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Purification of Pig Renin

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1. A new method of purification of renin is described. This method employs the following procedures: ethanol precipitation; saline extraction; precipitation of renin with 40% ammonium sulphate; precipitation of impurities with 3% ammonium sulphate at pH 2·5; chromatography on DEAE-cellulose and CM-Sephadex; gel filtration on Sephadex G-100 (both normal and superfine grade); finally, starch-gel electrophoresis. 2. The final renin preparation had a specific activity 10^4 times that of the initial saline extract. 3. A single band of stained protein corresponding to the renin activity was present on starch-gel electrophoresis in the final step and a single precipitin line was obtained to this material with rabbit anti-(pig renin) serum. 4. Double diffusion in agar with rabbit anti-(pig renin) serum showed one major precipitin line, probably due to renin–anti-renin complex, and in addition two minor components.

The purification of renin was undertaken by Haas, Lamfrom & Goldblatt (1953) with methods mainly dependent on selective denaturation and salt precipitation. Though this has been of value in the study of some of the properties of renin, the final product was of uncertain purity.

In the present work newer methods of protein purification were 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). Preliminary reports of certain of these techniques have been given (Peart, 1965; Peart *et al.* 1965).

EXPERIMENTAL

Materials and methods

Materials. Pig kidneys were obtained on the day of slaughter and stored at 4° for up to 24hr. before use.

Celite 545 was obtained from Johns-Manville Co. Ltd., London, S.W. 1. The DEAE-cellulose was Whatman Chromedia DE11. CM-Sephadex C-50 (lot no. To160M), Sephadex G-100 (140–400 mesh) and Sephadex G-100 (superfine grade; particle size 10–40 μ; lot no. To4634) were obtained from Pharmacia, Uppsala, Sweden.

Assay of renin. Two methods were used. The rat blood-pressure method described by Peart (1955, 1957–58) was employed for following the increase in specific activity of renin obtained in the purification steps. Rats were anaesthetized with pentobarbitone sodium (6mg./100g. body wt.) and pentapyrrolidinium tartrate (4mg./100g. body wt.) was administered. All assays were performed by using the bracketing technique with a standard pig renin. This standard was made up from a single batch of pig renin taken through the first four steps of the purification method and was found to be stable for more than 2 years at −10°. It was diluted with 0·15M-NaCl to contain 80 units of renin/ml. One such arbitrary unit gave an average elevation of blood pressure of 10–20mm. Hg. This renin unit bears no relation to the other unit used in this Laboratory (Lever, Robertson & Tree, 1964) for the assay of plasma renin. The other method used was 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. This technique is much more sensitive for small amounts of renin and was applied to some of the gel-diffusion experiments.

Protein estimation. Protein was estimated either by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine albumin as a standard, or by measuring E_{280} in a Unicam SP.500 spectrophotometer. Specific activity was expressed in terms of units of renin (as defined above)/mg. of protein.

Electrophoresis. Starch-gel electrophoresis was carried out vertically by using the discontinuous system of buffers suggested by Poulik (1957) and the 'split' starch method of Scopes (1963) (tray measured 36cm. × 12cm. × 0·6cm.). Hydrolysed starch (Connaught Medical Research Laboratory, Toronto, Canada; lot no. 210–1) was used in a concentration of 12g. of starch/100ml. of buffer. The initial gel was made in 0·081M-tris–citrate buffer (0·076M-tris; 0·005M-citric acid), pH 8·65. The starch was allowed to stand for 4hr. and then the whole of the starch 5cm. proximal to the sample slots was cut away and replaced with gel made up with 0·35M-borate buffer (0·3M-boric acid; 0·05N-NaOH), pH 8·1. This was allowed to stand overnight before use. Electrophoresis was carried out at room temperature by employing a constant current of 22mA and a voltage gradient of 6–7v/cm. Diluted human serum stained with bromophenol blue was used as a marker and

the run was stopped when the borate front was 1 cm. in advance of the albumin, after approx. 3 hr. The starch slab was placed at 4° for 15 min. immediately after electrophoresis and then marked at measured distances by sucking out vertical holes with a capillary pipette so that corresponding areas on horizontal slices of the starch slab could be identified. The slab was then sliced into two or three layers. Usually the top slice was stained with 1% Naphthalene Black in methanolic acetic acid (methanol–acetic acid–water, 4:1:1, by vol.). The middle slice was used for immunological studies. The bottom slice was cut in 5 mm. sequential strips that were eluted with 0·15 M-NaCl and assayed for renin activity.

Electrolysis. This method was employed to recover high-specific-activity renin after starch-gel electrophoresis. A Perspex tube (2·5 cm. × 8 cm.) was occluded at the lower end by Visking cellophan (36/32) and immersed in 0·35 M-borate buffer, pH 8·1, containing the negative pole. It was filled to within 1 cm. of the top with the same buffer. Strips of starch for elution were laid on the cellophan at the bottom of the chamber. The positive pole was provided by a short length of glass tubing (0·8 cm. diam.) occluded at the lower end by Visking cellophan filled with the same buffer and dipping into the electrolysis chamber. It was connected by a gauze wick to a reservoir containing 0·35 M-borate buffer, pH 8·1, and the positive pole. Electrolysis was carried out at 22 mA constant current for 2 hr. at room temperature. Platinum-wire electrodes were used.

Anti-renin. Antisera were raised in rabbits by using step 4 renin as antigen. This material contained 20 mg. of protein/ml. and approx. 50000 units of renin/ml. Three intramuscular injections of 0·5 ml. of step 4 renin mixed with 0·5 ml. of complete Freund's adjuvant were given to each rabbit at weekly intervals. Higher amounts of antibodies were produced in some rabbits by three subsequent weekly intramuscular injections of alum-precipitated step 4 renin. Blood was taken 1 week after the last injection, the serum separated and anti-renin titre assayed by mixing serial dilutions with standard renin and measuring the amount of renin neutralized (Lamfrom, Haas & Goldblatt, 1954) in the rat blood-pressure assay. One unit of anti-renin was that which completely inhibited 1 unit of renin. The production of antibody was very variable and not all rabbits produced anti-renin. The antisera used in these studies were obtained from rabbit 3, which had both renin–adjuvant and alum-precipitated renin and yielded 5000 units of anti-renin/ml., rabbit 12, which received renin–adjuvant alone and yielded 2500 units of anti-renin/ml., and rabbit 11, which had renin–adjuvant alone and produced no demonstrable anti-renin but did produce antibodies to other antigens in step 4 renin, including those contaminants still present in material from step 8 of the purification procedure.

Immunoelectrophoresis. A strip of starch gel containing two adjacent protein separations was placed on a glass plate (10 cm. × 13 cm.) that was coated with a thin layer of agar [1% agar (Oxoid no. 3) in barbitone buffer (Oxoid barbitone–acetate buffer for electrophoresis), pH 8·6 and I 0·1]. A wide trough was then cut out of the starch strip to include half of each adjacent protein run for comparison. This trough was filled with agar and when it was set a central longitudinal slot 2 mm. wide was cut out, antiserum was introduced and the plate was placed in a humid chamber at room temperature for up to 72 hr. Most of the precipitin lines had reached a maximum by 24–48 hr.

Double diffusion in agar. Double-diffusion studies were performed in an agar plate (Ouchterlony, 1958). Wells 5 mm. in diameter were used approx. 12 mm. apart from the centre of each well.

Purification

The main steps are shown in Table 1. Each batch of pig kidneys was taken up to step 4. Material from this step was stored at −10° for various times and portions were used for the further steps.

The steps up to and including step 4 described below are for each 18 kg. batch of pig kidneys.

Step 1: saline extraction. The kidneys were cut into halves longitudinally, fat and connective tissue were removed, and then they were minced in an electrical grinder. The mince was suspended in 30 l. of ethanol at 4° and stirred overnight. The liquid was then filtered through a single layer of muslin and dried by spinning in a linen bag in a domestic spin-drier (Hoover) to remove the ethanol. The part-dried kidney mince was then suspended in 30 l. of 0·9% NaCl and stirred overnight at 4°. The suspension was placed in a linen bag and spun to dryness in the spin-drier. The saline extract was retained and the solid discarded.

Step 2: 40% (w/v) ammonium sulphate precipitation. A 12 kg. portion of ammonium sulphate was dissolved in the 30 l. of saline extract and allowed to stand for at least 3 hr. Then 700 g. of Celite 545 was sprinkled on to carry the precipitate down on overnight settling. The supernatant was decanted off and the residue filtered on Whatman no. 50 filter paper. The precipitate was scraped off the filter paper and dissolved in a minimum of cold tap water. The filter paper was washed again with tap water and this was added to the slurry (approx. 3 l.), which was then dialysed in Visking cellophan sacs (36/32) against daily changes of 30 l. of tap water for 3 days. A fairly heavy precipitate was usually present in the dialysed material. This was removed by centrifugation (1800g for 30 min.) and the supernatant decanted. The precipitate was washed with 3 l. of tap water, centrifuged off (1800g for 30 min.) and discarded, the second supernatant being added to the first. The material throughout this step was kept at 4°.

Step 3: 3% (w/v) ammonium sulphate precipitation (pH 2·5). Ammonium sulphate (30 g./l.) was stirred into the renin solution from step 2 and then adjusted to pH 2·5 by the dropwise addition of 6 N-HCl. This was left to stand for no longer than ½ hr. and the precipitate was then centrifuged off (1800g for 30 min.) and discarded. The supernatant was immediately adjusted to pH 7·0 by the dropwise addition of 5 N-NaOH and dialysed in Visking cellophan sacs (36/32) against three daily changes of 0·005 M-phosphate buffer, pH 7·0 (40 l.). This step was carried out at 4°.

Step 4: DEAE-cellulose chromatography. DEAE-cellulose was prepared by equilibrating with 0·005 M-phosphate buffer, pH 7·0. Columns (3 cm. × 75 cm.) were packed under a pressure of 16 mm. Hg to a constant volume. The solution from step 3 (9 g. of protein in 6 l.) was applied to the column, which was then successively washed with 0·005 M-phosphate buffer, pH 7·0 (1 l.), and 0·05 M-phosphate buffer, pH 7·0 (1 l.), and finally elution of renin was achieved with 0·15 M-phosphate–saline buffer (0·05 M-phosphate buffer; 0·1 M-NaCl), pH 7·0. The first two washes were performed at 4° over 2 days and the final elution was made at room temperature on the third day. Fractions (25 or

50ml.) were collected and assayed for renin. The fractions containing renin were pooled, all those containing less than 160 units/ml. being discarded. This gave a volume of about 1l., which was dialysed in Visking cellophan sacs (28/32) against three daily changes of 10l. of distilled water (4°). The dialysed material was then freeze-dried to about 10–20ml. Precipitate at this stage was centrifuged off (1800*g* for 30min.) and discarded. Protein concentration was measured (Lowry *et al.* 1951) and sufficient M-phosphate buffer, pH6·0, was added to give a final protein concentration of 20mg./ml. in 0·1M-phosphate buffer, pH6·0. This was stored (−10°) in 5ml. fractions. For convenience five or more batches of post-DEAE-cellulose renin were pooled, resulting in about 5000000 units of renin of the same specific activity.

Step 5: CM-Sephadex chromatography. A 20g. portion of CM-Sephadex was prepared as usual and equilibrated with 0·2M-acetate buffer, pH4·9, packed under a pressure of 20cm. H_2O in a column (2cm. × 17cm.) and run at 4°. A 40ml. sample of step 4 renin (800mg. of protein) was dialysed overnight in Visking cellophan sacs (28/32) against 0·2M-acetate buffer, pH4·9, and applied to the column at a rate not exceeding 0·25ml./min. The column was then washed with 220ml. of 0·2M-acetate buffer, pH4·9, at the same rate. Elution of the renin was achieved with 0·2M-acetate buffer, pH6·0, 10ml. fractions being collected. The fractions containing renin were then concentrated by pressure dialysis (740mm. Hg) against 0·1M-phosphate buffer, pH6·0 (Visking cellophan 8/32), to approx. 5ml.

Step 6: Sephadex G-100-gel filtration. Sephadex G-100 was sieved and all particles over 200 mesh were discarded. Particles of 140–200 mesh were used. A 50g. portion was allowed to swell in 0·5M-phosphate buffer, pH6·0, for at least 5 days at 4°. A column (2·5cm. × 240cm.) was packed under hydrostatic pressure by continuous electrical stirring of the Sephadex slurry in a funnel at the top of the column. A 100mg. sample of protein from step 5 in a volume of approx. 5ml. was applied to the column at room temperature, and elution was achieved with 0·5M-phosphate buffer, pH6·0, at a rate of 20–25ml./hr.; 10ml. fractions were collected. The renin was usually eluted in a volume of 100–120ml., which was divided into two approximately equal fractions, the front half and the back half, the latter having a lower protein content and a higher specific activity. This second renin fraction was pooled and concentrated to about 1–2ml. by pressure dialysis (400mm. Hg) in Visking cellophan (8/32) at 4° against 0·1M-phosphate buffer, pH6·0. The first half of the renin was also pooled, similarly concentrated and either added to a subsequent run on Sephadex G-100 or used for other studies.

Step 7: Sephadex G-100 (superfine grade)-gel filtration. Sephadex G-100 (superfine grade) was prepared for use in the same way as the ordinary Sephadex G-100. A column (2·2cm. × 180cm.) was packed to a constant height (36hr.). A 10–15mg. sample of protein in 1–2ml. of 0·1M-phosphate buffer, pH6·0, from the back half of the renin peak in step 6 was applied. Elution was performed at room temperature with 0·5M-phosphate buffer, pH6·0. A pressure of 15mm. Hg applied to the top of the column was required to maintain a flow rate of 7ml./hr.; 5ml. fractions were collected. Pooled renin fractions were concentrated by pressure dialysis (400mm. Hg) in Visking cellophan (8/32) at 4° against 0·35M-borate buffer, pH8·1, to a concentration of about 4mg. of protein/ml. (1–2ml.).

Step 8: starch-gel electrophoresis. A 0·03ml. sample of material from step 7 was introduced into each of six slots in the starch gel and human serum stained with bromophenol blue was introduced into the two outermost slots. At the end of electrophoresis a strip of starch corresponding to the zone between the borate front and the leading edge of the serum albumin in the adjacent marker was cut out and eluted by electrolysis. The material from several runs was pooled and concentrated by pressure dialysis (400mm. Hg) in Visking cellophan (8/32) at 4° against 0·35M-borate buffer, pH8·1, to about 0·3ml. Small amounts of insoluble starch were removed by centrifugation (1000*g* for 15min.).

RESULTS

Purification

Table 1 gives the recoveries and increase in specific activity for renin from each step in the purification procedure. The values represent average results from a number of experiments for steps 1–4, and for steps 5–7 the values represent the results of a single batch of step 4 renin. Step 8 represents the results obtained with a portion of the step 7 material.

Step 1: saline extraction. This yielded about 30l. of solution having an activity of 40–80 units/ml. and a total renin content of 1200000–2400000 units. Assay at this stage was difficult as the shape of the blood-pressure curve obtained in the rat suggested that not all the pressor activity could be ascribed to renin. The specific activity was 2–20 units/mg. (average about 8 units/mg.). The total protein recovered was usually 200g.

Step 2: 40% (w/v) ammonium sulphate precipitation. After this stage the volume was approx. 6l. The renin activity was 150–300 units/ml. and the total renin was 1000000–2000000 units. About 36g. of protein was recovered. This resulted in a specific activity of about 40 units/mg. and a recovery of over 75%. There was usually a fivefold purification. The rat blood-pressure response was now similar to that of purer renin.

Step 3: 3% (w/v) ammonium sulphate precipitation (pH2·5). The volume was 6l. at this stage. The renin recovery was nearly complete but only about 6–12g. of protein was recovered, resulting in a three- to four-fold increase in specific activity to about 130 units/mg. of protein.

Step 4: DEAE-cellulose chromatography (*Fig.* 1). Approx. 20–30ml. of material containing 400–600mg. of protein and about 1000000 units of renin was recovered from this stage. The specific activity of the material was 1600–3900 units/mg. of protein and recovery of renin was over 80%. Purification was ten- to 20-fold.

Step 5: CM-Sephadex chromatography (*Fig.* 2). Of the 4000000 units applied to this step about 3000000 were recovered (75%). The specific

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

Steps 1–4 relate to 18kg. 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.

		Protein			Renin			
			Recovered			Recovered		Specific activity
Step	Description	Applied (g.)	(g.)	(%)	Applied (units × 10^6)	(units × 10^6)	(%)	(units/mg. of protein)
1	Extraction	18000	210	1·2	Unknown	1·8		8
2	40% (w/v) $(NH_4)_2SO_4$	210	36	17	1·8	1·4	77	40
3	3% (w/v) $(NH_4)_2SO_4$	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

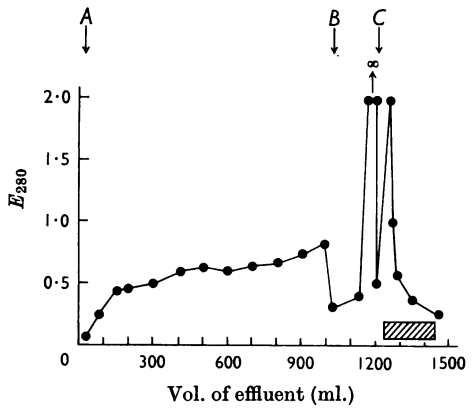

Fig. 1. Step 4 of the purification of pig renin: DEAE-cellulose chromatography. Step 3 material was applied to a column (3cm. × 75cm.) of DEAE-cellulose as described in the text and eluted with: *A*, 0·005M-phosphate buffer, pH7·0; *B*, 0·05M-phosphate buffer, pH7·0; *C*, 0·15M-phosphate–saline buffer, pH7·0. Renin fractions are shown under the hatched block.

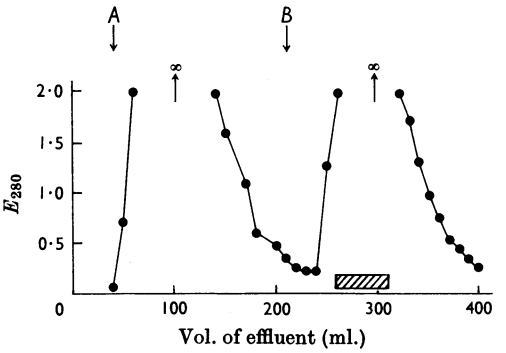

Fig. 2. Step 5 of the purification of pig renin: CM-Sephadex chromatography. Step 4 material was applied to a column (2cm. × 15cm.) of CM-Sephadex as described in the text and eluted with: *A*, acetate buffer, pH4·9; *B*, acetate buffer, pH6·0. Renin fractions are shown under the hatched block.

activity increased three- to four-fold from about 2500 to about 9000 units/mg. of protein.

Step 6: Sephadex G-100-gel filtration (*Fig.* 3). The renin from this stage was divided into two fractions. The front fraction contained about 770000 units of renin, a recovery of 26%. The specific activity was 32000 units/mg., a three- to four-fold increase. The back fraction contained 1200000 units of renin, a recovery of 40%. The specific activity was 80000 units/mg. of protein, an eight- to ten-fold increase.

Step 7: Sephadex G-100 (superfine grade)-gel filtration (*Fig.* 4). Of the 1100000 units of renin applied to the step, 660000 units were recovered (60%) with a specific activity of about 100000 units/mg. of protein.

Step 8: starch-gel electrophoresis (*Fig.* 5). Of the 180000 units of renin applied to this step, 70000 units were recovered (39%). The specific activity was increased to 110000 units/mg.

Criteria of purity

In the later stages of purification certain other methods besides increase in specific activity were used to demonstrate the increase in purity. These

methods were starch-gel electrophoresis, immunoelectrophoresis on starch gel and double diffusion in agar.

Starch-gel electrophoresis. Fig. 6 shows the results of starch-gel electrophoresis of renin recovered from steps 4, 5 and 6 of the purification method. Step 4 renin shows dense staining at the borate front, a sharp dense band in the pre-albumin region, a well-defined band opposite albumin and some staining extending into the post-albumin region. Renin activity was confined to a narrow zone corresponding to the stained pre-albumin band. After chromatography on CM-Sephadex, step 5 renin showed a loss of the pre-albumin band on the starch gel, whereas the albumin band became denser, as did the post-albumin staining. Renin activity, however, was still confined to a narrow zone in the pre-albumin position. (This demonstrates that most of the visible staining in the pre-albumin region in step 4 renin is not due to renin.) Step 6 renin shows

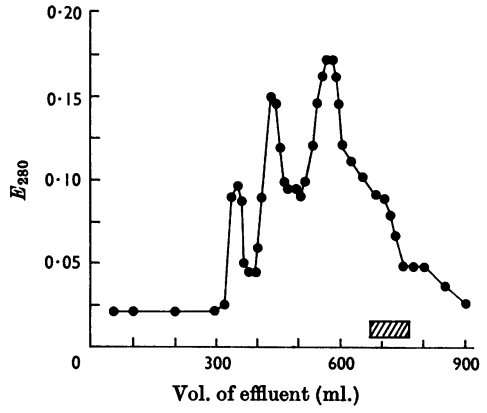

Fig. 3. Step 6 of the purification of pig renin: Sephadex G-100-gel filtration. Step 5 material was applied to a column (2·5 cm. × 240 cm.) of Sephadex G-100 as described in the text. Renin fractions are shown under the hatched block.

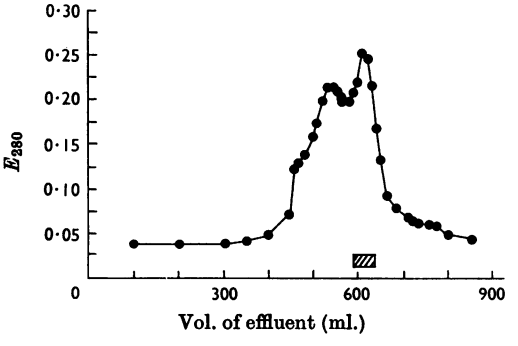

Fig. 4. Step 7 of the purification of pig renin: Sephadex G-100 (superfine grade)-gel filtration. Step 6 material was applied to a column (2·2 cm. × 180 cm.) of Sephadex G-100 (superfine grade) as described in the text. Renin fractions are shown under the hatched block.

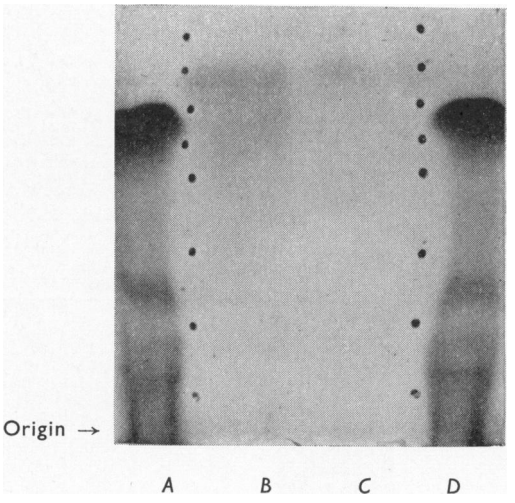

Fig. 5. Starch-gel electrophoresis of step 7 (*B*) and step 8 (*C*) renin compared with human serum (*A* and *D*). Details are given in the text. In the final preparation (step 8, *C*) only one protein band is visible, corresponding to the biological activity in the pre-albumin position.

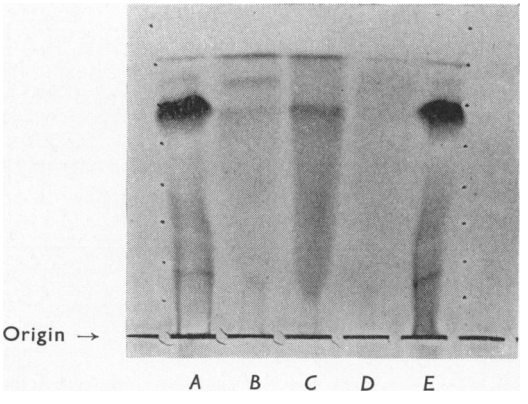

Fig. 6. Starch-gel electrophoresis of step 4, step 5 and step 6 renin (*B*, *C* and *D* respectively) compared with human serum (*A* and *E*). Details are given in the text. Renin was always recovered from the pre-albumin region and the removal of various contaminants by the different procedures can be seen.

some staining at the borate front, a faint band in the pre-albumin region and even fainter staining at the albumin level and post-albumin region. Renin activity again corresponded to the faint pre-albumin band.

Fig. 5 shows the results of starch-gel electrophoresis of renin from steps 7 and 8 of the purification method. The borate front is not visible. A well-defined band was present in the pre-albumin position in both samples. The slower-moving material in step 7 material has been eliminated in step 8. Step 8 renin showed a single stained band on electrophoresis that corresponded exactly to the pressor activity on assay.

Immunoelectrophoresis. Fig. 7 illustrates the results of immunoelectrophoresis on starch gel applied to step 7 and step 8 renin with rabbit anti-(pig renin) serum (rabbit 3). In this system the material subjected to electrophoresis in the starch diffuses into the agar to meet antiserum diffusing from the central slot. Precipitin lines form in the agar. The introduction of serum into the central well always resulted in a non-specific white line in the agar adjacent to the starch. The precipitin lines appeared nearer to the central slot. Step 7 renin produced three visible precipitin lines in this system, one of which was opposite the pre-albumin band and renin activity in the starch. Step 8 renin produced only one visible precipitin line at the same level corresponding to the renin activity.

In this system immunological purity seemed to have been achieved, if the visible precipitin line could be ascribed to the renin–anti-renin complex. Portions of agar were removed from both sides of this precipitin line and assayed for renin. No renin was detected on the anti-renin side of the precipitin line but large amounts were easily detected on the side nearer to the starch (Fig. 8).

Double diffusion in agar. Double diffusion in agar by using step 8 renin and anti-renin (rabit 3) produced one precipitin line after 24hr. followed in 24–48hr. by at least two further fainter precipitin lines closer to the renin well. Step 8 renin and antiserum containing no anti-renin (rabbit 11) did not produce the major precipitin line. The two later-appearing precipitin lines were seen and these showed reaction of identity with the later-appearing

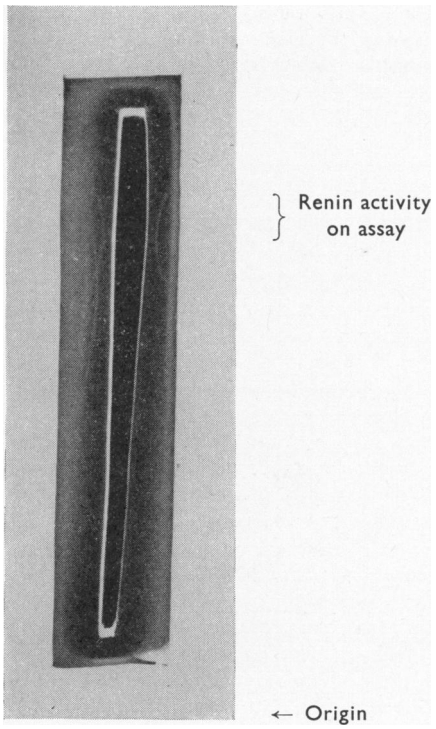

Fig. 7. Immunoelectrophoresis on starch gel of step 7 and step 8 renin. Details are given in the text. Between the slot containing rabbit antiserum in central agar and the starch, curved precipitin lines are visible. At least three arcs can be seen on the left that correspond to step 7 renin, and only one on the right corresponding to step 8 renin. Both the stained protein band and the biological activity were present in the starch opposite the precipitin arc.

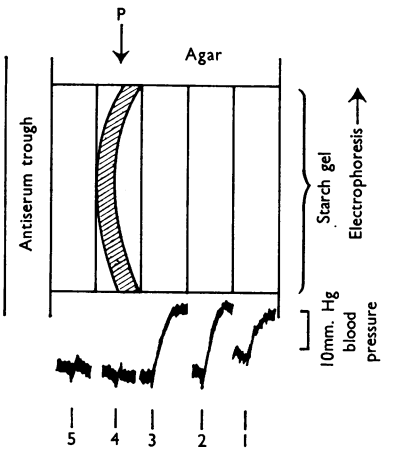

Fig. 8. Semi-diagrammatic representation of immuno-electrophoresis on starch gel of step 8 renin. The starch gel lies to the right of the agar and the antiserum trough. Successive sections between the starch and the antiserum trough were cut out as shown 1–5. The precipitin arc shown in Fig. 7 lay in segment 4. Direct pressor assay of renin eluted from each segment of agar separately was carried out and is shown below each. Renin was present in segments 1–3 inclusive and absent from the segment (4) containing the precipitin line (P) or beyond it (segment 5).

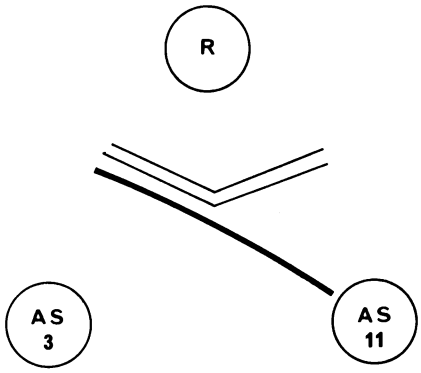

Fig. 9. Double diffusion in agar of step 8 renin (R), antiserum from rabbit 3 containing anti-renin (5000 units/ml.) (AS3) and antiserum from rabbit 11 containing no anti-renin (AS11). The diagram shows the precipitin lines that developed at 12–48hr. Three are present between R and AS3, whereas only two are present between R and AS11. These latter two, which are the later-appearing lines, show reaction of identity with the corresponding lines between R and A S3.

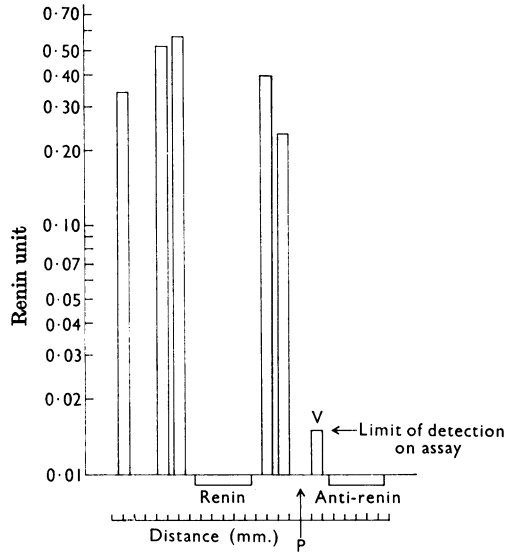

Fig. 10. Diagrammatic representation of double diffusion in agar of renin and anti-renin. The position and exact dimensions of the wells in the agar are shown on the abscissa and the site of the major precipitin arc is represented by the arrow marked P. Cylinders of agar (1mm. diam.) were punched out with a needle at points corresponding to the vertical bars and the pressor assay shown by the height of the vertical bars was measured on a logarithmic scale (ordinate).

precipitin lines between anti-renin (rabbit 3) and step 8 renin when these were put up simultaneously (Fig. 9). This strongly suggested that the early precipitin line was due to a renin–anti-renin complex. To demonstrate that this was the case, renin was assayed in portions of agar removed from the plate. When the precipitin lines were fully developed at 48hr., portions of agar were removed with a needle whose internal bore was 1mm. These were assayed by the enzyme-kinetic technique. It was easily shown that renin was present only up to the main precipitin line and could not be detected beyond it, whereas on the side of the renin well opposite to the antiserum well renin was present in large quantities beyond the point corresponding to the precipitin line, showing that renin could diffuse freely on this side in the absence of antiserum (Fig. 10). Allison & Humphrey (1960) described this type of approach to antigen–antibody precipitation in gel diffusion.

Renin stability

Renin up to step 4 is fairly stable and withstands warming to room temperature for short periods without significant loss. It can be stored for up to 2 years at $-10°$. Beyond step 5 the renin is much less stable and large and variable losses occurred when the material was frozen and thawed, freeze-dried, dialysed against large volumes of distilled water, warmed to room temperature, diluted to a concentration of less than 80 units/ml. or mixed so as to produce frothing. For these reasons all material beyond step 4, particularly when in dilute solution, was stored at 4°. Under these circumstances it was stable for up to 2 months. Concentration by freeze-drying was avoided and pressure dialysis employed instead. Material from step 5 withstood pressure dialysis at 740mm. Hg without significant loss. More purified renin was occasionally partially lost during pressure dialysis at these high pressures. A pressure of about 400mm. Hg was found to give good recovery. Presumably the loss was through the cellophan although no direct evidence was obtained, and as the molecular weight of renin is probably about 40000 this was a surprising finding. The approximate molecular weight was obtained by comparing the elution volumes of bovine albumin and renin on a Sephadex G-100 column (Andrews, 1964).

No preservatives were added to the renin at any of the stages of purification. Reliance was placed more on preventing gross contamination by cooling and avoiding delays between the steps. However, Merthiolate (thiomersal) could safely be added to renin solutions in a concentration of 3·3mg./100ml. without detectable loss of activity. Neomycin

sulphate (0·02%) has been used similarly, and the renin of the final step has been stored with either of these preservatives.

DISCUSSION

The extraction of crude renin from pig kidney described above is somewhat modified from that described by Peart (1957–58). The use of DEAE-cellulose chromatography in the purification of renin was also modified from the description by Peart (1959) and Lever & Peart (1962).

The CM-Sephadex step and the Sephadex G-100 step are completely interchangeable in order. It was found more convenient to employ CM-Sephadex first as up to 800mg. of protein could be applied to each column whereas the larger Sephadex G-100 column would separate effectively only about 150mg. of protein. Both CM-Sephadex and Sephadex G-100 columns can be run either at 4° or at room temperature with high recovery. Great care was exercised in adjusting flow rates on the CM-Sephadex columns as too high a flow rate during application and washing resulted in incomplete adsorption on the column at this stage. Elution could safely be carried out at a higher flow rate. The same column could be used for as little as 40mg. of protein with good recovery.

Sephadex G-100 was found to be superior to both Sephadex G-75 and Sephadex G-200 in the separation of renin from other proteins in the molecular-weight range 30000–100000.

Renin recovered from step 7 was clearly not pure as demonstrated on starch-gel electrophoresis and immunoelectrophoresis. However, the clear separation by electrophoresis of renin from the major slower-migrating impurities, as demonstrated by immunoelectrophoresis, led to the attempt to produce higher purification by means of starch-gel electrophoresis.

Better recovery of renin after starch-gel electrophoresis than that obtained by Nairn *et al.* (1960) has been achieved by reduction of the running time, possible because of the better early resolution resulting from the use of Poulik's (1957) discontinuous buffer system together with the advancing of the borate front as described by Scopes (1963).

The major difficulty in comparing the present purified renin with that of Haas *et al.* (1953) is that they employed different assay techniques. Renin assay has not as yet been standardized. It is hoped that this will soon be remedied by using the enzyme-kinetic technique of Lever *et al.* (1964) and an international standard renin.

Passananti (1959), using DEAE-cellulose chromatography, did not achieve as high a purification as Haas *et al.* (1953). His experience, however, with relatively crude kidney extracts confirmed the value of this method in renin purification. Nairn *et al.* (1960), using renin from step 6 of the method of Haas *et al.* (1953), showed significant increases in specific activity by starch-gel electrophoresis, kaolin adsorption and immunological purification. However, none of their final preparations had as high a specific activity as the final preparation of Haas *et al.* (1953), even allowing for the difference in the assay technique. Electrophoresis in their hands gave a high degree of purification but with very low recovery, too low to be of practical value. The renin obtained by Haas *et al.* (1953) has not been subjected to comparable immunological or chromatographic studies either, so that comparison of the degree of purity with the present preparation is not possible.

Though Haas *et al.* (1953) expressed the purity of their samples in terms of specific activity defined in units of renin (Goldblatt dog units)/mg. dry wt., we have found it more convenient to express purity in terms of specific activity defined as units of renin (rat units, as defined)/mg. of protein (Lowry *et al.* 1951).

The purest renin recovered from step 8 of our purification procedure had a specific activity of 110000 units of renin/mg. of protein, which is a purification of at least 10^4 times that of the original saline extract. It gave a single stained band on starch-gel electrophoresis, and on double diffusion in agar against antiserum prepared to step 4 renin produced one major and two minor precipitin lines.

The present results of double-diffusion studies on the more purified renin samples are of particular interest in view of the studies of Lamfrom *et al.* (1954) and Nairn *et al.* (1960), both of whom found that the renin–anti-renin reaction was non-precipitating. Our findings give evidence for production of renin–anti-renin precipitation both in immunoelectrophoresis on starch gel and double diffusion in agar.

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