent of each reaction without angiotensinase activity (pmol of renin substrate) was determined by enzyme-kinetic and mathematical analyses. (a) Heavy-granule fraction; (b) light-granule fraction; (c) microsomal fraction.

However, since the maximum amount of angiotensin I produced by each fraction was approximately proportional to the rate of production, it appears that the contribution of endogenous renin was too small to be of significance.

Angiotensin I was generated by each subcellular fraction, reaching a maximum by 2h. Thereafter, however, the amount of angiotensin I progressively decreased (Fig. 1). The loss of angiotensin I was most likely due to hydrolysis by angiotensinases present in the kidney fractions. Such a loss is to be expected, because kidney tissue contains an extremely high activity of angiotensinases (Page & Bumpus, 1961), which no one has as yet been able to inhibit completely by available chemical inhibitors (Nishimura & Sokabe, 1968; Matsunaga, 1971; Orth *et al.*, 1971). The degree of inhibition of renal angiotensinases by the combined use of EDTA + di-isopropyl phosphorofluoridate + 2,3-dimercaptopropanol was tested under the present assay conditions. The percentage degradation of 0.15pmol of exogenous [Asp¹, Ile⁵]angiotensin I in the presence and absence of chemical inhibitors is shown in Table 1. These results indicate that EDTA + di-isopropyl phosphorofluoridate + 2,3-dimercaptopropanol only partially inhibit renal angiotensinase. The partial inhibition of renal angiotensinase activity in the heavy-granule fraction under the conditions of the renin-substrate assay was

Table 1. *Angiotensinase activity of subcellular fractions of rat kidney cortex*

Exogenous angiotensin I (0.15 and 1.5 pmol) was incubated at 37°C for 1 h with 10 μ l of subcellular fractions in the presence of 10 mM-EDTA, 10 mM-di-isopropyl phosphorofluoridate and 25 mM-2,3-dimercaptopropanol in a total volume of 250 μ l with PSC buffer, pH 7.4. Values in parentheses show degradation of 0.15 pmol of angiotensin I in the absence of EDTA, di-isopropyl phosphorofluoridate and 2,3-dimercaptopropanol.

Fraction	Percentage degradation of [Asp ¹ ,Ile ⁵]angiotensin I	
	0.15 pmol	1.5 pmol
Heavy-granule	95 (100)	71
Light-granule	80 (87)	30
Microsomal	57 (77)	14
Soluble	41 (69)	0

Fig. 2. *Paper-chromatography test for inhibition of angiotensinases*

¹²⁵I-labelled [Asp¹,Ile⁵]angiotensin I (280 nCi, 1.0 pmol) was incubated at 37°C for 1 h with 10 μ l of the heavy-granule fraction in the presence and absence of 10 mM-EDTA, 10 mM-di-isopropyl phosphorofluoridate and 25 mM-2,3-dimercaptopropanol in a total volume of 250 μ l with PSC buffer, pH 7.4. Then 5 μ l of ethanolic extract was applied to Whatman no. 1 paper and descending chromatography performed with butan-2-ol/3% NH₃ (25:11, v/v) until the solvent front had migrated 40 cm past the sample zone. ○, No inhibitors; ●, with EDTA + di-isopropyl phosphorofluoridate + 2,3-dimercaptopropanol. The position of ¹²⁵I-labelled angiotensin I standard is also shown (R_F = 0.48). In the presence of angiotensinase inhibitors there was a greater recovery of radioactivity in the position of migration of the angiotensin I standard, and a decrease in the size of the first peak (radioactive fragments).

further demonstrated by paper chromatography after incubation with ¹²⁵I-labelled angiotensin I in the presence and absence of EDTA + di-isopropyl phosphorofluoridate + 2,3-dimercaptopropanol (Fig. 2). In the presence of the chemical inhibitors there was a greater recovery of radioactivity in a position corresponding to ¹²⁵I-labelled angiotensin I standard (R_F = 0.48) and a decrease in the size of the first peak, which presumably represented ¹²⁵I-labelled metabolites. Thus EDTA + di-isopropyl phosphorofluoridate + 2,3-dimercaptopropanol was partially effective in decreasing renal angiotensinase activity.

The decrease in angiotensin I between 2 and 5 h (Fig. 1) was due to angiotensinase activity and not the converting enzyme that liberates the octapeptide angiotensin II from decapeptide angiotensin I, since EDTA was present during assay. At the concentrations used, EDTA has been conclusively shown to produce complete chemical inhibition of renal converting enzyme (Yang *et al.*, 1971; Oshima *et al.*, 1974), so that hydrolysis of angiotensin I by converting enzyme is highly improbable. It is also noteworthy that Fig. 2 shows no evidence of a radioactive peak in the position of ¹²⁵I-labelled angiotensin II standard (R_F = 0.52) after paper chromatography.

The subcellular distribution of renal angiotensinase activity is evident from Table 1. The concentration of angiotensinase activity was: heavy-granule fraction > light-granule fraction > microsomal fraction > soluble fraction. This is consistent with the relative rates of degradation of angiotensin I between 2 and 5 h apparent in Fig. 1. The heavy-granule fraction therefore contains the highest concentration of renal angiotensinases. The type of subcellular particles that contain angiotensinases in the heavy-granule fraction will be examined later.

Table 1 also indicates that breakdown of angiotensin I is proportionately greater for 0.15 pmol than 1.5 pmol of angiotensin I, i.e., is not first-order in peptide concentration. This phenomenon has also been observed by Regoli *et al.* (1963) and may be due to saturation of enzyme.

The aim of the present study was to determine the subcellular distribution of renin substrate in rat kidney cortex. This requires the measurement of renin substrate in subcellular fractions. Measurement of renin substrate involved estimation of the amount of end product (angiotensin I) produced by complete hydrolysis of renin substrate by renin. However, although the amount of angiotensin I produced by subcellular fractions was distributed thus: heavy-granule fraction > light-granule fraction > microsomal fraction, concomitant metabolism of angiotensin I also occurred during assay, since complete chemical inhibition of renal angiotensinases is not possible, so that the amounts of angiotensin I measured are less than would have been measured had complete inhibition of angiotensinases been possible. However, this

does not affect the relative distribution of renin substrate, since the activity of angiotensinases was also distributed thus: heavy-granule fraction > light-granule fraction > microsomal fraction > soluble fraction. Notwithstanding, our data are sufficient for us to estimate the amount of angiotensin I that would have been generated from each fraction in the absence of angiotensinase activity. This was done by calculating the rate constant for the renin–renin substrate reaction in the microsomal fraction, which has low angiotensinase activity. Since the rate constant for a first-order reaction is independent of substrate, the rate constant so obtained could be used to determine the extent of reaction (amount of substrate) for the heavy- and light-granule fractions. The reaction in each fraction was indeed first-order, because the actual concentrations of angiotensin I produced in the reaction mixture during assay were 2.3, 2.4, 1.0, and 0.8 pmol/ml per g of kidney for the heavy-granule, light-granule, microsomal and soluble fractions respectively, values which are less than one-sixth of the K_m value (14.6 pmol/ml; see the Materials and Methods section). The equation of the renin–renin substrate reaction is: $\ln S(t) = -kt + \text{constant}$, where S = pmol of renin substrate, k = rate constant for the renin–renin substrate reaction and t = time. Since a plot of $\ln [\text{angiotensin I (pmol/g of kidney)}] (t)$ against t is a straight line of slope k , the data between 0 and 2 h for the microsomal fraction (Fig. 1) were used to determine the rate constant for the renin–renin substrate reaction. This value was $0.55 \pm 0.12/\text{h}$ (mean $\pm$ S.E.M.; $n = 6$). As a comparison k was also determined for the soluble fraction, which likewise contains low angiotensinase activity, and was $0.75 \pm 0.27/\text{h}$, which is not significantly different to the above value (' t ' test). Therefore particle-bound reactants are not involved, and since buffer, temperature, enzyme concentration and other reaction conditions were similar in each fraction it is fairly safe to assume that the rate constant for the renin–renin substrate reaction in the heavy- and light-granule fractions is similar to the value obtained for the microsomal fraction. At about $t = 0$, $d[\text{angiotensin I}]/dt = -d(S)/dt$. Because the velocity at zero time cannot be measured, the velocity between 0 and 0.5 h was used. Since $-(dS/dt)_0 = kS_0$, where S_0 = amount of renin substrate at zero time, S_0 is therefore equal to $-(dS/dt)$, evaluated $t = 0$ to $t = 0.5$, divided by k . For the light-granule fraction, $S_0 = (2.84 \pm 0.40)/0.55 = 5.2 \pm 0.7$ pmol (mean $\pm$ S.E.M.; $n = 6$) and for the heavy-granule fraction $S_0 = (6.12 \pm 1.26)/0.55 = 11.1 \pm 2.3$ pmol. This method of derivation examines the renin–renin substrate reaction alone and not the reaction of angiotensin I with angiotensinases. The values obtained are close approximations and would be slightly less than the true S_0 value because of the obvious difficulty in measuring velocity close to zero time and the small amount of hydrolysis of angiotensin I by angio-

tensinases between 0 and 0.5 h. Of all the samples assayed for renin substrate, only measurements for the heavy-granule and light-granule fractions were sufficiently affected by angiotensinase activity to warrant correction.

The data for the concentrations of angiotensin I produced in the heavy- and light-granule fractions were also analysed by fitting them to a two-term exponential equation (Wagner, 1967) of the form $C = Be^{-\beta t} - Ae^{-\alpha t}$ where C is the observed concentration of angiotensin I, β and α are rate constants and t is time. The initial concentrations of renin substrate were calculated as described by Wagner (1967) from the constants obtained. Although estimates of the concentration of angiotensin I were not made sufficiently frequently to make application of this approach to all the fractions worth while, it has the advantage that it does not require the assumption that the angiotensinase reaction does not significantly interfere in the determination of the initial concentrations of renin substrate. The values found for these concentrations did not differ significantly from those given above, so lending support to the assumption.

The specific concentration of renin substrate (pmol/mg of protein) in subcellular fractions obtained by differential centrifugation of the cytoplasmic extract of rat kidney cortex is shown in Fig. 3, plotted in the manner recommended by de Duve *et al.* (1955). The distribution of renin substrate differed from the distribution of renin, acid phosphatase, fumarase, cytochrome oxidase, glucose 6-phosphatase, lactate dehydrogenase and protein. Renin granules, lysosomes and mitochondria sedimented principally in the heavy-granule fraction. The heterogeneous distribution of lysosomal acid phosphatase has also been reported by others (Shibko & Tappel, 1965; Wattiaux-de Coninck *et al.*, 1965; Beaufay, 1972). Renin and acid phosphatase in the soluble fraction represented, to a large extent, enzyme released by rupture of vesicles during homogenization (Wattiaux-de Coninck *et al.*, 1965). However, fumarase present in the soluble fraction reflected the genuine occurrence of this enzyme in cytoplasm due to leakage from mitochondria within the cell (de Duve *et al.*, 1955). The distribution of cytochrome oxidase, which is tightly bound to the mitochondrial inner membrane, was similar to the distribution of fumarase among the particulate fractions. Most of the microsomal marker, glucose 6-phosphatase, was found in the 'high-speed' pellet (P_4). Lactate dehydrogenase, a specific cytoplasmic marker, occurred almost exclusively in the soluble fraction.

Contribution of renin substrate in renal plasma

The generation of angiotensin I from renin substrate in subcellular fractions of kidneys previously perfused to remove possible plasma renin substrate

Fig. 3. Differential centrifugation of rat kidney cortex

Distribution of (a) renin substrate (pmol/mg; mean $\pm$ s.e.m., $n = 6$), (b) renin (pmol angiotensin I/h per mg; $n = 6$), (c) acid phosphatase (munits/mg; $n = 6$), (d) fumarase (units/mg; $n = 3$), (e) cytochrome oxidase (units/mg; $n = 2$), (f) glucose 6-phosphatase (μ mol P₁/h per mg; $n = 2$), and (g) lactate dehydrogenase (munits/mg; $n = 2$) in subcellular fractions obtained by differential centrifugation of a cytoplasmic extract of rat kidney cortex. Specific activity on the ordinate is plotted against percentage of total protein in the cytoplasmic extract (S₁). P₂=Heavy-granule fraction; P₃=light-granule fraction; P₄=microsomal fraction; S₄=soluble fraction.

Table 2. *Effect of prior perfusion of kidneys with 0.9% NaCl on generation of angiotensin I from renin substrate in subcellular fractions*

Samples were incubated at 37°C with renin, EDTA, di-isopropyl phosphorofluoridate and 2,3-dimercaptopropanol in PSC buffer for 0, 0.5, 1.0 and 2.0 h and angiotensin I was measured by radioimmunoassay of ethanol-extracted samples.

		Angiotensin I (pmol/g wet wt. of renal-cortical tissue)											
Fraction	...	Heavy-granule (P ₂)				Light-granule (P ₃)				Microsomal (P ₄)			
Time (h)	...	0	0.5	1.0	2.0	0	0.5	1.0	2.0	0	0.5	1.0	2.0
Non-perfused		0	3.0	4.9	4.8	0	2.1	2.8	3.9	0	0.7	1.0	1.3
Perfused		0	2.9	4.4	5.1	0	1.4	2.7	3.4	0	0.6	0.9	1.6

Table 3. *Comparison of subcellular distribution of renin substrate in kidney-cortical tissue and liver tissue fractionated simultaneously*

Fractions were incubated with renin, EDTA, di-isopropyl phosphorofluoridate and 2,3-dimercaptopropanol in PSC buffer and angiotensin I was measured by radioimmunoassay after ethanol extraction. Values have not been corrected for angiotensinase activity. Also shown is the distribution of acid phosphatase, a marker for lysosomes that contain, in kidney at least, the major angiotensinase activity under the conditions of assay of renin substrate. Relative specific concentration (% of substance/% of protein in fraction) is shown in parentheses. P₂ = Heavy-granule fraction; P₃ = light-granule fraction; P₄ = microsomal fraction; S₄ = soluble fraction.

Fraction ...	Kidney				Liver			
	P ₂	P ₃	P ₄	S ₄	P ₂	P ₃	P ₄	S ₄
Renin substrate (pmol/mg of protein)	0.60 (3.5)	0.40 (1.6)	0.29 (1.1)	0.12 (0.6)	0.17 (0.8)	0.45 (1.9)	0.26 (1.3)	0.23 (0.9)
Acid phosphatase (munits*/mg of protein)	85 (2.6)	73 (2.3)	50 (1.6)	20 (0.6)	117 (2.3)	152 (3.0)	54 (0.5)	35 (0.7)
Protein (%)	9.1	7.8	6.9	76.2	10.5	7.2	7.6	74.7

* As defined in Andersch & Szczypinski (1947).

Table 4. *Results of preliminary experiments to determine time required for renin–renin-substrate reaction of gradient and chromatography samples to reach its maximum extent*

A variety of gradient and chromatography samples (designated at random A–H below) were incubated for different periods and angiotensin I was measured (pmol/ml). From these data a 1 h incubation period was chosen for assay of renin substrate in fractions from gradient centrifugation and chromatography.

		Angiotensin I (pmol/ml)							
Time (h)	Sample ...	A	B	C	D	E	F	G	H
0		0	0	0	0	0	0	0	0
0.5		0.37	2.00	0.65	0.24	0.27	0.70	1.70	0.59
1		0.54	2.80	0.93	0.40	0.40	1.10	2.80	0.84
2		0.52	2.80	0.98	0.36	0.46	1.20	2.10	0.82

is shown in Table 2. Values were not corrected for uninhibited angiotensinase activity. The amounts of angiotensin I produced by fractions from perfused tissue were similar to the amounts produced by fractions of non-perfused tissue.

Subcellular distribution of renin substrate in kidney and liver

Since plasma renin substrate is thought to be synthesized by the liver (Page *et al.*, 1941) the subcellular location of renin substrate in saline-perfused rat liver

was investigated and compared with its distribution in rat renal cortex. Results are shown in Table 3 and have not been corrected for angiotensinase activity. The concentration of uninhibited angiotensinase activity in kidney was shown in Table 1 to be in the order: heavy-granule fraction > light-granule fraction > microsomal fraction > soluble fraction. The distribution of the lysosomal marker also followed this order. Table 3 shows the distribution of the lysosomal marker in kidney and liver. Whereas the heavy-granule fraction contained the highest specific concentration of renin substrate in kidney-cortical tissue, the highest specific concentration of renin substrate in liver occurred predominantly in the light-granule fraction. A peak of lysosomal, and presumably uninhibited, angiotensinase activity, corresponded to the renin-substrate peaks.

Isopycnic gradient centrifugation of renin-substrate granules from kidney

To characterize further the granules containing renin substrate, isopycnic gradient centrifugation of

the heavy-granule fraction (P_2) was performed. The renin-substrate assay here used a 1 h period of incubation, since preliminary experiments showed that for gradient samples (and samples from chromatography) the maximum extent of the renin-renin substrate reaction was attained by 1 h (Table 4). A single peak of renin substrate was observed at a density of 1.190 g/ml (Fig. 4). This did not correspond to the distribution of renin granules (equilibrium density 1.201), mitochondrial marker (1.177), lysosomal marker (1.170 and 1.230), or protein (1.176). A dark-brown band in the region of density 1.170–1.180 corresponded to the peak of protein and also fumarase, and almost certainly represented mitochondria. In this experiment the light-granule fraction was also centrifuged simultaneously on a similar gradient. The equilibrium density of renin-substrate granules in the light-granule fraction was also 1.190. However, the equilibrium density of particulate renin was 1.195, suggesting that renin in this fraction is enclosed in less dense granules than those in the heavy-granule fraction. Renin substrate in this gradient also differed from the distribution of mitochondrial marker (1.165), lysosomal marker (1.140 and 1.170), and protein (1.143 and 1.165). The distribution of angiotensinase activity in the heavy-granule fraction consisted of two peaks in similar regions of density to the peaks of lysosomal marker enzyme (Fig. 5). The difference in equilibrium density of renin granules and renin-substrate granules was confirmed in a further experiment involving isopycnic gradient

Fig. 4. Isopycnic gradient centrifugation of heavy-granule fraction of rat kidney cortex

The heavy-granule fraction (P_2) obtained by differential centrifugation was resuspended in sucrose and a portion centrifuged at 70000g_{av} for 120 min on a linear continuous gradient of 1.0–2.0M-sucrose (radial distance 7.4–15.9 cm). Approximately equal fractions were isolated with a tube slicer and their position in the density gradient determined by measuring the dimensions of the cut tube segments. Percentage distribution of renin substrate (●), renin (▲), acid phosphatase (△), fumarase (■) and protein (○) along the centrifuge tube is shown.

Fig. 5. Angiotensinase activity in gradient fractions

Angiotensinase activity (□) was determined by the percentage degradation of 0.15 pmol of angiotensin I incubated at 37°C with 50 µl of each fraction in 200 µl of PSC buffer, pH 7.4. Ethanol extraction was performed before radioimmunoassay. The distribution of the lysosomal marker, acid phosphatase (△), was similar to that of angiotensinase activity.

centrifugation of the heavy-granule fraction on a narrower gradient of 1.4–1.7M-sucrose.

Molecular weight of renin substrate in kidney and liver granules, and plasma

The elution profiles of renin substrate after Sephadex G-100 chromatography of extracts of the heavy-granule fraction of kidney cortex and liver and of a sample of rat plasma are shown in Fig. 6. The elution of renin in the kidney sample is also shown. A major peak of protein was eluted in the void volume for each sample, with subsequent smaller peaks for kidney and liver, and a second major peak in the position of albumin for the sample of rat plasma. The molecular weight of renin substrate, determined from its K_{av} on the column, was estimated in two series of experiments where the sequence in which the samples were run down the column was reversed (Table 5).

The position of elution of renin in the kidney sample differed from the elution profile of renin substrate and the mol.wt. of renin was calculated as 37000. The specific concentration of renin substrate in the peak fraction (50 ml of eluate) was calculated as 2.8 pmol/mg of protein for kidney, 1.0 for liver and 2.7 for plasma.

Discussion

The present study constitutes the first measurement of renin substrate in kidney tissue. Renin substrate was detected in rat kidney cortex in particles having a lower sedimentation velocity than renin granules. The density of the granules containing renin substrate, determined by isopycnic gradient centrifugation, was also less than the density of renin granules, and differed from the density of equilibration of mitochondria, lysosomes and protein in the heavy-granule

Fig. 6. *Sephadex G-100 column chromatography of an extract of the heavy-granule fraction of rat kidney (a) and liver (b) and rat plasma (c)*

The elution profile of renin substrate (●) was similar for each tissue, but differed from that of protein (○) and the position of renin (▲) in the kidney sample.

Table 5. Molecular weights of renin substrate determined by Sephadex G-100 chromatography of extracts of the heavy-granule fraction of kidney cortex and liver and of rat plasma

Values were calculated from a plot of partition coefficient (K_{av}) against molecular weight for a series of protein standards. The mean $\pm$ S.E.M. of two separate chromatographic determinations is shown.

	Kidney	Liver	Plasma
K_{av}	0.1358 ± 0.0020	0.1349 ± 0.0046	0.1382 ± 0.0005
Mol.wt.	$61\,580 \pm 150$	$61\,700 \pm 400$	$61\,380 \pm 40$

fraction and light-granule fraction of a cytoplasmic extract of rat kidney cortex. The results suggest that within the renal cortex there are cells that contain renin substrate in a single population of granules which differ from the renin-storage granules, as well as the other major intracellular constituents.

The granules containing renin substrate in kidney appeared to have a higher sedimentation velocity than the granules containing renin substrate in liver. Other work, based on experiments *in vitro* with isolated perfused rat liver (Nasjletti & Masson, 1971, 1972) and slices of rat liver (Freeman & Rostorfer, 1972), suggests that renin substrate is continuously synthesized and released by rat liver, which is thought to be the source of the plasma protein. The presence in liver of renin substrate in smaller vesicles rather than large heavy vesicles, such as those in which secretory products such as renin are stored before secretion, is consistent with this concept. Since renin substrate in kidney was detected in a heavier, and presumably larger, type of vesicle than that in liver it is possible that in the kidney the protein may be stored by renal cells rather than continuously synthesized and released. The specific concentration of renin substrate in kidney granules, estimated from the results of differential centrifugation, was 3–6 pmol/mg of protein after correction for losses owing to rupture of granules during homogenization, and was 2–3 for liver granules and 2–5 for plasma. The amount of renin substrate in kidney granules was estimated as 15–20 pmol/g wet wt. of tissue and in liver granules was 10–15 pmol/g. However, since the weight of liver tissue in the rat is ten times the weight of renal cortical tissue, the total liver content of renin substrate per animal may be six to eight times the amount in the renal cortex. If the cells containing renin substrate in the renal cortex are localized in a specific region such as the juxtaglomerular apparatus, then the local concentration may be quite high.

The molecular weight of renin substrate in kidney, liver and plasma was similar (61 000–62 000) and is preliminary evidence for a similarity in the molecular nature of the protein in these three tissues. The molecular weight was slightly greater than the value of 57 000 reported by Skeggs *et al.* (1963) for renin substrate in pig plasma. The similarity in size of renin

substrate in rat kidney, plasma and liver could be explained on the basis of uptake of the plasma protein by renal cells. However, in preliminary experiments where rats were injected intravenously with Triton WR-1339, which binds to plasma proteins and lowers the equilibrium density of subcellular organelles which accumulate the complex from the circulation (Wattiaux-de Coninck *et al.*, 1965; Beaufay, 1972), we have been unable to demonstrate an alteration in the equilibrium density of the renin-substrate granules. The results are consistent with an alternative explanation, namely synthesis *de novo* and storage of renin substrate by cells in the renal cortex.

The type of cells containing renin substrate in the kidney cortex is suggested by a report of the production of angiotensin I by glomeruli isolated from rat kidney (Finkelman & Nahmod, 1969). This supports the concept that renin substrate may be present in, and react with, renin in the juxtaglomerular cells of the renal cortex. Since puromycin and actinomycin D abolished this effect, it has been suggested that renin substrate may be continuously synthesized in small amounts and serve as the rate-limiting molecule for the production *in vitro* of angiotensin I by the juxtaglomerular cells (Goldstein *et al.*, 1973). Production of angiotensin I by isolated glomeruli has also been observed in this laboratory (B. J. Morris, unpublished work).

In the present studies renin substrate has been demonstrated in specific subcellular granules in rat kidney cortex, and these granules may serve as a site of storage for the protein. Renin was also found in heavy granules, but these appeared to be slightly more dense. If the renin-substrate granules are located in the juxtaglomerular cells, as is renin itself, then stimuli such as changes in renal haemodynamics or ions in the distal tubule may act on vesicle membranes to increase their capacity for fusion with other vesicles. Fusion of renin granules with renin-substrate granules would result in the formation of angiotensin I within the juxtaglomerular cell. The rate of formation of angiotensin I within the fused granules would then depend on the activity of renin. In previous studies we have suggested that renin granules may contain an inactive form of renin which may be activated by a specific proteinase subsequent to fusion of

renin granules with lysosomes (Morris & Lumbers, 1972; Morris & Johnston, 1973). Thureau *et al.* (1972) have further provided evidence that the concentration of Na^+ in the distal tubule may regulate the process of activation of inactive renin. If stimuli also increase the capacity of fusion of the cell membrane, this would cause increased secretion of renin, renin substrate and angiotensin I into interstitial fluid, where further reaction may occur.

Converting enzyme has been demonstrated in the microsomal fraction of kidney (Yang *et al.*, 1971), where it is present in amounts eight times greater than in the microsomal fraction of lung (Oshima *et al.*, 1974). Renal converting enzyme may be located in the cell membrane, as it is in the lung, and Oshima *et al.* (1974) suggest that it may be physically separated from the renal circulation to serve a local intrarenal function. Further, since isolated glomeruli have been shown to convert angiotensin I into angiotensin II (Leckie *et al.*, 1972) and converting enzyme activity has been measured in the juxtaglomerular apparatus (Granger *et al.*, 1972) it is likely that the juxtaglomerular cells may contain converting enzyme in the cell membrane. Conversion of angiotensin I to angiotensin II may occur here or in more distal vessel walls, e.g. in the efferent arteriole, subsequent to transport of angiotensin I in renal plasma, lymph or interstitial fluid. Angiotensin II is believed to constrict vascular smooth-muscle cells by binding to a specific receptor (Devynck *et al.*, 1973) with resultant release of Ca^{2+} from the cell membrane (Baudouin *et al.*, 1972) and contraction of myofibrils. The juxtaglomerular cells contain contractile elements (Biava & West, 1966) and locally produced angiotensin II may constrict the afferent arteriole to decrease glomerular filtration rate. Alternatively, constriction of the efferent arteriole would increase glomerular filtration rate. However, the exact site or sites of action of angiotensin II formed intrarenally remains to be resolved. On the basis of the present work, mechanisms may also be proposed in which renin substrate is elaborated by cells other than those that produce renin.

Renin substrate in specific cytoplasmic granules therefore may provide yet another control mechanism in the renin-angiotensin system. Thus the rate of fusion of these granules with renin granules, conversion of inactive renin into active renin and the anatomical site within the kidney provide areas where control could be exerted.

The present study was supported by Grants-in-Aid from the National Health and Medical Research Council and the National Heart Foundation of Australia.

References

Andersch, M. A. & Szczypinski, A. J. (1947) *Am. J. Clin. Pathol.* **17**, 571–574

- Anderson, N. G. (1955) *Exp. Cell Res.* **9**, 446–459
- Baillie, M. D., Rector, F. C., Jr. & Seldin, D. W. (1971) *J. Clin. Invest.* **50**, 119–126
- Baudouin, M., Meyer, P., Fermandjian, S. & Morgat, J. L. (1972) *Nature (London)* **235**, 336–338
- Beaufay, H. (1972) in *Lysosomes: A Laboratory Handbook* (Dingle, J. T., ed.), pp. 1–45, North-Holland Publishing Co., Amsterdam and London
- Biava, C. G. & West, M. (1966) *Am. J. Pathol.* **49**, 679–721
- Brightwell, R. & Tappel, A. L. (1968) *Arch. Biochem. Biophys.* **124**, 333–343
- Cooperstein, S. J. & Lazarow, A. (1951) *J. Biol. Chem.* **189**, 665–670
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Devynck, M.-A., Pernollet, M.-G., Meyer, P., Fermandjian, S. & Fromageot, P. (1973) *Nature (London) New Biol.* **245**, 55–58
- Finkelman, S. & Nahmod, V. E. (1969) *Nature (London)* **222**, 1186–1188
- Freeman, R. H. & Rostorfer, H. H. (1972) *Am. J. Physiol.* **223**, 364–370
- Goldstein, D. J., Diaz, A., Finkelman, S., Nahmod, V. E. & Fischer-Ferraro, C. (1973) *Proc. Soc. Exp. Biol. Med.* **142**, 793–795
- Granger, P., Dahlheim, H. & Thureau, K. (1972) *Kidney Int.* **1**, 78–88
- Guyton, A. C., Langston, J. G. & Navar, B. (1964) *Circ. Res.* **15**, Suppl. 1, 187–197
- Haas, E., Goldblatt, H., Gipson, E. C. & Lewis, L. (1966) *Circ. Res.* **19**, 739–749
- Hofbauer, K. G., Zschiedrich, H., Rauh, W., Orth, H. & Gross, F. (1973) *Proc. Soc. Exp. Biol. Med.* **142**, 796–799
- Horky, K., Rojo-Ortega, J. M., Rodriguez, J., Boucher, R. & Genest, J. (1971) *Am. J. Physiol.* **220**, 307–311
- Johnston, C. I., Mendelsohn, F. A. O. & Doyle, A. E. (1972) *Circ. Res.* **30/31**, Suppl. 2, 203–213
- Kanarek, L. & Hill, R. L. (1964) *J. Biol. Chem.* **239**, 4202–4206
- Krahé, P., Hofbauer, K. G. & Gross, F. (1970) *Life Sci.* **9**, 1317–1320
- Leckie, B., Gavras, H., McGregor, J. & McElwee, G. (1972) *J. Endocrinol.* **55**, 229–230
- Lever, A. F. & Peart, W. S. (1962) *J. Physiol.* **160**, 548–563
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Matsunaga, M. (1971) *Jpn. Circ. J.* **35**, 333–338
- Morris, B. J. & Johnston, C. I. (1973) *Proc. Aust. Soc. Med. Res.* **3**, 177 (Abstr.)
- Morris, B. J. & Lumbers, E. R. (1972) *Biochim. Biophys. Acta* **289**, 385–391
- Nasjletti, A. & Masson, G. M. C. (1971) *Can. J. Physiol. Pharmacol.* **49**, 931–932
- Nasjletti, A. & Masson, G. M. C. (1972) *Circ. Res.* **30/31**, Suppl. 2, 187–202
- Nishimura, H. & Sokabe, H. (1968) *Jpn. Heart J.* **9**, 494–503
- Nordlie, R. C. & Arion, W. J. (1966) *Methods Enzymol.* **9**, 619–625
- Orth, H., Krahé, P., Steigelmann, C. & Gross, F. (1971) *Pfluegers Arch.* **329**, 125–135
- Oshima, G., Gecse, A. & Erdös, E. G. (1974) *Biochim. Biophys. Acta* **350**, 26–37

- Page, I. H. & Bumpus, F. M. (1961) *Physiol. Rev.* **41**, 331–390
- Page, I. H., McSwain, B., Knapp, G. M. & Andrus, W. de W. (1941) *Am. J. Physiol.* **135**, 214–222
- Parvin, R. & Smith, R. A. (1969) *Anal. Biochem.* **27**, 65–72
- Regoli, D., Riniker, B. & Brunner, H. (1963) *Biochem. Pharmacol.* **12**, 637–646
- Shibko, S. & Tappel, A. L. (1965) *Biochem. J.* **95**, 731–741
- Skeggs, L. T., Lentz, K. E., Hochstrasser, H. & Kahn, J. R. (1963) *J. Exp. Med.* **118**, 73–98
- Skinner, S. L. (1967) *Circ. Res.* **20**, 391–402
- Thurau, K. (1964) *Am. J. Med.* **36**, 698–719
- Thurau, K. W. C., Dahlheim, H., Grüner, A., Mason, J. & Granger, P. (1972) *Circ. Res.* **30/31**, Suppl. 2, 182–186
- Thurau, K., Mason, J., Takabatake, T. & Olbricht, Ch. (1974) *Coll. Inst. Nat. Santé Rech. Méd.* **30**, 121–130
- Wagner, J. G. (1967) *Clin. Pharmacol. Ther.* **8**, 201–218
- Wattiaux-de Coninck, S., Rutgeerts, M.-J. & Wattiaux, R. (1965) *Biochim. Biophys. Acta* **105**, 446–459
- Wróblewski, F. & LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90**, 210–213
- Yang, H. Y. T., Erdös, E. G. & Levin, Y. (1971) *J. Pharmacol. Exp. Ther.* **177**, 291–300