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RENIN AND ANGIOTENSIN-LIKE ACTIVITY IN RENAL LYMPH

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Doubts have been expressed about the presence of renin in renal vein blood during the development of renal hypertension (Peart, 1959; Gross & Turrian, 1960). For this reason the renal lymphatic system was examined as a possible alternative exit for renin.

In the present investigation pressor activity has been found in the renal lymph of the dog. Several of its biochemical and pharmacological characteristics have been compared with dog renin and studies have been made of the effect of renal artery stenosis upon its concentration in renal lymph. A preliminary report of these findings has been given (Lever & Peart, 1961).

METHODS

The collection of renal lymph. Dogs were anaesthetized with thiopentone sodium (May and Baker) 10 mg/kg and pentobarbitone sodium (Abbott Laboratories) 3–12 mg/kg/hr intravenously. Blood pressure was measured from the femoral artery by mercury manometer and intravenous infusions of 2–6 l. of 2·5 % glucose in 0·45 % sodium chloride were given via the femoral vein during experiments which lasted from 8 to 26 hr. These large amounts were necessary to maintain blood pressure and urine flow in the face of considerable fluid loss from the exposed abdominal cavity. A long transverse abdominal incision was made to expose both kidneys and their lymphatics. The gut was removed from rectum to duodenum, with ligation of the mesenteric vessels at their origins. In a few experiments the stomach and spleen were also removed, in the remainder a large polythene tube was passed through the duodenal stump to drain the stomach.

The anatomical arrangement of the renal lymphatics corresponded with the description by Sugarman, Friedman, Barrett & Addis (1942). A double system exists in the dog, with several larger lymphatic trunks passing from the hilum of the kidney along the vessels to the mid-line collecting duct. In addition, a smaller group of lymphatics lies in a plexus visible beneath the capsule of the kidney. These drain to the poles of the kidney and thence through the perinephric fat to the mid line.

In most dogs the renal lymphatics were identified by an intravenous injection of 5 ml. of 10 % Pontamine Blue (Gurr). The dye appeared in the renal lymphatics within 15 min, as described by Barer & Ward-McQuaid (1957) in the rabbit. A single hilar lymphatic from each kidney was cannulated with 00 nylon tubing (Portex), or polyethylene of 0·8 mm external diameter, and the lymph was collected in silaned glass tubes kept in iced water.

Thoracic duct collection. In eight dogs the thoracic duct was cannulated in the neck, and the concentrated lymph examined before and after nephrectomy by direct and indirect

pressor assay. Three of these dogs had been eviscerated before the collection. In the remaining five experiments the kidneys were removed retroperitoneally through bilateral loin incisions, without opening the anterior abdomen.

Non-renal lymph. In several of the dogs lymph was collected from various sources other than the kidneys; the thoracic duct in the neck after nephrectomy, the main left cervical duct, the testicular lymphatics and from a trunk lying on the ventral surface of the inferior vena cava below the kidneys.

Anticoagulants were not used, except for heparinization of the arterial cannula.

Hourly collections of lymph from both kidneys were made, together with lymph from a non-renal source.

Urine measurements. Urine was collected from each renal pelvis by ureteric cannulation with hourly measurement of individual volumes. Urinary sodium concentrations were measured by flame photometer and creatinine by a standard technique (Owen, Iggo, Scandrett & Stewart, 1954).

Stenosis of the renal artery was produced by tying a fine silk ligature around the vessel and a length of polyvinyl tubing of 2 mm external diameter. The tubing was withdrawn, leaving the ligature in position. The adequacy of this stenosis was assessed by the reduction in urine flow on the stenosed side compared with the control. A 50–75 % reduction was regarded as satisfactory. In some experiments the stenosis was adjusted on account of oliguria or inadequate reduction in urine flow.

Renal vein samples were collected in three experiments through a polyvinyl tube inserted via the femoral vein and inferior vena cava into the renal vein.

Assay of lymph

Lymph was assayed by its pressor effect on intravenous injection into rats anaesthetized with pentobarbitone sodium and treated with pentolinium (Peart, 1955, 1957). Two methods of assay have been used.

Direct assay. The lymph was injected into the rat without previous incubation with plasma. This is essentially a test for renin-like activity; injected renin reacts with the plasma of the test rat to give a pressor substance. The pressor activity of the lymph was assayed, as in the example of Fig. 1*A*, by bracketing with the pressor effect of a standard preparation of dog renin. Figure 2 shows a further comparison of the pressor effects of renal lymph and dog renin, and illustrates the similarity of response and duration of action. The standard preparation of dog renin used in these experiments was prepared by the method described by Peart (1955). It contained 1·6 mg protein/ml. as measured by the method of Lowry, Rosebrough, Farr & Randall (1951). An intravenous injection of 0·01 ml. of the material produced a pressor response of 8–20 mm Hg in the usual anaesthetized rat preparation. No loss of activity was detected after 9 months storage at −20° C.

Indirect assay. The lymph was incubated at 37° C for 20 min with dog plasma as substrate. In most experiments 0·5 ml. of lymph was added to 0·5 ml. of the substrate. In the example illustrated in Fig. 1*A*, the proportions were plasma substrate 0·7 ml. and lymph 0·3 ml. The pooled plasma used as a source of substrate in all these experiments was obtained by bleeding two heparinized dogs. Samples from the incubated mixture of plasma and lymph were injected intravenously in the rat and the pressor effect was assayed by comparison with the effect of a standard solution of synthetic angiotensin Val. 5 octapeptide ('Hypertensin', Ciba). This standard contained 0·1 μg/ml. Figure 1*A* is an example of an indirect assay. The indirect assay results are expressed in terms of angiotensin μg/ml. produced on incubation.

Lymph nitrogen concentrations were measured by a micro-Kjeldahl method in three experiments.

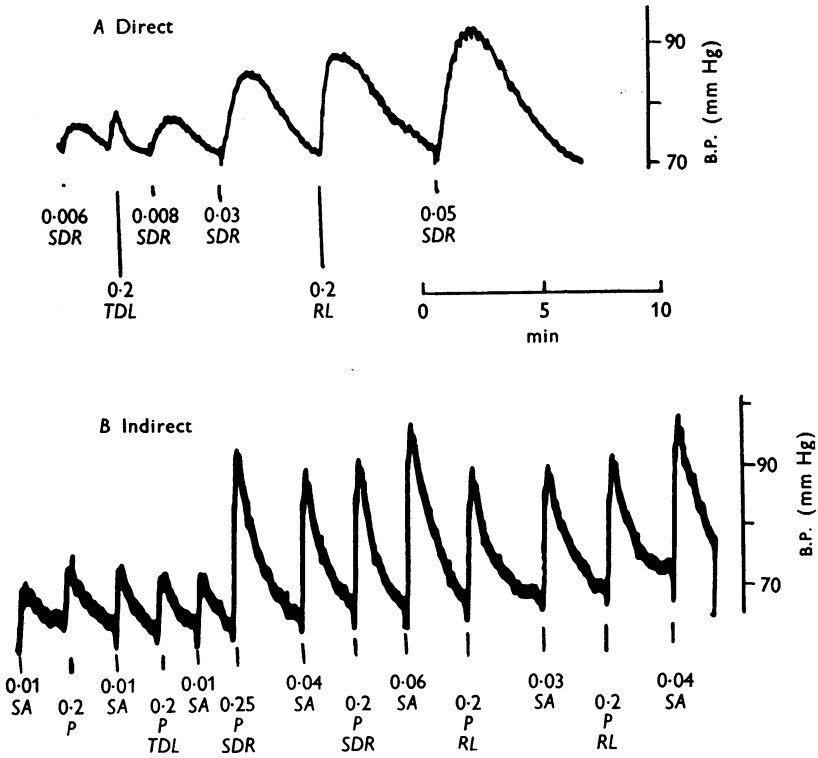

Fig. 1. Direct and indirect assay on the rat blood pressure preparation.

A Direct: samples of lymph; (1) renal lymph (*RL*) and (2) concentrated thoracic duct lymph after a bilateral nephrectomy (*TDL*), assayed by direct injection against standard dog renin (*SDR*). All doses in ml. (0·2 ml. *RL* = 0·03–0·05 ml. *SDR*).

B Indirect: the following mixtures were incubated at 37° C for 20 min and then assayed on the rat against a standard solution of angiotensin (*SA*) containing 0·1 μg/ml. (1) 0·7 ml. dog plasma substrate (*P*) + 0·3 ml. 0·9 % NaCl (*P*); (2) 0·7 ml. (*P*) + 0·3 *TDL* (*P-TDL*); (3) 0·7 ml. (*P*) + 0·27 ml. 0·9 % NaCl + 0·03 *SDR* (*P-SDR*); (4) 0·7 ml. (*P*) + 0·3 *RL* (*P-RL*). All doses are in ml. (0·2 ml. *P-RL* = 0·03–0·04 ml. *SA*: ∴ *P-RL* ≡ 0·015–0·02 μg/ml. as angiotensin.) The same time scale applies to both *A* and *B*.

Separation of pressor activity

Sephadex gel filtration. Grade G.75 Sephadex (Pharmacia) was washed with 10 changes of tap water, equilibrated with 0·05 M-NaCl, adjusted to pH 6 with N/10 NaOH, and used in two ways:

(1) A short column 18 × 0·8 cm was used to separate the renin-like protein from the angiotensin-like peptide activity. The initial material was applied in 1 ml. and fractions of 1·2 ml. were collected.

(2) A long column 3·1 m × 1·2 cm was used to separate the renin-like material from other proteins in lymph, and to compare its mobility with standard dog renin. Activity was

applied in 1 ml., and 1·6 ml. fractions were collected. The position of the main protein peak in the effluent was identified by estimation of the 280 mμ absorption of the effluent fractions in a Unicam spectrophotometer (SP 500).

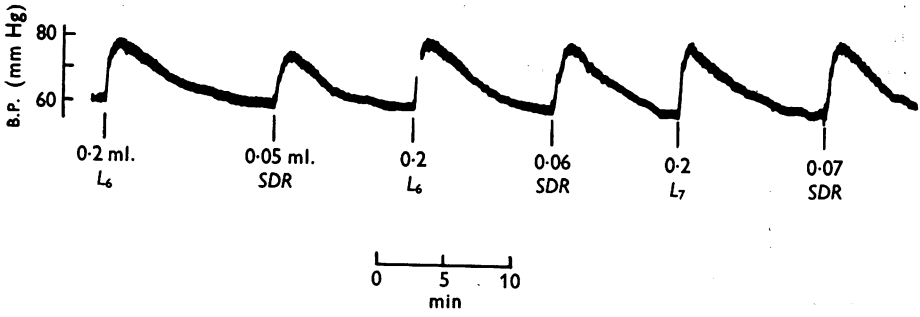

Fig. 2. Comparison of pharmacological response of renal lymph and standard dog renin. Two samples of renal lymph L_6 and L_7 collected during consecutive hours are compared with standard renin on the rat blood-pressure preparation.

DEAE cellulose chromatography

A diethylaminoethyl-cellulose column (10 × 1 cm) (Sober, Gutter, Wyckoff & Peterson, 1956) was used as an anionic exchanger in the adsorption and elution of renin and lymph pressor material. The column was equilibrated with 0·05 M pH 7 phosphate buffer. The activity was applied as either renin or lymph which had been dialysed against this buffer. Elution was achieved with a 0·15 M pH 7 buffer (0·05 M phosphate, 0·1 M-NaCl).

RESULTS

The presence of activity in renal lymph

Direct assay. 194 samples of renal lymph from sixteen dogs were assayed for their pressor activity against standard dog renin, as in the examples of Fig. 1*A*. The distribution of these results is shown in Fig. 3. Eighteen samples of non-renal lymph from eight dogs were assayed by the same technique and are also shown in Fig. 3 for comparison. The threshold value for the average rat blood-pressure preparation is at about 0·025 ml. of the standard solution of renin/ml. lymph. The majority of the renal lymph samples assay above this level, while all the non-renal samples fall below it.

Indirect assay. Likewise, the indirect assays against angiotensin after incubation with substrate show a similar distribution between the renal and non-renal lymphs (Fig. 4). Again all the non-renal samples fall below the limit of sensitivity of the rat, which is at about 0·01 μg angiotensin/ml. incubation mixture, whereas the majority of renal lymphs are above this level.

In both direct and indirect assays some of the renal and thoracic-duct samples fell below the threshold level for the assay preparation. Twelve such samples of renal lymph and 51 samples of thoracic-duct lymph were

concentrated five- to twentyfold by ultrafiltration or freeze drying and compared by indirect assay with nineteen samples of non-renal lymph concentrated to a comparable degree. After concentration, all the renal and thoracic-duct lymph samples were above the threshold level in the indirect assay (Fig. 4*C*), whereas the concentrated non-renal samples were all, as before, below this limit (Fig. 4*D*).

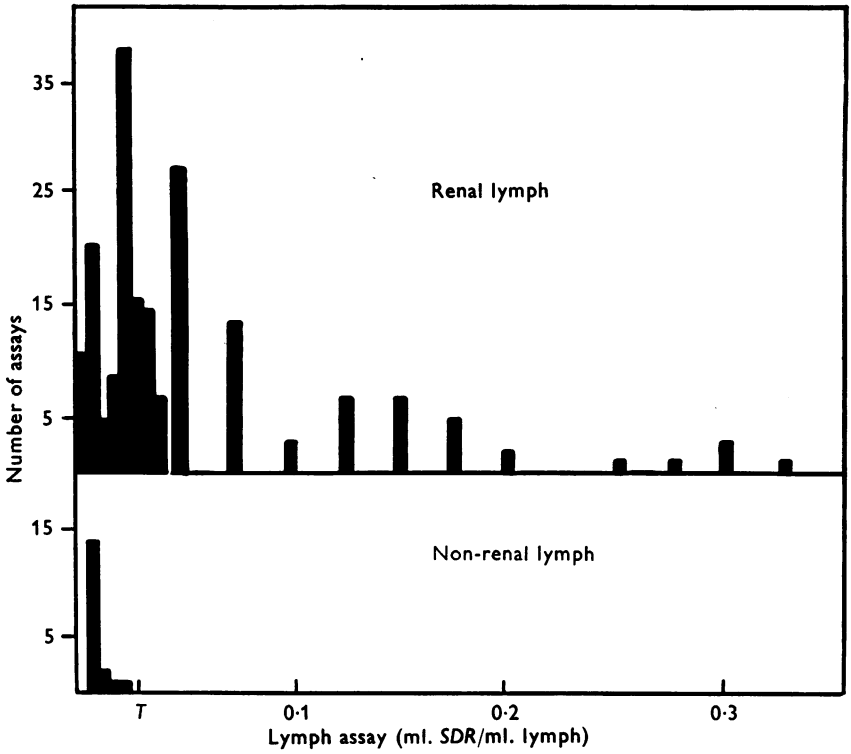

Fig. 3. Renin-like activity of lymph (direct method). 194 samples of renal lymph and 18 samples of non-renal lymph from 16 dogs were assayed against standard dog renin (*SDR*) by the direct method. *T* shows the threshold sensitivity of the usual assay.

Evidence of the source of pressor activity in thoracic-duct lymph

In eight dogs the thoracic duct was cannulated in the neck and lymph collected in silaned measuring cylinders surrounded by ice. After collection of two 90 min control samples, both kidneys were removed and, after a 20 min interval, two further 90 min collections of thoracic-duct lymph were made. Each of these collections was freeze-dried and dissolved in 15 ml. of distilled water. Figure 5 shows an experiment in which 0·2 ml. of this concentrated thoracic duct lymph had a large pressor effect in the

rat assay (TDL_1, TDL_2) but samples collected after nephrectomy (TDL_3, TDL_4) had no significant effect. In this experiment the pressor effect on direct injection has the rapid time course of angiotensin rather than the slower effect of renin (Figs. 1*A*, 2). As will be observed later, this can be explained by the presence in lymph of a substrate for renin in addition to a renin-like material.

Figure 6 shows the results of the remaining seven experiments where the individual lymph samples were concentrated to a constant volume and assayed against angiotensin, after incubation with substrate. Again in

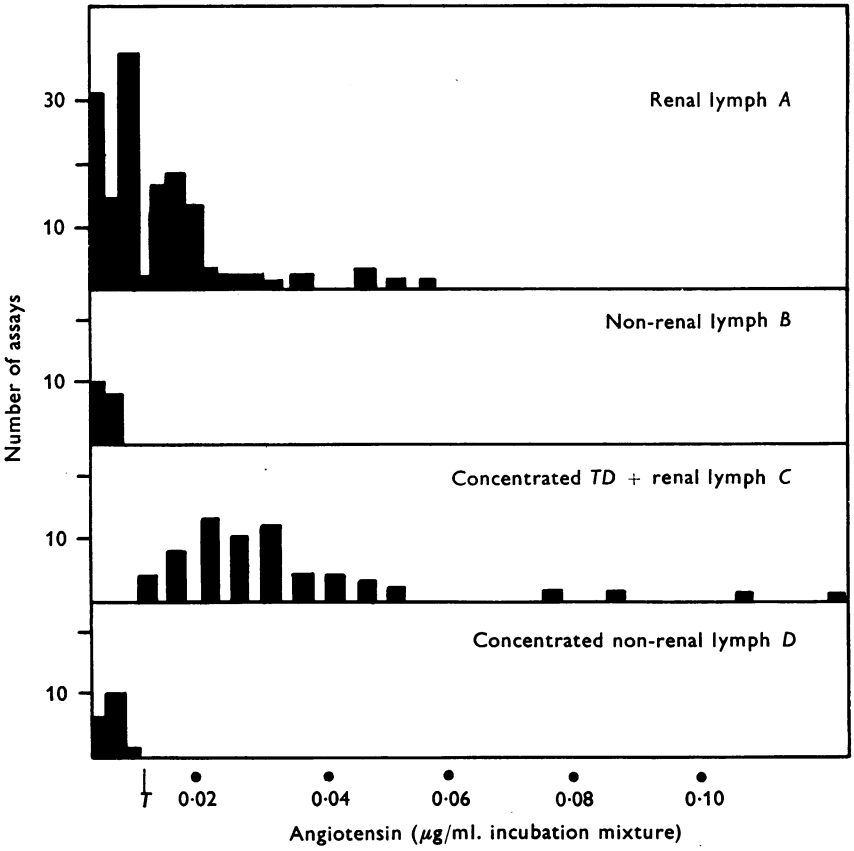

Fig. 4. Angiotensin-like activity of lymph (indirect method). *A*, 147 samples of renal lymph from 15 dogs assayed by the indirect method against standard angiotensin as in Fig. 1*B*. *B*, 18 samples of non-renal lymph from the same 15 dogs assayed by the same method. *C*, 12 of the least pressor renal samples and 51 non-pressor samples of thoracic duct lymph (*TD*) were concentrated and re-assayed by the same method. *D*, 19 samples of non-renal lymph similarly concentrated and re-assayed. *T* shows the threshold sensitivity of usual assay preparation for angiotensin.

each case bilateral nephrectomy was associated with a reduction of pressor activity to unassayable levels.

It was noted in each of these experiments that the bilateral nephrectomy was followed by a fall in the rate of thoracic-duct flow to about 50 % of its previous level, the flow before nephrectomy varying between 30 and 120 ml./hr. Three of the eight dogs were eviscerated before the thoracic-duct cannulation. As an approximate index of total renal lymph flow, this would give values considerably higher than those reported by Le Brie & Mayerson (1960) and Sugarman *et al.* (1942) derived from the flow in single cannulated renal lymphatics.

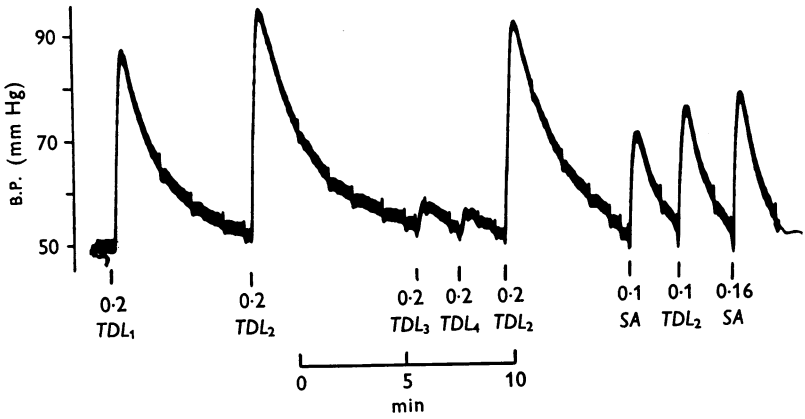

Fig. 5. Activity of thoracic duct lymph before and after bilateral nephrectomy. Direct assay on rat blood pressure. Thoracic duct lymph from 4 successive 90 min collections was concentrated to 15 and 0·2 ml. injected at $TDL_{1, 2, 3, 4}$; after TDL_2 both kidneys were removed. *SA* shows the injection (ml.) of a solution containing standard angiotensin 0·1 μg/ml.

Characterization of the pressor activity of renal lymph

Sephadex gel filtration. Some of the samples of renal lymph showed on injection into the rat a form of pressor response which had a fast initial response like angiotensin together with a slower decline like renin. It seemed likely that these samples might contain a mixture of slow- and fast-acting substances. As angiotensin, unlike renin, diffuses through cellophane, diffusible and non-diffusible fractions of renal lymph were obtained in five experiments by ultrafiltration of renal lymph derived from three dogs. The ultrafiltrate was then concentrated by vacuum distillation. The diffusible and non-diffusible fractions were run separately on an 18 cm Sephadex column, which had been previously shown to be capable of separating renin from angiotensin. The effect on the rat's blood pressure

of injecting 0·2 ml. of successive effluent fractions from one of these experiments is shown in Fig. 7. In the control mixture (Fig. 7*A*) the protein renin appears in the earlier fractions, the peptide angiotensin later. Recoveries of both substances were usually about 80%. With the diffusible fraction of renal lymph (Fig. 7*B*) material with the same pressor characteristics and mobility as angiotensin was obtained. The non-diffusible fraction (Fig. 7*C*) contained material with the same characteristics as renin.

Renal lymph can be shown to contain considerable amounts of renin substrate by incubating it with an excess of renin, when angiotensin-like material is made in larger quantities than in incubated samples of lymph without added renin. If the pressor material in lymph were renin therefore, it would be expected that a diffusible component like angiotensin would be formed without the addition of exogenous substrate.

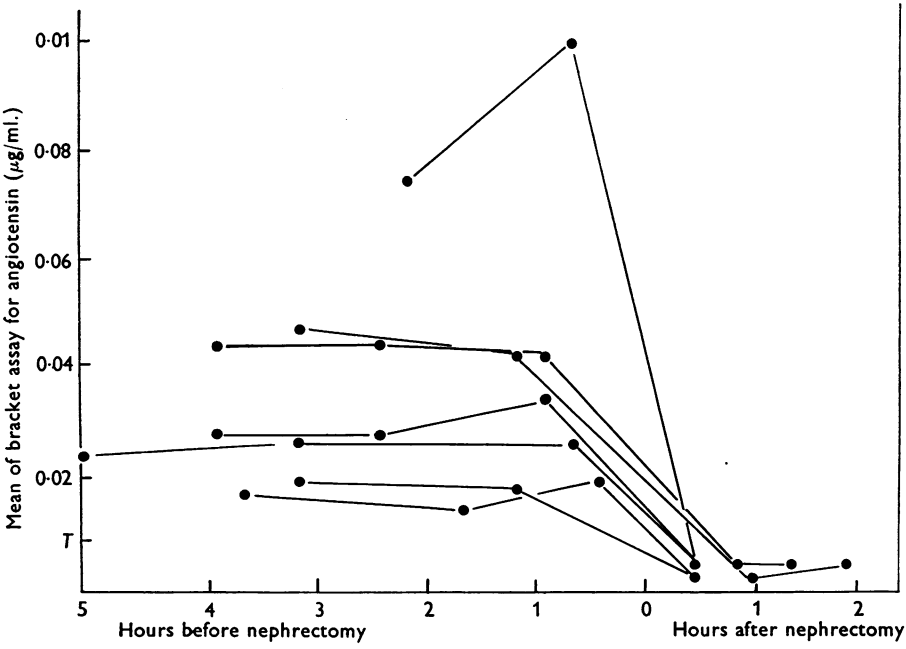

Fig. 6. Effect of bilateral nephrectomy on pressor activity of concentrated thoracic duct lymph. In seven dogs the pressor activity of concentrated thoracic duct lymph was assayed indirectly before and after a bilateral nephrectomy which in all cases was followed by a fall in pressor activity to undetectable levels. In each experiment the collections were made for equal times, and were then concentrated to the same volume, thereby enabling pressor activity of individual samples to be compared on the basis of hourly output of pressor material. Each point represents the mean value of an indirect assay as angiotensin of the samples. (*T* = threshold sensitivity of usual assay.)

The diffusible material in lymph has been further studied. Like angiotensin it is heat-stable, withstanding a temperature of 80° C for 30 min. It is rapidly destroyed by solutions of dog plasma of high angiotensinase activity.

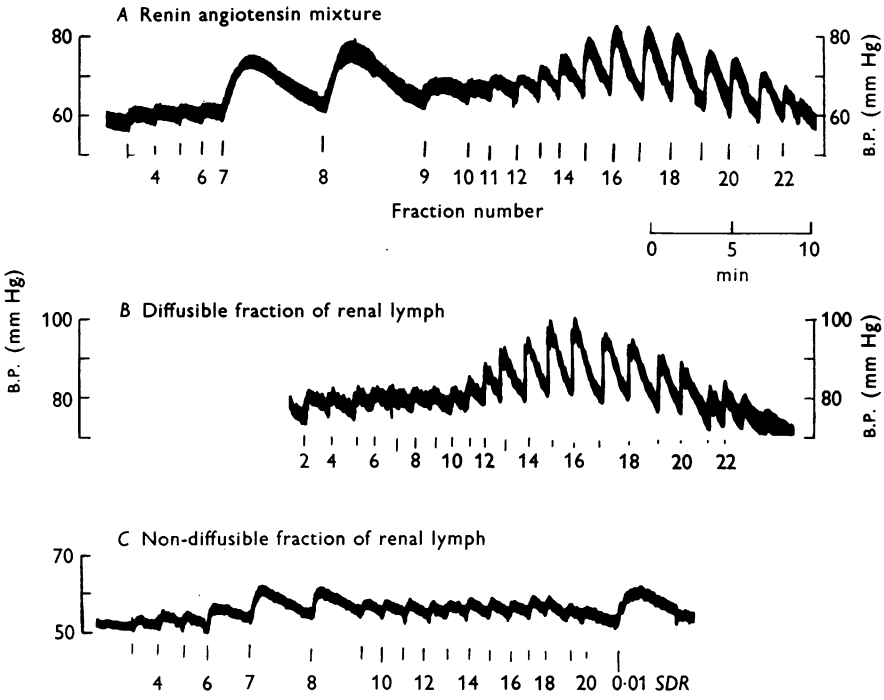

Fig. 7. Sephadex gel filtration, rat B.P. assay. *A*: Injection of 0·2 ml. of successive effluent fractions from an 18 cm Sephadex column after the application of 1 ml. of a mixture of standard dog renin and synthetic angiotensin. Renin appeared in fractions 7 and 8 and angiotensin in fractions 13–22. *B*: Injection into the same rat of 0·2 ml. of successive effluent fractions from the same column after the application of 1 ml. of a concentrate of the diffusible material in renal lymph. *C*: Injection into the same rat of 0·2 ml. of successive effluent fractions from the same column after the application of 1 ml. of the concentrated non-diffusible material in renal lymph. A final injection of standard dog renin (0·01 ml. *SDR*) was given. Column temperature 4° C.

Chromatography on a long Sephadex column. By using a 3·1 m G.75 Sephadex column it was possible to separate the activity in a crude preparation of dog renin from some of the protein impurities, the renin appearing after the main protein peak, which was identified by estimation of the 280 mμ absorption in the effluent fractions. A four- to eightfold purification, in terms of the activity:nitrogen ratio, was achieved in two experiments.

With the same column in three experiments the renin-like activity of lymph appeared in a similar delayed position relative to the main protein peak (Fig. 8).

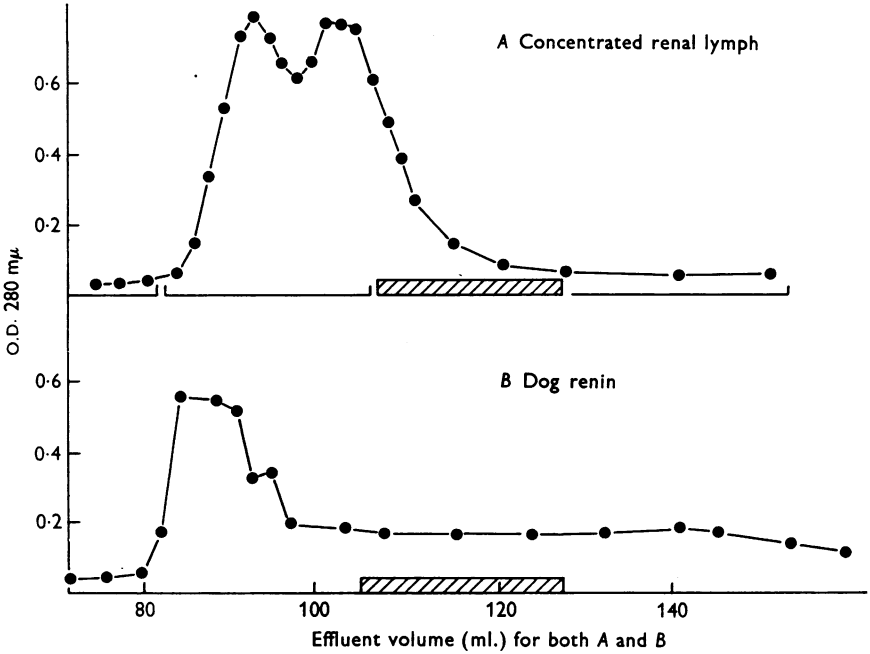

Fig. 8. Chromatography on a long Sephadex column (3·1 m). *A*: concentrated renal lymph and *B*, crude dog renin, applied separately to same column. ●—● individual effluent fractions. In *A* the short uprights indicate the effluent fractions which were combined for concentration and separate direct assay. The hatched area shows the only set of fractions in which renin-like activity was detected. In *B* the hatched area shows the fractions in which 70–80 % of the renin was present.

Diethylaminoethyl cellulose chromatography. With a 10 × 1 cm DEAE column, dog renin was adsorbed from a solution dialysed against 0·05 M phosphate buffer at pH 7. It was eluted with 0·15 M buffer (0·05 M phosphate, 0·1 M-NaCl). A fourfold purification was achieved. With a similar column in four experiments the pressor material in renal lymph was adsorbed and eluted with the same molarities of buffer solutions.

Dialysis. The slow-acting pressor activity in renal lymph was not diffusible. In two experiments there was no loss of activity of the slow-acting pressor material after dialysis for 24 hr in cellophane tubing against 0·9 % NaCl adjusted to pH 7 with N/10 NaOH.

Effect of heat. Heating renal lymph to 80° C for ½ hr in two experiments resulted in a complete loss of slow-acting pressor activity.

Enzyme kinetic properties. The velocity of the reaction between standard dog renin and plasma has been compared with that of renal lymph added to the same substrate. Both reactions produce maximal concentrations of angiotensin-like material after incubation at 37° for 15 min. Insufficient data have, however, been obtained to calculate their Michaelis constants.

Pressor activity of renal lymph after unilateral renal artery stenosis

In fifteen dogs bilateral renal lymph and urine collections were made before and after unilateral renal artery stenosis. The results in these fifteen experiments are shown in Table 1, and the details of one, showing an increase of pressor activity after stenosis, in Fig. 9. In this experiment the direct assay of renal lymph showed an increase in concentration of pressor activity on the side of the stenosis; the non-stenosed control side remained unchanged. The indirect assay after incubation showed a similar increase in the lymph from the stenosed side.

TABLE 1. The effect of stenosis of the renal artery on the pressor activity in lymph from that kidney, and the occurrence of anuria

	Change in assay bracket for renin after stenosis. No. of expts.	Distribution of experiments with anuria. No. of expts.
No change	4	3
1–2-fold increase	5	2
1·5–2-fold increase	2	–
2–3-fold increase	2	–
3–4-fold increase	2	–
Decrease	0	–

It seemed possible that the increase in pressor activity could represent an over-all increase in the concentration of renal lymph proteins by simple reduction in the water content of the lymph secondary to the renal artery stenosis. Although the actual flow of lymph in a single cannulated lymphatic did not usually change after the stenosis, it could not be inferred from this that the total renal lymph flow remained unchanged, as collection of all lymph from one kidney could never be achieved. For this reason the total nitrogen of renal lymph was measured as an index of protein concentration on both sides in three experiments. In none was stenosis followed by an increase in the concentration of nitrogen in the lymph from the ischaemic kidney, although in two instances (e.g. Fig. 9) definite increases in pressor material occurred. It would seem likely, therefore, that the increases in pressor activity represent an increased rate of secretion.

The urine was studied in thirteen of the fifteen dogs as an index of the effectiveness of the stenosis. Figure 9 shows the effect of renal-artery

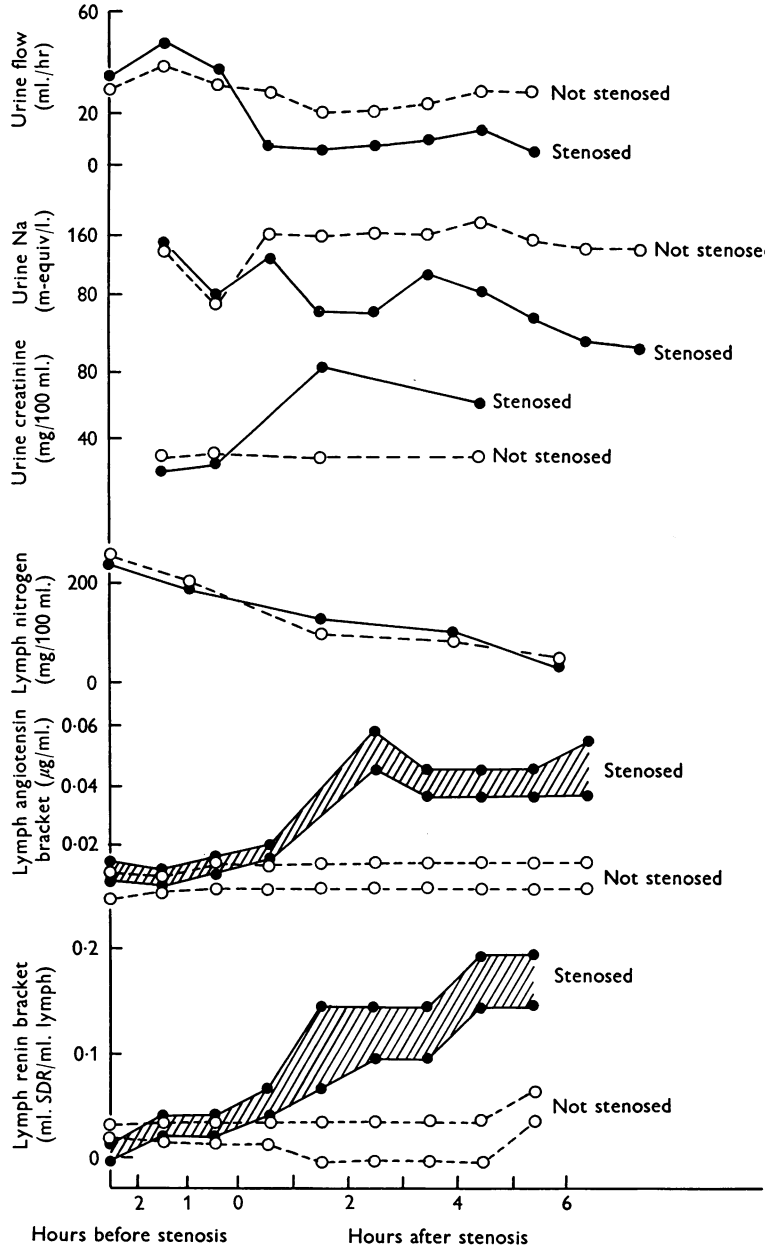

Fig. 9. Urine and lymph changes after renal artery stenosis; shows fall in urine flow and sodium concentration with rise in creatinine concentration on the stenosed side. The assay values for renin and angiotensin rise on the stenosed side without an increase in nitrogen concentration. ●—● side stenosed at time 0; ○—○ side not stenosed.

stenosis upon the urine volume, sodium and creatinine concentrations. The fall of urine volume and sodium concentration with a rise in creatinine concentration form the basis of a method of identifying renal-artery stenosis in man by bilateral ureteric studies (Howard, Berthrong, Gould & Yendt, 1954; Brown, Owen, Peart, Robertson & Sutton, 1960). Earlier Mueller, Surtshin, Carlin & White (1951) had described similar changes in urine volume and sodium concentration in the dog with chronic renal-artery stenosis.

An analysis of the fifteen experiments is shown in Table 1. In four there was no increase in pressor activity after stenosis, five showed a small increase of questionable significance and six a definite increase. The pressor activity of renal lymph from the stenosed side never fell below that of the control kidney.

In five of these experiments anuria lasting for 1 hr or more developed on the stenosed side without the flow of lymph ceasing. It is of interest to note the findings in these five experiments. In three there was no change in pressor activity and in two a doubtful change. None of the six experiments showing a definite increase in pressor activity developed such anuria. It would seem therefore that an increase in pressor activity was more likely to occur with moderate than with extreme renal ischaemia.

Renal-vein plasmas were assayed in three experiments. In none was renin-like material identifiable before or after stenosis, although no attempt was made at concentration of the plasma.

Control experiments

Cell-rich and cell-free fractions of lymph were obtained by centrifuging lymph and decanting the supernatant. There appeared to be no difference in the pressor properties of the two fractions. No difference appeared after freezing and thawing the cell-rich fraction.

Effect of blood. Several of the lymph samples contained small quantities of blood when collected, irrespective of whether they possessed pressor activity. In two separate experiments therefore 0·5 ml. of fresh dog blood was added to 5 ml. of blood-free but pressor renal lymph. No difference between the renin-like activity of this sample and its control was noted after 12 hr at 20° C.

Pontamine Blue. No effect on renin, angiotensin, or the ability of renin to form angiotensin from substrate, was noted in controls to which Pontamine Blue was added.

Sterility. In two dogs samples of renal lymph from a late stage of the experiment were cultured aerobically and anaerobically. No bacterial growth occurred.

DISCUSSION

The appearance of greater enzyme activity in lymph than blood is not unknown. Carlsten (1950) examined the thoracic-duct lymph of the cat for its histaminase activity and found it markedly reduced by bilateral nephrectomy. It is of interest that histaminase was not assayable in the peripheral blood and that the kidney was the richest source of extractable enzyme.

In these experiments a pressor material has been found in renal lymph. This material was not detectable in lymph derived from other organs or in the renal-vein blood. As renin itself has only been partially purified, the actual identification of the pressor material could not be taken beyond a comparison with a standard preparation of renin in respect of its biochemical, pharmacological and enzyme-kinetic characteristics. In nine of these respects, the material in renal lymph was indistinguishable from dog renin. In the majority of the experiments with renal-artery stenosis there was an increase in the pressor activity in renal lymph.

These results may indicate a method by which renal-artery stenosis could cause hypertension. It is emphasized, however, that none of these estimations were done on hypertensive animals, and that no animal became hypertensive during the experiment. Moreover, the conditions of prolonged anaesthesia with evisceration are very different from those in which experimental renal hypertension is usually produced. No absolute measure of the amount of pressor material leaving the kidney in the lymphatics has been obtained in these experiments. From the limited data available on renal lymph flow, however, it would seem that the absolute amounts of activity in lymph under these circumstances are small.

The possibility of pressor material leaving the kidney in both renal vein and renal lymph arose. In the three cases where negative results were obtained in the assay of renal-vein blood, it is still possible that pressor material was leaving the kidney in the renal vein in amounts absolutely greater than in the renal lymph by virtue of the difference in flow rates in the two systems, particularly as the renal-vein plasmas had not been concentrated before assay.

Before the renal lymphatic system could be implicated as the route by which renal hypertension is mediated, it would be necessary to extend these studies to animals with renal hypertension and a total collection of renal lymph to determine quantitatively whether the output of pressor material was sufficient to cause hypertension in an animal of the same species.

It is, nevertheless, possible that the small amounts of renin-like activity could be of considerable importance if their action was not a direct pressor

one but depended upon the activation of some non-renal pressor mechanism. The recent experiments of Laragh, Angers, Kelly & Lieberman (1960), where infusions of angiotensin in man produced a considerable increase in aldosterone secretion, are suggestive of such a mechanism, and could indicate a physiological link between kidney and suprarenal concerned with electrolyte and water metabolism. Alternatively, the presence of renin in renal lymph may indicate that it gains access to renal extracellular fluid where its main action as a local enzyme could be exerted.

SUMMARY

1. Renal lymph of the anaesthetized dog contains pressor material which is not detectable in lymph derived from other organs.

2. This pressor activity has been studied by several biochemical and pharmacological methods and is indistinguishable from dog renin.

3. Stenosis of one renal artery during the course of bilateral lymph collection in the dog is in the majority of cases followed by increased concentration of this material in the lymph coming from the ischaemic kidney.

This work was done during the tenure by one of us (A.F.L.) of a Sandoz Research Fellowship, and subsequently of a Wellcome Trust Fellowship.

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