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STUDIES ON THE RELATIONSHIP BETWEEN RAT RENAL MEDULLARY CELL VOLUME AND EXTERNAL ANION CONCENTRATION IN HYPEROSMOLAL MEDIA

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SUMMARY

1. The volumes of cells in slices of rat renal outer medulla have been examined following incubation for 25 min in hyperosmolal media (650 and 950 m-osmole/kg H_2O) containing independently variable concentrations of Cl (70–235 mM) and HCO_3 (10–60 mM) (gas phase 95% O_2/5% CO_2).

2. For any given level of external Cl concentration cell volumes were reduced by increasing the external HCO_3 concentration. These reductions were accompanied by net loss of cellular K and Cl. In confirmation of earlier findings, cell volumes were also reduced by increasing external Cl concentration.

3. Experiments in which the HCO_3 concentration and pH of the incubation media were independently varied by the use of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)/100% O_2 showed that it is the HCO_3 anion *per se* which influences cell volume.

4. The anion exchange inhibitor 4-acetamido-4'-*iso*thiocyanatostilbene-2,2'-disulphonic acid (SITS, disodium salt, 1 mM) abolished the dependence of cell volume upon HCO_3 but not upon Cl.

5. Acetazolamide (1 mM) influenced (reduced) cell volumes only in the presence of low (10 mM) HCO_3.

6. CNS (25 mM) also markedly reduced cell volumes in media containing 10 mM-HCO_3 and, to a lesser extent, 25 mM-HCO_3. It was without effect on cell volume when external HCO_3 was 60 mM.

7. The presence of CNS was associated with the significant cellular net accumulation of Cl in media in which either Cl or HCO_3 concentration (or both) was low (70 or 130 mM and 10 mM respectively).

8. The outer medullary [^{35}S]CNS space at 25 min, determined for slices incubated in a representative selection of the various media employed in this study, exceeded the [^{14}C]inulin space by 1·77 μl./10 mg wet weight.

INTRODUCTION

Over a century has elapsed since Nasse (1878) first described Cl and HCO_3 movement across red cell membranes, an occurrence now more commonly associated with his compatriot who 'rediscovered' the phenomenon a few years later (Hamburger, 1891). It is only comparatively recently, however, that this and related anion

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exchanges have been studied in detail, not only in red blood cells (Deuticke, 1972; Cousin & Motais, 1976; Lambert & Lowe, 1978; Cabantchik, Knauf & Rothstein, 1978; Wieth, 1979) but also in excitable tissues (Thomas, 1977; Vaughan-Jones, 1979) and a wide variety of transporting epithelia including the gastric mucosa (Sanders, O'Callaghan, Butler & Rehm, 1972), turtle urinary bladder (Leslie, Schwartz & Steinmetz, 1973), the collecting duct of the exocrine pancreas (Swanson & Solomon, 1975), colon (Frizzell, Koch & Schultz, 1976) and gall-bladder (Heintze, Petersen, Olles, Saverymuttu & Wood, 1979).

One tissue which has not hitherto attracted experimental attention in this respect is the mammalian renal medulla. Several investigations however, have shown that net transport of anions occurs across the plasma membranes of the diverse cell population in this region. Cells in the diluting segment (at least, in the rabbit) actively transport Cl ions (Rocha & Kokko, 1973), as do cells in the human collecting duct (Jacobson, Gross, Kawamura, Waters & Kokko, 1976). Moreover, although it is beyond dispute that cortical parts of the nephron are responsible for the majority of HCO_3 reabsorption, it is likely that reabsorption can also occur in medullary segments (see Malnic & Steinmetz, 1976), and Levine, Byers, McLeod & Luisello (1979) have recently shown that under appropriate conditions HCO_3 accumulation can take place in the short loops of Henle in rat kidney. Stoner, Burg & Orloff (1974) have obtained evidence of electrically neutral chloride reabsorption in rabbit cortical collecting ducts, which might represent Cl-HCO_3 exchange. Whether or not this also occurs in medullary segments is not known.

The present study is concerned with the inter-relationship between Cl and HCO_3 as determinants of outer medullary cell volume and composition under conditions of controlled extracellular osmolality. It is probable that the ratio of intracellular to extracellular Cl concentration is in part balanced by a counter-distribution of HCO_3 (Law, 1977) and that Cl ions influence cell volumes in this region (Law, 1979). Some of the present findings have already been published in abstract form (Law, 1980).

METHODS

The basic format of the present experiments closely resembles that of Law (1979).

Duplicates slices (4–14 mg, thickness not exceeding 0·3 mm) from the outer medulla of normally hydrated adult male Wistar rats were individually weighed to the nearest 50 μg and then incubated for 5 min at 37 °C in a medium constituted as follows (mM): Na^+ 180, K^+ 5·9, Ca^{2+} 2·6, Mg^{2+} 1·2, choline 45, Cl^- 189, HCO_3^- 25, $H_2PO_4^-$ 2·2, SO_4^{2-} 1·2, pyruvate 4·8, glutamate 4·8, fumarate 5·3, glucose 10, urea 54, calculated osmolality 540 m-osmole/kg H_2O. The pH of this medium when gassed with 95 % O_2/5 % CO_2 was approximately 7·35. The object of this incubation was to endeavour to achieve uniformity of slice composition before the beginning of the various experimental incubations described below.

After blotting and re-weighing, slices were transferred to the medium (2·5 ml.) whose effect on cell volume it was desired to investigate. Such incubations were performed for a period of 25 min at 37 °C with vigorous agitation in a shaking water-bath. Gassing was achieved through a pin-hole in the parafilm seal of the incubation vessel. Incubation media were based on the medium described above, with the following modifications and/or additions.

(1) *Anion concentrations and osmolality.* By appropriate equimolar substitution of Na gluconate for NaCl and/or $NaHCO_3$, of $NaHCO_3$ for NaCl, and of choline chloride for NaCl and/or urea, media were prepared which contained all possible combinations of the following variables: (*a*) HCO_3 (mM), 10, 25 or 60 (the approximate pH values for these being, respectively, 7·0, 7·35 and 7·75), (*b*) Cl (mM) 70, 130, 190 or 235, (*c*) calculated osmolality (m-osmole/kg H_2O), 650 or 950.

On the basis of previously available data these values may be considered to reflect the osmolalities (although not necessarily the solute composition) of rat outer medullary fluids during moderate and severe antidiuresis respectively (Saikia, 1965; Hai & Thomas, 1969; Lee & Williams, 1972; R. O. Law, unpublished observations). In the present report, in order to avoid unwieldy descriptions of media, these will be referred to in terms of the three variables in the order quoted above, e.g. 25/130/950.

One object of the present investigation has been to provide relevant information for projected studies of the effect of external osmolality on the activity of certain medullary enzymes. However, in respect of the results presented here, there were no significant qualitative differences between the responses of cells to media of the two osmolalities. In the Results section, therefore, attention will be focussed on observations made in media of 950 m-osmole/kg H_2O together with a simple statement of the response noted in media of the lower osmolality.

(2) *Adjustments of pH.* It was necessary to establish whether the observed effects of differing concentrations of HCO_3 upon cell volume and composition were due to this anion *per se* or to the accompanying alterations of pH which have already been referred to. Two series of incubations were performed in order to examine this. In one, the pH of media containing 10 and 60 mM-HCO_3 was adjusted to 7·35, and the findings compared with those made in media containing 25 mM-HCO_3. In the other, the pH of media containing 25 mM-HCO_3 was adjusted to 7·0 or 7·75, and findings compared with those made in media containing 10 or 60 mM-HCO_3 respectively. These adjustments of pH were made by the dropwise addition of a molar aqueous solution of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), the gas phase being 100 % O_2. pH was checked at the start and finish of incubation, and it was observed that the use of this buffer caused the pH to increase by approximately 0·05 pH unit over 25 min. Accordingly, sufficient HEPES was added to bring the pH to 0·025–0·05 pH unit below the desired pH. While this method would clearly be unsuitable in a detailed examination of the effects of pH as such on cell volume, it proved adequate in terms of the present objectives. All measurements and adjustments of pH were made at 37 °C, with appropriate gassing, using a Philips PW 9148 pH meter.

(3) *Addition of SITS.* Incubations were performed in the presence of the anion exchange inhibitor 4-acetamido-4'-*iso*thiocyanato-stilbene-2,2'-disulphonic acid (SITS, disodium salt, 1 mM).

(4) *Addition of acetazolamide* (*Diamox*). In a further series of incubations acetazolamide (American Cyanamid Co.) (1 mM) was added to the media.

(5) *Addition of CNS.* A final series of incubations was carried out with the incorporation of 25 mM-NaCNS. This necessitated further equimolar adjustments of media constituents, in the manner already described, in order to maintain the specified Cl, HCO_3, and osmolal concentrations. It was not possible by these means to prepare solution 60/235/650 in the presence of 25 mM-NaCNS.

Following a random selection of incubations under each of the headings above, medium [HCO_3] was checked by means of a volumetric van Slyke apparatus. Within the limits of accuracy of this method no decrements were observable.

Determination of cell volume and composition

(*a*) *Cell volume, Na and K.* Methods for the estimation of cell volume from tissue wet weight and distribution of [^{14}C]carboxyl inulin (The Radio Chemical Centre, Amersham) and cellular Na and K content and concentration, together with certain inherent assumptions, have been described in detail previously (Law, 1979). Cell volume was expressed as percentage swelling or shrinkage (volume at the start of incubation = 100). Cellular Na and K were expressed as mM (concentration) and μequiv/g solute-free dry weight (SFDW) (content).

(*b*) *Chloride.* During the initial part of the period over which the present investigation was carried out, Cl was estimated using a Corning-EEL 920 Chloride Meter. As previously pointed out (Law, 1979) the sensitivity of this instrument effectively precludes the possibility of estimating Cl in the same leachates, and hence in the same slices, as those used for inulin, Na and K. It was necessary to carry out separate incubations, after which slices were leached in very small volumes of fluid (250 μl.) and the cellular Cl content calculated by reference to data obtained from the incubations incorporating inulin (see Law, 1979).

Subsequently, a Cl-sensitive electrode became available (EIL Chloride Electrode Model 8004-2

coupled to a Corning-EEL 109 pH/mV Meter). Due to the lower limit of sensitivity of this instrument it was possible to estimate Cl in the same slices as those used for determinations of inulin, Na and K. 1 ml. aliquots of leachate containing Cl were mixed with 100 μl. buffer comprising 0·5 M-ammonium acetate in 0·5 M-acetic acid in a shallow Perspex dish designed to allow satisfactory immersion of the electrodes in so small a volume of fluid. The fluid was vigorously stirred with a magnetic micro-stirrer during analysis. The electrodes were calibrated using standard solutions of NaCl within the concentration range 50–500μM. All earlier Cl estimations were repeated using this method: the opportunity was also taken to repeat the related estimations of inulin, Na and K. Values for Cl content obtained by these two methods were found to be in good agreement. This repetition of earlier estimations explains why the number of individual values (n) under certain experimental conditions (notably in the presence of 25 or 60 mM-HCO_3 without additions) conspicuously exceed those under others (see Results).

The solubility product of AgCNS is lower than that of AgCl, and CNS seriously interferes with estimation of Cl when the two anions are present in comparable concentrations. In order to make allowance for this, the following method was adopted.

The first step was to estimate the volume of distribution of CNS in outer medullary slices (the CNS space) at 25 min in order to establish the concentration of CNS likely to be present in any given slice leachate. Slices were incubated for 25 min in media incorporating [^{35}S]KCNS (New England Nuclear) (*ca.* 0·5 μCi/ml.). The media used were those known to produce (within the context of the present investigation) the greatest cell swelling (10/70/650) and shrinkage (60/235/950), and two media having an intermediate effect on cell volume (10/190/650 and 60/130/950) (see Fig. 1). ^{35}S in the subsequent leachate was estimated using a Packard TriCarb Liquid Scintillation Spectrometer Model 3320. It was found (see Results) that in each of these media the 25 min CNS space exceeded the inulin space by approximately 1·77 μl./10 mg wet weight of tissue. It was assumed that this value should also be applicable to slices incubated in other media. This indirect approach might have been avoided by estimating the [^{14}C]inulin space and [^{35}S]CNS space simultaneously in the same slices. However, the soft β-radiations from both isotopes have closely similar maximum energies, and they cannot therefore be conveniently counted by scintillation spectrometry when present in the same solution.

Two consequences follow from the above. First, from a knowledge of the inulin space previously determined in any slice, it should be possible to calculate the probable CNS space and hence the concentration of CNS which would have been present in the leachate obtained from that slice. Secondly, bearing in mind that the concentration of CNS employed was 25 mM, it was estimated that the CNS concentrations encountered in the leachates should fall within the range 8–80 μM.

Accordingly, a series of standard solutions were prepared containing differing concentrations of Cl (50, 125, 250, 375 and 500 μM) in combination with differing concentrations of CNS (0, 10, 40, 70 and 100 μM), i.e. 25 solutions covering the concentration ranges for both anions likely to be present in the leachates. The apparent Cl concentration present in each solution was determined using the Cl electrodes as described above. By comparing these apparent concentrations with the true concentration (i.e. in the absence of CNS) a series of calibration curves was constructed from which it was possible, directly or by interpolation, to convert apparent Cl concentration to true Cl concentration, given the CNS concentration of the leachate.

It was appreciated that this indirect and rather cumbersome method to Cl estimation in the presence of CNS contains several steps which might be subject to error. Each experimental stage was exhaustively checked in an attempt to minimize this possibility.

RESULTS

Effects of Cl and HCO_3 on cell volume and composition

The effects upon cell volume (here referred to as the cell volume response) of independantly altering external Cl and HCO_3 concentrations ($[Cl]_o$ and $[HCO_3]_o$) in media containing 650 and 950 m-osmole/kg H_2O are shown in Fig. 1. It may be seen that for each value of $[Cl]_o$ shrinkage is enhanced as $[HCO_3]_o$ is increased. The range of values for $[Cl]_o$ in the present experiments (70–235 mM) somewhat exceeds that

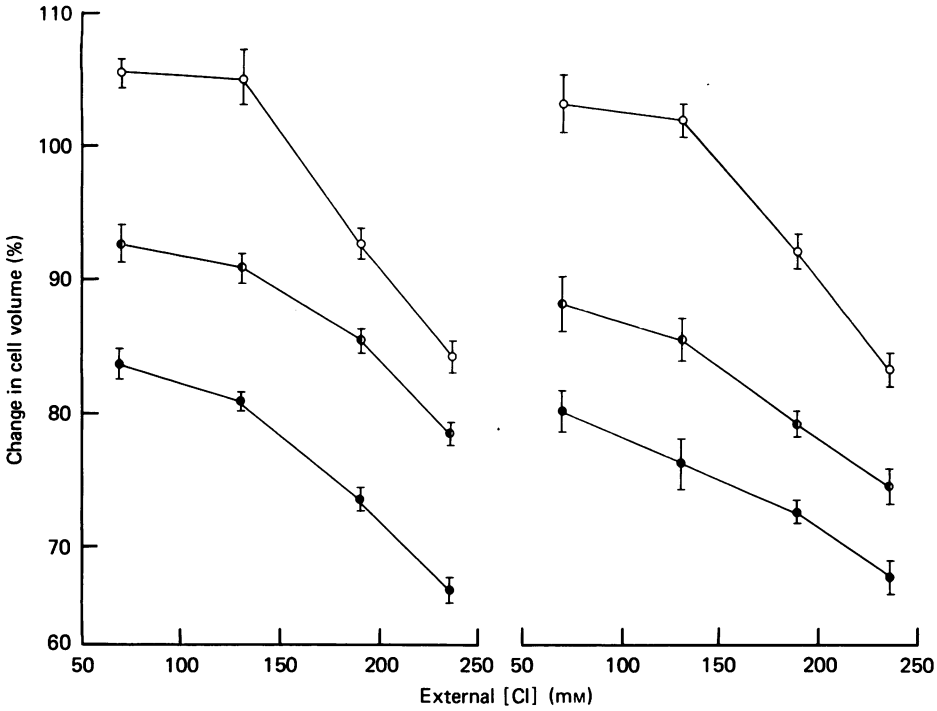

Fig. 1. The volume responses of outer medullary cells incubated in media of 650 or 950 m-osmole/kg H_2O containing 10 (○), 25 (◐) or 60 mM (●) HCO_3 and 70, 130, 190 or 235 mM-Cl (abscissa). Points represent mean ± S.E. of mean ($32 \geqslant n \geqslant 8$).

TABLE 1. The K and Cl contents of cells incubated in media (950 m-osmole/kg H_2O) containing variable concentrations of Cl and HCO_3. Values are mean ± S.E. of mean (n). The significance of differences in content between cells incubated in 10 or 60 mM-HCO_3 and those incubated in 25 mM-HCO_3 are indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

	$[HCO_3]_o$ (mM)		
	10	25	60
$[Cl]_o$ (mM)	K content (μequiv/g SFDW)		
70	415 ± 24 (8)***	290 ± 7 (8)	256 ± 8 (24)*
130	404 ± 19 (16)***	290 ± 9 (23)	267 ± 9 (23)
190	386 ± 15 (8)**	298 ± 13 (24)	266 ± 6 (32)*
235	382 ± 7 (7)***	306 ± 9 (31)	260 ± 8 (29)***
	Cl content (μequiv/g SFDW)		
70	240 ± 6 (7)**	186 ± 9 (15)	153 ± 6 (15)**
130	312 ± 15 (16)**	250 ± 11 (14)	213 ± 11 (15)*
190	416 ± 13 (16)**	346 ± 20 (12)	292 ± 16 (13)*
235	463 ± 20 (10)	419 ± 21 (12)	329 ± 18 (8)**

used in previous studies (90–215 mM) (Law, 1979) and reveals the slight curvilinearity of the relationship between volume and $[Cl]_o$. This is particularly noticeable in the presence of 70 mM-Cl and 10 mM-HCO_3 (in which media slight cell swelling occurred) but it is unlikely that the curvilinearity represents cells achieving their maximum distensibility, since previously cells have been shown to swell to over 125% of their original volume under appropriate conditions (Law, 1979).

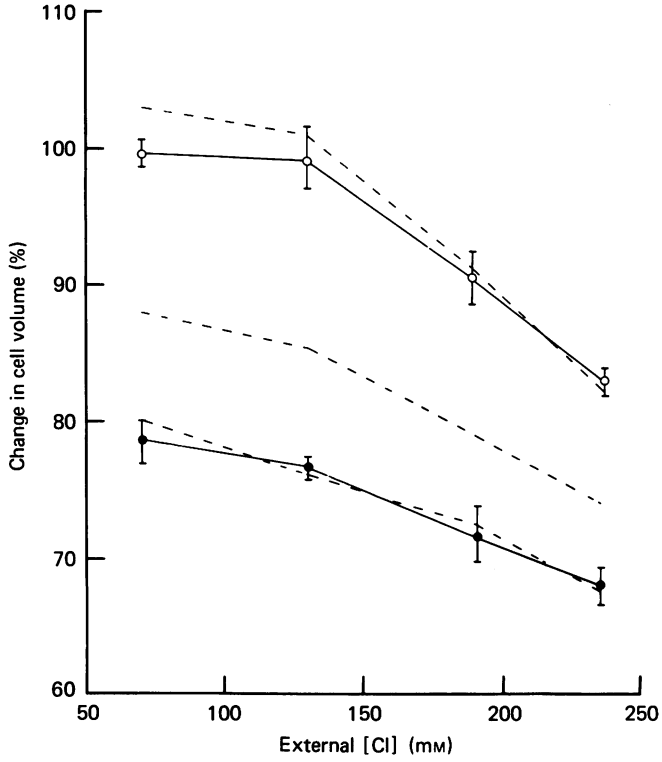

Fig. 2. The volume responses of outer medullary cells incubated in media of 950 m-osmole/kg H_2O containing 10 (○) or 60 mM (●) HCO_3 and 70, 130, 190 or 235 mM-Cl (abscissa). The pH of all media was adjusted to approx. 7·35 by the addition of HEPES. Points represent mean ± S.E. of mean ($24 \geqslant n \geqslant 8$). The dashed lines represent the volume responses in the absence of HEPES, taken from Fig. 1.

The increased cell volumes in the presence of 10 mM-HCO_3 (by comparison with volumes in 25 mM-HCO_3) and decreased volumes in 60 mM-HCO_3 were associated with net retention and loss of cellular KCl respectively. The relevant values for cells incubated in media containing 950 m-osmole/kg H_2O are shown in Table 1. In all save a single instance for each ion the amounts of K and Cl retained or lost are statistically significant at the level $P < 0{\cdot}05$ or less. Results for cells incubated in media containing 650 m-osmole/kg H_2O were comparable.

There were no significant Cl^- or HCO_3^- dependant variations in cellular Na content, and these values have therefore been omitted from Table 1. In conformity with previous observations (Law, 1979) cell Na content was consistently slightly lower in media of the higher osmolality.

Effects of pH adjustment using 1 M-HEPES

In Fig. 2 are shown the effects on medullary cell volume responses of adjusting the pH of media containing 10 or 60 mM-HCO_3 to 7·35 by means of dropwise addition of 1 M-HEPES. Only results in media containing 950 m-osmole/kg H_2O are shown: those in 650 m-osmole/kg H_2O were qualitatively similar.

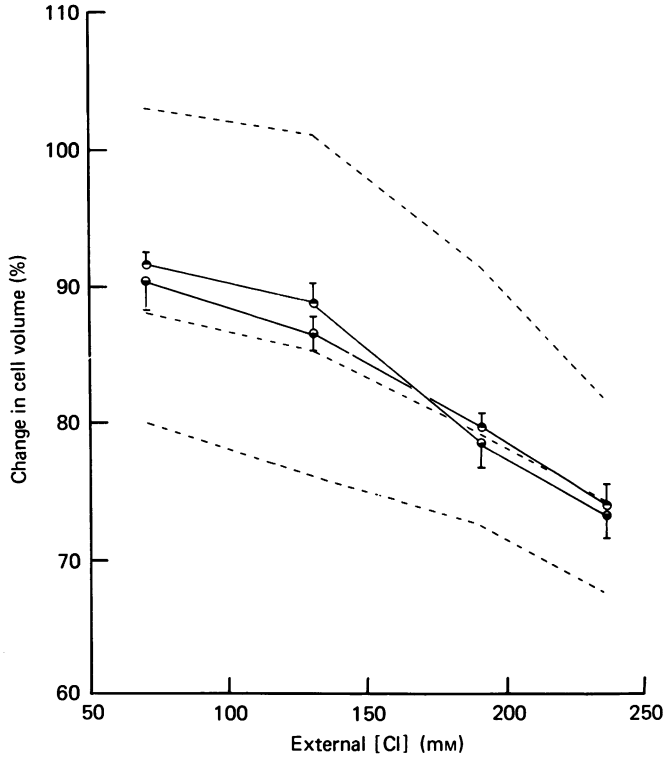

Fig. 3. The volume responses of outer medullary cells incubated in media of 950 m-osmole/kg H_2O containing 25 mM-HCO_3 and 70, 130, 190 or 235 mM-Cl (abscissa). The pH of all media was adjusted either to approx. 7·75 (◓) or to approx. 7·0 (◒) by the addition of HEPES. Points represent mean ± S.E. of mean (+ or − S.E. of mean where error bars would otherwise overlap) ($15 \geqslant n \geqslant 7$). The dashed lines represent the volume responses in the absence of HEPES, taken from Fig. 1.

The volume responses in these media following the adjustment of pH with HEPES can be seen closely to correspond with those occurring in the absence of such adjustment.

The results of adjusting the pH of media containing 25 mM-HCO_3 to 7·0 or 7·75 by the dropwise addition of 1 M-HEPES are shown in Fig. 3.

As in Fig. 2, it is clear that alteration of pH has no discernible effect upon cell volume responses, and that these continue to depend upon $[HCO_3]_o$ *per se*.

In media of 650 and 950 m-osmole/kg H_2O cellular electrolyte composition following adjustment of external pH remained, like the cell volume responses, typical of $[HCO_3]_o$ (as shown in Table 1).

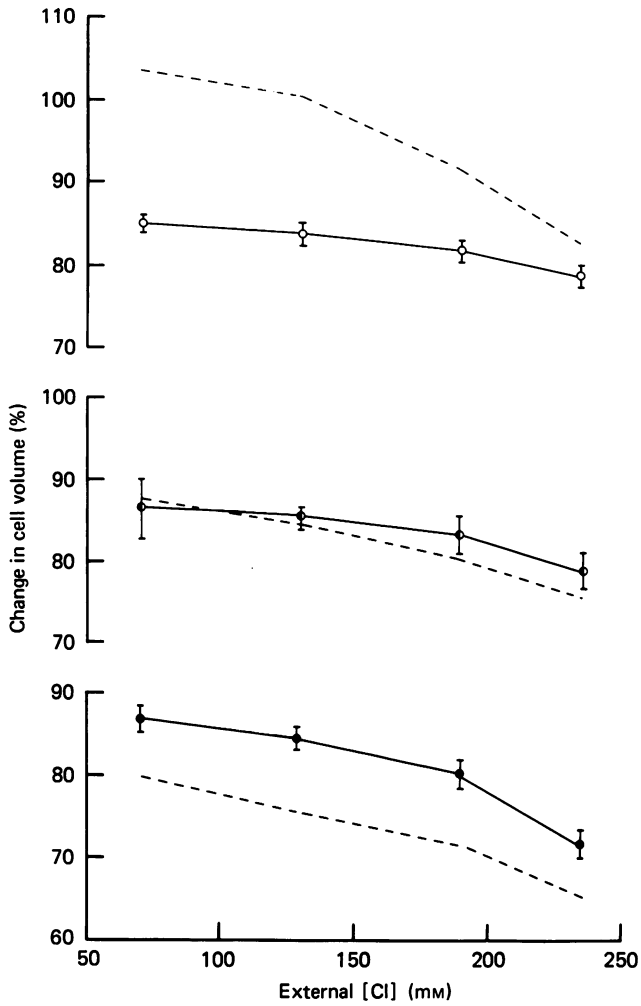

Fig. 4. The volume responses of outer medullary cells incubated in media of 950 m-osmole/kg H_2O containing 10 (○), 25 (◐) or 60 mM (●) HCO_3, 70, 130, 190 or 235 mM-Cl (abscissa) and 1 mM-SITS. Points represent mean ± S.E. of mean ($16 \geqslant n \geqslant 6$). The dashed lines represent the volume responses in the absence of SITS, taken from Fig. 1.

Effects of 1 mM- SITS

The concentration (1 mM) of SITS was chosen because this has been shown significantly to affect anion movement in rat proximal tubule (Ullrich, Capasso, Rumrich, Papavassiliou & Kloss, 1977; Bishop & Green, 1978).

The result of adding 1 mM-SITS to incubation media was to abolish the dependence of cell volume upon $[HCO_3]_o$ while leaving dependence upon $[Cl]_o$ unaltered This is illustrated (for media containing 950 m-osmole/kg H_2O) in Fig. 4. Similar results were obtained following incubation in media of 650 m-osmole/kg H_2O.

Cell volumes in the presence of 10 and 60 mM-HCO_3 are reduced and increased, respectively, in the presence of 1 mM-SITS. Although, in the interests of clarity,

volume responses (and their dashed controls) in the various media have not been superimposed in the construction of Fig. 4, it may be seen that these responses in the presence of SITS closely resemble the response obtained in the presence of 25 mM-HCO_3, which is not itself significantly affected by the addition of SITS to the medium. Comparable volume responses were noted in the presence of 650 m-osmole/kg H_2O.

TABLE 2. The K and Cl contents of cells incubated in media (950 m-osmole/kg H_2O) containing variable concentrations of Cl and 10 or 60 mM-HCO_3, in the presence of SITS (1 mM). Values are mean ± S.E. of mean (n). The significance of differences between the contents shown here and those in the absence of SITS (see Table 1) are indicated as follows: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$

	$[HCO_3]_o$ (mM)	
	10	60
$[Cl]_o$ (mM)	K content (μequiv/g SFDW)	
70	342 ± 13 (7)*	260 ± 8 (16)
130	342 ± 15 (16)**	280 ± 12 (14)
190	319 ± 12 (16)**	278 ± 8 (16)
235	369 ± 19 (6)	317 ± 15 (8)**
	Cl content (μequiv/g SFDW)	
70	173 ± 11 (7)***	172 ± 14 (8)
130	284 ± 10 (8)	261 ± 9 (8)**
190	386 ± 11 (16)	384 ± 20 (6)**
235	438 ± 8 (8)	440 ± 6 (8)***

The effects of SITS on cellular K and Cl contents in media of 950 m-osmole/kg are shown in Table 2.

In media containing 10 mM-HCO_3 there was a net loss of cellular K and Cl, whereas in the presence of 60 mM-HCO_3 net cellular uptake of these ions was observed. Although such loss or gain was consistently seen in media of given $[HCO_3]_o$ (as they also were in media of 650 m-osmole/kg H_2O) it is apparent from Table 2 that these did not always attain statistical significance at the level $P < 0{\cdot}05$. Addition of SITS produced negligible changes in cell K and Cl content in media containing 25 mM-HCO_3.

Effects of 1 mM-acetazolamide

As may be seen from Fig. 5, acetazolamide significantly reduced cell volumes in the presence of 10 mM-HCO_3 (the dashed line being the control response from Fig. 1). This was observed both in media of 650 and 950 m-osmole/kg H_2O. It was without effect in media containing higher concentrations of HCO_3, and these results have therefore been omitted from Fig. 5.

The reductions in cell volume in media containing 10 mM-HCO_3 were accompanied by net cellular loss of K and Cl (Table 3).

Effects of 25 mM-CNS

CNS belongs to the group of anions which occupy an 'extracellular' space considerably in excess of that available to inulin (see Elkington & Danowski, 1955). Table 4 shows a comparison of the 25 min inulin and CNS spaces obtained in four of the media used in the present work, as described in Methods. It can be seen that

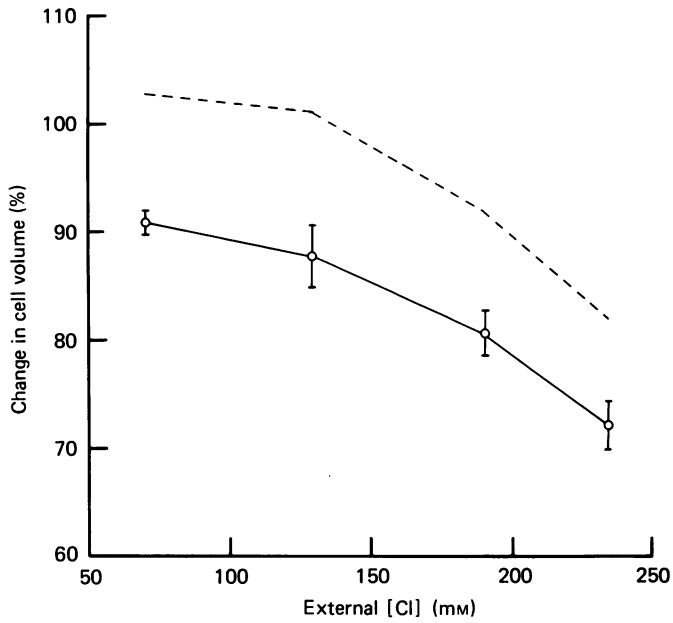

Fig. 5. The volume response of outer medullary cells incubated in media of 950 m-osmole/kg H_2O containing 10 mM-HCO_3, 70, 130, 190 or 235 mM-Cl (abscissa) and 1 mM-acetazolamide. Points represent mean ± S.E. of mean ($n = 7$ or 8). The dashed lines represent the volume response in the absence of acetazolamide, taken from Fig. 1.

TABLE 3. The K and Cl contents of slices of incubated in media (950 m-osmole/kg H_2O) containing variable concentrations of Cl and 10 mM-HCO_3, in the presence of acetazolamide (1 mM). Values are mean ± S.E. of mean (n). The significance of differences between the contents shown here and those in the absence of acetazolamide (see Table 1) are indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (for medium 10/130/950, $0.1 > P > 0.05$)

$[Cl]_o$ (mM)	K content (μequiv/g SFDW)	Cl content (μequiv/g SFDW)
70	334 ± 10 (8)**	191 ± 12 (8)**
130	323 ± 13 (7)***	268 ± 6 (7)
190	333 ± 17 (8)*	355 ± 24 (8)*
235	330 ± 12 (8)**	381 ± 24 (8)*

TABLE 4. Comparison of the [^{14}C]inulin and [^{35}S]CNS spaces in outer medullary slices incubated for 25 min in selected media. Values are mean ± S.E. of mean (n). Also shown are the arithmetic differences between the spaces (and the mean value for the four media) and their ratios

Composition of medium	Space (μl./10 mg wet weight)			CNS space
	[^{14}C]inulin	[^{35}S]CNS	CNS-inulin	inulin space
10/70/650	2.37 ± 0.08 (16)	4.14 ± 0.23 (10)	1.77	1.75
10/190/650	2.70 ± 0.10 (15)	4.48 ± 0.21 (10)	1.78	1.67
60/130/950	3.16 ± 0.17 (8)	4.95 ± 0.17 (10)	1.79	1.56
60/235/950	3.49 ± 0.11 (16)	5.24 ± 0.21 (10)	1.75	1.50
			Mean = 1.77	

whereas the ratio of the CNS space: inulin space decreased as the inulin space increased (and cell shrinkage became more pronounced), the arithmetical difference between the spaces remained approximately constant (mean = 1·77 μl./10 mg wet weight of tissue), and it was this value which was used in calculating the extent of interference of CNS ions in Cl estimation (see Methods).

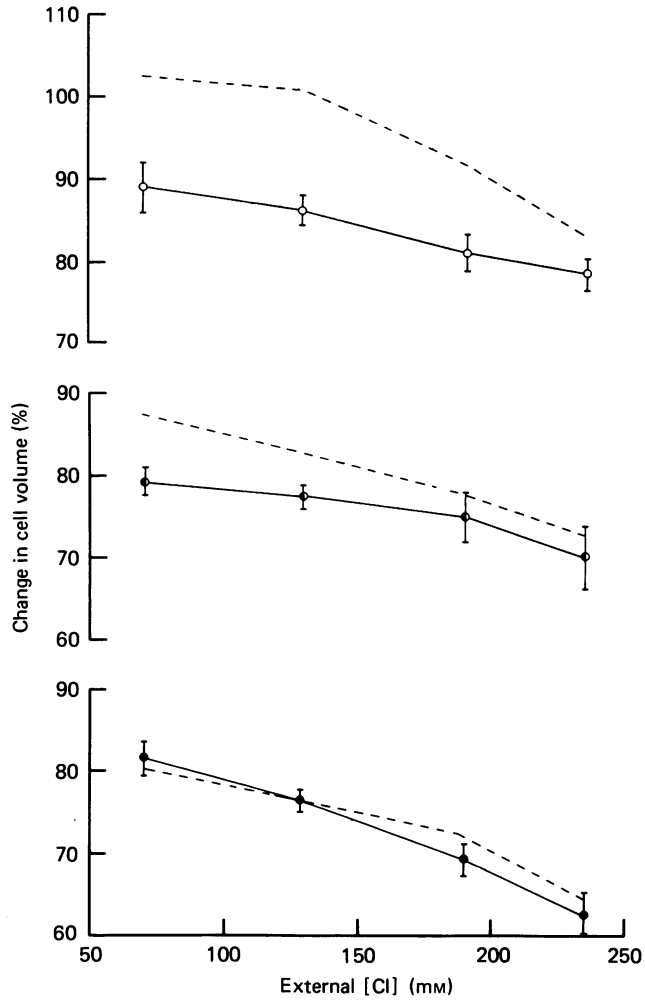

Fig. 6. The volume responses of outer medullary cells incubated in media of 950 m-osmole/kg H_2O containing 10 (○), 25 (◐) or 60 mM (●) HCO_3, 70, 130, 190 or 235 mM-Cl (abscissa) and 25 mM-CNS. Points represent mean ± S.E. of mean ($8 \geqslant n \geqslant 6$). The dashed lines represent the volume responses in the absence of CNS, taken from Fig. 1.

The effects upon medullary cell volume responses of incorporating 25 mM-CNS in incubation media are shown in Fig. 6. Volumes in media containing 10 and 25 mM-HCO_3 were respectively markedly and slightly reduced by comparison with control values (dashed lines, from Fig. 1): volumes in the presence of 60 mM-HCO_3 were unaltered. In media containing 10 mM-HCO_3, CNS in common with other additives causing reduction in cell volume (SITS, Table 2; acetazolamide, Table 3) brought

about a net loss of cellular K (Table 5). K content was not significantly affected in other media. The most striking effect of CNS, in terms of cellular composition, was that upon Cl content. In media containing 70 mM-Cl cellular Cl accumulation occurred to such an extent that the ratio $[Cl]_i/[Cl]_o$ (normally within the range 0·4–0·5 (Law, 1977, 1979)) approximated to unity. This is shown in Table 5. In other media, the

TABLE 5. The K and Cl contents, and intracellular Cl concentrations, of cells incubated in media (950 m-osmole/kg H_2O) containing variable concentrations of Cl and HCO_3, and CNS (25 mM). Values are mean ± S.E. of mean (n). The significance of differences between the contents shown here and those in the absence of CNS (Table 1) are indicated as follows: $^{**}P < 0.01$, $^{***}P < 0.001$. Other differences are not significant at the level $P < 0.05$. Cl concentrations are shown as mM and in the form of the ratio, $R = [Cl]_i/[Cl]_o$.

	$[HCO_3]_o$ (mM)					
	10		25		60	
$[Cl]_o$ (mM)	K content (μequiv/g SDFW					
70	278 ± 13 (8)***		264 ± 7 (8)		263 ± 10 (8)	
130	288 ± 13 (8)***		314 ± 14 (7)		253 ± 9 (7)	
190	302 ± 11 (8)***		299 ± 20 (8)		255 ± 12 (8)	
235	296 ± 11 (8)***		303 ± 11 (8)		250 ± 13 (8)	
	Cl content (μequiv/g SFDW)					
70	365 ± 6 (8)***		315 ± 11 (8)***		300 ± 18 (8)***	
130	437 ± 24 (8)***		358 ± 18 (7)***		322 ± 15 (7)***	
190	548 ± 30 (8)***		381 ± 11 (8)		329 ± 6 (7)	
235	588 ± 20 (8)**		434 ± 24 (8)		375 ± 9 (6)	
	Cl concentration (mM)					
		R		R		R
70	67 ± 5 (8)	0·95	73 ± 6 (8)	1·04	66 ± 6 (8)	0·94
130	86 ± 8 (8)	0·66	76 ± 4 (7)	0·59	75 ± 4 (7)	0·56
190	120 ± 10 (8)	0·63	86 ± 11 (8)	0·45	81 ± 2 (7)	0·43
235	136 ± 9 (8)	0·64	108 ± 11 (8)	0·46	106 ± 3 (6)	0·45

presence of CNS affected cellular Cl in a manner which may be summarized as follows (see also Table 5). In 10 mM-HCO_3 the increase in cell Cl content was significant at the level $P < 0.01$ or 0·001 for each level of $[Cl]_o$; the value of $[Cl]_i/[Cl]_o$, while less than unity in the presence of external Cl concentrations of more than 70 mM, remained considerably in excess of the normal range quoted above. In the presence of higher external HCO_3 concentrations (25 and 60 mM) the net increase of cellular Cl content were significant only in the presence of 130 mM external Cl. Net accumulation of Cl in the presence of higher levels of $[Cl]_o$ were trivial, and the value of $[Cl]_i/[Cl]_o$ in the presence of $[Cl]_o$ 190–235 mM lay within or only slightly outside the normal range. Results in the presence of 650 m-osmole/kg H_2O, both as regards cellular Cl accumulation and the ratio $[Cl]_i/[Cl]_o$, agreed well with those in the presence of 950 m-osmole/kg H_2O as shown in Table 5.

DISCUSSION

The majority of cells in the mammalian body are normally bathed by fluid of uniform and scarcely variable osmolality, and possess mechanisms enabling them to

regulate their volume with great precision (for reviews see Hoffman, 1977; Macknight & Leaf, 1977). Those in the renal medulla are exceptional in that the osmolality of their external environment exceeds that of plasma and is, moreover, widely variable. They are thus faced with unique problems in regard to volume control. The object of the present study has been to examine the participation of Cl and HCO_3 ions in this regulation under conditions simulating those found in the medulla of the concentrating kidney. Most of the previous studies on medullary Cl and HCO_3 referred to in the present report concern transport and exchange processes which may or may not be directly implicated in the problem of cell volume regulation. But it should not be overlooked that in terms of double Donnan equilibrium any such process must effect cell volume.

The results of experiments in which the pH of media were adjusted with HEPES/O_2 suggest that the volume responses in the presence of variable concentrations of HCO_3 depend upon this anion *per se* and not upon ambient pH. It might be argued that since the pH of media changed during the course of incubation the buffer system could not have been in equilibrium. Although this must be conceded, it is also true that the alterations in pH were trivial within the context of the experimental design, and were not accompanied by detectable alterations in medium HCO_3 concentration (see Methods). Whatever strictly quantitative constraints this disequilibrium may impose upon the results obtained, the material presented in Figs. 2 and 3 clearly favour volume dependence upon HCO_3 rather than upon pH. This does not preclude the possibility that the composition of the external medium might influence cell volume through an effect on intracellular pH. Factors affecting medullary intracellular pH have not been examined, but in excitable cells they are known to include Cl/HCO_3 exchange (Aickin & Thomas, 1977; Thomas, 1977; Vaughan-Jones, 1979).

The dependence of medullary cell volume upon $[HCO_3]_o$, but not upon $[Cl]_o$, was abolished by SITS, a substance best characterized as an anion exchange inhibitor in red cells (Cabantchik & Rothstein, 1972; Aubert & Motais, 1975; Lambert & Lowe, 1978) (see Fig. 4). It has recently been shown to exert an inhibitory effect upon anion exchange in sheep Purkinje fibres by impairing HCO_3-stimulated inwardly directed Cl pumping (Vaughan-Jones, 1979). It is difficult to envisage an analogous process in medullary cells, since in the present experiments SITS was associated both with net Cl loss (when $[HCO_3]_o$ was low) and with net gain (when $[HCO_3]_o$ was high). The observed changes in cell Cl content, and also of K content (Table 2), may reflect uptake or extrusion of KCl consequent upon, rather than as a cause of cell volume adjustment. One possibility is that SITS acts primarily by interfering with medullary HCO_3-HCO_3 self-exchange. The closely related anion-exchange inhibitor 4,4′-di*iso*thiocyanatostilbene-2,2′-disulphonic acid (DIDS) is known to have this effect in red cells (Wieth, 1979) but since in the present experiments there were no measurements of intracellular $[HCO_3]$ this possibility cannot be pursued.

Acetazolamide does not affect Cl-HCO_3 exchange across red cell membranes (Cousin, Motais & Sola, 1975) nor, uniquely among a wide range of carbonic anhydrase inhibitors studied, does it impair Cl-Cl self-exchange (Cousin & Motais, 1976). If these findings are applicable also to renal medullary cells, the effects of acetazolamide upon cell volume (Fig. 5) would be more reasonably ascribed to altered rates of cellular HCO_3 production than to influence upon the properties of the plasma

membranes. Medullary carbonic anhydrase has not been studied in the rat. It is present, however, in the medulla of the dog (Maren, 1963) and the human (Mattenheimer, Pollack & Muehrcke, 1970), and since for most tissues a good degree of correspondence exists between the activity of this enzyme in different mammalian species (Maren, 1967) it is likely that this enzyme is present in rat outer medulla, and susceptible to the inhibitory influence of acetazolamide. It was notable that acetazolamide affected cell volume and composition only in the presence of low (10 mM) external HCO_3. If it exerts an effect on cell volume through impairment of carbonic anhydrase-mediated cellular HCO_3 production, as here suggested, then the simplest explanation of this finding would be that in the presence of higher concentrations of HCO_3 (25 or 60 mM) sufficient HCO_3 can enter cells to compensate for this deficit.

There is considerable evidence that CNS ions inhibit a component of ATPase activity, present in a variety of tissues, which is stimulated by oxyanions such as SO_3 and HCO_3 (for references see Cole, 1979). HCO_3-stimulated ATPase is present in renal medulla, and is maximally stimulated by HCO_3 within the concentration range 25–50 mM (Cole, 1979). The activity of the medullary enzyme in the presence of lower concentrations has not been determined, but by analogy with the cortical enzyme might be expected to fall by 50% or more in the presence of 10 mM-HCO_3 (Kinne-Saffran & Kinne, 1974; Cole, 1979). Whether under the present experimental conditions this enzyme was active or, if active, inhibited by 25 mM-CNS was not established, and the only conclusion which can be drawn, therefore, is that there exists a volume-regulatory process in renal medullary cells which is markedly inhibited by CNS when $[HCO_3]_o$ is low and unaffected by this anion when $[HCO_3]_o$ is high (Fig. 6).

In terms of cellular composition, a medullary CNS-sensitive process appears to play a part in limiting Cl content and concentration. Both of these increase in the presence of CNS, the increments being most striking when $[HCO_3]_o$ or $[Cl]_o$ are low (Table 5). The massive influx of Cl in the presence of 70 mM-$[Cl]_o$ is noteworthy since this occurred not only in medium 10/70/950 (accompanied by marked cell skrinkage) but also in media 60/70/950 (Table 5) and 60/70/650 (not shown) in which there was no change in cell volume. $[Cl]_i$ thus appears to have no influence on cell volume under these conditions.

These results, if afforded the most logical explanation consistent with what has been observed in other tissues, provide evidence for medullary cell volume regulatory mechanisms dependant upon external HCO_3, anion exchange or self-exchange, intracellular HCO_3 production and for a CNS-sensitive process which may have a distinct function as a regulator of intracellular Cl. Clearly a number of hypothetical models could be constructed to accommodate these possibilities. Equally clearly there is at present insufficient evidence to justify so doing. Further studies, particularly on cellular metabolic activity, the electrical gradient across the cell membranes, and a knowledge of relevant ionic activity at the sites involved in volume regulation, are required before the present findings can be incorporated into a self-consistent model.

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