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THE EXTRACELLULAR SPACE IN RAT RENAL CORTICAL SLICES INCUBATED AT 0.5° AND 25°

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SUMMARY

1. Inulin has been used to measure the extracellular spaces in rat renal cortical slices incubated at 0.5° and 25° in ordinary media, K^+-free media containing 10 mM ouabain and ordinary media containing 1 mM sodium iodoacetate.

2. Neither the composition of the media, nor the temperature of incubation affected the initial equilibration of inulin in approx. 26% of the tissue wet weight.

3. Slices which were moderately swollen at 0.5° showed little change in their extracellular spaces, but the percentage of the tissue wet weight which was extracellular decreased significantly as slices became grossly swollen.

4. Slices incubated at 25° showed a gradual increase in the percentage of their wet weights occupied by inulin, irrespective of their water contents. This was shown not to be due to any increase in their freely accessible extracellular space but was the result either of inulin diffusing into a relatively inaccessible extracellular compartment or, more probably, into cells.

INTRODUCTION

The polysaccharide inulin, has been used by many workers to provide a measure of the extracellular space in renal slices[1–9], and it seems reasonably well established that inulin is in fact suitable for this purpose in both kidney[6] and liver[10] slices. However there are discrepancies between reported changes in extracellular spaces measured by inulin in kidney slices. Thus while ROBINSON[1], WHITTAM[3] and WHITTEMBURY[8] found no difference in extracellular spaces, expressed as a percentage of tissue wet weight, when the spaces in swollen tissue and in tissue incubated at 0–4° were compared with the spaces in tissue incubated at 25° or 37° in oxygenated media, CONWAY AND GEOGHEGAN[2], KLEINZELLER AND CORT[5] and SWAN, ELLINGTON AND MILLER[4] all reported that the extracellular space at 0° to 4° was smaller than that at 25° or 37°. The results of SWAN, ELLINGTON AND MILLER[4] also suggested that swollen tissue had a smaller percentage of its wet weight extracellular than did tissue of a more normal water content incubated at the same temperature. FOX *et al.*[7] observed that slices incubated at 37° in media in which potassium replaced all the sodium showed a consid-

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erable decrease in the percentage of the wet weight of the tissue which was extracellular, although whether this decrease was a result of the modification of the media or of the swelling of the tissue is uncertain.

Experiments have been reported previously[11] in which changes in tissue water and ions were observed in rat renal cortical slices leached at 0.5° and subsequently reincubated at 25° in either balanced saline media or media modified by the removal of potassium, addition of ouabain, or addition of iodoacetate. Because of the possibility that the size of the extracellular space may change when renal cortical slices are incubated under varying conditions, inulin has been used to measure the extracellular space in rat renal cortical slices in an attempt to decide whether (a) changes in the composition of the incubation media, (b) changes in the temperature at which slices were incubated, or (c) swelling of the slices, affected the size of the extracellular space, and thereby invalidated any conclusions previously reported.

METHODS

Media

The media had the following composition in mequiv/l. (1) Ordinary media: Na^+, 146; K^+, 5; Ca^{2+}, 5; Mg^{2+}, 2; Cl^-, 134; SO_4^{2-}, 2; acetate, 10; buffered with phosphate (8 mM) at pH 7.26. (2) K^+-free media: These had the same composition as the ordinary media except that they contained 150 mequiv/l of Na^+, and no K^+. In addition they contained ouabain, 10 mM (obtained from B.D.H.).

The slices were prepared as described previously[11].

Inulin was obtained from Merck and Co. Darmstadt. This preparation is claimed to contain less than 0.2 % fructose and its purity was checked by paper chromatography, which failed to reveal any low molecular weight fructosans or fructose. It therefore seems reasonable to accept that the space occupied by inulin was not overestimated as a result of low molecular weight contaminants diffusing across cellular membranes.

Inulin was dissolved in the media to give a final concentration of 0.5 % (w/v). Since inulin in solution tends to precipitate if left to stand over several days, and also may undergo polymerization on standing[12], inulin was dissolved in the media not more than 18 h before use, by rapidly heating the media to just less than 60°. This has been shown to provide the best compromise between considerable solubility and slight breakdown of the inulin[12]. After heating, the media were filtered and stored at 0–4° before use.

Analytical methods

The water contents of the slices were determined gravimetrically, sodium and potassium were measured in 0.1 M HNO_3 extracts using an EEL flame photometer, and chloride was measured by the method of COTLOVE, TRANTHAM AND BOWMAN[13].

The inulin content of each incubated slice was determined separately by extracting the inulin overnight from the dried slice into 5 ml 6.25 % (w/v) trichloroacetic acid at 55°. Next morning two 2-ml aliquots were taken from each tube for analysis of inulin content by the method of KULKA[14], the absorbance of the samples being measured using a Beckman DU spectrophotometer. Appropriate dilutions of the media were also analysed in each experiment. In preliminary experiments it was established that extraction of wet tissue and of dried homogenised tissue by this

method provided the same recovery of inulin. It was also established that extraction overnight (14–18 h) was long enough to recover all of the inulin in the slice.

Slices leached at 0.5° for 150 min and reincubated for 60 min at 25° in the three media, but without inulin, were also analysed to allow correction of the measured absorbances of the slices incubated in media containing inulin for substances present in the slices and reacting in the same way as inulin. Correction factors were obtained by dividing the measured absorbance for each slice by the wet weight of that slice; this gave a correction factor in terms of absorbance per mg tissue wet weight. There were no important differences between the correction factors under these differing conditions and so the mean of these correction factors was used in all subsequent experiments unless otherwise stated. (This mean was obtained from observations on 29 slices from the kidneys of 5 rats.)

Presentation of results

The amount of inulin in the extract from any one slice was obtained by subtracting from the measured absorbance the product of the mean correction factor as calculated above and the wet weight of the slice. The subtracted absorbance was approx. 17 % of the total absorbance. The inulin or extracellular space, α, was then calculated as under:

$$\alpha = \frac{\text{concentration of inulin in tissue wet weight}}{\text{concentration of inulin in medium}} \times 100\,\%$$

The extracellular, or inulin, space is expressed as a percentage of the tissue wet weight. The values shown in the figure and given in the text and table are the mean $\pm$ S.D. of the number of observations shown. The statistical significances of differences between groups have been determined by STUDENT's t test.

RESULTS

The extracellular space in slices leached at 0.5°

Slices leached anaerobically at 0.5° in either ordinary media, K^+-free media containing 10 mM ouabain, or ordinary media containing 1 mM sodium iodoacetate have been shown previously[11] to swell to the same extent. Thus the extracellular spaces of slices leached in these media should be the same after the same period of leaching unless the changes in composition of the media produced changes in the extracellular space. Slices were first equilibrated at 25° for 15 min in oxygenated ordinary media containing 0.5 % (w/v) inulin. The water content of equilibrated slices was 2.52 $\pm$ 0.12 kg water per kg tissue dry matter, and the extracellular space 26.2 $\pm$ 3.1 %. Four slices were then leached in each of the three media for either 60, 90, 120, 150 or 210 min. The slices swelled at comparable rates and to the same extent, the mean water contents of all slices after 210 min being 3.28 $\pm$ 0.15 kg water per kg dry matter. The mean values of the extracellular spaces in all slices lay between 24.2 $\pm$ 0.8 % and 26.9 $\pm$ 2.4 %, and there were no significant differences in the extracellular space of slices leached in any of the media for the same time. It therefore seems reasonable to conclude that the changes in composition of the media did not of themselves affect the size of the extracellular space. When the extracellular space of all slices after 60 min of leaching (26.6 $\pm$ 2.0 %) was compared to that measured after 210 min (24.9 $\pm$ 1.7 %)

there was a small, barely significant, decrease ($P < 0.05$) in the extracellular space.

The possibility that the extracellular space expressed as a percentage of the wet weight was decreasing as the slices became more swollen during leaching, was therefore examined by leaching slices anaerobically at 0.5° for up to 20 h in ordinary media containing 0.5 % (w/v) inulin. Slices were first equilibrated at 25° for 15 min in oxygenated ordinary media but without inulin. This allowed observation of the time taken for inulin to equilibrate in the slices at 0.5°. Because the period of incubation in these experiments was much greater than that in any other experiments, some slices were leached anaerobically at 0.5° for 20 h in ordinary media without inulin to allow accurate correction of the total measured absorbance in the slices containing inulin. The mean correction factor per mg tissue wet weight was significantly lower after 20 h, and this lower factor was used to correct the measured absorbance in slices leached for this time. This had the effect of making the calculated extracellular space at 20 h higher than it would otherwise have been. In spite of this, the extracellular space as a percentage of the tissue wet weight was very significantly lower after 20 h of leaching (19.5 ± 1.6 %, 13 slices) when compared with the value measured at 60 min (24.0 ± 2.3 %, 12 slices, $P < 0.001$), 120 min (23.6 ± 2.1 %, 11 slices, $P < 0.001$) and 240 min (21.2 ± 2.0 %, 12 slices, $P < 0.05$). The spaces in slices leached for 30 min (21.3 ± 2.0 %, 6 slices) and 20 h did not differ significantly ($P > 0.30$), but this was almost certainly due to the fact that inulin had not equilibrated in the extracellular space by 30 min at 0.5°. At this temperature it appeared to take between 30 min and 60 min for inulin to become distributed through the freely accessible extracellular space.

Slices leached for 20 h showed a very considerable swelling, their water content (4.76 ± 0.27 kg water per kg tissue dry matter) having increased by approx. 90 % from that found in slices equilibrated at 25° for 15 min in oxygenated ordinary media (2.52 ± 0.12 kg water per kg tissue dry matter), and the results do suggest that at 0.5°, while a moderate increase in water content has little effect on the percentage of tissue wet weight which is extracellular, gross swelling does decrease this percentage. It can be calculated that under these conditions the swelling of tissue reflected, almost entirely, a cellular swelling.

The extracellular space in slices incubated at 25°

Fig. 1 shows the apparent changes in the extracellular space in rat renal cortical slices leached at 0.5° and subsequently reincubated at 25°. The pattern of behaviour of the water contents of the slices was the same as that reported previously[11]. When the results during reincubation were analysed there was a suggestion that in the early minutes the extracellular space might have increased more rapidly in the slices which had lost water during aerobic reincubation, but apart from the spaces after 2 min of reincubation, the differences were not significant and this possibility has not been examined further. However, there was no doubt that the extracellular spaces did appear to increase very significantly ($P < 0.001$ in each case) and to the same extent after 60 min of reincubation when compared to the space after 150 min of leaching. A similar steady increase in the extracellular space has been reported in rat liver slices reincubated after leaching[15,16].

While it was surprising to find that the apparent extracellular space increased during reincubation when slices lost water, it was even more surprising to find that the space increased, and to the same extent, in slices which were continuing to swell.

The temperature at which swelling occurred had not been expected to make such a difference to the extracellular space.

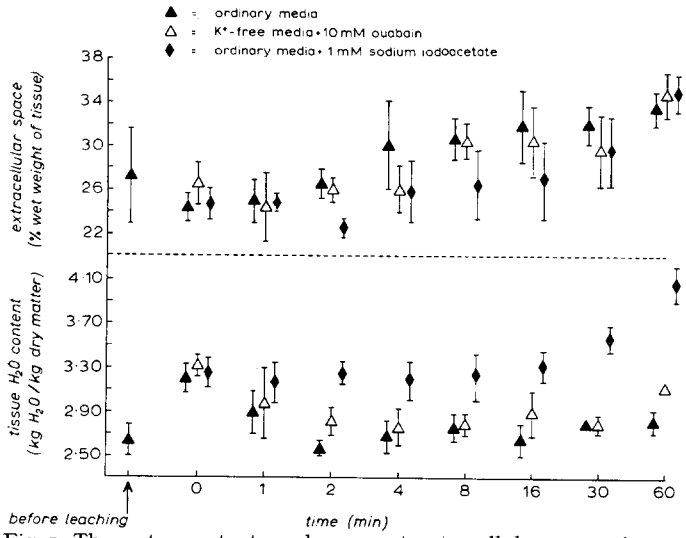

Fig. 1. The water contents and apparent extracellular spaces in rat renal cortical slices leached anaerobically at 0.5° for 150 min and subsequently reincubated at 25° for up to 60 min with 0.5 % (w/v) inulin present throughout. Before leaching slices were equilibrated at 25° for 15 min in ordinary medium containing 0.5 % (w/v) inulin. Slices were reincubated in oxygenated media except for those reincubated anaerobically in media containing 1 mM sodium iodoacetate. Each value represents the mean ± S.D. of 4 observations on slices from the kidneys of 20 rats. Composition of slices at end of leaching plotted at 0. ▲, slices incubated in ordinary media; △, slices incubated in K^+-free media containing 10 mM ouabain; ◆, slices incubated in ordinary media containing 1 mM sodium iodoacetate.

Slices were incubated at 25° in oxygenated ordinary media containing 0.5 % (w/v) inulin for up to 240 min to determine whether the same slow increase in extracellular space occurred in slices incubated throughout at 25°. The water contents of these slices showed only a relatively slight increase from 2.60 ± 0.14 kg water per kg tissue dry matter after 15 min to 3.09 ± 0.08 kg water per kg tissue dry matter after 240 min and this slight swelling would not have been expected appreciably to alter the size of the extracellular space. During the first 15 min of incubation at 25°, inulin rapidly entered 26.9 ± 3.8 % of the tissue wet weight (12 slices). This rapid penetration was followed by a slow, steady, highly significant increase in the inulin space to 34.4 ± 2.8 (8 slices) over the next 225 min ($P < 0.001$). It appears therefore that the inulin space increased during incubation at 25° both in slices previously leached at 0.5°, and in slices incubated throughout at 25°.

This observed increase might theoretically have been caused by (i) an actual increase in that part of the extracellular space which was freely accessible to inulin, (ii) a more rapid diffusion of inulin at the higher temperature into a relatively inaccessible, but nevertheless extracellular, compartment, or (iii) diffusion of inulin into cells, a process which might be expected to occur much more rapidly at 25° than at 0.5° both because of the direct effect of temperature on the rate of diffusion of inulin and also because any tendency for the cellular membranes to lose their selectivity due to autolytic changes might occur more rapidly at the higher temperature.

An effort was made to distinguish between these possibilities.

The results of an experiment in which slices were incubated at 25° for up to 240 min in oxygenated ordinary media appear to exclude the possibility that the freely accessible extracellular space increased at 25°. Slices incubated in media containing 0.5 % (w/v) inulin for either the first 60 min (10 slices) or the last 60 min (10 slices) only, of a 240-min incubation, showed no significant difference in their extracellular spaces ($P > 0.30$), the values being 29.3 ± 2.1 % and 30.3 ± 2.5 % respectively, whereas slices incubated in the presence of inulin throughout the 240 min had a considerably higher extracellular space (36.9 ± 3.2 %, 10 slices, $P < 0.001$). Since inulin rapidly equilibrates at 25° in the freely accessible extracellular space, the inulin spaces after 240-min incubation, whether measured for the four hours or only for the last hour, should have been similar, and both significantly different from the space measured in the first 60 min of the incubation, if the freely accessible extracellular space actually increased during incubation at 25°.

The conclusion that the freely accessible extracellular space did not increase at 25° after slices were leached at 0.5° was supported by the results of an experiment in which slices were incubated at 25° for 120 min in oxygenated ordinary media containing 0.5 % (w/v) inulin before being leached at 0.5° for 60 and 150 min. The space after 120 min at 25° was 29.3 ± 3.3 % (9 slices) and after leaching for 60 min and 150 min was 29.5 ± 2.5 % and 29.6 ± 2.0 % respectively. If the extracellular space actually increased when slices were transferred from media at 0.5° to media at 25°, than it should have decreased when slices which had been incubated at 25° were leached at 0.5°. In fact the extracellular space showed no significant change under these conditions, but remained significantly higher at 0.5° after incubation at 25° for 120 min than the space measured in leached slices which had been incubated at 25° for no more than 15 min before leaching ($P < 0.001$).

It appears then that the freely accessible extracellular space was not increasing either in slices transferred to media at 25° after leaching at 0.5° or in slices incubated throughout at 25°, but that the increase in inulin space was the result of penetration of inulin either into cells or into a restricted extracellular compartment. In the experiments already described it was not possible to distinguish between these possibilities. However the results of a further experiment do suggest that, at least in this experiment, inulin was diffusing into cells.

Slices were incubated at 25° in ordinary media containing 1 mM sodium iodoacetate and after 2 h had the same water content (4.81 ± 0.19 kg water per kg tissue dry matter) as slices leached at 0.5° for 20 h (4.76 ± 0.27 kg water per kg tissue dry matter).

The inulin space in slices incubated at 25° was 39.1 ± 3.5 % compared with 19.5 ± 1.6 % at 0.5°. Since earlier experiments had provided no evidence that the freely accessible extracellular space increased at the higher temperature, it seemed reasonable to accept that the freely accessible space was 19.5 % at both temperatures. This would mean that 1.14 kg water per kg tissue dry matter, or 50 % of the water in which inulin was distributed at 25°, was either in a relatively inaccessible extracellular compartment or was intracellular. The first possibility would imply that 50 % of the extracellular water was inaccessible to inulin even after 20 h of incubation at 0.5° but became accessible within 2 h at 25°. This seems improbable and it appears more reasonable to postulate that under these conditions inulin was diffusing into cells.

The effects of inulin on the tissue contents of water and ions

PARSONS AND VAN ROSSUM[10] have reported that a concentration of 0.5 % (w/v) inulin in balanced saline media did not affect the water contents of rat liver slices, though with higher concentrations of inulin slices became less swollen. Table I shows that in rat renal cortical slices 0.5 % (w/v) inulin had no significant effect on the water and ion contents of slices leached at 0.5° in ordinary media for 150 min, and, apart from a barely significant difference in K^+ content ($P < 0.05$), no effect on the water and ion contents of leached slices reincubated at 25° for 60 min in oxygenated ordinary media.

TABLE I

THE EFFECTS OF INULIN ON THE WATER AND ELECTROLYTE CONTENTS OF RAT RENAL CORTICAL SLICES

Slices were leached anaerobically at 0.5° for 150 min in ordinary media either with or without 0.5 % (w/v) inulin, and then reincubated at 25° for 60 min in oxygenated ordinary media with or without 0.5 % (w/v) inulin. Each value represents the mean ± S.D. of 8 slices from the kidneys of 5 rats.

Tissue content of	*Slices leached at 0.5° for 150 min*			*Slices reincubated at 25° for 60 min*		
	Without inulin	*With inulin*	*t test*	*Without inulin*	*With inulin*	*t test*
Water*	3.23 ± 0.20	3.20 ± 0.20	$P > 0.70$	2.73 ± 0.07	2.68 ± 0.05	$P > 0.05$
Na^{+}**	461 ± 23	457 ± 27	$P > 0.70$	297 ± 24	283 ± 11	$P > 0.10$
K^{+}**	131 ± 12	138 ± 12	$P > 0.20$	252 ± 23	274 ± 14	$P < 0.05$
Cl^{-}**	299 ± 23	298 ± 21	$P > 0.90$	247 ± 15	242 ± 6	$P > 0.30$

* kg water/kg tissue dry matter.
** mequiv/kg tissue dry matter.

DISCUSSION

The present results have shown that changing the composition of the incubation media by omitting potassium, adding ouabain or adding sodium iodoacetate did not of itself alter the size of the extracellular space in rat renal cortical slices.

It has also been shown that changing the temperature at which slices were incubated from 0.5° to 25° did not alter the freely accessible extracellular space. At both temperatures inulin became equilibrated in about 26 % of the tissue wet weight. However as incubation continued at 25°, inulin continued to enter the tissue. Whether this increasing inulin space was caused by inulin diffusing into a relatively inaccessible extracellular compartment or into cellular water was not established, though when metabolism was suppressed at 25° it appeared more likely that inulin was diffusing into cells.

It was also found that when slices became considerably swollen at 0.5°, the percentage of the tissue wet weight which was extracellular decreased significantly. This was not observed at 25° probably because at this temperature inulin was diffusing into cells.

It seems reasonable to accept that the freely accessible extracellular space of slices incubated in the three media at both 0.5° and 25° was approx. 26 % of the tissue wet weight in all but grossly swollen slices. This space is of the same order as that reported by others in guinea pig[3,8] and rat[1] at 0° to 4°, and in guinea pig[3,4,8], rabbit[5], rat[1,6,7], and sheep[9], at 25° or 37°. But the measured inulin space may not necessarily

provide a measure of the true extracellular water. There may for instance be extracellular compartments which are relatively inaccessible to a molecule the size of inulin. Alternatively some inulin may always be diffusing across cell membranes or entering damaged cells, and the extracellular space may always be overestimated. Because of this uncertainty it is important to emphasise that the calculated intracellular contents and concentrations of different ions in rat renal cortical slices would not always vary greatly if the size of the extracellular space were wrongly estimated. Irrespective of the water content of the tissue, the true extracellular space in rat renal cortical slices probably lies between 19 and 35 % of the tissue wet weight. Within these limits it can be calculated, using the values given in Table I, that the intracellular concentrations of Na^+ and Cl^- would remain relatively constant both in leached and in reincubated slices, as would the K^+ concentration in leached slices. Similarly the calculated intracellular K^+ content would be independent of the size of the extracellular space. Though the amounts of water, Na^+ and Cl^- in the cells would vary considerably with the size of the extracellular space, what is important for the interpretation of the results previously published[11], is that only if the size of the extracellular spaces actually decreased appreciably when slices were warmed from 0.5° to 25° could any decrease in the amounts of water and ions in the tissue have been due solely to a change in the size of the extracellular space rather than to loss of water and ions from the cells. There was no evidence at all in the present experiments to suggest that the extracellular space did in fact decrease when slices were reincubated after being leached. Thus it can be confidently accepted that the changes in tissue composition reported earlier[11] reflected changes in intracellular composition.

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