protein (5.64×10^5) prepared from isolated chloroplasts is much greater than the value of 3.75×10^5 reported by Eggman *et al.* (1953) for Fraction I protein derived from a cell-free protoplasmic extract of spinach leaves (*Spinacia* sp.), but is in closer agreement with the value of 5.15×10^5 for ribulose diphosphate carboxylase from the chloroplasts of spinach leaves (Trown, 1965).

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Purification of Pig Renin

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The major previous work on purification of pig renin was that of Haas, Lamfrom & Goldblatt (1953), who used methods mainly dependent on selective denaturation and salt precipitation. In the present work newer methods of protein purification have been used in an attempt to isolate the pure enzyme. Some of these have previously been applied as single procedures (Peart, 1959; Passananti, 1959; Nairn, Chadwick & Fraser, 1960; Lever & Peart, 1962). The steps in the preparation are shown in Table 1; a standard renin and method of assay were needed to follow changes in specific activity. The standard renin consisted of a large batch of renin taken through to the post-DEAE-cellulose stage and kept frozen (-10°) in 5ml. volumes. In these circumstances it was stable for at least 2 years without deterioration. An arbitrary unit was used based on the biological assay and which has no relation to any other renin unit reported from this Laboratory (Lever, Robertson & Tree, 1964) for the measurement of renin in body fluid. For the direct assay a solution of 80 units/ml. was used, and the pressor effect of the unknown was compared with the standard by intravenous injection into the anaesthetized rat (Peart, 1957).

One unit usually raised the blood pressure by 10–20 mm. Hg. The other assay method was by an enzyme velocity measurement (Lever *et al.* 1964), where standard renin or unknown was incubated with a standard substrate and the rate of production of angiotensin was measured. The final pressor assay was again performed in the rat. The protein concentration of solutions was measured by the technique of Lowry, Rosebrough, Farr & Randall (1951). In the preparation of a single batch, 18 kg. of fresh pig kidney was minced and then extracted with 30l. of cold (4°) ethanol overnight; the ethanol was removed by centrifugation and the residue again extracted overnight with 30l. of cold (4°) 0.9% NaCl. The supernatant containing the renin was recovered by centrifugation and the renin was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (40g./100ml.). After settling overnight, the precipitate was filtered off on Whatman no. 50 filter paper. It was dissolved in a minimum amount of cold tap water and dialysed in Visking cellophan sacs (36/32) against daily changes of tap water for 3 days until free of NH_3 . The resultant precipitate was centrifuged off, solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to a concentration of 3% (w/v),

Table 1. *Outline for purification of pig renin showing procedures employed and results obtained*

Steps 1-4 relate to 18 kg. batches of pig kidneys and give average results obtained from a number of batches. Steps 5-7 relate to a single pooled batch of step 4 renin. Step 8 shows results obtained from a limited amount of step 7 renin purified by electrophoresis.

Step	Description	Protein			Renin			Specific activity (units/mg. of protein)
		Applied (g.)	Recovered		Applied (units × 10 ⁶)	Recovered		
			(g.)	(%)		(units × 10 ⁶)	(%)	
1	Extraction	18000	210	1.2	Unknown	1.8		8
2	40% (w/v) (NH ₄) ₂ SO ₄	210	36	17	1.8	1.4	77	40
3	3% (w/v) (NH ₄) ₂ SO ₄	36	9	25	1.4	1.2	86	130
4	DEAE-cellulose	9	0.4	4.4	1.2	1.0	83	2500
5	CM-Sephadex	1.6	0.32	20	4.0	3.0	75	9000
6	Sephadex G-100							
	Front	0.32	0.024	7.5	3.0	0.77	26	32000
	Back		0.015	4.7		1.2	40	80000
7	Sephadex G-100 (superfine grade)	0.014	0.0065	46	1.1	0.66	60	100000
8	Electrophoresis	0.002	0.00064	34	0.18	0.07	39	110000

and the pH was adjusted to 2.5 by the dropwise addition of 6N-HCl. After standing for 30 min., the precipitate was centrifuged off and the supernatant adjusted to pH 7 by the dropwise addition of 5N-NaOH. The solution was dialysed in Visking cellophan (36/32) against three daily changes of 5mm-phosphate buffer, pH 7. The solution (61.) was then run on to a DEAE-cellulose column (3 cm. $\times$ 75 cm.) previously equilibrated with 5mm-phosphate buffer, pH 7, and washed with 5mm-phosphate buffer, pH 7, followed by 50mm-phosphate buffer, pH 7, to elute much of the impurity and a little renin. The main batch of renin was eluted with 0.15M-phosphate-saline buffer, pH 7 (50mm-phosphate-0.1M-NaCl). The renin fractions were pooled and dialysed against three daily changes of distilled water in Visking cellophan (28/32). The dialysed material was then freeze-dried. The renin preparations from five or more pooled batches derived from 90 kg. of kidney were combined at this stage and, to avoid deterioration of the purer material in the later stages, convenient amounts were taken from this pool through the final stages. Portions of this post-DEAE-cellulose material, equivalent to 800 mg. of protein, were next dialysed in Visking cellophan (28/32) against 0.2M-acetate buffer, pH 4.9, and applied to a CM-Sephadex column (2 cm. $\times$ 15 cm.) equilibrated with the same buffer. After application the column was washed with the same buffer and elution of the renin performed with 0.2M-acetate buffer, pH 6. Renin was then concentrated by pressure-dialysis against 0.1M-phosphate buffer, pH 6. From this stage on it was important to avoid exposing dilute solutions of renin either to large surfaces or to warmth and freeze-drying, since large and unpredictable losses

in activity occurred. Pressure-dialysis gave the highest recoveries. Post-CM-Sephadex material (100 mg. of protein) was next applied to a Sephadex G-100 column (2.5 cm. $\times$ 240 cm.) equilibrated with 0.5M-phosphate buffer, pH 6. The renin peak was divided into a front and back half and the back half was carried further, being first concentrated by pressure-dialysis against 0.1M-phosphate, pH 6. This renin fraction (14 mg.) was re-run on a Sephadex G-100 (superfine grade) column (2.2 cm. $\times$ 180 cm.) equilibrated with 0.5M-phosphate buffer, pH 6, and eluted in the same buffer. The renin fractions were again concentrated by pressure-dialysis against 0.35M- H_3BO_3 -NaOH buffer (0.3M- H_3BO_3 -0.05N-NaOH), pH 8.1, to a concentration of 4 mg./ml. Table 1 shows that all these stages gave a recovery of 60-80% of the applied renin. The increase in specific activity from the initial stage was 10⁴-fold. Apart from the increase in specific activity, further assessment of increased purity was made by starch-gel electrophoresis. This was carried out in vertical trays by using the discontinuous system of Poulik (1957). The renin ran in the pre-albumin region in a well-defined band, easily stained with Naphthalene Black. It was recovered from the starch by electrolysis of portions cut out of the block. It was convenient to compare the purities of different stages of the preparation by comparison of the stained protein pattern. The purest preparations of renin as determined by immunological techniques and specific activity were prepared from similar starch-gel electrophoretic runs. Antibodies to pig-renin preparations were raised in rabbits by using the post-DEAE-cellulose material with Freund's complete adjuvant. Starch-gel immunoelectrophoresis

was carried out. A slot was cut in the block parallel to the direction of running after the usual starch-gel electrophoresis and filled with agar. A 2mm. slot was then cut along the length of this in the centre and filled with antiserum. Precipitin lines appeared and could be compared with the position of the stained protein bands and the biological activity. The preparation of renin obtained from the Sephadex G-100 (superfine grade) step had three precipitin lines on starch-gel immunoelectrophoresis: one was opposite the renin activity; the other two were further back in the post-albumin region. Repeat electrophoresis of renin combined from a number of similar starch-gel electrophoreses resulted in a preparation with about the same specific activity but which had one stained band and one precipitin line with the anti-renin serum. The contaminants that ran behind the renin had been removed on the re-run. Double-diffusion techniques in agar gel revealed the presence, not only of this one major precipitin line which appeared early, but, after 48–72 hr., of two fainter lines closer to the renin well. Therefore immunologically this preparation is not completely pure. By assaying consecutive fractions of the gel cut out between the renin well and antiserum well, it was possible to show that the renin stopped abruptly at the main precipitin line and it is most likely that this represents the renin-

anti-renin precipitin line. The renin finally obtained was stable in the cold (4°), but losses occurred if the solution was diluted to the usual concentration used for direct pressor assay, even in the cold, or if the solution was warmed to room temperature (20°), or if it was frozen and thawed, or if frothing was caused in the solution.

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Isolation and Identification of 15 α -Hydroxyoestrone from the Urine of Pregnant Women

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Knuppen & Breuer (1964) have reported the isolation of 15 α -hydroxyoestrone after the incubation of oestrone with bovine adrenal glands. Since 15 α -hydroxyoestrone is the main product of oestrogen metabolism in the adrenal gland, it was suggested that this compound might also be present in urine (Breuer, 1964). Although this suggestion was rather speculative, it appeared to be sufficiently plausible to justify an intensive search in pregnancy urine for 15 α -hydroxyoestrone. Accordingly, the work described in the present communication was undertaken.

Specimens (1l.) of late-pregnancy urine were acidified to pH 4.8 with acetic acid, buffered to this

pH by the addition of 0.1vol. of M-acetate buffer and incubated at 37° with an extract from *Helix pomatia* [2ml., containing 100 000 Fishman units (Talalay, Fishman & Huggins, 1946) of β -glucuronidase and 50 000 Whitehead units (Whitehead, Morrison & Young, 1952) of sulphatase/ml.] for a period of 48 hr.; half of the total amount of enzyme used was added initially and half after 24 hr. The hydrolysed mixture was extracted twice with 0.67 vol. of ether; the extracts were combined and washed once with 0.1vol. of 8% (w/v) NaHCO₃ and then extracted twice with 0.17 vol. of N-NaOH. The NaOH extracts were neutralized to pH 7–8 with 5N-H₂SO₄. The extraction with NaOH and