Isolation and Characterization of Renin-like Enzymes from Mouse Submaxillary Glands†

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ABSTRACT: The isolation, in stable and pure form, of a family of potent hypertensive, renin-like enzymes from the submaxillary gland of adult male mice is reported. The enzymes are chromatographically and electrophoretically distinct, but are antigenically related. The chemical and physical properties of two of the enzymes, salivary renins A and C, have been examined. Molecular weight values of 43,000 and 36,000–37,000 were obtained by gel filtration and by equilibrium sedimentation, respectively. The two enzymes differ in their isoelectric points; values of 5.4 and 5.6 for renins A and C, respectively, were obtained by isoelectric focusing. The amino acid compositions of the two enzymes were very similar. Positive periodate–Schiff tests indicate both are glycoproteins. Both salivary renins react with synthetic renin substrates to yield the expected products, and with whole plasma renin substrate to yield angiotensin-like pressor material. Both have direct and sustained pressor effects *in vivo*. Renin A has been obtained in crystalline form.

The morphological appearance and biochemical composition of the submaxillary glands of the mouse depend to a great extent on the hormonal status of the animal (Sreebny and Meyer, 1964). The administration of testosterone to female mice induces both tubular cell growth in the submaxillary gland and a marked increase in the levels of a number of specific proteolytic enzymes and hormone-like proteins. These include a hypertensive renin-like enzyme (Werle *et al.*, 1957, 1968; Bing and Farup, 1965; Oliver and Gross, 1967; Chiang *et al*, 1968; Takeda *et al.*, 1969), arginine esterases (Angeletti *et al.*, 1967), the nerve growth factor (Cohen, 1960), and the epidermal growth factor (Cohen, 1962). It has been suggested that the growth factors present in the gland may originate by specific proteolytic cleavages of larger precursor molecules (Taylor *et al.*, 1970).

In this paper we are reporting the isolation and characterization of a family of renin-like enzymes from the submaxillary gland of the adult male mouse. Although the marked hypertensive properties of renin and renin-like enzymes (due to the enzymatic formation of angiotensin) have been known for many years, renin has not yet been obtained in a stable pure form (Lee, 1969). Our purpose was to define biochemically the very potent hypertensive activity present in the salivary gland, thus permitting further enzymological, immunological, and physiological studies. In addition, these studies may help in the understanding of the interrelationships of the various hormone-like peptides and specific proteolytic enzymes induced by testosterone in the salivary gland.

Experimental Section

Materials and Methods. Renin activity was assayed with the fluorogenic renin substrate (benzyloxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-β-naphthylamine) of Reinharz and Roth (1969). The procedure measures the extent of cleavage of the Leu–Leu bond in terms of the quantity of β-naphthylamine liberated by subsequent digestion with aminopeptidase M. The assay mixture contained 0.05 M pyrophosphate buffer (pH 6.2), 0.1 mM zinc acetate, 150 μg of bovine albumin (Sigma, type F), 10 μg of substrate in 20 μl of dimethylformamide, and 0.1–1.0 μg of the renin preparation in a final volume of 250 μl. A blank without renin was run in each series of assays. The mixture was incubated for 1 hr at 37°, and the reaction terminated by heating the mixture for 5 min at 100°. After cooling, 10 μl of 2 M Tris-Cl buffer (pH 8.6) and 100 μg of aminopeptidase M (10 EU) were added. The final mixture (pH 7.75) was further incubated for 2 hr at 37° and the amount of free β-naphthylamine liberated from the substrate was measured by fluorometry as described by Reinharz and Roth (1969). All estimations were carried out in the range in which the amount of renin present was directly proportional to the quantity of β-naphthylamine liberated. Aminopeptidase M was obtained from Carl Roth Co., Karlsruhe, West Germany. The fluorogenic renin substrate was very kindly provided by Dr. M. Roth.

The enzymatic cleavage of the synthetic tetradecapeptide renin substrate (Schwarz-Mann) by salivary gland renin was examined in the following manner. The tetradecapeptide (500 μg) was incubated with 70 μg of the renin preparations for 4 hr at 37°. The incubation was carried out in 0.1 M sodium pyrophosphate-HCl buffer (pH 6.8) in a final volume of 250 μl. The reaction was terminated by the addition of 2 μl of glacial acetic acid. Thin-layer chromatography was performed on microcrystalline cellulose sheets obtained from Eastman-Kodak. Peptides were detected with the Pauly reagent.

For extinction coefficient determinations, measurements of protein concentrations were performed with the Spinco Model E analytical ultracentrifuge equipped with a Rayleigh interference optical system, in a synthetic boundary cell at 20° according to the conditions described by Chervenka (1969). Renin was dissolved in 0.1 M sodium acetate (pH 5.60). A value of 4.1 fringes/mg per ml was used as the average refractive increment for a typical protein solution (Babul and Stellwagen, 1969). Optical densities were measured at 280

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nm with a Beckman DU spectrophotometer in 0.1 M sodium acetate (pH 5.60), and in glass-distilled water, using a 1-cm path-length cuvet. No differences in protein absorbance were observed between the two solvents.

Sedimentation equilibrium studies were performed with a Spinco Model E analytical ultracentrifuge equipped with a split-beam photoelectric scanning system and a multiplex accessory. Scans were made at 280 nm using the slow scanning speed. Solutions of renin were exhaustively dialyzed against 0.1 M sodium acetate (pH 5.60), and a 3-mm liquid column of sample was layered over perfluorotributylamine (FC 43, Minnesota Mining and Manufacturing Co.) in the solution section of the cell with dialysate being added to the solvent sector. Determinations were made at 20° with a rotor velocity of 13,000 rpm for low-speed sedimentation equilibrium studies, and 32,000 rpm for high-speed (meniscus depletion) studies. Runs were made for 24–36 hr before scanning. Different protein solutions were examined over a 3- to 5-fold concentration range.

The apparent weight-average molecular weights were calculated from the sedimentation equilibrium data by the equation

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2}$$

where c is directly proportional to the recorder deflections of the scanner. The terms of the equation are M, apparent weight-average molecular weight; R, gas constant; T, absolute temperature, $\bar{v}$, partial specific volume; ρ, solution density; ω, angular velocity in radians per second; r, distance in centimeter from the center of rotation; c, absolute protein concentration at r. Base-line absorption corrections were obtained by the method described by Chervenka (1969). The partial specific volume was assumed to be 0.74 for both proteins.

Sedimentation velocity studies were performed on a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system, using 12-mm double-sector cells with a synthetic boundary centerpiece. Measurements were made at 20° with a rotor velocity of 56,000 rpm. Renin solutions were in 0.1 M sodium acetate (pH 5.60). Observed sedimentation velocity coefficients were corrected to standard conditions ($s_{20,w}$).

Gel filtration was performed at 4° by the upward-flow technique using Sephadex G-100 (Pharmacia). The columns were calibrated as described by Whitaker (1963). Ion-exchange chromatography was performed with DE-52-cellulose and CM-52-cellulose (Whatman). All column eluates were monitored by an ISCO uv flow monitor at 280 nm. Protein content was estimated by the procedure of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Protein solutions were concentrated by pressure ultrafitration using Amicon equipment. Buffer changes were made by the addition of the appropriate buffer and reconcentration.

Polyacrylamide gel disc electrophoresis was performed as described by Davis (1964), but with the omission of the stacking gel, using prewashed gels according to the directions of Mitchell (1967). Protein bands were stained with Amido Black, and the procedure of Zacharius *et al.* (1969) was used for the detection of glycoproteins.

Isoelectric focusing was performed at 5° on LKB equipment according to the manufacturer's directions in 1.2% ampholyte solutions. The pH was measured at 1–3° using a Sargent-Welch pH meter (Model NX).

TABLE I: Recoveries of Renin-like Activity[a] during Purification.

Preparation	Total Protein (mg)	Total Act. (μmoles/hr)	Sp Act. (μmoles/hr per mg of Protein)	Yield (%)
Homogenate	5400	880	0.16	100
Ammonium sulfate	2600	900	0.35	102
Sephadex G-100	500	440	0.88	50
First CM-52	150	260	1.73	30
DE-52	65	180	2.77	20
Fraction A				
Final CM-52	15	100	6.67	11
Fraction C				
Final CM-52	3	18	6.00	2

[a] Assayed with the fluorogenic renin substrate of Reinharz and Roth (1969), see Materials and Methods.

Amino acid analysis was done according to the method of Spackman *et al.* (1958) on a Spinco Model 120C analyzer, equipped with an Infotronics integrator.

Antiserum to the renin-like enzyme of fraction C was prepared by injecting 0.5 mg of the preparation in Freund's complete adjuvant (Difco) into the footpads of a rabbit. After 3 weeks the injection was repeated and the animals were bled from the ear vein 1–2 weeks after the second injection.

Isolation of the Renin-like Enzymes. Adult male Swiss–Webster mice weighing 40 g or more were killed with chloroform. The submaxillary glands were excised and stored frozen until a sufficient quantity had been accumulated. Approximately 25–30 g wet weight of glands was obtained from 150 mice. The recovery of enzyme activity at each step of the isolation procedure may be followed by referring to Table I.

EXTRACTION PROCEDURE. For each 25 g of frozen tissue was added 100 ml of cold distilled water, and the mixture was homogenized in a Waring Blendor for 3 min. The homogenate was centrifuged for 10 min at 3° and 16,000*g*. The supernatant fluid was decanted and the residue was stirred with 90 ml of cold distilled water and recentrifuged. The combined supernatants contained approximately 5 g of protein.

To nine volumes of extract was added one volume of a stock streptomycin solution (1.46 g of streptomycin sulfate, adjusted to pH 9.0, in a final volume of 20 ml). The final pH of the mixture was between 6.8 and 7.1. The mixture was allowed to stand at 2–5° overnight, centrifuged for 5 min, and the residue was discarded. Solid ammonium sulfate was added to the supernatant liquid (56 g of ammonium sulfate/100 ml of supernatant) and, after solution, the mixture was allowed to stand at 0° for 30 min. The precipitate was then separated by centrifugation (as described above), and the supernatant liquid was discarded. The residue was suspended in 20 ml of water and dialyzed with stirring for 24 hr against five changes of 2-l. amounts of distilled water at 2–5°. This dialyzed preparation contained 2.4–2.8 g of protein in approximately 110 ml and over 95% of the initial activity.

GEL FILTRATION ON SEPHADEX G-100. The dialyzed preparation was concentrated through a Diaflo membrane (UM-

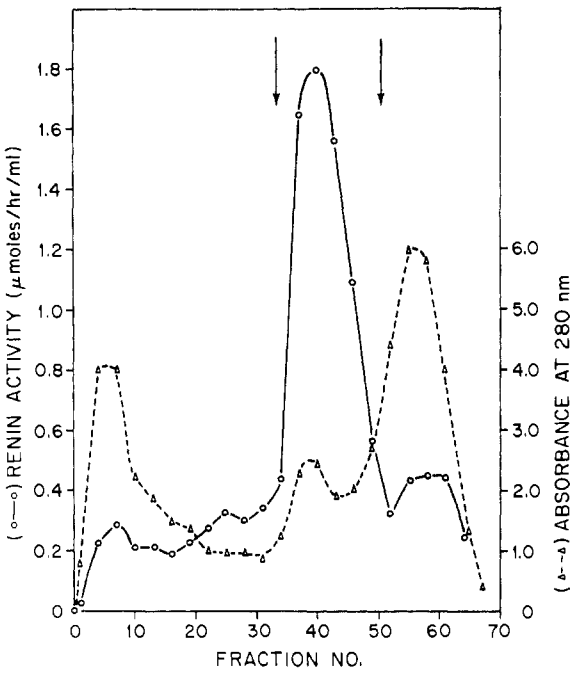

FIGURE 1: Gel filtration of submaxillary gland extract. Sample volumes of 20 ml containing 1.2–1.4 g of protein were applied to a Sephadex G-100 column (5.0 × 90 cm) and eluted with 0.01 M sodium acetate (pH 5.9) buffer containing 0.1 M NaCl. The flow rate was 2.0 ml/cm² per hr and 10-ml fractions were collected.

10) to a final volume of 40 ml, and was centrifuged at 20,000*g* for 3 hr. The supernatant was divided into two equal portions (1.2–1.4 g of protein) and was applied, in separate experiments, to a Sephadex G-100 column and eluted as described in the legend to Figure 1. Using the fluorogenic substrate method of Reinharz and Roth (1969), the major peak of renin-like activity was found in the fraction shown between the arrows (Figure 1). This fraction, containing approximately 50% of the applied activity, was taken for further purification. The remaining tubes, having relatively small amounts of renin-like activity, were not examined further. They may represent other forms of renin, or may be artifacts due to the

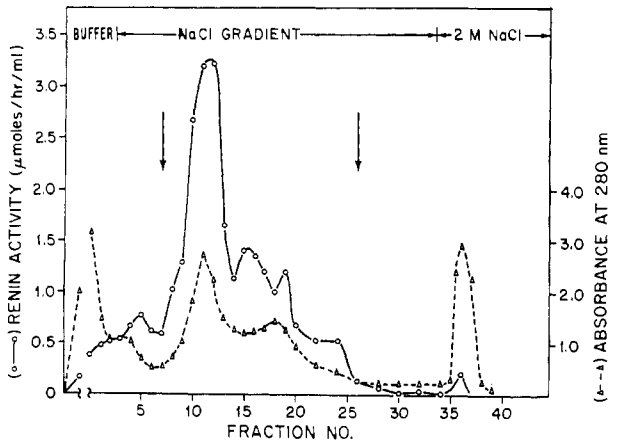

FIGURE 2: Chromatographic elution pattern of the DE-52-treated Sephadex peak on CM-52-cellulose. The sample was applied to a column (1.5 × 15 cm) of CM-52-cellulose and eluted with a salt gradient (see text for details). The flow rate was 0.2 ml/min, and approximately 6-ml fractions were collected.

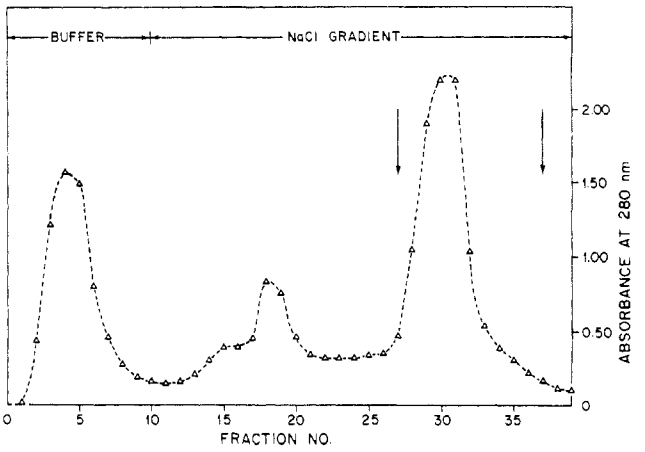

FIGURE 3: Chromatographic elution pattern of the CM-52-cellulose fraction (Figure 2) on DE-52-cellulose. The sample was applied to a column (1.5 × 15 cm) of DE-52-cellulose and eluted with a salt gradient (see text for details). The flow rate was 0.2 ml/min and approximately 6-ml fractions were collected.

detection of non-renin-like enzymatic activity by the assay method employed.

ION-EXCHANGE CHROMATOGRAPHY OF THE G-100 PEAK. The pooled fraction was concentrated in a Diaflo ultrafiltration cell to approximately 20 ml. The pH of this material was lowered to 4.5 by ultrafiltration with 0.1 M acetate buffer (pH 4.5), followed by 0.01 M buffer. The sample was then reconcentrated to 20 ml and centrifuged at 2500 rpm for 10 min. The supernatant (400–500 mg of protein) was applied to a small column (1.5 × 10 cm) of DE-52-cellulose equilibrated with 0.01 M sodium acetate (pH 4.5), and eluted with the same buffer. Under these conditions some protein (about 30%) but very little of the renin-like activity was adsorbed to the cellulose. The pH of the eluate (containing 300–350 mg of protein and most of the renin-like activity) was adjusted to pH 5.4 with 0.05 M sodium acetate by ultrafiltration and was concentrated to approximately 15 ml. (Recovery of renin at this step is not shown in Table I.)

The sample was applied to a column of CM-52-cellulose equilibrated with 0.05 M sodium acetate buffer (pH 5.4) and eluted with the same buffer. More than 80 per cent of the applied activity remained adsorbed and was subsequently eluted with a sodium chloride gradient. The salt gradient was prepared by allowing 0.05 M sodium acetate buffer (pH 5.4), containing 0.15 M NaCl to flow into a 125-ml constant-volume mixing chamber filled with the sodium chloride free buffer. The protein remaining on the column was eluted with 2 M NaCl. A typical elution pattern is shown in Figure 2. The multiple-peaked fractions between the arrows, containing 130–180 mg of protein and approximately 80% of the applied activity, were pooled.

This material was concentrated to approximately 15 ml in an ultrafiltration cell, adjusted to pH 7.5 with 0.02 M Tris-HCl buffer, and reconcentrated to 15 ml. The sample was then applied to a column of DE-52-cellulose equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) and eluted with the same buffer. All of the renin-like activity remained adsorbed, and was then eluted with a 0–0.3 M sodium chloride gradient prepared as described above. The result is shown in Figure 3. The peak between the arrows contained 60–75 mg of protein and essentially all of the applied activity.

This material was concentrated to 10 ml in an ultrafiltration cell, adjusted to pH 5.4 with 0.05 M acetate buffer (pH

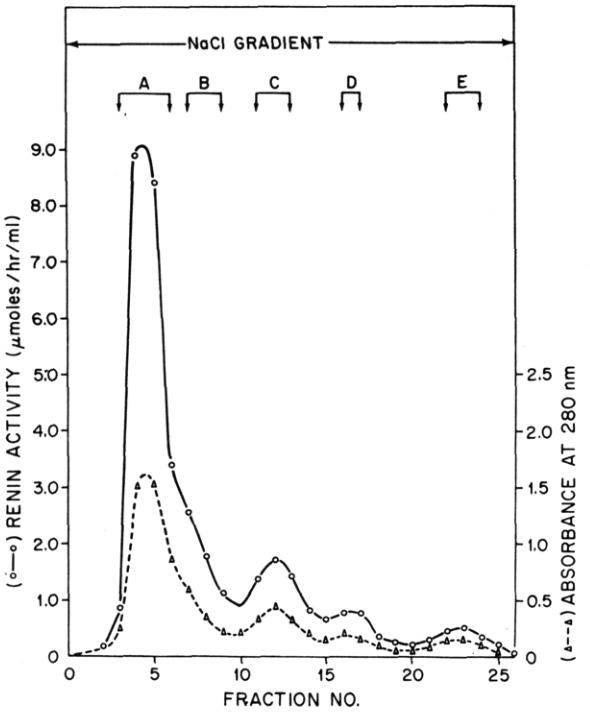

FIGURE 4: Chromatographic elution pattern of the DE-52-cellulose fraction (Figure 3) on CM-52-cellulose. See text and Figure 2 for procedure.

5.4) and reconcentrated to 15 ml. The sample was again applied to a CM-52 column, and the activity eluted with a sodium chloride gradient prepared as described with the first CM-52 column. A typical elution profile is shown in Figure 4. All of the observed peaks detectable by their absorbance at 280 nm were enzymatically active; the specific activities of all the fractions were almost identical, suggesting the existence of a family of renin-like enzymes.

Gel Electrophoresis of Isolated Fractions. Samples containing approximately 40 μg of protein obtained from individual tubes or pooled tubes shown in Figure 4 were examined by polyacrylamide gel disc electrophoresis. Tubes in fractions A, C, D, and E (Figure 4) each contained only one electrophoretically detectable band, with different distances of migration. Tubes from fraction B contained a mixture with one band clearly different from any of the others detected. Typical electrophoretic results are shown in Figure 5. Thus both chromatographically and electrophoretically we have been able

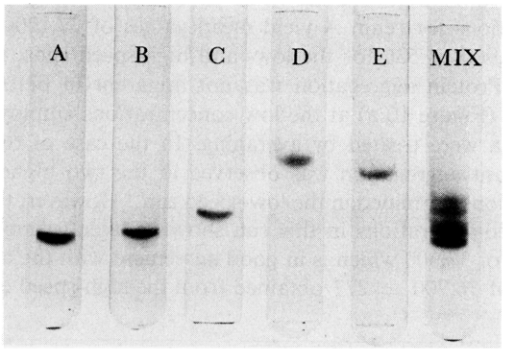

FIGURE 5: Disc gel electrophoresis of renin-containing fractions A–E (Figure 4). The pH of the resolving gel was pH 9.5. The cathode was at the top of the column.

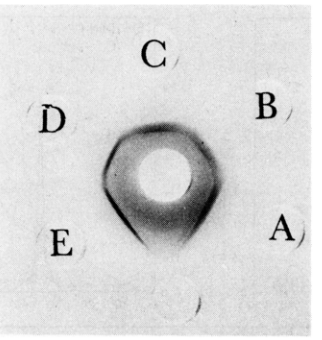

FIGURE 6: Immunodiffusion pattern of renin-containing fractions A–E (Figure 4). The center well contained antiserum to renin C. The outside wells contained the renin fractions A, B, C, D, and E, respectively, at concentrations of 0.4–0.5 mg/ml.

to separate four renin-like enzymes, with one form predominating. The evidence suggests a fifth minor form appearing as a shoulder of the main first peak (fraction B, Figure 4).

Immunological Studies. Rabbit antiserum was prepared against the pooled enzyme contained in fraction C (Figure 4). By means of immunodiffusion in agarose gels, the reaction of the antiserum to each of the fractions (A, B, C, D, and E) was examined. The result is shown in Figure 6. The precipitin bands of all of the fractions examined form a line of identity indicating a close antigenic similarity. (The inhibition of renin activity by the antiserum will be reported in a later publication.)

Final Purification of Two of the Renin-like Enzymes. The pooled fractions A and C, respectively, were again chromatographed on CM-52-cellulose (pH 5.4) in a manner identical with that described for the first CM-52 fractionation. Typical elution patterns are shown in Figure 7. In each instance a single peak was observed with an almost constant specific activity throughout the elution profile. All subsequent experiments were performed on the enzymes which were purified in this manner.

Aliquots of the two preparations, containing 30–40 μg of protein, were subjected to polyacrylamide gel electrophoresis at pH 8.9 and 4.5. In each instance only one band of material staining with Amido Black could be detected. Since both preparations stained clearly with a periodic acid–Schiff reagent, these renin-like enzymes appear to be glycoproteins.

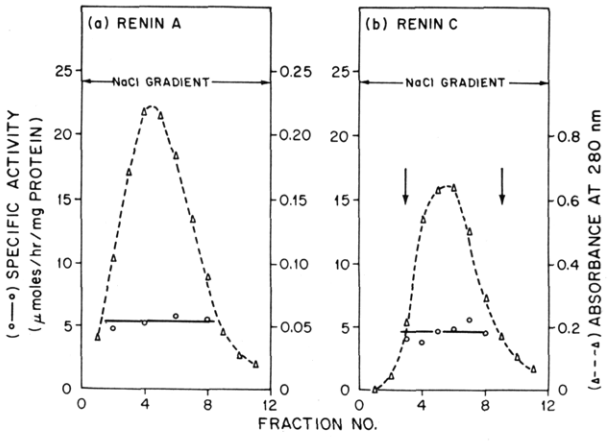

FIGURE 7: Rechromatography of renin fractions A and C (Figure 4) on CM-52-cellulose. See text and Figure 2 for procedure.

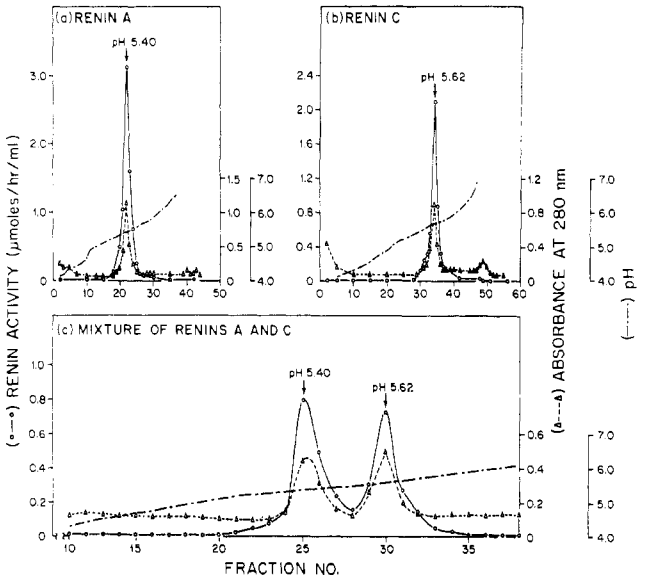

FIGURE 8: Isoelectric focusing of renin A, renin C, and a mixture of the two enzymes. Approximately 2 mg of each enzyme was added to a pH 4–6 range ampholyte solution. Fractions of 1.8 ml were collected.

The final yields of the two preparations were approximately 12–15 mg of protein from fraction A and 3–4 mg from fraction C, representing about 12 and 2%, respectively, of the original enzymatic activity of the homogenate. The specific activities of the two preparations were similar and indicated a purification of approximately 40-fold. The recoveries of renin-like activity at each step in a representative purification are summarized in Table I.

Isoelectric Focusing. Aliquots (1.5–3 mg) of the two preparations were subjected to isoelectric focusing in the pH 4–6 range as shown in Figure 8. In each instance only one major protein peak coinciding with the renin-like activity was noted. A mixture of the two enzymes were resolvable by this procedure (Figure 8). The isoelectric points were determined to be 5.40 and 5.62 for fraction A and C, respectively.

Amino Acid Composition. The amino acid compositions of the salivary gland renins A and C were quite similar. The data are summarized in Table II. Further analysis is required to determine the significance of the observed small variations.

TABLE II: Amino Acid Composition of Salivary Gland Renins A and C.

	Residues/1000 Residues[a]			
	Renin A		Renin C	
	Time of Hydrolysis:			
Amino Acid	24 hr	48 hr	24 hr	48 hr
Lys	37.6	37.4	38.5	37.9
His	25.9	26.9	27.5	27.0
Arg	28.1	27.7	30.1	30.3
Asp	82.0	82.0	84.1	82.2
Thr	73.4	70.9	72.5	72.4
Ser	98.8	90.8	97.3	97.3
Glu	83.0	83.5	79.9	79.5
Pro	57.7	59.0	56.1	58.8
Gly	105.2	105.0	104.0	104.0
Ala	43.4	42.9	44.0	43.7
Half-Cys	13.0	10.9	14.8	14.2
Val	82.0	93.7	85.6	85.5
Met	18.4	18.6	14.1	15.5
Ile	43.0	44.8	43.0	43.1
Leu	101.0	99.2	100.0	99.7
Tyr	48.4	48.9	48.4	47.8
Phe	50.5	49.4	51.2	52.2
Trp	8.3	8.4	8.8	8.7

[a] Uncorrected for hydrolytic losses. Tryptophan was determined by the spectrophotometric method of Goodwin and Morton (1946).

Molecular Weights by Gel Filtration. The two enzymes were applied (in separate experiments) to a column of Sephadex G-100 which had been previously calibrated with various known marker proteins. In each instance a symmetrical elution profile was obtained. By this method (Figure 9) the molecular weights of both renin-like enzymes were calculated to be approximately 43,000.

Extinction Coefficient. The extinction coefficients ($E^{1\%}_{1\,cm,\,280\,nm}$) for renin A and renin C were determined to be 10.5 and 10.8, respectively. These values were used to estimate the protein concentration in many of the studies described here.

Sedimentation Equilibrium. The results of the sedimentation equilibrium studies are shown in Table III. Molecular weight calculations for renin A yield mean values of 37,100 ± 818 and 35,900 ± 536 for the low- and high-speed runs, respectively. Protein aggregation was not apparent in plots of ln *c vs.* r^2 (Figure 10 a) at the low concentrations employed, so the data were treated by averaging. In the case of renin C, significant aggregation was observed at the two higher concentrations examined in the low-speed run.[1] However, the two lower concentrations in this run show an average molecular weight of 37,300 which is in good agreement with the average value of 36,700 ± 277 obtained from the high-speed equilib-

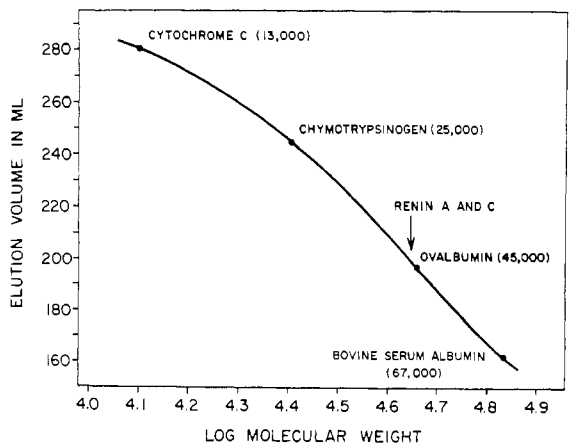

FIGURE 9: Estimation of the molecular weights of renin A and renin C by gel filtration. A 4-ml sample containing 2–4 mg of protein was applied to a 2.5 × 90 cm Sephadex G-100 column and eluted with 0.01 M sodium acetate (pH 5.9) containing 0.1 M NaCl. Flow rate was 4.3 ml/cm² per hr.

[1] The sedimentation velocity coefficient ($s_{20,w}$) for renin A was found to be 6.54 S and for renin C, 6.42 S. These unexpectedly high values may indicate dimer or aggregate formation under the conditions of high protein concentration (4–5 mg/ml) employed for these studies.

TABLE III: Molecular Weight Data from Sedimentation Equilibrium Studies.

	Renin A		Renin C	
Rpm	Initial Concn (mg/ml)	App Mol Wt	Initial Concn (mg/ml)	App Mol Wt
13,000	0.321	37,500	0.281	40,100
	0.256	36,400	0.213	39,600
	0.192	36,500	0.142	37,400
	0.128	38,100	0.085	37,200
	Av	37,100 ± 818	Av[a]	
32,000	0.256	36,600	0.142	36,800
	0.192	36,100	0.085	36,900
	0.128	35,600	0.056	36,800
	0.064	35,400	0.029	36,300
	Av	35,900 ± 536	Av	36,700 ± 277

[a] An average value was not calculated for renin C because of the apparent aggregation observed at the two higher concentrations examined.

rium data. A tendency toward aggregation at the relatively higher protein concentration near the base of the solution column is indicated by the plot of ln c *vs.* r^2 as illustrated in Figure 10b. This phenomenon became increasingly prominent at higher protein concentrations, and dictated the use of dilute solutions for molecular weight studies. The linearity of the slope over the major portion of the plot, however, indicates a relatively homogeneous protein preparation, for both proteins.

Crystallization of Renin A. An aqueous solution containing 15 mg of renin A was concentrated in an ultrafiltration cell to approximately 1.5 ml. The material was then dialyzed against 0.1 M sodium acetate buffer (pH 5.6) for 3 days at 3–6°. During this period a portion of the renin preparation (approximately one-fourth) crystallized. The crystals are illustrated in Figure 11. To check for enzymatic activity, the crystals were freed of mother liquor by four rapid washes with cold 0.2 M sodium acetate buffer (pH 5.6) and centrifugation. The crystalline material, after solution in 0.1 M Tris buffer (pH 7.5), had a specific activity of 6.5 μmoles/hr per mg of protein, identical with that of the starting material.

Biological and Enzymatic Activities of the Renin-like Enzymes. Both enzymes are biologically active *in vivo*. The intravenous injection of 1 ng of either preparation into 24-hr nephrectomized, anesthetized rats, treated with ganglionic (pentolinium) and adrenergic (phenoxybenzamine) blocking agents, resulted in a clear and prolonged (over 30 min) elevation of the mean blood pressure. The enzymes also liberated an angiotensin-like pressor substance when incubated *in vitro* with the renin substrate of rat plasma according to the method of Boucher *et al.* (1964). The details of these experimental results will be presented in a later publication.

Both enzymes (renin A and C) were studied for their ability to cleave the tetradecapeptide renin substrate of Skeggs *et al.* (1958) according to the procedure given in Materials and Methods. The amino acid sequence of this substrate is

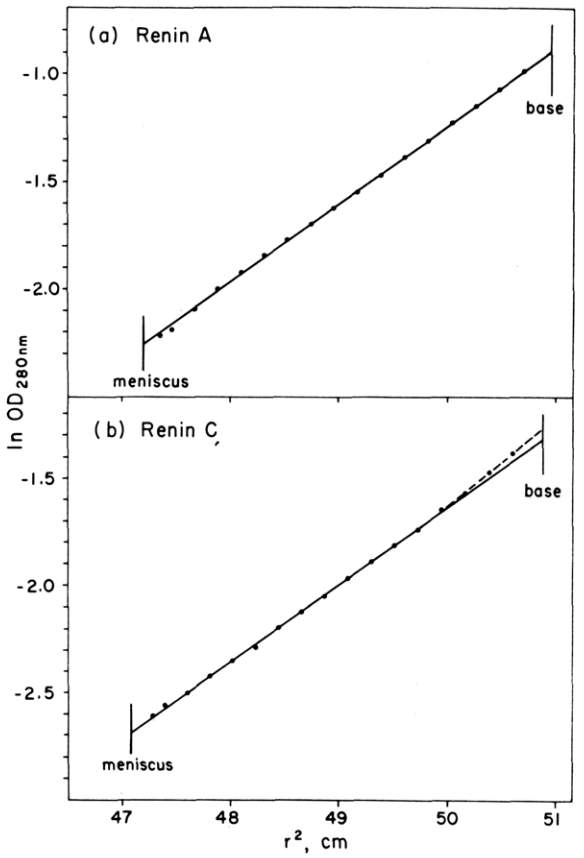

FIGURE 10: Sedimentation equilibrium plot of ln OD against r^2 at 13,000 rpm. (a) Renin A at an initial concentration of 0.192 mg/ml. (b) Renin C at an initial concentration of 0.142 mg/ml.

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser, and kidney renin, from a variety of animal species, has been shown to cleave the Leu–Leu peptide bond to yield two peptide reaction products (Skeggs *et al.*, 1967a). After the incubation, the resulting mixtures were chromatographed on thin-layer cellulose plates with a solvent consisting of a 1-butanol–acetic acid–water (4:1:5) mixture. In each instance only two spots with R_F values of 0.49 and 0.72 were detected with the Pauly reagent for the identification of histidine and tyrosine residues. The peptide material in areas corresponding to these spots on duplicate chromatograms (renin C incubation) were

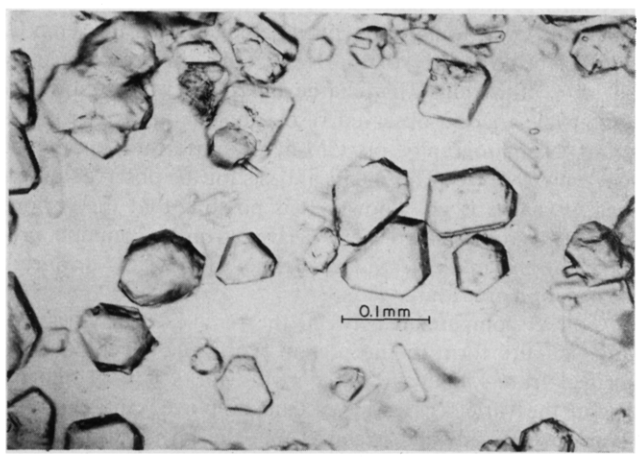

FIGURE 11: Crystals of renin A.

TABLE IV: Amino Acid Composition of Peptides Liberated from the Synthetic Renin Substrate by Salivary Renin C.

	Decapeptide (Residues/Mole)[a]		Tetrapeptide (Residues/Mole)	
Amino Acid	Obsd	Theor	Obsd	Theor
Asp	1.00	1		
Arg	1.09	1		
His	1.84	2		
Pro	1.03	1		
Phe	1.09	1		
Ile	0.66	1		
Leu	1.20	1	1.09	1
Val	0.86	1	0.92	1
Tyr	0.90	1	0.84	1
Ser	0.09	0	1.15	1

[a] Assuming one aspartic acid residue per mole.

eluted with 5% acetic acid, taken to dryness, and hydrolyzed in 6 N HCl for 24 hr according to the method of Spackman *et al.* (1958). The results of the amino acid analysis of the two isolated peptides are shown in Table IV. It can be seen that the salivary gland renin hydrolyzed the tetradecapeptide into the expected products of the reaction: a tetrapeptide, and the decapeptide, angiotensin I.

Discussion

The isolation of a family of renin-like enzymes from the submaxillary gland of the adult male mouse has been accomplished. All cleave the synthetic renin substrate of Reinharz and Roth (1969) with approximately the same specific activity and appear to be antigenically related.

The preparations of both renin A and renin C were homogeneous. They appeared as single peaks (with constant specific activity) when examined by ion-exchange and Sephadex chromatography. Disc gel electrophoresis and isoelectric focusing of the two enzymes again indicated that each is composed of one molecular species. In ultracentrifugal studies, linear plots of log concentration *vs.* r^2 revealed no significant heterogeneity. The molecular weights and the amino acid compositions of the two enzymes were similar.

Renins A and C differ in their isoelectric points; the p*I* for renin A was found to be at pH 5.40 and that for renin C, at pH 5.62. Mixtures of the two renins were resolvable by isoelectric focusing. This charge difference was presumably responsible for the observed resolution of the two enzymes upon chromatography on CM-52-cellulose and upon disc gel electrophoresis. The chemical basis for the observed difference in charge is not known. It is possible that the various forms of renin isolated were derived from a common precursor by enzymatic degradation either in the gland or during the isolation procedure.

A direct comparison between the renin-like enzymes isolated in pure form in the present work and those partially purified from kidney by other investigators is very difficult due to the variety of assay systems employed and the relatively few data available concerning the physical characteristics of the preparations employed. Waldhausl *et al.* (1970) reported that human kidney renin had a molecular weight of 42,000 by gel filtration and that the isoelectric point was at pH 5.25. Peart *et al.* (1966) reported that the molecular weight of hog renin, as determined by gel filtration, was about 40,000. Skeggs *et al.* (1967a) was able to detect four forms of hog renin by chromatographic procedures. The preparations of salivary gland renin obtained by Werle *et al.* (1968) presumably contain one or more of the renin-like enzymes isolated in our studies.

The possibility that additional forms of the renin-like enzyme (or complexes of the enzyme with other proteins) exist in the salivary gland is suggested by the observation (Figure 1) that some renin-like activity was noted throughout the elution profile on the Sephadex column. However, since the fluorogenic substrate is potentially susceptible to nonspecific endopeptidase action, it is not certain that the unidentified activity was truly due to the renin-like enzyme. It must be emphasized that the fluorogenic substrate was able to detect the renin-like activity in preference to the nonspecific peptidase activity primarily because of the low substrate concentration which may have minimized the action of nonspecific enzymes.

The salivary gland renin-like enzymes resemble kidney renin in their enzymatic and biological activities. They appear to be the purest preparations with renin-like activity from any source yet reported, and will facilitate our planned studies on the specificity, kinetic parameters, and physiology of this enzyme.

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Subtilisin; a Stereochemical Mechanism Involving Transition-State Stabilization†

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ABSTRACT: The difference-Fourier method was used to determine the binding geometry for various polypeptides to subtilisin BPN′. The principal finding was that polypeptides corresponding to the acylating portion of good substrates bind in a fashion very similar to that seen previously for polypeptide chloromethyl ketone derivatives. The binding of these virtual substrate polypeptides was assumed to represent a model for the Michaelis complex between the enzyme and actual polypeptide substrates. The carbonyl oxygen of the susceptible bond is near two potential hydrogen-bond donating groups, the backbone NH of Ser-221 and Nδ2 of Asn-155, but the distance is too large for the bonds to be formed in the Michaelis complex. The focus of these two hydrogen bonds is termed the "oxyanion hole." In addition the amido nitrogen of the substrate specificity residue P_1 (Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27*, 157) is poised near to, but again too far from, the carbonyl oxygen of Ser-125 for a hydrogen bond, termed the "S_1–P_1 hydrogen bond," to be formed. Model-building experiments readily led to models for the two other likely intermediates, a tetrahedral addition compound and the acyl-enzyme. In the tetrahedral addition compound construction of a covalent linkage between the Ser-221 Oγ and the carbonyl carbon of the susceptible bond results in distortion of that carbon to a tetrahedral conformation, and closer contact between the substrate specificity residue P_1 and the enzyme. Consequently, the S_1–P_1 hydrogen bond is formed in the addition compound, and the oxygen of the specificity residue, now carrying a formal negative charge, is stabilized in the oxyanion hole by formation of two additional hydrogen bonds. These three hydrogen bonds, plus a fourth between the leaving group and His-64, stabilize the tetrahedral intermediate and thereby lower the free energy of activation for rate-limiting transition states. Furthermore, model building also indicates that when the tetrahedral intermediate collapses to the acyl-enzyme, steric constraints imposed on a specific substrate by the enzyme prevent the carbonyl oxygen of the resulting ester linkage from being maintained in the oxyanion hole. Finally, the stereochemical similarity between subtilisin and chymotrypsin previously described for the charge relay system and the binding site is shown to extend as well to the oxyanion hole, apparently so important for catalysis. This provides further evidence that the two enzymes have converged in an evolutionary sense to the same mechanism of action.

In the previous paper of this series we described the binding to subtilisin BPN′ of four chloromethyl ketone analogs of good polypeptide substrates, as seen by the difference-Fourier method (Robertus *et al.*, 1972). All four inhibitors contained an L-phenylalanine residue at P_1. The polypeptide portion in each case was hydrogen bonded to an extended segment, Ser-125–Leu-126–Gly-127, of the enzyme backbone to form an anti-parallel β structure, with the P_1 phenylalanine side chain fitting snugly into a hydrophobic crevice. One wall of this crevice consisted of the extended backbone segment 125–127.

This geometry represented a plausible model for binding of the P_1–P_3 residues, that is, the acylating portion of polypeptide substrates. Evidence for the validity of this model included the convincing manner in which it explained various aspects of the enzyme's specificity at P_1–P_3 (Morihara *et al.*, 1970) and the virtual identity of the model with that proposed independently for substrate binding to γ-chymotrypsin on the basis of similar experiments by Segal *et al.* (1971).

Several basic questions remained to be answered, however, regarding subtilisin's mechanism of action. What was the effect on the observed binding due to covalent attachment of the inhibitors to His-64 *via* a methylene carbonyl linkage? Would a Michaelis complex with a polypeptide substrate be appreciably different? Is the binding mode the same when P_1 is other than phenylalanine? How would the leaving portion (P_1', P_2', etc.) be bound in the Michaelis complex? What is the

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