The rapid purification and partial characterization of human serum angiotensinogen

Callum J. CAMPBELL, Peter A. CHARLTON, Christine J. GRINHAM, Christopher J. MOONEY and Jane E. PENDLEBURY

Mammalian Biochemistry Department, Glaxo Group Research Limited, Greenford Road, Greenford, Middx. UB6 0HE, U.K.

Human angiotensinogen has been purified 390-fold from serum by a rapid high-yielding procedure that involved chromatography on Blue Sepharose, phenyl-Sepharose, hydroxyapatite and immobilized 5-hydroxytryptamine (5-HT). Angiotensinogen was specifically bound to immobilized 5-HT, which effected a partial resolution into multiple forms, which were also evident when analysed by SDS/polyacrylamide-gel electrophoresis (M_r 59400, 60600, 62600 and 63800). This heterogeneity was confirmed by resolution into six main bands on isoelectric focusing, ranging from pI 4.40 to 4.82. *N*-terminal analysis, digestion with human renal renin and deglycosylation studies implied that the preparation comprised several forms of angiotensinogen, varying in their degree of glycosylation. The presence of sialic acid was shown to be a major factor in determining the heterogeneity.

INTRODUCTION

Angiotensinogen (renin substrate), an α_2 -globulin glycoprotein, is synthesized mainly by the liver and secreted into the bloodstream, where, in the human (Printz *et al.*, 1977; Lentz *et al.*, 1978; Faiers *et al.*, 1978; Kokubu *et al.*, 1980; Tewksbury, 1983) and other species (Skeggs *et al.*, 1963; Printz & Skidgel, 1981; Hilgenfeldt & Hackenthal, 1982), it has been shown to exist in multiple forms. The decapeptide angiotensin I is then released by the action of circulating renin (EC 3.4.23.15) and subsequent conversion by a dipeptidyl carboxypeptidase (angiotensin-converting enzyme, EC 3.4.15.1) produces angiotensin II, which plays a major role in the control of blood pressure and in fluid and electrolyte homeostasis (Reid *et al.*, 1978; Skeggs *et al.*, 1980).

In order to study the kinetics of inhibition of the renin-angiotensinogen reaction, both purified renin and substrate were required. Renal renin could be purified efficiently by a single affinity-chromatography step (McIntyre *et al.*, 1983), and human angiotensinogen could be purified from plasma to apparent homogeneity by extended procedures (Slater & Strout, 1981; Tewksbury, 1983; Sato *et al.*, 1984; Wintroub *et al.*, 1984; Fyhrquist *et al.*, 1985). Our requirement for the rapid purification of angiotensinogen in good yield prompted us to optimize existing procedures. Subsequent characterization of human angiotensinogen purified by this improved procedure confirmed that varying degrees of glycosylation contributed to the micro-heterogeneity of angiotensinogen (Campbell *et al.*, 1985) and unequivocally implicated sialic acid as the major contributor.

MATERIALS AND METHODS

Materials

Biochemicals and 6-aminohexanoic acid-activated Sepharose-4B were obtained from Sigma Chemical Co., Poole, Dorset, U.K., glycopeptidase F was from Boehringer Mannheim, Lewes, East Sussex, U.K., and

BDH Chemicals, Dagenham, Essex, U.K., supplied $(\text{NH}_4)_2\text{SO}_4$ (specially low in heavy metals) and Electran grade electrophoresis reagents. LKB Instruments, South Croydon, Surrey, U.K., supplied Ampholines, Pharmacia, Milton Keynes, Bucks., U.K., Blue Sepharose CL-6B and phenyl-Sepharose CL-4B, and Bio-Rad Laboratories, Watford, Herts., U.K., hydroxyapatite (Bio-Gel HTP). Anti-AI antibody was purchased from Miles Laboratories, Stoke Poges, Berks., U.K., and Aldrich Chemical Co., Gillingham, Dorset, U.K., supplied TFMS. Purified human renin was prepared from cadaver kidney essentially by the method of McIntyre *et al.* (1983), and fresh blood was obtained from female volunteers ingesting oral contraceptives.

Methods

Assays. Angiotensinogen in column fractions was determined by incubation with purified human renal renin (0.0016 Goldblatt unit; 37 °C, pH 7.4) for 1 h, whereas angiotensinogen concentrations were determined by an end-point assay by incubation as above, but for 20 h. Liberated AI was measured by an antibody-trapping radioimmunoassay technique essentially as described by Poulsen and Jorgensen (1974) and Szelke *et al.* (1982). Protein concentration was measured as described by Bradford (1976), with bovine serum albumin as standard.

Preparation of 5-HT-Sepharose. Activated 6-aminohexanoic acid-Sepharose 4B (45 g) was coupled (16 h) in 0.1 M- NaHCO_3 /0.5 M- NaCl , pH 8.0 (300 ml), to 5-HT (392 mg), the remaining active groups were blocked (3 h) with 1 M-ethanolamine, pH 8.5, and the gel was washed according to the manufacturers' (Pharmacia) instructions. All steps were undertaken at 4 °C.

Preparation of serum. Blood (600 ml) was allowed to coagulate on ice (1–1.5 h) and then centrifuged (10000 *g*, 30 min, 4 °C). Serum (300 ml) was stored at –30 °C until required, when it was re-centrifuged as above.

Abbreviations used: AI, angiotensin I; 5-HT, 5-hydroxytryptamine; IEF, isoelectric focusing; TFMS, trifluoromethanesulphonic acid.

Preparation of des-AI-angiotensinogen. Purified angiotensinogen (140 μ g) was incubated (20 °C, 16 h) in 50 mM-sodium phosphate buffer, pH 7.0 (0.12 ml), with human renal renin (0.05 Goldblatt unit).

Periodate oxidation. Purified angiotensinogen (250 μ g) was incubated (4 °C, 0.5 h or 2 h) in 80 mM-sodium acetate buffer/20 mM-sodium periodate, pH 5 (0.63 ml). The reaction was stopped by the addition of 600 mM-glucose (0.13 ml) and dialysed against 2 mM-sodium phosphate buffer, pH 6.9 (500 ml).

Desialylation by neuraminidase (*Clostridium perfringens*; EC 3.2.1.18). Purified angiotensinogen (130 μ g) was incubated (20 °C, 20 h) with neuraminidase (0.5 unit) in 80 mM-sodium acetate buffer, pH 5 (0.1 ml). Liberated sialic acid was measured by the periodic acid–thiobarbituric acid method of Aminoff (1961).

Deglycosylation by glycopeptidase F (*Flavobacterium meningosepticum*; EC 3.2.2.18). Angiotensinogen (40 μ g) was incubated (37 °C, 18 h) with glycopeptidase F (4 munits) and 100 mM-sodium phosphate buffer/0.5% Triton X-100/5 mM-mercaptoethanol, pH 7.4, in a final volume of 0.1 ml.

Deglycosylation by TFMS. This was carried out essentially as described by Kalyan & Bahl (1981). Freeze-dried angiotensinogen (130 μ g) was made to react (0–2 °C, 5 h) with TFMS (0.1 ml) and anisole (0.1 ml) under nitrogen, and the reaction was terminated by the rapid addition of ice-cold aq. 37% (v/v) pyridine (0.27 ml). The reaction mixture was thoroughly dialysed against several changes of water (3 $\times$ 2 l) and freeze-dried.

N-Terminal sequence analysis. Automated Edman degradation was carried out on an Applied Biosystems model 470A gas-phase protein sequencer in series with a model 120A PTH analyser.

Purification methods. All purification, concentration (Amicon, PM10) and dialysis steps were performed at 4 °C. Small samples were removed at each purification step and stored at –30 °C before the determination of specific activities.

Serum (265 ml) was loaded on to a Blue Sepharose column (4.4 cm $\times$ 43.5 cm) previously equilibrated in 50 mM-Tris/HCl, pH 8. The column was washed with equilibration buffer until the A_{280} approached zero. Fractions containing angiotensinogen were pooled (378 ml) and adjusted to 20% satn. with $(\text{NH}_4)_2\text{SO}_4$ and to pH 7 with 1 M-HCl. This solution (390 ml) was chromatographed on phenyl-Sepharose (3.2 cm $\times$ 56.5 cm) previously equilibrated with 25 mM-sodium phosphate buffer/0.8 M- $(\text{NH}_4)_2\text{SO}_4$, pH 7.2. After the column was washed with equilibration buffer (800 ml), followed by a gradient from equilibration buffer to water (1.5 l total), angiotensinogen was eluted with water (800 ml). The active fractions (57 ml) were chromatographed on hydroxyapatite (4.4 cm $\times$ 29 cm) after equilibration with 2 mM-sodium phosphate buffer, pH 6.8. The column was washed with equilibration buffer until the A_{280} approached zero (700 ml). Angiotensinogen was eluted by a 2–60 mM-sodium phosphate buffer gradient, pH 6.8 (2 l total), followed by 60 mM-sodium phosphate buffer, pH 6.8 (1 l). Active fractions were pooled

(1082 ml) and concentrated (198 ml) before being loaded on to 5-HT-Sepharose (3.2 cm $\times$ 7 cm) previously equilibrated with 50 mM-sodium phosphate buffer, pH 7. The column was washed consecutively with equilibration buffer (500 ml), 100 mM-sodium phosphate buffer, pH 7 (600 ml), 100 mM-sodium phosphate buffer/250 mM-NaCl, pH 7 (600 ml), and then 200 mM-sodium formate buffer, pH 3 (200 ml). Angiotensinogen was selectively eluted during the 250 mM-NaCl wash. After concentration and dialysis against 50 mM-sodium phosphate buffer, pH 7, the angiotensinogen was stored at –30 °C.

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970), with vertical slab gels [stacking 3% (w/v) and resolving 7.5% (w/v) acrylamide; 0.1% SDS]. Analytical IEF was undertaken on both narrow- (3.5–5.2) and broad- (3.5–9.5) pH-range gels [5% (w/v) acrylamide, 2% (w/v) ampholyte]. The gels were prepared, fixed and stained, and focusing was performed at 10–14 °C, as described by Winter *et al.* (1977). The pH gradient was measured with a flat surface combination micro-electrode (Orion Research). The presence of carbohydrate in the angiotensinogen sample after IEF was detected by periodic acid–Schiff stain [see Polyacrylamide Gel Electrophoresis Laboratory Techniques (Pharmacia, 1980)].

RESULTS

5-HT-Sepharose

Angiotensinogen, in 50 mM-sodium phosphate buffer, pH 7, was bound by 5-HT immobilized to Sepharose via a spacer arm, and higher-ionic-strength buffer (100 mM-sodium phosphate buffer/250 mM-NaCl, pH 7) was required for desorption. A control gel, devoid of 5-HT, failed to bind angiotensinogen under identical conditions, implying that 5-HT contributed significantly to binding. Non-specific binding to the spacer arm seemed unlikely, as the conditions employed for elution (high ionic strength) should favour binding through hydrophobic interactions. Furthermore, angiotensinogen could be eluted from 5-HT that was immobilized directly to Sepharose by buffers of low ionic strength, suggesting the spacer arm was promoting favourable interactions between 5-HT and angiotensinogen. The chromatographic behaviour of angiotensinogen treated with periodate or neuraminidase on 5-HT-Sepharose suggested that the mode of binding was complex; treated angiotensinogen bound to the gel although the dimensions of the eluted peak, but not the area, changed, and occasionally a shoulder appeared. Similar binding characteristics were exhibited by fetuin and asialofetuin.

The potential use of the interaction between 5-HT and angiotensinogen was highlighted by preliminary chromatographic studies. Human plasma angiotensinogen was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation and chromatography by ion-exchange and hydroxyapatite. The hydroxyapatite column resolved angiotensinogen into two activity peaks, which subsequently were chromatographed on 5-HT-Sepharose. A considerable degree of purification was observed (Fig. 1).

Purification of angiotensinogen from serum

Serum, rather than plasma, was used as a source of angiotensinogen to lessen the possibility of fibrin deposition during chromatography on Blue Sepharose.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of angiotensinogen partially purified from plasma

Hydroxyapatite chromatography resolved the preparation into two peaks of activity. Each peak was then chromatographed separately on 5-HT-Sepharose. Tracks 1 and 3, before 5-HT-Sepharose (5 µg); tracks 2 and 4, after 5-HT-Sepharose (1.5 µg); track 5, standards (2 µg each).

There was no significant difference between the plasma and serum concentrations of angiotensinogen, implying that serum was a slightly enriched source. The purification is summarized in Table 1. The Blue Sepharose and phenyl-Sepharose steps removed approx. 97% of total serum protein, with a concomitant recovery of approx. 95% angiotensinogen. Hydroxyapatite chromatography gave a 7-fold purification, and 5-HT-Sepharose removed all residual contaminating proteins and resulted in a pure preparation.

Homogeneity of purified angiotensinogen

SDS/polyacrylamide-gel-electrophoretic analysis confirmed the success of the purification (Fig. 2). Purified

Fig. 2. SDS/polyacrylamide-gel electrophoresis of angiotensinogen through the purification procedure

Tracks: 1, serum (52 µg); 2, Blue Sepharose (16 µg); 3, phenyl-Sepharose (9.8 µg); 4, hydroxyapatite (5 µg); 5, 5-HT-Sepharose (5 µg); 6, as track 5 (1.3 µg); 7, standards (2 µg each).

angiotensinogen was resolved into four bands of M_r 63 800, 62 600, 60 600 and 59 400. The species of M_r 63 800 and 62 600 were routinely found to be the predominant forms. This heterogeneity was confirmed by IEF analysis on a broad-pH-range gel, which resolved the preparation into six major and two minor bands (Fig. 3). A similar phenomenon was observed on a narrow-pH-range gel (Fig. 3). Subsequent studies were therefore designed to prove that the heterogeneity observed was due to multiple forms of angiotensinogen rather than to contaminating protein.

Transient-state IEF

Angiotensinogen and des-AI-angiotensinogen reportedly differ slightly in pI value, and can be separated by transient-state IEF (Auzan *et al.*, 1985). Discernible differences between samples of angiotensinogen and des-AI-angiotensinogen, when focused through the transient state to the steady state, were maximal after

Table 1. Summary of angiotensinogen purification

Specific activities in excess of theoretical (26.1 µg of AI/mg of protein) were routinely obtained. The quantitative end-point estimation of angiotensinogen concentration is thought to be responsible for this discrepancy.

Purification step	Vol. (ml)	Protein (mg)	Activity (µg of AI)	Sp. activity (µg of AI/mg)	Yield (%)	Purification (fold)
1. Serum	265	18285	1961	0.11	100	1
2. Blue Sepharose	378	3954	1845	0.47	94.1	4.2
3. Phenyl-Sepharose	57	468	1870	4.00	95.4	36.4
4. Hydroxyapatite	63	36.4	1052	28.9	53.6	263.1
5. 5-HT-Sepharose	10.5	14.4 (16.7)*	617	42.9 (36.9)*	31.5	390.2

* Protein measured by quantitative amino acid analysis.

Fig. 3. IEF of purified angiotensinogen on broad- and narrow-pH-range analytical gels

(a) Angiotensinogen (6.6 μ g) on a broad-pH-range gel. Tracks 1 and 2, before and after treatment with neuraminidase. (b) Transient-state IEF of angiotensinogen (18 μ g) on a narrow-pH-range gel. Tracks 1 and 2, before and after exhaustive incubation with renin as described in the text. Samples were applied at the cathode and focused for 30, 60, 120 and 200 min.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of angiotensinogen treated with renin, neuraminidase, glycopeptidase F and TFMS

Samples were incubated as described in the text. Tracks 1, control; 2, renin; 3, neuraminidase; 4, neuraminidase plus renin; 5, glycopeptidase F; 6, glycopeptidase F plus renin; 7, TFMS; 8, standards (2 μ g each). Angiotensinogen loaded: tracks 1-4 and 7, 8 μ g; tracks 5 and 6, 4 μ g.

120 min (Fig. 3). Scanning densitometry suggested that all the species comprising des-AI-angiotensinogen had moved slightly to more acidic pI values, and implied that the heterogeneity was due to multiple forms of angiotensinogen. The range of pI values at the steady (200 min) state shifted from 4.82-4.40 (angiotensinogen) to 4.75-4.34 (des-AI-angiotensinogen). Periodic acid-Schiff staining of the separated forms suggested that each was a glycoprotein, although the carbohydrate content, as judged by stain intensity, appeared to differ.

Deglycosylation of angiotensinogen

Desialylation with neuraminidase resulted in an average decrease in M_r of approx. 3000, with a small but significant decrease in heterogeneity on SDS/polyacrylamide-gel electrophoresis (Fig. 4). More profound differences were observed on analytical IEF after neuraminidase treatment (Fig. 3). Not only was

angiotensinogen converted into less acidic forms, with major bands at pI 6.02 and 6.09 and minor bands at pI 5.80, 6.26 and 6.32, but the degree of heterogeneity had decreased significantly.

Deglycosylation with TFMS markedly decreased the heterogeneity evident on SDS/polyacrylamide-gel electrophoresis, and resulted in two main species, of M_r 50600 and 52400, and treatment with glycopeptidase F resulted in main species of M_r 47600, 50400 and 53700, with minor bands, possibly representing deglycosylation intermediates, at M_r 54600 and above (Fig. 4). These changes on average represented a decrease in M_r of approx. 10000, with no evidence of any residual protein at approx. M_r 60000. Treatment of angiotensinogen with glycopeptidase F or glycopeptidase F plus renin gave identical patterns on SDS/polyacrylamide-gel electrophoresis, except that the latter gave species that were all approx. M_r 1000 greater. This unexpected increase in apparent M_r was also observed after treating native angiotensinogen with renin. Glycopeptidase F treatment did not significantly alter the IEF pattern, and the insoluble nature of TFMS-treated angiotensinogen precluded analysis by IEF.

N-Terminal sequence analysis

The first 20 amino acid residues of the published sequence were confirmed by Edman degradation, except for the asparagine in position 14, which is thought to be a potential glycosylation site (Kageyama *et al.*, 1984). There was no evidence for any secondary sequence.

DISCUSSION

The interaction of *N*-acetylneuraminic acid with 5-HT has been described in some biologically important systems (Dette & Wesemann, 1978), and the interaction of sialoglycoconjugates, sialic acids and their derivatives with immobilized 5-HT has been thoroughly investigated (Sturgeon & Sturgeon, 1982; Corfield *et al.*, 1985), although the importance of certain functional groups in sialic acids and 5-HT required for specific binding is disputed. Human angiotensinogen used in this study exhibited a high affinity for 5-HT-Sepharose and, although modification of the C_7 - C_9 side chain of *N*-acetylneuraminic acid by periodate oxidation and desialylation with neuraminidase altered the characteristics of angiotensinogen binding, the ability to bind was not abolished. With neuraminidase treatment, the exposure of new terminal residues (e.g. β -galactose), capable of interacting with 5-HT, may have contributed to the altered elution profile. The possibility that the multiple forms of angiotensinogen have different degrees of resistance to periodate oxidation and desialylation (see Hilgenfeldt & Hackenthal, 1982) should not be overlooked. Nevertheless, assuming an average content of 7 mol of sialic acid/mol of angiotensinogen (Tewksbury, 1983), not only was a quantitative recovery of sialic acid observed, but there was evidence from SDS/polyacrylamide-gel electrophoresis and IEF studies that the heterogeneity decreased after neuraminidase treatment. The contribution of other sugar residues to the binding of angiotensinogen to immobilized 5-HT could be established by monitoring the affinity, after the sequential removal of sugar residues by various exoglycosidases. To elucidate the mode of binding, further studies would be required.

The purification procedure described has several advantages over those in the literature (Slater & Strout, 1981; Tewksbury, 1983; Sato *et al.*, 1984; Wintroub *et al.*, 1984; Fyhrquist *et al.*, 1985). Firstly, serum rather than plasma was chosen because it is a cleaner source of angiotensinogen and, practically, is easier to handle. Secondly, careful design of the protocol enabled purification to homogeneity in only four steps, and dispensed with the requirement for intermediate dialysis stages, resulting in a rapid high-yielding purification. Thirdly, the introduction of 5-HT-Sepharose affinity chromatography as a final step proved very successful in removing all residual contaminating protein. Introduction of this step earlier in the protocol proved less successful, owing to the interaction of 5-HT with other sialoglycoconjugates (as discussed above). There was some evidence that chromatography on 5-HT-Sepharose partially resolved angiotensinogen into the different molecular forms observed in both IEF and SDS/polyacrylamide-gel-electrophoresis studies.

Analysis by SDS/polyacrylamide-gel electrophoresis resolved angiotensinogen into four species in the range M_r 59400–63800. These values corresponded well to those reported by Wintroub *et al.* (1984) and Tewksbury (1983). Surprisingly, the multiple forms present in our angiotensinogen preparation, after exhaustive incubation with renin, routinely showed a slight increase in M_r (approx. 1000–1300) on SDS/polyacrylamide-gel electrophoresis, although a quantitative release of AI could be demonstrated by radioimmunoassay. The removal of AI, which under the conditions used will not be a highly charged species, should not adversely affect the electrophoretic behaviour of des-AI-angiotensinogen in SDS unless gross conformational changes are induced. In agreement with Auzan *et al.* (1985), des-AI-angiotensinogen appeared less basic than angiotensinogen during the transient state of IEF. At the steady state, however, in contrast with the small cathodic shift (0.1 pH unit) noted by Auzan *et al.* (1985), we observed a small anodic shift in pI value (0.04–0.10 pH unit), implying that des-AI-angiotensinogen was slightly less positively charged.

The M_r difference on SDS/polyacrylamide-gel electrophoresis between the pairs of bands observed at M_r 63800 and 62600 and M_r 60600 and 59400 was identical (1200). This implied that the latter and former pairs were related by a change in M_r of 3200, possibly owing to altered carbohydrate content. The involvement of *O*-glycosylation was suggested by the failure of glycopeptidase F (which should effect complete *N*-deglycosylation) to decrease the observed heterogeneity of angiotensinogen. Treatment of angiotensinogen with TFMS produced extensive, although incomplete, deglycosylation. The M_r values of 50600 and 52400 observed after this treatment were in good agreement with a total carbohydrate content of 14% reported for the glycosylated form (Tewksbury, 1983) and with the M_r value deduced, from the cloned cDNA sequence, for the unglycosylated form (Kageyama *et al.*, 1984). In contrast with the results of Faiers *et al.* (1978), neuraminidase treatment of angiotensinogen showed a significant decrease in heterogeneity. Incomplete removal of resistant sialic acids (Hilgenfeldt & Hackenthal, 1982) may account for several of the minor bands observed on IEF analysis after neuraminidase treatment. These IEF studies suggested that sialic acid was a major determinant of human angiotensinogen's apparent heterogeneity.

Furthermore, as *N*-deglycosylation with glycopeptidase F did not significantly alter the heterogeneity, it would appear that sialic acid may be located preferentially on carbohydrate chains linked *O*-glycosidically to the polypeptide. This concept is supported by Campbell *et al.* (1985), who noted that some forms of angiotensinogen secreted from tunicamycin-treated Hep G2 cells were modified by neuraminidase treatment.

IEF studies of angiotensinogen and *N*-terminal sequence analysis suggested that the apparent heterogeneity was due to multiple forms varying in carbohydrate content, rather than to protein contamination, and the range of pI values observed support the results quoted in the literature (Printz *et al.*, 1977; Faiers *et al.*, 1978; Lentz *et al.*, 1978; Kokubu *et al.*, 1980; Wintroub *et al.*, 1984).

The four-step purification of angiotensinogen described was rapid, high-yielding and involved a novel use of 5-HT-Sepharose as an affinity ligand. The product, by the criteria described above, appeared to be homogeneous.

The *N*-terminal sequence analysis was undertaken by Dr. A. Aitken (London School of Pharmacy) and is gratefully acknowledged.

REFERENCES

- Aminoff, D. (1961) *Biochem. J.* **81**, 384–392
- Auzan, C., Genain, C., Corvol, P., Menard, J. & Chrambach, A. (1985) *Electrophoresis* **6**, 201–206
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Campbell, D. J., Bouhnik, J., Coezy, E., Menard, J. & Corvol, P. (1985) *J. Clin. Invest.* **75**, 1880–1893
- Corfield, A. P., Winterburn, P. J., Clamp, J. R., Bolton, C. H. & Martin, J. (1985) *Biochem. Soc. Trans.* **13**, 956–957
- Detle, G. A. & Wesemann, W. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 399–406
- Faiers, A. A., Loh, A. Y. & Osmond, D. H. (1978) *Can. J. Biochem.* **56**, 892–899
- Fyhrquist, F., Rosenlöf, K., Grönhagen-Riska, C., Hortling, L. & Tikkanen, I. (1985) *Scand. J. Urol. Nephrol.* **90**, Suppl 1, 41–44
- Hilgenfeldt, U. & Hackenthal, E. (1982) *Biochim. Biophys. Acta* **708**, 335–342
- Kageyama, R., Ohkubo, H. & Nakanishi, S. (1984) *Biochemistry* **23**, 3603–3609
- Kalyan, N. K. & Bahl, O. P. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1246–1253
- Kokubu, T., Hiwada, K. & Sogo, Y. (1980) *Jpn. Circ. J.* **44**, 274–282
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lentz, K. E., Dorer, F. E., Kahn, J. R., Levine, M. & Skeggs, L. T. (1978) *Clin. Chim. Acta* **83**, 249–257
- McIntyre, G. D., Leckie, B., Hallett, A. & Szelke, M. (1983) *Biochem. J.* **211**, 519–522
- Poulsen, K. & Jorgensen, J. (1974) *J. Clin. Endocrinol. Metab.* **39**, 816–825
- Printz, M. P. & Skidgel, R. A. (1981) in *Heterogeneity of Renin and Renin-Substrate* (Sambhi, M. P., ed.), pp. 271–279, Elsevier/North-Holland, New York
- Printz, M. P., Printz, J. M., Lewicki, J. A. & Gregory, T. (1977) *Circ. Res.* **41**, Suppl. II, II-37–II-43
- Reid, I. A., Morris, B. J. & Ganong, W. F. (1978) *Annu. Rev. Physiol.* **40**, 377–410
- Sato, Y., Hiwada, K. & Kokubu, T. (1984) *Jpn. Circ. J.* **48**, 1236–1242
- Skeggs, L. T., Jr., Lentz, K. E., Hochstrasser, H. & Kahn, J. R. (1963) *J. Exp. Med.* **118**, 73–98

- Skeggs, L. T., Dorer, F. E., Levine, M., Lentz, K. E. & Kahn, J. R. (1980) in *The Renin-Angiotensin System* (Johnson, J. A. & Anderson, R. R., eds.), pp. 1-27, Plenum Press, New York
- Slater, E. E. & Strout, H. V., Jr. (1981) *J. Biol. Chem.* **256**, 8164-8171
- Sturgeon, R. J. & Sturgeon, C. M. (1982) *Carbohydr. Res.* **103**, 213-219
- Szelke, M., Leckie, B. J., Tree, M., Brown, A., Grant, J., Hallett, A., Hughes, M., Jones, D. M. & Lever, A. F. (1982) *Hypertension* **4**, Suppl. II, II-59-II-69
- Tewksbury, D. A. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 2724-2728
- Winter, A., Ek, K. & Andersson, U.-B. (1977) *LKB Application Note* 250
- Wintroub, B. U., Klickstein, L. B., Dzau, V. J. & Watt, K. W. K. (1984) *Biochemistry* **23**, 227-232

Received 22 July 1986/22 October 1986; accepted 1 December 1986