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CHARACTERISTICS OF IONIC BINDING BY RAT RENAL TISSUE *IN VITRO*

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SUMMARY

1. A study has been made of Na^+ and Cl^- binding in metabolically inhibited slices of rat renal cortex and outer medulla incubated in modified Krebs phosphate–bicarbonate Ringer solution.

2. At pH 7·35 in control media (cortex, 147 mmol Na^+/l, 105 mmol Cl^-/l; outer medulla, 187 mmol Na^+/l, 145 mmol Cl^-/l) cortical slices bound (mean) 171 nmol Na^+ and 56·7 nmol Cl^-/mg solute-free dry weight; outer medullary slices bound 188 nmol Na^+/mg and negligible amounts of Cl^-.

3. In both regions, Na^+ was exchangeable on a 1:1 basis for K^+ or Li^+ in media containing equal concentrations of each cation: Na^+ was completely displaced by La^{3+}.

4. In cortical slices in media containing equimolar Cl^- and other monovalent anions, binding occurred according to the sequence acetate $\leqslant$ salicylate $\leqslant Cl^- <$ SCN^-; Cl^- was completely displaced by PO_4^{3-}.

5. When medium pH was lowered, Na^+ binding was markedly reduced in both regions, whereas Cl^- binding increased (and became significant in outer medulla).

6. In NaCl solutions, Na^+ binding capacity was saturated at control Na^+ concentrations. When [Na^+] was progressively reduced (iso-osmolality being maintained by addition of urea), bound Na^+ in both regions was nearly linearly related to log medium [Na^+].

7. Raising medium osmolality with urea caused decreased Na^+ binding and increased Cl^- binding in both regions.

8. Na^+ binding in both regions was significantly reduced by pre-treatment with chondroitinase ABC.

9. Binding of both ions was independent of temperature within the range 2–37 °C.

10. The possibility is raised that renal ionic binding might influence vectorial ion transport by affecting free ion activity in the region of the transporting cells.

INTRODUCTION

The possibility that bound, osmotically inactive ions may be functionally significant in terms of whole-body electrolyte balance has been recognized for many years (for references see Farber & Schubert, 1957). One major site of cation binding is probably

polyanionic glycosaminoglycans (GAGs): whether polycationic anion binding occurs physiologically is unknown. The electrochemical aspects of micro-ion–macro-ion interaction in connective tissue GAGs has been reviewed by Comper & Laurent (1978), and the nature of interaction between GAGs and extracellular and cell-surface macromolecules by Chvapil (1967) and Lindahl & Hook (1978).

Binding or sequestration of Na^+ and Cl^- has been studied in considerable detail in mammalian vascular smooth muscle (Villamil, Rettori, Barajas & Kleeman, 1968; Palaty, Gustafson & Friedman, 1969; Siegel, Roedel, Nolte, Hofer & Bertsche, 1976; Somlyo, Somlyo & Shuman, 1979), but little is known regarding ionic binding, either to GAGs or other macromolecules, in other tissues, and the functional role of bound ions remains speculative.

One class of tissue offering potentially rewarding study in this respect is epithelium involved in the vectorial transport of ions. Two such tissues, in which the chemical nature of GAGs has been studied, are colon (for references see Thornton, Hunt & Huckerby, 1983) and the kidney (Allalouf, Ber & Sharon, 1964; Castor & Greene, 1968; Constantopoulos, Louie & Dekaban, 1973; Murata, 1975) of several species. It has been suggested (Macknight, Mason, Rose & Sherman, 1982) that in rabbit colon binding not only might explain the apparent distribution of tissue Na^+ but that binding within crypts might facilitate Na^+ absorption from Na^+-poor fluids. But the only functional role which has been suggested for GAGs in kidney is that hyaluronic acid gel (probably not itself a major site of cation binding (Palaty *et al.* 1969)) may affect the rate of water withdrawal out of the medullary collecting duct (e.g. see Dicker, 1970; Law & Rowen, 1981; Rowen & Law, 1981).

The object of the present study has been to determine the levels of bound Na^+ and Cl^- in metabolically inhibited slices of rat renal cortex and medulla *in vitro*, and to examine some characteristics of binding relative to the composition of the external bathing medium. While no firm proposals can be made at present, it is suggested that certain of the findings, when viewed relative to the properties of intrarenal fluids *in vivo*, are compatible with a physiological significance for renally bound ions as modulators of epithelial ion transport through their effect on the activity of ions present in free solution. A short account of some of these has been published previously (Law, 1984*a*).

METHODS

Estimations of bound ions were made in slices of cortex and outer medulla (thickness approx. 0·25 mm, weight 5–15 mg) cut free-hand from the kidneys of freshly sacrificed male Wistar rats which had been maintained on a normal laboratory pellet diet and tap water *ad lib.*

The principle of the method upon which these studies depend is that prolonged incubation under conditions of total metabolic blockade allows the entire fluid moiety of the tissue to become identical in composition with the incubation medium. Subsequent analysis of the tissue in respect of fluid and ionic content readily permits the estimation of apparently bound ions as the excess of the total ions determined over the total which would have been expected had ions existed only within the fluid moiety (viz. in free solution at concentrations identical to those in the incubation medium). This method has been applied to arterial wall (e.g. see Villamil *et al.* 1968; Law, 1982).

In the present experiments, slices were blotted on hard filter paper (Whatman no. 542), weighed to the nearest 25 μg on a torsion balance, and incubated in sealed vessels for 18–24 h (in batches of five to seven (cortex) and two or three (outer medulla)) in 2·5 ml incubation medium (control medium) whose composition (modified from Krebs, 1950) was as follows (mmol/l): cortex, Na^+, 147; K^+, 6; Ca^{2+}, 2·6; Mg^{2+}, 1·2; Cl^-, 105; HCO_3^-, 25; $H_2PO_4^-$, 2·2; SO_4^{2-}, 1·2; CN^-, 5; iodoacetate,

1; pyruvate, 4·8; glutamate, 4·8; fumarate, 5·3; glucose, 10; calculated osmolality 321 mosmol/kg H_2O. Outer medullary incubation medium was identical except that Na^+ was increased to 187 and Cl^- to 145 mmol/l; urea (8·7 g/l) was added in order to increase the calculated osmolality of this medium to approx. 540 mosmol/kg H_2O, a figure corresponding to the calculated osmolality of fluid normally present in rat outer medulla (Law, 1975*b*). Media were gassed to pH 7·35 with 95 % N_2/5 % CO_2 at 37 °C. The reason for including metabolic substrates in media designed to abolish cellular metabolism is that binding of major ions may be influenced by other ions present (e.g. see Palaty *et al.* 1969), and it was intended in the present experiments to include all ions which are normally present in medium used for the incubation of metabolically active renal slices.

Following incubation, slices were briefly blotted and reweighed. They were then individually leached for 24 h in 2·5 ml deionized water. This process is adequate for the complete leaching of ions out of very small tissue samples (cf. Law, 1975*a*; George & Solomon, 1981). Ion contents of leachates were estimated using the methods described below.

Cortical slices were dried to constant weight (24 h at 105 °C) on tared aluminium foil. Post-incubatory fluid content was calculated by subtraction and expressed as μl/mg solute-free dry weight (s.f.d.w.). The low solid content of outer medullary tissue renders this process rather unreliable, especially for very small slices, and for this region a pre-incubatory figure of 12 mg dry weight/100 mg wet weight (i.e. 7·33 μl fluid/mg s.f.d.w.) was assumed: this value was the mean obtained by drying larger segments of outer medullary tissue to constant weight in a series of preliminary investigations.

Cation substitution. By appropriate equimolar substitution, media for cortical incubations were prepared containing 76·5 mmol Na^+/l plus 76·5 mmol K^+/l, and 73·5 mmol Na^+/l plus 73·5 mmol Li^+ or La^{3+}/l. The corresponding media for outer medullary incubation contained Na^+ and K^+ at 96·5 mmol/l or Na^+ and Li^+ or La^{3+} at 93·5 mmol/l. Anions precipitating La^{3+} (viz. HCO_3^-, SO_4^{2-}, $H_2PO_4^-$ and fumarate) were omitted from these media (replaced by Cl^- and glutamate). pH was adjusted to approx. 7·8 using 2-amino-2-(hydroxymethyl)-propane-1,3-diol (Tris)/N_2. Over several hours a slight, cloudy suspension formed in these media. It has been suggested that this is due to reaction between La^{3+} and tissue phosphate (Miledi, Molenaar & Polak, 1980). It is also possible that colloidal $La(OH)_3$ might be a contributory factor (Pressler, Elharrer & Bailey, 1982). In the present experiments, however, it was found that formation of the suspension was mainly due to the presence of Tris. pH fell to approx. 7·3 by the end of incubation.

Alteration of pH. pH was raised to approx. 8·9 with Tris, or reduced within the range 3·5–6·5 by dropwise addition of conc. HNO_3. In the latter media HCO_3^- was replaced by NO_3^-. The gas phase in all incubations involving pH adjustment was 100 % N_2.

Effects of altering medium ion concentrations. Cortical slices were incubated in solutions containing 165 mmol NaCl/l (i.e. iso-osmolal with control medium) and solutions in which NaCl was progressively reduced to a minimum of 8·25 mmol/l, iso-osmolality being maintained by equimolar addition of urea. pH was adjusted to approx. 7·35 by dropwise addition of 1 M-3-(*N*-morpholino)-propanesulphonic acid (MOPS), gas phase 100 % N_2. The effect on ionic binding of increasing medium NaCl was not studied in the cortex, since it is not possible to compose such media without concomitantly increasing calculated osmolality, which (see below) itself affects binding. In the case of outer medullary slices, medium [Na^+] was altered within the range 13·5–270 mmol/l. The increase in concentration above that in control medium is possible in this case; additional NaCl was added in place of the urea which is present in outer medullary control incubation medium, there thus being no alteration in calculated osmolality. Cl^- binding was not studied in this region.

Effects of increasing medium osmolality. The calculated osmolality of fluids for incubation of both regions was increased up to 2-fold by the addition of urea (max. values 640 mosmol/kg H_2O, cortex; 1080 mosmol/kg H_2O, outer medulla).

Action of chondroitinase ABC. In one series of experiments slices were incubated for 6 h at 37 °C in medium containing Na acetate (60 mmol/l), bovine serum albumin Fraction V (BDH Biochemicals; 5 g/l) and chondroitinase ABC (Sigma Chemical Company; 0·2 u./ml), adjusted to pH 8·0 with Tris (Yamagata, Saito, Habuchi & Suzuki, 1968) before being subjected to metabolic inhibition.

Effect of temperature. In a final series of incubations, the ambient temperature was reduced to 2 °C.

Analytical methods. Na^+ and K^+ in leachates were estimated by flame photometry, Li^+ by atomic emission spectrophotometry (Pye Unicam SP-90) and La^{3+} by inductively coupled plasma

spectrometry (La^{3+} analyses were carried out in the Department of Geology, King's College, London). Cl^- was measured using a EIL Chloride Electrode Model 8004-2 coupled to a Corning-EEL 109 pH/mV meter. Acetate, SCN^- and salicylate were estimated by incorporation in the media of [U-^{14}C]acetate, [^{14}C]SCN^- (both obtained from Amersham International Ltd.) and [7-^{14}C]salicylate (New England Nuclear) (final medium activity approx. 20 kBq/ml). Neither acetate nor salicylate interferes with the electrometric estimation of Cl^-, but SCN^- does so due to the very low solubility product of AgSCN. In order to overcome this an interpolative method, using a series model NaCl/NaSCN solution, was used as previously described (Law, 1980). PO_4^{3-} also interferes with Cl^- estimations. In these experiments [^{36}Cl]NaCl (Amersham International Ltd.) was added to the incubation medium (20 kBq/ml). All radioactivity measurements were made using a Packard TriCarb Liquid Scintillation Spectrometer Model 3320, the activity of leachates being compared with that of a 1:1000 dilution of original incubation medium. PO_4^{3-} was estimated by the colorimetric method of Fiske & Subbarow (1925), allowance being made for the fact that $H_2PO_4^-$ also participates in the reaction.

Calculations. Estimation of slice water and s.f.d.w. have been described above. Bound ions were in the first instance calculated as notional 'concentration' (mmol/l), i.e. the concentration in excess of that which could be accounted for on the basis of post-incubatory slice fluid content and the known ionic concentration of the incubation medium. This was converted to ionic content (nmol/mg s.f.d.w.), i.e. the product of concentration (mmol/l) and fluid content (μl/mg s.f.d.w.).

Unless otherwise stated, values were determined as mean $\pm$ S.E. of mean (n). The specific gravity of the renal tissue is taken as 1·0, i.e. μl and mg are considered interchangeable.

RESULTS

Slice weight changes

Under all incubatory conditions slices underwent highly variable changes in weight, in most cases weight loss. The cause of this was not investigated: its relevance in the present context is that loss might involve reduction of slice binding capacity. In order to resolve this a computer program was written which enabled slice ion content, at any value of post-incubatory slice water content, to be compared with the content which would have been expected if no binding occurred. If the level of ionic binding is independent of weight gain or loss, the difference between the measured and expected ionic contents (which represents the level of binding) should be constant at any level of post-incubatory slice water content. Note that this assumes slice s.f.d.w. to remain constant, weight changes being solely due to gain or loss of water and solutes.

A typical print-out is shown in Fig. 1. In this experiment cortical slices were incubated in the presence of 0·25 × normal medium Na^+, viz. 36·75 mmol/l. The lines drawn are statistically parallel (for details see legend) and the difference between them at $\bar{x}$ (mean water content 2·55 μl/mg s.f.d.w.), 73·0 nmol/mg s.f.d.w., represents the level of Na^+ binding under these conditions (this value is incorporated in Fig. 5). Had slice weight (i.e. fluid) loss incurred loss of binding capacity, the two lines would have been significantly convergent towards the origins of the axes.

Similar plots were prepared for all incubatory conditions, and in no case was significant lack of parallelism observed.

Bound ions at pH 7·35

Fig. 2 shows the levels of bound Na^+ and Cl^-, relative to s.f.d.w., in rat renal cortex and outer medulla in control media at pH 7·35. The level of outer medullary Cl^- binding (9·5 $\pm$ 2·2 (25) nmol/mg s.f.d.w.) is not statistically significant, and no

attempt was made to study inter-anionic competitive binding in this region (see below).

Competitive binding by cations

Fig. 3 shows the levels of bound cation, relative to s.f.d.w., in rat renal cortex and outer medulla incubated at pH 7·35 in the presence of equimolar Na^+ and either K^+, Li^+ or La^{3+}. In both regions Na^+ binds at a level not significantly different from that of K^+ or Li^+. Na^+ is completely displaced by La^{3+}. It may be noted, however, that the amount of bound La^{3+} is considerably less than the sum of the amounts of bound monovalent cations.

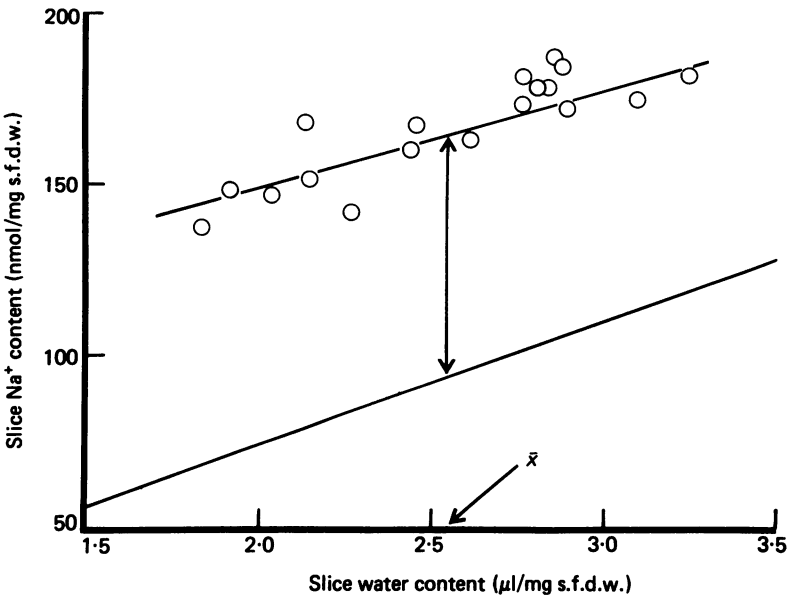

Fig. 1. Levels of cortical bound Na^+ (nmol/mg s.f.d.w.) as a function of slice water content (µl/mg s.f.d.w.) in medium containing 36·75 mmol Na^+/l at pH 7·35. The rationale for the construction of this Figure is explained in the accompanying text. The lower line represents the slice Na^+ content which would have been expected if no Na^+ binding had occurred, i.e. all Na^+ had been present in free solution at 36·75 mmol/l. The upper line is the mean of the experimentally determined Na^+ contents ($n = 18$). The slope of this line, 34·47, with a S.D. of 4·97, is not significantly different from 36·75. The mean water content ($\bar{x}$) was 2·55 µl/mg s.f.d.w. The vertical arrow indicates the mean amount of bound Na^+ (73·0 nmol/mg s.f.d.w.) at $\bar{x}$.

Competitive binding by anions

Fig. 4 shows the results of comparable experiments in which slices were incubated in the presence of equimolar concentrations of Cl^- and either acetate, salicylate, SCN^- or PO_4^{3-}. Cl^- binds to a greater extent than acetate ($P < 0·001$), somewhat more than salicylate ($0·1 > P > 0·05$), and to a lesser extent than SCN^- ($P < 0·005$); it is completely displaced by PO_4^{3-}. As with La^{3+} (Fig. 2) the amount of trivalent ion bound is less than the sum of the bound monovalent ions. No incubations were performed in which all four monovalent anions were present in the same media, thus

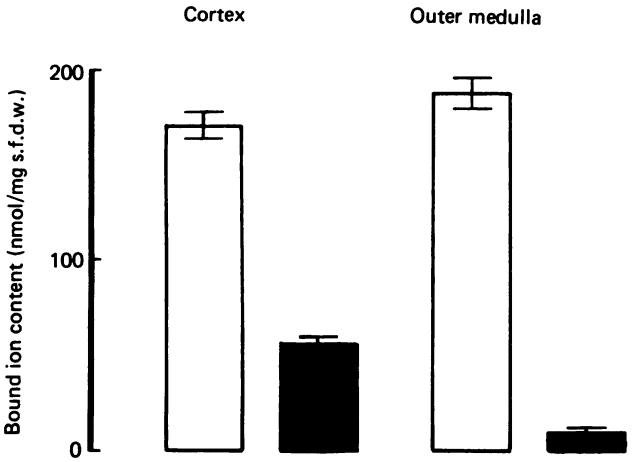

Fig. 2. Levels of cortical and outer medullary bound Na^+ (open bars) and Cl^- (filled bars) (nmol/mg s.f.d.w.) under control conditions at pH 7·35. Error bars are ±S.E. of mean ($25 \leqslant n \leqslant 75$).

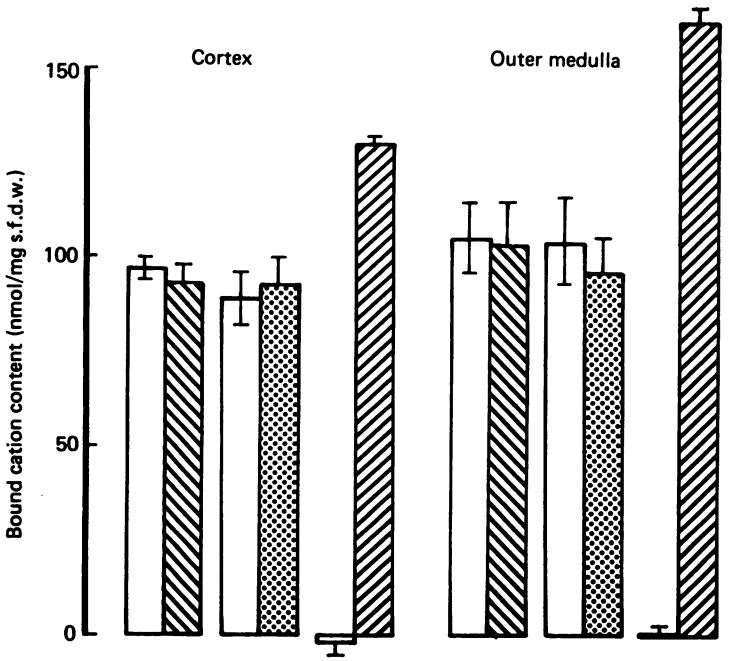

Fig. 3. Levels of cortical and outer medullary bound K^+ (left-inclined hatched bars), Li^+ (stippled bars) and La^{3+} (right-inclined hatched bars) (nmol/mg s.f.d.w.) in the presence of equimolar concentrations of Na^+ (open bars) at pH 7·35. Error bars are ±S.E. of mean ($15 \leqslant n \leqslant 24$).

no direct comparison can be made of the binding propensities of such anions in direct competition with each other. However, if the amounts of non-Cl^- anion bound (in the presence of constant [Cl^-]) are compared, it is found that (i) the amount of bound acetate is not significantly lower than that of salicylate, whereas (ii) bound salicylate $< SCN^-$ ($P < 0{\cdot}025$). In conjunction, the findings presented in Fig. 4

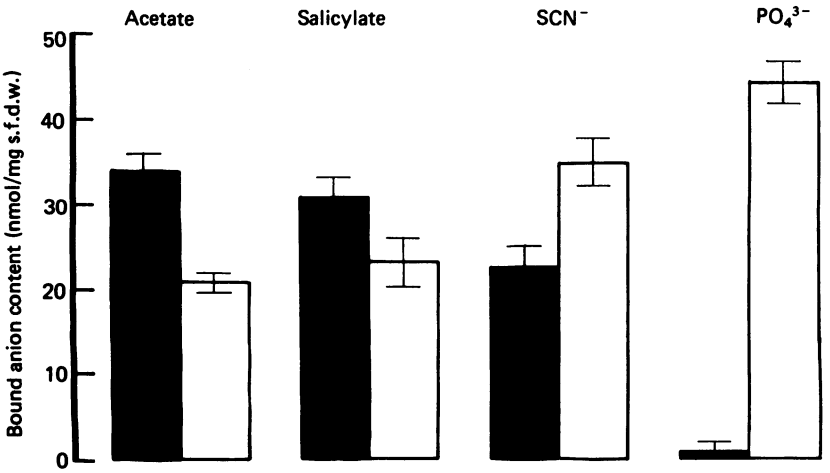

Fig. 4. Levels of cortical bound acetate, salicylate, SCN^- and PO_4^{3-} (open bars) (nmol/mg s.f.d.w.) in the presence of equimolar concentrations of Cl^- (filled bars) at pH 7·35. Error bars are ±S.E. of mean ($n = 25$).

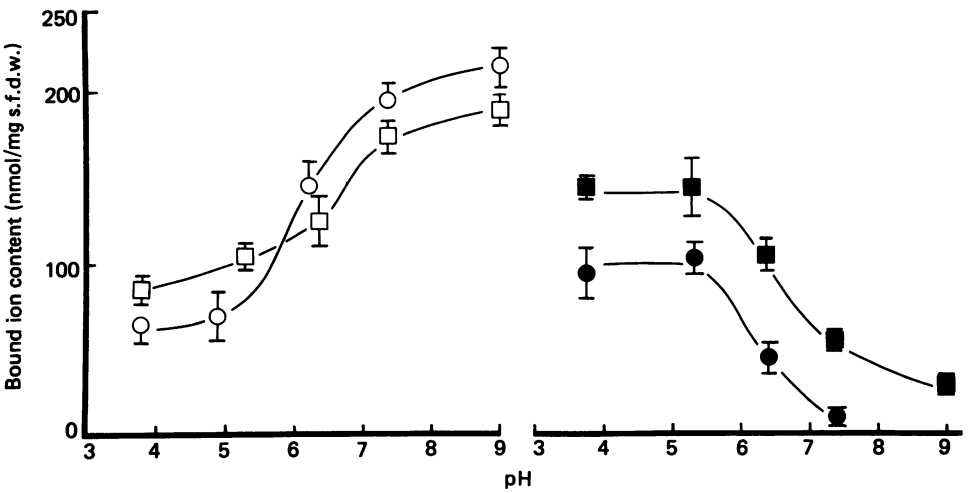

Fig. 5. Levels of cortical and outer medullary bound Na^+ and Cl^- (nmol/mg s.f.d.w.) within the pH range 3–9. Error bars are ±S.E. of mean (single bars where these would otherwise conflict) ($13 \leqslant n \leqslant 29$ at pH > or < 7·35; at pH 7·35 n is quoted in the legend to Fig. 2). Cortical Na^+ (□), Cl^- (■); outer medullary Na^+ (○), Cl^- (●).

indicate that monovalent anions probably bind according to the sequence acetate $\leqslant$ salicylate $\leqslant Cl^- < SCN^-$.

Effects of altering pH

When the pH of the incubation media was altered there were marked changes in the level of bound Na^+ and Cl^-. These are shown in Fig. 5. Na^+ binding in both regions increases only slightly as pH is increased to 8·9, suggesting that the pH-dependent component of Na^+ binding is near-saturated at pH 7·35 (i.e. normal physiological pH). Binding declines sharply as pH is reduced below pH 7·35, more conspicuously in outer

medulla than in cortex, approaching constancy at approx. pH 4. Conversely, levels of bound Cl^- increase as pH is reduced and attain a maximum value in both regions below approx. pH 5.

Dependence of ionic binding upon medium ion concentration

The dependence of levels of bound cortical and outer medullary Na^+, and of cortical Cl^-, upon the concentrations of ions in solutions containing NaCl (with or without urea) at pH 7·35 is shown in Fig. 6. The following points may be noted.

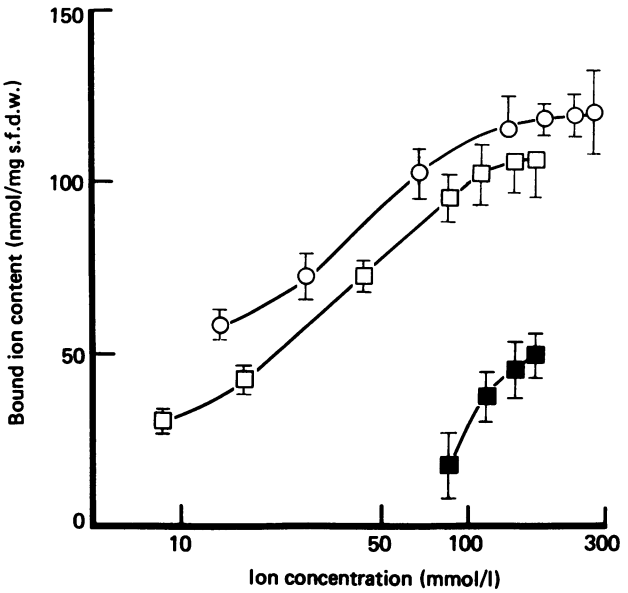

Fig. 6. Levels of bound cortical Na^+ (□) and Cl^- (■) and outer medullary Na^+ (○) (nmol/mg s.f.d.w.) as a function of medium ionic concentration (mmol/l, log scale) in media of constant calculated osmolality containing NaCl with or without urea at pH 7·35. Error bars are ±S.E. of mean (single bar where these would otherwise conflict) ($10 \leqslant n \leqslant 20$).

(i) Although the relationship between bound Na^+ and $[Na^+]$ is described by an S-shaped curve, over at least a 10-fold concentration range Na^+ binding is very nearly linearly related to log $[Na^+]$; (ii) the slopes of binding *vs.* log $[Na^+]$ in the two regions are approximately parallel, the difference between them (~15 nmol/mg s.f.d.w.) being comparable to the difference shown in the relevant histograms in Fig. 2; (iii) concentration-dependent binding is saturated at the levels of $[Na^+]$ present in control media (cortex, 147 mmol/l; outer medulla, 187 mmol/l); (iv) these levels are markedly lower than those which were found in the presence of fully constituted incubation media (Fig. 2); (v) bound Cl^- is not shown for slices incubated in media containing less than 50 mmol NaCl/l as leachate Cl^- concentrations were too low to measure reliably; unlike Na^+, Cl^- binding shows little tendency to saturate at control medium $[Cl^-]$(105 mmol/l).

Effect of altering medium osmolality

As may be seen from Fig. 7, raising medium osmolality to a maximum of 2-fold with urea markedly reduces cortical and, to a greater extent, outer medullary Na^+ binding. Conversely, Cl^- in both regions is enhanced.

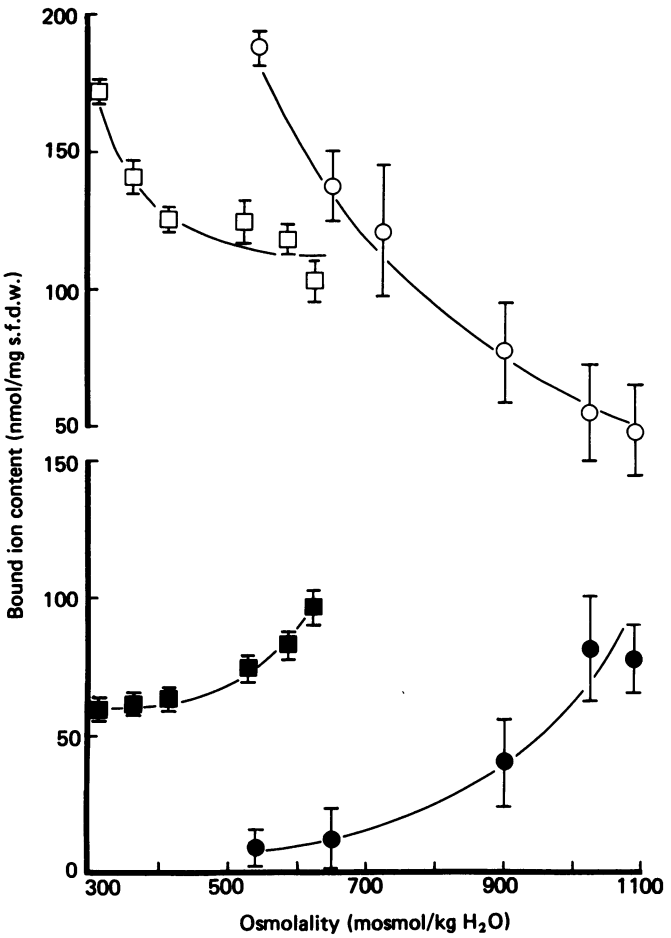

Fig. 7. Levels of bound cortical Na^+ (□) and Cl^- (■) and outer medullary Na^+ (○) and Cl^- (●) (nmol/mg s.f.d.w.) as a function of calculated medium osmolality at pH 7·35. Error bars are ±S.E. of mean ($11 \leqslant n \leqslant 20$). In this Figure the mean values at control osmolality are derived from incubations distinct from those summarized in Fig. 2.

Action of chondroitinase ABC

Pre-treatment of slices with chondroitinase ABC caused marked reductions in cortical and outer medullary Na^+ binding. For the cortex, the control level of $171 \pm 6(58)$ nmol/mg s.f.d.w. (see Fig. 2) was reduced to $112 \pm 11(20)$ nmol/mg s.f.d.w. ($P < 0{\cdot}001$). The corresponding values for outer medulla were $188 \pm 8(33)$ nmol/mg s.f.d.w. (Fig. 2) and $86 \pm 13(11)$ nmol/mg s.f.d.w. ($P < 0{\cdot}001$).

Effect of incubation at 2 °C

Cortical and outer medullary Na^+ binding were not significantly affected by incubation in the cold: respective values were $182 \pm 12(19)$ and $202 \pm 12(12)$ nmol/mg s.f.d.w. (n.s. *vs.* control values shown in the preceding subsection). Cortical Cl^- binding was also unaffected ($56{\cdot}7 \pm 2{\cdot}8(75)$ (Fig. 2) *vs.* $51{\cdot}7 \pm 4{\cdot}3(20)$ nmol/mg s.f.d.w., n.s.).

DISCUSSION

The present findings may be considered under two headings. First, what do they reveal about the nature and possible sites of ionic binding? Secondly, what is the potential physiological significance of ionic binding?

Regarding the latter question it must be recognized that although the present findings clearly demonstrate a capacity for ion binding by renal tissue they do not necessarily reflect levels which may obtain *in vivo*. The main difference between living tissue and tissue subjected to metabolic blockade concerns intracellular composition. Cells become loaded with Na^+ and severely depleted of K^+ (in pilot experiments, which have not been reported here, extremely low levels of tissue K^+ were found in blockaded slices). While it is probable that only low levels of Na^+ and K^+ are rigidly bound within animal cells, considerable intracellular compartmentalization is believed to occur (for discussion see Civan, 1981). Compartmentalization of ions in free solution can normally be maintained only by expenditure of metabolic energy. Thus both modes of sequestration are likely to be affected by metabolic blockade and reversal of the normal intracellular Na^+/K^+ ratio. Similar considerations apply to measured Cl^- binding (see below).

The constancy of Na^+ and Cl^- binding independent of slice weight loss, as exemplified in Fig. 1, suggests that binding takes place onto structural or intracellularly confined tissue components. In the case of Na^+, binding probably occurs through electrostatic attraction to sulphated GAGs and, less strongly, to hyaluronic acid (see Palaty *et al.* 1969). The marked reduction in binding following pre-treatment with chondroitinase ABC is consistent with this. The reduction was greater than might have been anticipated, since GAGs which are attacked by this enzyme, notably chondroitin 4- and 6-sulphate and dermatan sulphate, are reported to be scarce in rat kidney (Allalouf *et al.* 1964). But interaction between micro-ions and macromolecular polyions is not confined to direct or 'site' binding; it additionally involves charge-dependent Donnan-type inequalities of distribution of dissolved micro-ions in the immediate vicinity of the macromolecule (see Comper & Laurent, 1978), and these, as well as site binding, would be affected by enzymatic degradation of the macromolecule. Thus the apparent reduction in Na^+ binding should be regarded as indicating the involvement of GAGs rather than as a measure of the amount bound by them.

The one-to-one exchangeability of Na^+ for K^+ (Fig. 3) corresponds with what has been observed in various natural and synthetic polyanions (Wall & Doremus, 1954; Farber & Schubert, 1957; Comper & Preston, 1975). Presumably Li^+ binds in a similar fashion. The displacement of Na^+ by La^{3+} is compatible with electrostatic binding

dependent on cation surface charge density, and that the amount of bound La^{3+} (on a molar basis) is less than that of monovalent cations probably reflects the fact that La^{3+} in aqueous solution may exhibit a coordination number as great as 6. The pH dependence of Na^+ (and of Cl^-) binding (Fig. 5) is presumably due to alteration in the surface charge density on the macromolecule: in the case of Na^+ a comparable dependence has been shown in arterial wall (Headings, Rondel & Bohr, 1960). Temperature should not significantly affect electrostatic binding: a similar observation has been made for Na^+ binding by polyacrylic acid (Wall & Doremus, 1954). The nature of the relationship between Na^+ binding and medium [Na^+] (Fig. 6) resembles a simple dissociation curve and suggests a relatively non-complex binding (cf. buffer titration curves). The low levels of binding of Na^+ (and of Cl^-) in NaCl solutions, by contrast with fully constituted media, stresses the significance of 'minor' ions in facilitating binding (notably K^+, Mg^{2+} and HCO_3^-, see Palaty *et al.* 1969).

The nature of Cl^- binding, although superficially comparable with Na^+ binding in some respects (e.g. displacement by a trivalent ion), is more difficult to interpret since the binding sites are unknown. They are much less abundant in outer medulla than in cortex under control conditions (Fig. 2), but their potential receptivity in the former region is revealed by lowering pH (Fig. 5) or raising external osmolality (Fig. 7). Cl^- sequestration has been examined in vascular smooth muscle and variously ascribed to extracellular (Villamil *et al.* 1968) and intracellular sites (Somlyo *et al.* 1979). It is reasonable to infer that any intracellular binding in the present experiments was metabolically independent, but this may not, of course, necessarily be true for such binding in metabolizing tissue. In rat aortic wall, Cl^- binding is greatly reduced by pre-treatment with proteolytic enzyme, but only after cell walls are rendered permeable to macromolecules by incubation in 50% (v/v) glycerol in saline (Law, 1984*b*). However, renal slices disintegrate if treated in this way. Polycationic intracellular proteins, e.g. histone- and protamine-rich nucleoprotein, are a possible site for pH-dependent anion binding.

The fact that monovalent anions (Fig. 4), unlike cations (Fig. 3), do not bind to equal extents when present in equal concentrations, suggests that their propensity to bind may depend upon their relative positions within a lyotropic series. A wide range of such series within biological systems has been reviewed by Wright & Diamond (1977). Anionic position within such a series is known to influence its absorption by proteins and other macromolecules (for recent discussion see Dani, Sanchez & Hille, 1983). But until the relevant binding sites are known the existence of lyotropic series in the present context must remain speculative.

The effects of raising medium osmolality upon binding of Na^+ and Cl^- are qualitatively opposed (Fig. 7) and are particularly difficult to interpret. Urea was chosen as the osmotic additive since it is the principal non-electrolyte in renal fluids. The concentration of urea in the outer medulla, where its effects on binding of both ions is more pronounced than in the cortex, as well as interstitial osmolality, normally fluctuates with the diluting or concentrating activity of the kidney (Hai & Thomas, 1969). It is difficult to accept that the increases in osmolality in the present experiments (not greater than 2-fold) were sufficient to alter macromolecular conformation to an extent which could account for the altered levels of binding shown in Fig. 7. It is possible that urea specifically affects the distribution of hydrophobic

groups within tissue macromolecules and hence their ion-binding capacities (for discussion see Chvapil, 1967; Dani *et al.* 1983). Detailed knowledge of the nature and physico-chemical properties of such molecules within the kidney would be required to establish this.

To turn briefly to the possible physiological significance of renally bound ions, three points may be made. First, the kidney is an organ in which Na^+ and Cl^- concentrations, osmolality and pH can alter locally (e.g. $[Na^+]$ within lateral cellular interspaces in the proximal tubule) or more generally (e.g. the osmolality of the medullary interstitium). Secondly, although it would be unjustifiably teleological to imply that such ions play a specific role in the regulation of renal function, it may be stressed that the levels of binding, particularly of Na^+, are by no means negligible. For example, it may readily be calculated that in a cortical slice with a fluid content of 2·50 μl/mg s.f.d.w. (a typical value) approx. 30 % of total tissue Na^+ is bound under control conditions. Thirdly, the variations in the levels of binding in response to alterations in ambient conditions such as occur intrarenally are mostly marked rather than trivial. In view of these considerations it is not unjustified to suggest that bound ions may influence renal function.

Of the roles tentatively assigned to bound ions in other tissues, the most directly relevant is that suggested by Macknight *et al.* (1982) whereby GAG-bound Na^+ on apical plasma membranes and in the crypts of colonic wall might facilitate Na^+ reabsorption by minimizing variations in local Na^+ activity. There is a clear parallel to be drawn between this and the situation in renal tubules, although a comparable role for Cl^- becomes more difficult to conceive if binding is mainly intracellular. Similarly, it has been suggested that polypeptide-bound Na^+ may act as a buffer stabilizing free $[Na^+]$ in cardiac muscle (Hickling, Barclay, White & White, 1976). In a very different biological context Treherne, Schofield & Lane (1982) have suggested that anionic GAGs may constitute a reservoir subserving the short-term maintenance of ionic homoeostasis within the central nervous system of an insect.

The possibility that intrarenally bound ions act in a comparable manner affords ample scope for valid speculation. Renal slices, however, although they form a simple and convenient preparation for measuring ion binding and its variations, are not suitable for assessment of possible functional consequences of such variations in respect of tubular ion transport, although these possibilities could be tested using the wide variety of isolated renal tubular preparations which are now obtainable (as reviewed by Burg, 1982).

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