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SOME RENAL ACTIONS OF TWO ANIONIC FRACTIONS OF PLASMA GLOBULINS

BY

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Two anionic globulins which modify the function of kidneys *in situ* are found in the plasma of normal man, sheep and rat at concentrations of approximately 20–30 and 50–60 mg/l. respectively. These proteins were separated from plasma by fractional precipitation with salt at pH 3.9 and were purified by ion-exchange chromatography on DEAE-Sephadex A-25 after dialysis and lyophilization (Siddiqui & Lockett, unpublished).

Our first objective was to discover whether these proteins had been isolated unchanged from plasma. Whereas one protein remained pharmacologically unchanged during purification, the other had acquired pharmacological activity attributable to the activation of a kininogenase. Secondly, the direct actions of these proteins were examined on perfused cat kidneys. The actions of one protein remained unchanged when a heart-lung circuit replaced the pump-oxygenator system. By contrast, the monophasic action of the second protein on kidneys perfused from a pump-oxygenator system became biphasic either when a heart was put into the system, or when a heart-lung replaced the pump-oxygenator circuit.

METHODS

A. Biochemical

Separation of anionic globulins from plasma by precipitation. All procedures were carried out at 3° C. For the first precipitation, plasma diluted with an equal volume of water was adjusted to pH 3.9 by addition of 0.1 then 0.01 N H_2SO_4, with stirring. The precipitate which formed overnight was discarded before sodium chloride (100 g/l.) was added, with stirring. The precipitate which formed overnight was collected, freed of electrolytes by dialysis and freeze-dried. The freeze-dried material was dissolved in 0.1 N acetate buffer pH 3.9 (1 g/140 ml.) for fractional precipitation with sodium chloride. The protein fraction, which was soluble in NaCl 60 g/l. and insoluble in NaCl 100 g/l., was collected, freed of electrolytes by dialysis and freeze-dried. This fraction is designated B_3. From E_3 four fractions I, II, III, and IV have been obtained by ion-exchange chromatography (Siddiqui & Lockett, unpublished).

Separation of anionic globulins from plasma by gel filtration. The buffer used throughout was 0.1 M Tris-HCl, pH 8.0 containing 1.0 M NaCl. Sephadex G-200 (Pharmacia, Uppsala, Sweden) was swollen in buffer and was packed into columns 30×2.5 cm to give settled gel beds of 150 ml. after equilibration with buffer. Protein samples (30 mg in 2 ml. buffer) or fresh plasma samples (2 ml.) were applied to these beds, and the percentage of transmission at 280 mμ was determined on

each successive 5 ml. eluate. Two absorption peaks appeared both from human plasma and from B_3 at 55–95 ml. and at 125–145 ml. of eluate. Serial eluates corresponding to each peak were pooled, dialysed and freeze-dried.

Disc electrophoresis. Disc electrophoresis was carried out as described by Ornstein (1964), using apparatus and chemicals supplied by the Canal Industrial Corporation, Bethesda, Maryland. β-Alanine-acetic buffer pH 5.0 was used in the electrode compartments. Large pore 3.5% acrylamide spacer and sample gels were buffered at pH 6.5. Lower gels, of standard 7.5% acrylamide, were stacked at pH 5.0. Polymerization of these large pore gels was assisted by addition of tetramethylene diamine (Lewis, 1963). All samples were run at a constant current of 5 mA per tube for 1 hr at pH 4.3 with reversed electrodes. Gels were stained by immersion in Amido-Schwartz reagent for 1 hr, destained electrophoretically and examined by means of a double-beam recording and integrating densitometer (Joyce, Loebl & Co. Ltd., England), speed 2 mm/sec; gain 5.

B. Physiological

Cats, anaesthetized by intraperitoneal injection of 8 ml. of 1.0% chloralose w/v in 0.9% NaCl, were used throughout. Blood to fill perfusion circuits was collected from male, female or neutered cats and contained creatinine 30–50 mg, *p*-aminohippuric acid 7.5–12.5 mg, atropine sulphate 0.25 mg (all British Drug Houses, Ltd., diphenhydramine hydrochloride 7.5 mg (Parke Davis & Co. Ltd.), heparin 3,000 units (Evans Medical Ltd.) and added glucose 25 mg/100 ml. In all experiments in which a pump-oxygenator circuit and in one in which a heart-lung preparation perfused the kidney, 0.05 ml. of 1.0% w/v methylene blue (E. Gurr & Co. Ltd.) in 0.9% NaCl was added per 100 ml. of blood.

A total of eleven experiments was performed; in three the kidneys, which weighed 6.9±0.41 g, were perfused by a heart-lung circuit (Davey & Lockett, 1960; Lockett, 1966). In the remaining eight experiments the kidneys, which weighed 7.6±0.62 g, were perfused from a pump-oxygenator circuit which was dual in one experiment to make possible the perfusion of a heart in parallel (Lockett, 1967b). The perfusion pressure was kept constant throughout every experiment by adjustment, when necessary, solely in the resistance on the spill-over flow way. Table 1 shows the mean control values for these preparations calculated per 10 g of kidney.

TABLE 1

SUMMARY OF THE CONTROL VALUES OF PERFUSION PRESSURE AND OF RENAL FUNCTION SHOWN BY CAT KIDNEYS PERFUSED AT CONSTANT PRESSURE EITHER FROM PUMP-OXYGENATOR OR FROM HEART-LUNG CIRCUITS

All values shown are means ± their standard errors.

	Perfusion circuit	
	Pump oxygenator	Heart-lung
No. of preparations	8	3
Mean perfusion pressure (mm Hg)	117·3± 0·99	121·2±0·36
Plasma filtration fraction (%)	19·1± 0·69	16·7±0·42
Values per 10 g renal tissue		
Renal blood flow (ml./min)	12·3± 0·94	14·6±0·28
Glomerular filtration rate (ml./min)	2·4± 0·21	2·5±0·38
Urine flow (ml./min)	0·09± 0·070	0·05±0·024
Sodium excretion (μ-equiv/min)	27·1± 9·02	5·2±1·60
(μ-equiv/ml.)	479 ±21·7	112 ±7·8
Sodium/potassium in urine	17·2± 2·53	8·4± 1·08

The pH and pO_2 of blood were measured on 0.4 ml. samples by means of oxygen and glass electrodes set in stacked constant temperature curvettes and coupled to a physiological gas analyser (Beckman Ltd.). Measurements of arterial pressure, haematocrit, renal blood and urine flows, clearances of creatinine and concentrations of sodium and potassium in plasma and urine were made as previously described (Lockett, 1966).

RESULTS

Chemical

The yields of B_3 obtained from sheep and human plasma by fractional precipitation with salt at pH 3.9 were 7.35 and 7.41 g/l., respectively. Cationic proteins, B_3II, constitute 80% of the crude B_3 fraction; the residual 20% of protein is anionic and can be separated into two sub-fractions, B_3III and B_3IV, by ion-exchange chromatography on DEAE-Sephadex A-25 (Siddiqui & Lockett, unpublished).

The proteins of highest molecular weight are the first to be eluted from Sephadex G-200 (Porath, 1959). The yield of dialysed freeze-dried protein responsible for the first absorption peaks eluted after application of 2 ml. of fresh plasma and 30 mg of B_3 to 150 ml. gel beds of Sephadex G-200 were 8 mg and 18 mg, respectively. The electrophoretic patterns of the anionic proteins contained in 20 μg of these elution fractions are shown in Fig. 1 (as C and D, respectively): both contain three bands, labelled 2, 3 and 4, and these bands are superimposable, and are also superimposable on bands 2, 3 and 4 for the anionic proteins contained in 4 μl. of untreated human plasma and 200 μg B_3. There is therefore good evidence that the anionic proteins present in crude B_3 and in the first elution peaks derived from application of plasma and of B_3 to Sephadex G-200 are present as such in normal plasma. Fractionation of B_3 by ion-exchange chromatography yields two elution fraction B_3III and B_3IV which appear as pure single bands on electrophoresis. The electrophoretic mobility of these bands exceeds that of the original anionic proteins of B_3: evidence presented in the next section demonstrates that the pharmacological properties of these fractions remain unchanged.

Effects of the various protein fractions on kidneys perfused from pump-oxygenator circuits

When 20–25 μg of the proteins of human plasma and of B_3 which were first to be eluted from Sephadex G-200 were injected into the renal arterial supply there was an immediate increase in the blood flow through kidneys perfused at constant temperature and pressure from pump-oxygenator circuits (Fig. 2). A rise in urine flow was delayed for 3 to 5 min after the rise in renal blood flow and was accompanied by a rise in glomerular filtration rate which was proportionately greater than that of the renal blood flow; hence the fraction of plasma filtered also rose. The concentration of sodium in the urine fell but the net loss of sodium into the urine always increased because of the large increase in urine flow. The net loss of potassium also increased but less markedly: therefore the ratio of sodium to potassium in the urine rose. Maximal effects of the proteins of plasma and of B_3 which were first eluted from Sephadex G-200 and of unfractionated B_3 were caused by the close arterial injection of approximately 100 μg of these unfractioned proteins, and were characterized by prolonged action (2–2.5 hr) and an enormous increase in the fraction of plasma filtered. Figure 2 shows the immediate and abrupt rises in renal blood flow and glomerular filtration rate which resulted from the arterial injection of 100 μg of those proteins of human plasma first eluted from Sephadex G-200. The glomerular filtration rate rose to three times the resting value whereas the renal blood flow increased only by 32%. The urine flow and the rate of excretion of sodium began to increase 3–5 min after the injection, and continued to rise steadily toward maxima which were reached in 1 hr and were maintained for 30–45 min.

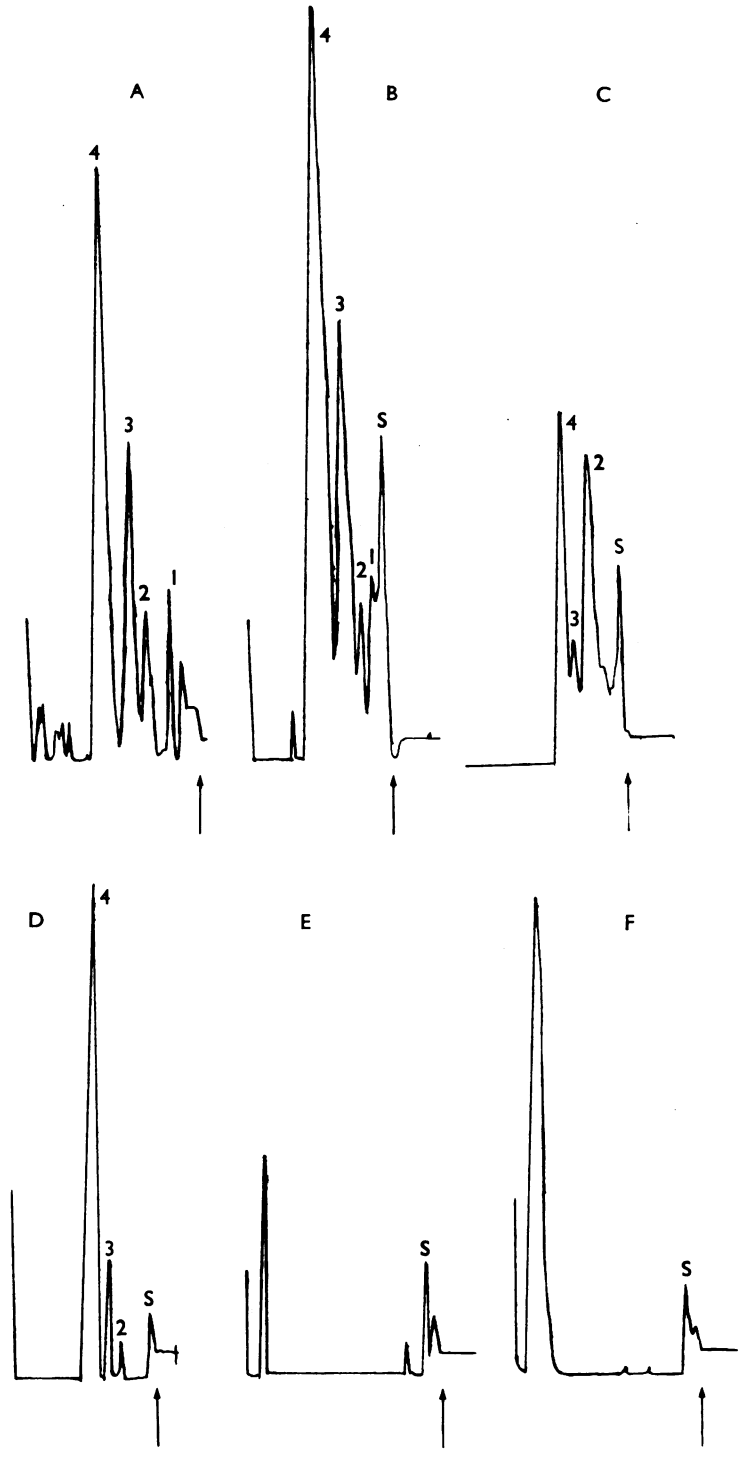

Fig. 1. Disc electrophoretic patterns of the anionic proteins in various fractions. Chart speed 2 mm/sec; gain 5. A, human plasma, 4 μl.; B, human B_3, 200 μg; C, high molecular weight proteins of human plasma; D, highest molecular weight fraction of human B_3; E, B_3III, 100 μg; F, B_3IV, 100 μg.

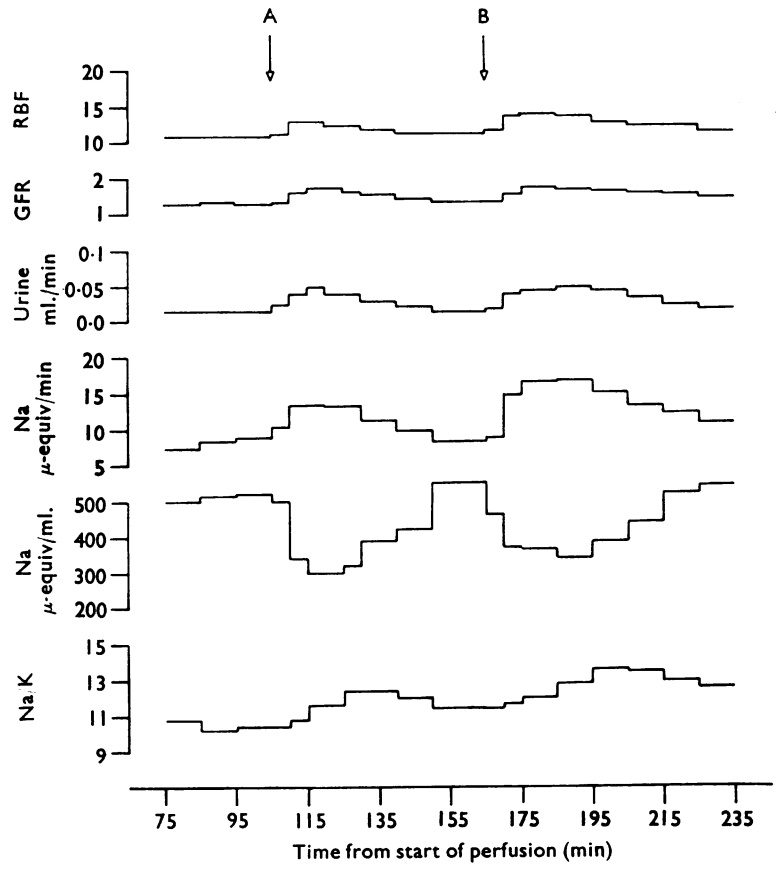

Fig. 2. Data provided by a kidney, 8.7 g, perfused at 120 mm Hg and 38° C from a pump-oxygenator system. 20 μg protein from human B_3 (arrow A) and 25 μg protein from human plasma (arrow B) eluted from Sephadex G-200 (see text) were injected into the renal arterial supply (0.05 ml.). Ordinates, from the top: renal blood flow (RBF), ml./min; glomerular filtration rate (GFR), ml./min; urine flow, ml./min; urinary sodium, μ-equiv/min; urinary sodium. μ-equiv/ml.; ratio in urine of sodium to potassium.

Thereafter diuresis and natriuresis abated gradually at a rate comparable with that of their onset and in parallel with the decline in renal blood flow. Restoration of the resting filtration rate invariably preceded the restoration of normal rates of urine flow. glomerular filtration rate and natriuresis: the enormous initial increase in the fraction of plasma filtered was therefore followed by a phase in which the fraction fell below the normal values. A normal fraction of plasma filtered was restored as the renal blood flow returned to resting levels. A slow rise in the ratio of sodium to potassium in the urine characterized the action of these proteins on kidneys perfused from pump-oxygenator circuits. This rise in the ratio of sodium to potassium was not attributable to the development of pH changes in the arterial blood and reversed spontaneously. The maximum actions of these proteins were not accompanied by measurable changes in the oxygen consumption of the kidneys.

In Fig. 3, and Table 2, the renal effects of unfractionated B_3 are compared with those of the single band anionic proteins separated from B_3 by ion-exchange chromatography. It is evident that the renal actions of B_3IV very closely resemble (Table 2) and are additive with (Fig. 3) those of unfractionated B_3. Figure 3 also shows that the intense effects of B_3 and B_3IV on the glomerular filtration rate are largely independent of effects on renal blood flow. The renal blood flow rose abruptly to the limiting rate of flow permitted by the orifice of the renal arterial cannula in use at the operative mean perfusion pressure (116 mm Hg) when B_3 50 μg was first added to the reservoir: the usual rises in the glomerular filtration rate and the fraction of plasma filtered were seen. When B_3IV was added to the contents of the reservoir, glomerular filtration rate and plasma fraction again rose abruptly, but the renal blood flow remained at the maximum attainable flow. Similarly, when B_3 was again added to the contents of the reservoir, the glomerular filtration rate and the plasma fraction again rose although renal blood flow remained unchanged. Figure 3 also demonstrates that the actions of B_3III and B_3IV differ. B_3III was without action on the glomerular filtration rate, the renal blood flow

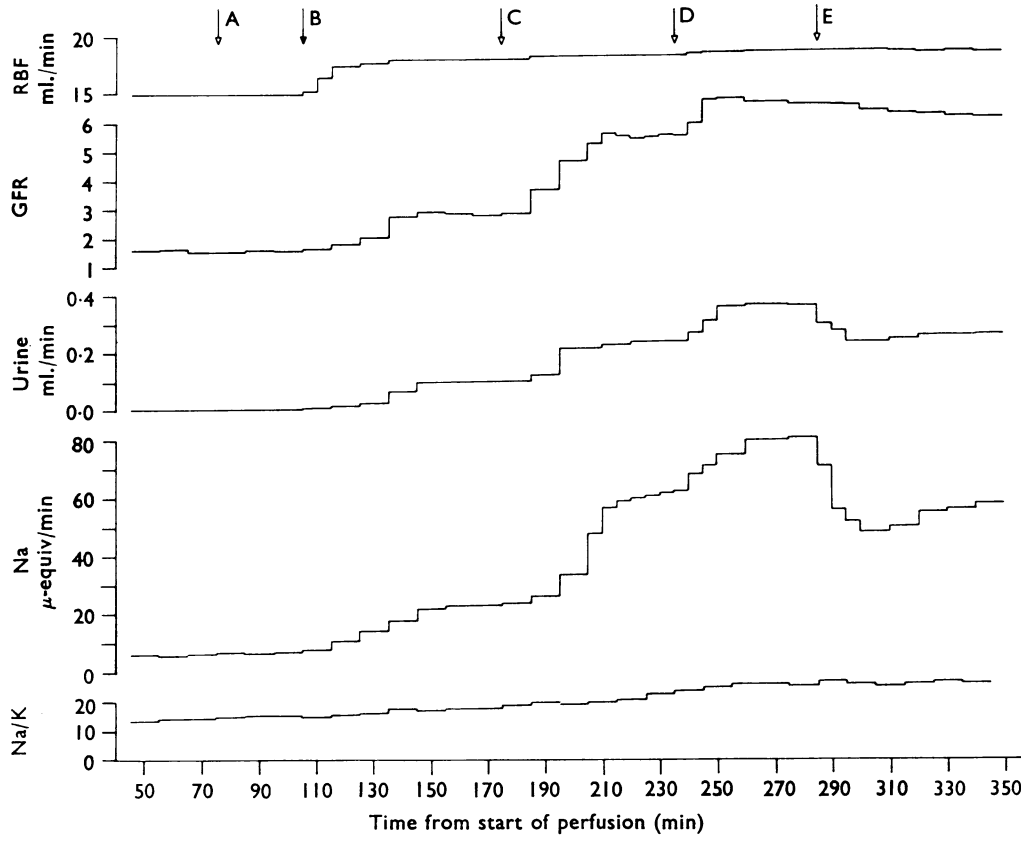

Fig. 3. Data provided by a kidney, 7.6 g, perfused at 116 mm Hg and 38° C from a pump-oxygenator system. The arrows A to E denote the following injections: A, 100 μg B_3II; B and D, 50 μg B_3; C, 40 μg B_3IV; E, 100 μg B_3III. A and E injected close arterially; B, C and D into the reservoir. Ordinates as in Fig. 2, omitting sodium, μ-equiv/ml.

TABLE 2

PERCENTAGE CHANGES INDUCED IN RENAL BLOOD FLOW (RBF) IN GLOMERULAR FILTRATION RATE (GFR) AND IN URINARY SODIUM BY VARIOUS PROTEIN FRACTIONS ISOLATED FROM PLASMA

All measurements were made on cat kidneys perfused from pump-oxygenator or from heart-lung circuits. All values shown are means ± their standard errors.

Protein fraction		No. of preparations	Percentage changes from resting values					
						Urinary sodium		
Designation	μg/120 ml.		RBF	GFR	Urine flow	Excretion rate	Concentration	Sodium/potassium
Pump-oxygenator								
Plasma peak I	20–30	2	18·6± 2·1	31·4± 3·7	214 ±17·6	93·4± 5·8	−40·7±4·4	16·2±2·3
B_3	50–100	3	13·7± 0·7	66·7±15·6	244 ±38·3	137·7±22·1	−53·3±9·2	13·3±4·8
B_3 IV	40–50	5	36·5±18·7	56·3±12·6	253 ±31·4	75·3±12·4	−38·7±6·9	21·0±4·7
B_3 III	100	6	0·8± 0·2	−0·3± 1·2	−31·3± 4·2	−36·2± 3·1	−22·3±6·2	1·8±1·2
Heart-lung								
B_3 III	100	3	0·2± 0·3	0·1± 2·4	−38·2± 3·7	−40·6± 6·2	−27·2±4·6	0·4±2·1
B_3 IV phase I	50	3	25·7± 4·7	37·4± 3·8	121·8±11·6	86·2± 7·4	−28·4±7·2	9·6±1·8
phase II	50	3	24·8± 5·1	35·2± 3·5	−14·6± 6·3	−18·2± 4·4	−26·6±5·8	−6·8±1·4

and the ratio of sodium to potassium in the urine: B_3III was antidiuretic and sodium retaining both by reason of the antidiuresis and because the concentration of sodium in the urine decreased. Additionally, Fig. 3 shows that the cationic proteins separated from B_3 by ion-exchange chromatography (B_3II) were without influence on any of the parameters of renal function under study when injected close arterially in doses up to 100 μg.

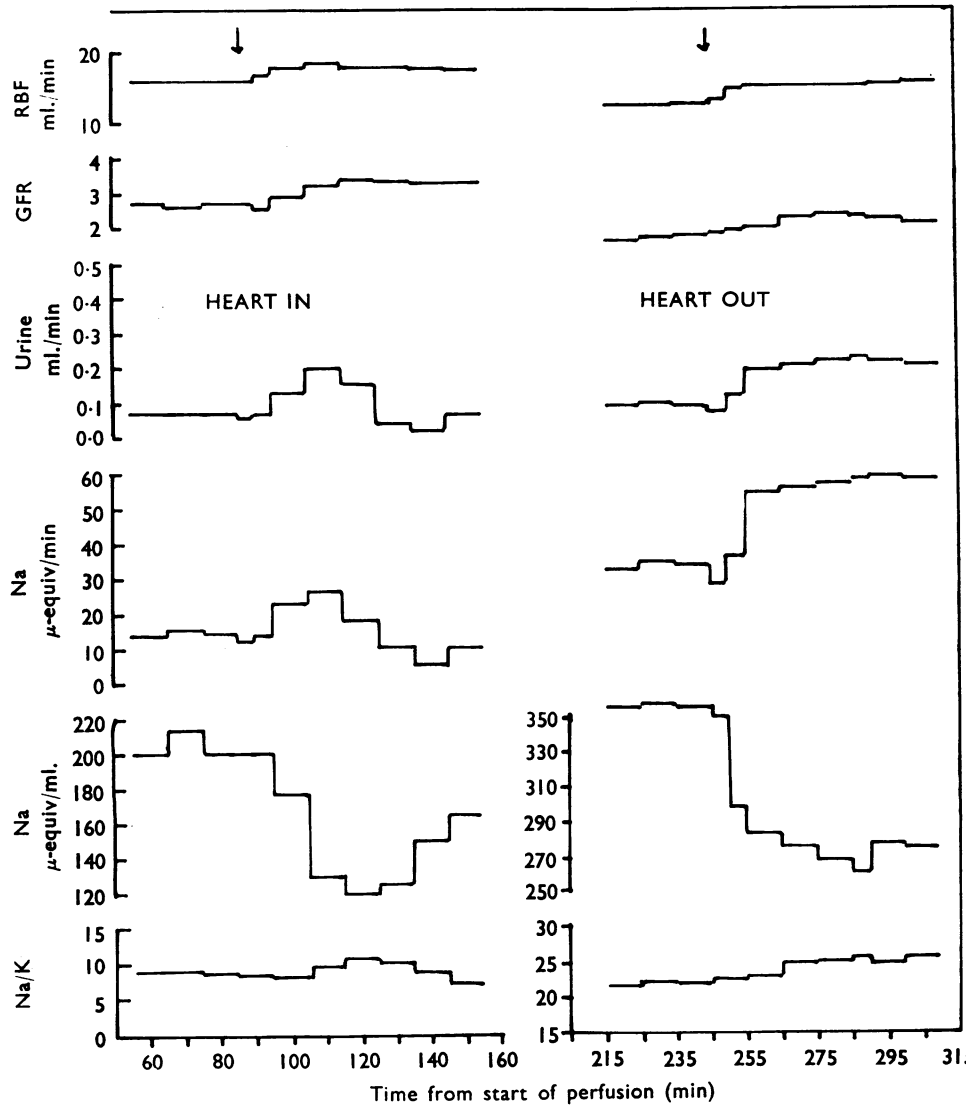

Fig. 4. Data provided by a kidney, 7.6 g, perfused at 38° C and 128 mm Hg from a pump-oxygenator system with a heart in circuit (left) and with no heart in circuit (right). The arrows denote additions of B_3IV 50 μg to the common reservoir.

Comparison of the actions of B_3III and B_3IV on kidneys perfused from heart-lung circuits

The actions of B_3III remained unchanged when a heart-lung preparation perfused the kidney in place of the pump-oxygenator system (Fig. 3). B_3III modified neither the renal blood flow, the glomerular filtration rate nor the ratio of sodium to potassium in the urine. This fraction caused antidiuresis and lowered the concentration of sodium in the urine. By contrast, the actions both of B_3 (Table 2) and of B_3IV (Table 2) became biphasic when the kidney was perfused from a heart-lung circuit and when a heart was perfused in parallel with the kidney from a pump-oxygenator circuit (Fig. 4) but were monophasic whenever the kidney was the sole organ perfused by the pump-oxygenator system (Figs. 2 and 3 and Table 2). The first phase of the action of B_3 and B_3IV on a kidney with a heart in circuit resembled the monophasic action of these proteins on kidneys perfused from pump-oxygenator circuits (compare Figs. 2 and 4). Renal blood flow, glomerular filtration rate and the fraction of plasma filtered rose with very short latency. Shortly afterwards an intense diuresis began which was accompanied by a decrease in the concentration of urinary sodium and a rise in the ratio of sodium to potassium. The diuresis, natriuresis and rise of the ratio of sodium to potassium were, however, almost abruptly reversed at 30–45 min after exposure to B_3IV or B_3 when a heart was in circuit, although the effects of these fractions on the renal blood flow, the glomerular filtration rate and the concentration of urinary sodium were unaltered. This antidiuresis, reduction in natriuresis and early reversal of the effect of B_3 and B_3IV on the sodium to potassium ratio constitute the second phase of the biphasic actions of these proteins seen when a kidney is perfused with a heart in circuit.

DISCUSSION

The anionic proteins of plasma can be partially purified either by simple filtration through Sephadex G-200 on Tris-HCl buffer at pH 8.0 or by salting out at pH 3.9 without change in solubility or in electrophoretic mobility (Fig. 1). Pharmacological properties which are not characteristic of plasma are, however, generated by these simple processes and remain associated with the anionic protein B_3IV during its separation by ion-exchange chromatography (Siddiqui & Lockett, unpublished) from the cationic proteins B_3II and a second anionic protein B_3III.

The diuretic natriuretic action of B_3IV is accompanied by increase in renal blood flow and increase in potassium excretion; qualitatively these renal actions resemble those of bradykin infused intravenously or intra-arterially into cats (Stürmer & Berde, 1963), dogs (Szakáll, 1932; Barraclough & Mills, 1965) and man (Mertz, 1964). Quantitatively the actions of B_3 and B_3IV on the isolated kidney differ from those of bradykin in the whole animal, for on the isolated preparation the diuresis evoked is so intense that the concentration of urinary sodium falls: in the whole animal the diuresis is less dramatic and the concentration of urinary sodium rises (Szakáll, 1932). These quantitative differences may, however, prove attributable to the release of vasopressin by bradykinin in the intact animal (Roche e Silva, Jr. & Malnic, 1964). It is therefore probable that B_3IV is or contains a kininogen contaminated by plasma kininogenase which readily activates spontaneously (Erdós, 1966). The appearance of an antidiuretic second phase in the renal action of B_3IV whenever a heart is included in the perfusion circuit suggests that

the very active antidiuretic sodium retaining steroid of heart muscle (Lockett, 1967a, b; unpublished results of Ilett & Lockett, 1967) is liberated either by action of the kinin formed in the plasma by or from B_3IV or in consequence of an increase in the haematocrit generated by the intense diuresis.

The actions of B_3III on the isolated kidney resemble those of growth hormone (Lockett & Roberts, 1964; Lockett, 1965). The concentration of B_3III protein in the plasma is 20–30 mg/l.: the concentration of growth hormone in plasma is normally less than 10 ng/ml. (Utiger, 1964): contamination of B_3III by growth hormone could therefore not be detected electrophoretically. The renal activity of B_3III is not, however, attributable to contamination with growth hormone because, weight for weight, B_3III has approximately one half of the renal activity of growth hormone.

SUMMARY

1. The anionic proteins of plasma are found in the first eluates of plasma from Sephadex G-200 by 0.1 M Tris-HCl 1.0 M NaCl buffer pH 8.0 and precipitate at pH 3.9 when the concentration of salt is raised from 60 to 100 g/l.

2. Neither these procedures nor subsequent dialysis and freeze-drying alter the solubility or the electrical motility of the anionic proteins of plasma. Pharmacological properties which are not characteristic of plasma are, however, generated.

3. The acquired pharmacological properties remain unchanged during purification of the anionic proteins by ion-exchange chromatography and are finally found associated with an electrophoretically homogenous anionic protein: yield 50–60 mg/l.

4. The pharmacological properties acquired by this protein during its isolation have been examined on perfused cat kidneys. In concentrations of 25–100 μg/120 ml. blood it causes an immediate increase in renal blood flow and glomerular filtration rate and an intense diuresis and natriuresis which builds up over 30–40 min and lasts for 60–120 min. A heart in circuit curtailed the diuresis and natriuresis but did not modify the changes in renal blood flow and glomerular filtration rate. These acquired actions of the anionic proteins are qualitatively similar to previously reported renal actions of bradykinin in intact animals.

5. A second electrophoretically pure anionic protein has been isolated from plasma, yield 20–30 mg/l. This protein, at a concentration of 100 μg/120 ml. blood causes antidiuresis and retention of sodium by the perfused cat kidney. Renal blood flow, glomerular filtration rate and the ratio of sodium to potassium in urine remain unchanged.

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REFERENCES

BARRACLOUGH, M. A. & MILLS, I. H. (1965). Effect of bradykinin on renal function. *Clin. Sci.*, **28**, 69–74.

DAVEY, M. J. & LOCKETT, M. F. (1960). Action and interaction of aldosterone monoacetate and neurohypophysial hormones on the isolated cat kidney. *J. Physiol., Lond.*, **152**, 206–219.

ERDÓS, E. G. (1966). Hypotensive peptides. *Adv. Pharmcc.*, **4**, 1–74.

LEWIS, U. J. (1963). Factors that affect fragmentation of growth hormone and prolactin by hypophyseal proteinases. *J. biol. Chem.*, **238**, 3330–3335.

LOCKETT, M. F. (1965). Comparison of the direct renal actions of pituitary growth and lactogenic hormones. *J. Physiol., Lond.*, **181**, 192–199.

LOCKETT, M. F. (1966). Perfusion of cat kidneys from heart-lung circuits. *Proc. Soc. exp. Biol. Med.*, **121**, 937–941.

LOCKETT, M. F. (1967a). The influence of the polar anastomotic vessels on autoregulation and the urinary excretion of water and sodium by perfused cat kidneys. *J. Physiol., Lond.*, in the Press.

LOCKETT, M. F. (1967b). Hormonal action of the heart and lung on the isolated kidney. *J. Physiol., Lond.*, in the Press.

LOCKETT, M. F. & ROBERTS, C. N. (1964). Some actions of growth hormone on the perfused cat kidney. *J. Physiol., Lond.*, **169**, 879–888.

MERTZ, D. P. (1964). Über die Wirkung von synthetischen Eledoisin auf die Nierenfunktion, den Wasser— und Ektrolythaushalt beim Menschen. *Arch. exp. Path. Pharmak.*, **246**, 338–354.

ORNSTEIN, L. (1964). Disc electrophoresis. *Ann. N.Y. Acad. Sci.*, **121**, 321–349.

PORATH, J. (1959). Fractionation of polypeptides and proteins on dextran gels. *Clin. Chim. Acta.*, **4**, 776–778.

ROCHE E SILVA, M. JR. & MALNIC, G. (1964). Release of antidiuretic hormone by bradykinin. *J. Pharmac. exp. Ther.*, **146**, 24–32.

STÜRMER, E. & BERDE, B. (1963). A comparative pharmacological study of synthetic eledoisin and synthetic bradykinin. *J. Pharmac. exp. Ther.*, **140**, 349–352.

SZAKÁLL, A. (1932). Uber die Wirkung intravenoser Kallikrein-zufuhr auf den tierischen Organismus. *Arch. exp. Path. Pharmac.*, **166**, 301–318.

UTIGER, R. D. (1964). Extraction and radioimmunoassay of growth hormone in human serum. *J. clin. Endocr. Metab.*, **24**, 60–67.