The pressor enzyme renin (EC 3.4.99.19) was isolated in a pure and stable form from hog kidney by affinity chromatography on a pepstatin/agarose gel followed by three additional steps of conventional chromatography. Destruction of the enzyme by proteolysis during isolation was prevented by chemically eliminating proteases in extracts. The pure preparation was used for the characterization of this enzyme. Renin was found to be a glycoprotein containing glucosamine and possessing binding affinity to concanavalin A. Contrary to previous reports, pure renin is stable at neutral pH either at 4 or -20°C for 3 to 8 weeks. It has a molecular weight of 36,100 as determined by equilibrium ultracentrifugation, an isoelectric point of 5.2 and \(E_{280}^\text{Cm} \text{ (280 nm)} \) of 9.1. In contrast to crude preparations, the enzyme activity has a broad pH optimum between pH 5.5 and 7.0 for both hog angiotensinogen and the synthetic octapeptide substrate benzoyloxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-beta-naphthylamide. The rate of formation of angiotensin I from hog angiotensinogen at pH 6.0 and 37°C was 267 \(\mu\text{g/h/\mu g of renin}\), or 2000 Goldblatt units/mg of renin. For the synthetic fluorogenic octapeptide substrate benzoxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-beta-naphthylamide, a \(K_m\) of 33 \(\mu\text{M}\) and a \(V_{max}\) of 0.94 \(\mu\text{mol/h/mg of enzyme}\) were obtained at pH 6.5 and 37°C.

The highly specific proteolytic cleavage of angiotensinogen by renin (EC 3.4.99.19) to produce angiotensin I is the first step in the series of reactions leading to the formation of the major pressor substances angiotensin II and angiotensin III and to the release of aldosterone. Furthermore, the secretion of renin from the kidney into the circulation is the rate-limiting step regulating the subsequent production of the angiotensins (1). Thus, renin plays the central role in blood pressure regulation. However, very little has been known about its molecular and enzymological properties primary because no pure renin preparation has been available. In spite of numerous attempts in the past (2-12), renin purification has encountered insurmountable difficulties due to a very low concentration of this enzyme in the kidney and also because renin is rapidly destroyed during purification.

The recent advent of affinity chromatographic techniques (13) aroused renewed interest in the purification of renin. However, many of the affinity gels for renin thus far prepared were not adequate for the isolation of this enzyme from a crude homogenate of the kidney (12, 14-16). Prompted by reports of the strong binding affinity of the bacterial N-acylated pentapeptide pepstatin (17) to renin (18-21), we were able to prepare an efficient affinity gel by coupling pepstatin to aminohexylagarose as previously reported (22). Affinity chromatography on this gel and subsequent application of conventional chromatography led to the isolation of an electrophoretically homogeneous and stable renin preparation. With this purified renin preparation, we have been able for the first time to determine some of the molecular and enzymological properties of renin.

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Acid analysis in a Durrum D500 analyzer after timed hydrolysis in 6 N HCl for 24, 48, and 96 h (28). Tryptophan was determined after alkaline hydrolysis (29), cysteine was determined as cysteic acid after performic acid oxidation (30), and amino sugars were determined after 5-h hydrolysis with 4 N HCl at 110° (31).

**Isoelectric Point**—The isoelectric point was determined by the isoelectric focusing method (32) on a 5% polyacrylamide gel plate with 3% cross-linkage containing 2.4% ampholine, pH 3.5 to 10 (LKB Instrument, Inc.), in a LKB Multiphor apparatus. Electrode voltage applied across the shorter edge was increased from 100 to 500 V over 60 min and the experiment was terminated after 4 h. The pH of gel slices cut at 5.5-cm intervals from both edges of the gel plate was determined after overnight extraction into 2 ml of deionized and degassed water at 4°.

**Extinction Coefficient**—Absorbance at 280 nm of renin solution exhaustively dialyzed against 0.18 M triethylamine/acetate buffer, pH 7.0, was determined, then the weight of protein in the solution was determined after drying to a constant weight in a glass cup placed in a drying pistol maintained at 110° with toluene refluX.

**Protein Concentration**—Protein concentration of dilute solutions was determined spectrophotometrically using an E:Ts of 9.1 at 280 nm obtained in this study. The biuret method (33) was used for very dense crude extracts using bovine serum albumin as reference standard. Kidney powder was extracted in 0.75 N NaOH for the determination of protein concentration by this method.

**Molecular Weight**—Sedimentation equilibrium studies were performed with a Spinco model E analytical ultracentrifuge equipped with a split beam photoelectric scanning system (280 nm) and electronic speed control using the short column method (34). The sample renin solution (0.1 ml), dialyzed against 0.05 M pyrophosphate buffer, pH 6.2, containing 0.1 M KCl, was layered over performic acid oxidized bovine serum albumin (Minnesota Mining and Manufacturing Co.) in the solution section of a sector cell with the dialysate being added to the solvent sector. Equilibrium scans were made at 6° at a rotor velocity of 13,000 rpm. In calculating the weight average molecular weight, the partial specific volume of protein was estimated to be 0.734 using 0.25% dithiothreitol (36, 37).

**RESULTS**

The outline and result of the purification of renin from hog kidneys are summarized in Table I. A 133,000-fold purification was obtained with a remarkably high yield of 19%. Two milligrams of pure renin can be obtained from 20 kg of hog kidney. The final product obtained after chromatography on CM-cellulose was used for subsequent characterization studies reported in this paper. The preparation after DEAE-cellulose chromatography already had a very high specific activity, almost comparable with that of the final product. The use of the mixture of protease inactivators seems to reduce multiple peaks, thus facilitating purification. The product obtained with the protease inactivators produced a clean band on polyacrylamide gel electrophoresis, whereas omission of the inactivators caused great difficulties in obtaining a pure product.

**Purity**— Electrophoresis of the purified hog renin on polyacrylamide gel at pH 6.5 (Fig. 5A) gave a discrete single band. Likewise, electrophoresis in SDS-polyacrylamide gel agarose 0.1% was layered over performic acid oxidized bovine serum albumin (Minnesota Mining and Manufacturing Co.) in the solution section of a sector cell with the dialysate being added to the solvent sector. Equilibrium scans were made at 6° at a rotor velocity of 13,000 rpm. In calculating the weight average molecular weight, the partial specific volume of protein was estimated to be 0.734 using 0.25% dithiothreitol (36, 37).

**FIG. 5 (left).** Polyacrylamide gel electrophoresis of hog renin obtained from the chromatography on the CM-cellulose column (A). The same preparation was electrophoresed in the presence of 0.1% SDS after heating without (B) and with 0.25% dithiothreitol (C). **FIG. 6 (right).** Isoelectric focusing of purified hog renin.

The final product obtained after chromatography on CM-cellulose column (A). The same preparation was electrophoresed in the presence of 0.1% SDS after heating without (B) and with 0.25% dithiothreitol (C).

**Stability**—The enzyme solution (0.2 mg/ml) was stored at various pH values in the presence of 0.02 M pyrophosphate buffer, pH 6.2, containing 0.1 M KCl, was layered over performic acid oxidized bovine serum albumin (Minnesota Mining and Manufacturing Co.) in the solution section of a sector cell with the dialysate being added to the solvent sector. Equilibrium scans were made at 6° at a rotor velocity of 13,000 rpm. In calculating the weight average molecular weight, the partial specific volume of protein was estimated to be 0.734 using 0.25% dithiothreitol (36, 37).

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**FIG. 6 (right).** Isoelectric focusing of purified hog renin.

The preparation after DEAE-cellulose chromatography already gave single bands upon disc electrophoresis and electrofocusing although the bands were less discrete.

**Extinction Coefficient**—An E:Ts of 9.1 was obtained at 280 nm. This value was used throughout the characterization studies.

**Molecular Weight**—Molecular weight was determined at 6° with a renin solution, 0.3 mg/ml in 0.05 M sodium pyrophosphate buffer, pH 6.2, containing 0.1 M KCl. The molecular weight estimated from the least squares slope of the plot of the natural logarithm of the absorbance at 280 nm against the square of the radius was 36,400. The plot was linear over the entire cell and did not give any sign of aggregation at the bottom of the cell. Passage of the enzyme solution through a calibrated column of Sephadex G-75 (2.5 x 110 cm) gave a molecular weight estimate of 42,000 (Fig. 7). Electrophoresis
Pure Hog Renin

Fig. 7. Molecular weight determination of hog renin by gel filtration on a calibrated Sephadex G-75 column (2.5 x 110 cm). BSA, bovine serum albumin; OA, ovalbumin; P, pepsin; CG, chymotrypsinogen; M, myoglobin.

Fig. 8. Molecular weight determination of hog renin by (0.2%) SDS-polyacrylamide gel (10%) electrophoresis without dithiothreitol (a) and with dithiothreitol (0.5%) (DTT) (b). See legend to Fig. 7 for definitions of abbreviations.

Table II

| Amino Acid | Hog Renin | Mouse Submaxillary Renin |
|------------|-----------|--------------------------|
|            | Weight (%)| Residues/protein molecule| Residues/protein molecule |
| Alanine    | 2.69      | 15.7                     | 14                       |
| Arginine   | 3.54      | 9.4                      | 9                        |
| Aspartic acid| 7.18    | 25.9                     | 27                       |
| 1/2 Cystine| 0.81      | 3.4                      | 4                        |
| Glutamic acid| 9.58   | 30.8                     | 27                       |
| Glycine    | 4.63      | 33.6                     | 35                       |
| Histidine  | 1.67      | 5.0                      | 8                        |
| Isoleucine | 3.34      | 12.2                     | 15                       |
| Leucine    | 8.26      | 30.3                     | 33                       |
| Lysine     | 3.20      | 10.6                     | 12                       |
| Methionine | 1.18      | 3.7                      | 7                        |
| Phenylalanine| 6.05   | 36.9                     | 17                       |
| Proline    | 3.93      | 17.6                     | 18                       |
| Serine     | 6.68      | 31.9                     | 33                       |
| Threonine  | 6.44      | 26.2                     | 27                       |
| Tryptophan | 2.24      | 5.0                      | 5                        |
| Tyrosine   | 6.44      | 16.4                     | 17                       |
| Valine     | 8.11      | 34.2                     | 39                       |
| Galactosamine | N.D.  | N.D.                     | N.D.                     |
| Glucosamine| 1.16      | 3.2                      | N.D.                     |

* Tentative values calculated on the basis of the renin molecular weight of 36,400.
* T. Imagami and K. Murakami, unpublished results.
* N.D., not determined.

in SDS-polyacrylamide gel columns in the absence of dithio- threitol produced a single discrete Coomassie blue-stained band (Fig. 5B) at a position very close to that of ovalbumin, corresponding to a molecular weight of 42,500 (Fig. 8A). On the other hand, the presence of 0.25% dithiothreitol in the same system produced a single band (Fig. 5C) corresponding to a molecular weight of 35,000, as shown in Fig. 8B.

Amino Acid Analysis—Extrapolation of the results of analyses of timed hydrolysates produced the amino acid composition of hog renin tabulated in Table II in comparison with the composition of mouse submaxillary gland renin (38). The recovered amino acids and glucosamine account for 87% of the total weight. Since the hog renin is a glycoprotein as indicated by the presence of glucosamine in the amino acid analysis and by the affinity to a concanavalin A/agarose column (see below), at least part of the remainder of the composition may be accounted for by carbohydrates. Amino sugars other than glucosamine could not be detected, although the analysis method employed (31) is capable of resolving glucosamine and galactosamine.

Affinity Chromatography on Concanavalin A—Since the amount of hog renin preparation was not sufficient for quantitative carbohydrates analysis, further investigation of the present of carbohydrates was made by studying the affinity of
renin to concanavalin A. Renin was completely retained by the column and could be rapidly eluted by 0.25 M α-methyl-D-glucose in the same buffer, as shown in Fig. 9. These observations indicate that hog renin contains carbohydrate residues at nonreducing termini which have a specific affinity to concanavalin A (39, 40).  

**Renin Activity** — The pH dependence of the rate of hydrolysis of the synthetic octapeptide substrate of Roth and Reinharz (25) was investigated at 37° at a fixed substrate concentration of 50 mm. A broad pH optimum ranging from pH 5.5 to 7 was observed, as shown by the *solid curve* in Fig. 10. Initial rates, determined at different substrate concentrations (0.081 to 0.01 M) in 10% dimethylformamide at 37° and pH 6.5 (0.05 M sodium/pyrophosphate buffer), gave a normal Lineweaver-Burk plot and a *Kₘ* value of 33 µM and *Vₘₐₓ* of 0.94 µmol/h/mg of enzyme.

The rate of angiotensin I formation by the pure hog renin from hog angiotensinogen was determined at 37° using 0.31 nmol of partially purified substrate and 0.05 ng of pure renin in 0.5-ml reaction mixtures. Again a broad plateau from pH 5.5 to 7 was observed, as shown by the *broken line* of Fig. 10. The renin activity reached a level higher than 90% of the plateau value as the angiotensinogen concentration was increased to 1.2 µM. The specific activity for renin determined at pH 6.0 and 37° at this substrate concentration, was 267 µg of angiotensin I formed/h/µg of enzyme. Comparison with the activity of standardized renin determined under identical conditions showed that the present preparation possesses 2000 Goldblatt units/mg.

**DISCUSSION**

Although a pure and stable preparation of renin has been isolated from the submaxillary gland of male mice in our laboratories (38), renin from kidney has never been available in a pure and stable form because of its very low concentration in kidney and also because of its alleged instability in the pure state, which seems to be due to contaminating proteases. In the present studies, by circumventing these difficulties through the use of affinity chromatography and protease inactivators, we were able to obtain a pure and stable renin preparation from the kidney for the first time since the discovery of this enzyme in 1898 (43). This preparation allowed us to determine properties that can be obtained only with a pure substance. Thus this study will provide a frame of reference for many studies conducted with crude preparations of renin.

The 300-fold purification attained at the affinity chromatographic step was no doubt the single most important factor contributing to the completion of the present purification studies with an overall yield of 18% and 133,000-fold purification. However, it must be pointed out that the affinity chromatography alone could not produce a pure preparation in one step. The purification method described in the present study incorporates a number of improvements over the preliminary method reported previously (22), making the isolation work much faster, larger in scale, and more dependable.

Adherence to a relatively neutral pH range throughout the purification studies and the use of protease inactivators were intended to minimize the proteolytic destruction and subsequent generation of multiple isoenzymes of renin in view of the reported observation of multiplicity upon acidification (44). Nevertheless, the presence or the generation of minor components was observed in the elution pattern of ion exchange chromatographic steps (Figs. 3 and 4). In the present investigation, no attempts were made to purify these minor components.

The additives used in the purification are inactivators for serine (iPr₃P-F and PhCH₂SO₃F), cysteine (sodium/tetraethylthiuram), and metalloproteases (EDTA). No inactivator for acidic proteases convenient for a large scale experiment was available. It is highly probable that acidic proteases such as cathepsins D and E in the kidney homogenate could have been active during the brief acidification required for the elution of renin from the affinity column, resulting in the formation of derivatives of renin by limited proteolysis.

The homogeneity of the present preparation is ascertained by the discrete single band observed in the polyacrylamide gel electrophoresis with (Fig. 5, B and C) and without (Fig. 5 A) SDS, by the single band upon isoelectric focusing (Fig. 6) as well as by the symmetrical chromatographic peak in the final purification step (Fig. 4). The preparation from the DEAE-cellulose chromatography seems to have a purity higher than 90% of the final preparation (Table I). Electrophoresis on polyacrylamide and electrofocusing gave single bands, although it was somewhat less discrete than the final preparation. In view of extensive loss of material and very little gain in purity in the final chromatographic step, after the DEAE-cellulose column, this preparation may be used for some experiments which do not require absolute purity of the enzyme. Nonspecific hydrophobic binding of glycoproteins to concanavalin A has been well known. Thus the binding of renin to concanavalin A/agarose may not be taken as the absolute proof of the glycoprotein nature of renin. Detection of glucosamine in renin in the present studies, however, provides unequivocal proof that hog renin is a glycoprotein.

Amino acids and glucosamine account for at least 87% of the total mass of the protein preparation (Table II). Its carbohydrate components, which have not yet been analyzed completely, could account for a significant proportion of the protein. Due to the extremely small amount of the present renin preparation, detailed carbohydrate analysis will have to await future studies.

The isoelectric point of 5.2, obtained for the present pure hog renin, is in reasonable agreement with values ranging from 4.9 to 5.1 obtained by Lauritzen et al. (45) and Devaux et al. (46) for partially purified hog renin preparations.

The molecular weight of 36,400 estimated by sedimentation equilibrium was considerably lower than 42,000 and 42,500 obtained by gel filtration and SDS-polyacrylamide gel electrophoresis without dithiothreitol. Since the latter two methods can give rise to erroneously high molecular weight values due to molecular asymmetry or the presence of carbohydrate in the molecule (47), the lowest value obtained by the sedimentation experiment should be the most reliable estimate of the molecular weight. The SDS electrophoresis in the presence of dithiothreitol gave a definitely lower value of 35,000 compared to 42,500 obtained from a similar SDS electrophoresis in the absence of dithiothreitol. In view of the small number of half-cystine residues in the renin molecule (Table II), the possibility that cleavage of disulfide bridges caused a drastic change in the degree of molecular asymmetry does not seem to be plausible. It is more likely that the renin molecule consists of

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3 This result was obtained independently from recent observations by other investigators (41, 42).

4 Standardized renin kindly supplied by Dr. Erwin Haas of Cleveland (Lot no. 119) produced 135 pg of angiotensin I Goldblatt unit/h.

Standard renin supplied by The National Institute for Biologic Standards and Controls, London (Lot no. 119) possessed practically identical activity.
two polypeptide chains which are linked by disulfide bridges. However, the minor polypeptide chain has not yet been detected on polyacrylamide gels after electrophoresis in the presence of SDS and dithiothreitol. Further studies are needed to clarify this point.

It is noteworthy that hog renal renin and mouse submaxillary gland renin (38) seem to be closely related in a number of properties, although they do not cross react immunologically (48). For example, amino acid compositions of the two enzymes given in Table I very closely resemble each other except for the absence of amino sugars in the submaxillary gland enzyme and significant differences in the content of half-cystine, histidine, and methionine. Molecular weights, 36,400 for the hog enzyme and 37,200 for the mouse submaxillary gland enzyme, are very similar. The isoelectric points, 5.2 for hog renin and 5.3 to 5.6 for the mouse enzymes, are also very close.

It is interesting to note that the pH dependence of renin activity determined with the pure preparation is considerably different from that with crude preparations. When the synthetic fluorogenic octapeptide substrate was used, Reinharz and Roth observed that hog renin activity was highest at pH 5.5 (24), whereas with the purified enzyme it has a broad plateau of activity extending to a neutral pH. With the plasma renin substrate crude renin preparations generally show optimum activity near pH 5.5 to 6.0 (49). The present results agree very well with the ratio of 2.4 between the extents of activation has a specific activity of 2000 Goldblatt units/mg of protein, 2.6 times as high as that of Haas et al. (2). This figure is noteworthy that hog renal renin and mouse submaxillary gland renin (38) seem to be closely related in a number of properties, although they do not cross react immunologically (48).

Acknowledgments - We are greatly indebted to Dr. L. Holmberg, J. W. Harbor, and Haber, E. (1975) Biochim. Biophys. Acta 400, 258-262

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SALIVARY BENGKEL AND AHMAD MARWAN

**Purification of renin from hog kidney**

Affinity chromatography:

1. The affinity gel, in which the peptide [T] was coupled to an aminoacyl-

3. The affinity column was equilibrated with approximately 300 ml of 0.3 M amide, followed by

4. Further purification by conventional chromatography:

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**TABLE 1: Purification of renin from the hog kidney**

| Purification Step | Total Protein | Specific Activity | Purification | Yield (%) |
|------------------|---------------|------------------|--------------|-----------|
| Hog Kidney       | 112.000       | 0.002            |              | 1.0       | 100      |
| Crude Extract    | 30.000        | 0.089            | 4.3          | 52        |
| DEAE-Dextran ClO4 | 63.208       | 0.035            | 13           | 74        |
| Sephadex G-75    | 16.7          | 0.05             | 50           | 90        |
| CM-Dextran ClO4  | 2.8           | 0.005            | 100          | 51        |

* as angiotensin I forming renin protein at pH 8.0
Pure Renin. Isolation from hog kidney and characterization.
T Inagami and K Murakami

J. Biol. Chem. 1977, 252:2978-2983.

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