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EXTRACELLULAR SPACE IN SOME ISOLATED TISSUES

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SUMMARY

1. The spaces occupied by isotopically labelled inulin, polyethylene glycol, mol. wt. 4000 (PEG 4000), polyethylene glycol, mol. wt. 1000 (PEG 1000) and sucrose in metabolizing mammalian kidney and liver slices and in toad bladder epithelial cell preparations incubated *in vitro* have been examined.

2. In slices of mammalian tissue, and in homogenized liver, it proved impossible to extract inulin completely from tissue which had been dried. However, inulin was recovered as completely from both dried and undried toad bladder epithelial cells scraped from hemibladders incubated *in vitro.*

3. PEG 4000 occupied a space in all preparations similar to that from which inulin was extracted in dried tissue.

4. PEG 1000 and sucrose entered cellular water in mammalian slices, but PEG 1000 occupied a similar space to inulin in toad bladder epithelial cell preparations.

6. It is concluded that inulin enters cellular water in mammalian slices from which after drying of the slices it cannot be extracted. It thus rather fortuitously provides a measure of extracellular water under these conditions. In preparations of toad bladder epithelial cells inulin seems to be a satisfactory extracellular marker. PEG 4000, which did not appear to enter cellular water also allows a reasonable estimate of extracellular water. PEG 1000 is a suitable extracellular marker for toad bladder epithelial cell preparations but not for mammalian slices. Sucrose entered cellular water in both slices and toad bladder epithelial cells and is not a satisfactory extracellular marker in these tissues.

INTRODUCTION

Isolated tissues are often employed in experiments to study mechanisms by which cells control their volumes and ionic compositions. The interpretation of such experiments requires a knowledge of the contribution of the

extracellular fluid to the values obtained and an appropriate correction of the data. Many workers have examined this problem and have employed a number of substances, among them inulin, sucrose, mannitol, polyglucose and sulphate as extracellular markers. The assumption underlying their use is that the marker equilibrates completely in the extracellular fluid and yet does not penetrate the cell membrane. Perhaps the most widely employed marker has been inulin which seems to have proved satisfactory in a variety of tissues.

Recently, polyethylene glycol, mol. wt. 6000 (PEG 6000) has been used to prevent tissue swelling in non-metabolizing tissue (Davey & Skegg, 1971; Little & Robinson, 1967; McIver & Raine, 1972; Robinson, 1971; Wiggins, 1964, 1965, 1967). Interpretation of these experiments has depended on the assumption that PEG 6000 equilibrates in the extracellular fluid but does not enter cells, its osmotic effect thereby balancing the colloid osmotic pressure of the intracellular macromolecules. We decided to test this hypothesis by directly measuring the PEG space using the recently available [^{14}C]PEG 4000. In the course of these experiments it became apparent that the interpretation of extracellular space measurement with inulin was more complicated than had previously been supposed. Difficulties in the extraction of PEG from dried tissue led us to measure the spaces occupied by both PEG and inulin in tissue extracted without drying. The finding that under these circumstances the inulin space was always higher than it was in dried tissue has led us to re-examine the problem of the measurement of extracellular space in some isolated tissues.

METHODS

Media. The medium in which mammalian tissue slices were incubated had the following composition (mM): Na^+, 145; K^+, 5; Ca^{2+}, 2·5; Mg^{2+}, 1; Cl^-, 133; SO_4^{2-}, 1; acetate$^-$, 9; buffered with phosphate, 8; at pH 7·35.

The medium in which toad hemibladders were incubated contained (mM): Na^+, 117; K^+, 3·5; Ca^{2+}, 1·0; Cl^-, 117; glucose, 10; buffered with HPO_4^{2-}, 2; at pH 7·8.

[Carboxy-^{14}C]inulin, [methoxy-^{3}H]inulin, [1,2-^{14}C]polyethylene glycol mol. wt. 4000, and [1,2-^{3}H]polyethylene glycol mol. wt. 1000, were obtained from New England Nuclear Corporation. [^{14}C]sucrose and [^{3}H]sucrose were obtained from the Radio Chemical Centre, Amersham. The final concentrations of radioactivity in the media were between 0·1 and 0·2 μc/ml. Unlabelled inulin was obtained from Merck and Co., Darmstadt. This preparation is claimed to contain less than 0·2 per cent fructose and this was confirmed by paper chromatography. Chromatography of solutions of the labelled inulins from New England Nuclear Corporation confirmed their purity.

To dissolve the relatively large concentrations of unlabelled inulin in medium (0·5 per cent w/v) or extraction fluid (0·025 per cent w/v) it was necessary to heat the solutions rapidly to just less than 60° C. This has been shown (Phelps, 1965) to provide the best compromise between considerable solubility and slight break-down of the inulin. It was found that the much smaller amounts of radioactive inulin used dissolved without heating the media.

Procedure. Adult male rats, rabbits and guinea-pigs were stunned by a blow on the head and bled from the carotid arteries. Kidneys and livers were removed and placed in ice-cold medium where they remained till sliced. Kidneys were sliced by Cohen's (1945) modification of the method of Deutsch (1936). The outermost slice was not discarded (Macknight, 1967) and two to three slices were obtained from each half kidney. Liver slices were prepared using a mechanical tissue chopper (McIlwain & Buddle, 1953). Both kidney and liver slices were 200–300 μm thick. All slices were immediately transferred to oxygenated medium at 25° C where they remained, with vigorous stirring, for at least 15 min (Robinson, 1961). They were then transferred to fresh oxygenated medium containing labelled marker and incubated at 25° C.

Female toads of the species *Bufo marinus* obtained from the Dominican Republic (National Reagents, Bridgeport, Conn., U.S.A.) were doubly pithed and their hearts were immediately perfused with medium. As the livers distended they were cut to allow drainage of blood from the animals. After several minutes vessels in the hemibladders were free of blood and the hemibladders were then removed and after washing in medium placed in beakers of oxygenated medium containing labelled marker. The oxygen was bubbled through the medium at a rate sufficient to produce continuous stirring of the hemibladders in the beakers.

Analytical methods. Slices of mammalian tissues were removed from media and immediately blotted. Toad hemibladders were removed from the medium, blotted, and the epithelial cells then scraped from the underlying tissue with a glass slide as previously described (Macknight, DiBona, Leaf & Civan, 1971). In kinetic studies, slices were removed from media at the times indicated. Otherwise tissues were incubated for 1–2 hr.

All samples were transferred to weighed tubes which were then reweighed to allow calculation of tissue wet weight. In some experiments tubes were subsequently dried in a hot air oven for at least 2 hr at 105° C, cooled and reweighed, the usual procedure for the determination of tissue water content (Little, 1964).

The 'extracellular' markers were extracted from both undried and dried tissues by adding 10 ml. 0·1 M nitric acid or 10 ml. distilled water to the tubes. The tubes then stood for at least 14 hr at room temperature (17–20° C). Previous work with unlabelled inulin (A. D. C. Macknight, unpublished observation) revealed that, though inulin is relatively insoluble at room temperature, heating to 55–60° C during extraction had no effect on the amount of inulin recovered from dried tissue. This finding was consistent with the fact that the actual quantity of inulin in the tissue was much less than that which will dissolve in 10 ml. water at room temperature (Phelps, 1965). In comparison with inulin the other markers used in the present study are all much more soluble. In representative experiments it was found that inulin, PEG 1000 and sucrose spaces were unaffected by the composition of the fluid used for extraction. However, for extraction of [^{14}C]PEG 4000, 1 per cent unlabelled PEG 4000 was present in the extraction fluid. This allowed a much more rapid extraction of the isotope from the tissues. All samples were extracted for a minimum of 14 hr.

After extraction, aliquots of the samples and of the medium, suitably diluted with the same fluid as had been used for the tissue extraction, were taken for analysis. In one series of experiments where spaces occupied by radioactive inulin and unlabelled inulin were directly compared, unlabelled inulin was analysed as previously described (Macknight, 1968) using the method of Kulka (1956). Otherwise all experiments were performed with isotopes, radioactivity being measured by liquid scintillation counting, using 2 ml. sample in 15 ml. dioxane fluor, in a three-channel Packard TriCarb Liquid Scintillation counter.

Experiments with homogenized tissue

A series of experiments was performed to determine whether or not inulin, PEG 4000, and PEG 1000 were fully extractable from homogenized tissue. Rat livers were homogenized using a Silverson mixer-emulsifier. Phase contrast microscopy of samples of homogenate did not reveal any intact cells after this procedure. Aliquots of the homogenate were transferred to borosilicate test tubes to provide in each tube a final dry weight of homogenate of approximately the same weight as that of a dried slice. Some of these tubes contained a small volume (25 μl.) of medium in which was dissolved an amount of isotope comparable to that which the average slice would normally contain. Other tubes contained only homogenate. All tubes stood at room temperature for 2 hr. Some tubes containing homogenate with and without isotope were then dried in a hot air oven at 105° C overnight (the same way that slices were dried). Some tubes containing only medium were also dried in this way, while other samples of medium were diluted and counted without being dried. After cooling at room temperature, the small dried pellet of homogenate in each tube was broken up into small fragments with a glass rod to ensure adequate extraction of substances from the tissue. These fragments of tissue were thinner and smaller than the normal dried slices used in these experiments. In one experiment in this series, tubes containing homogenate were not dried. Instead they stood for 2 hr with or without isotope and were then simply extracted. They were then centrifuged at 3000 rev/min and the supernatant gently decanted. In all experiments tissue and medium were extracted for up to 10 days with distilled water which in experiments with [^{14}C]-PEG 4000 contained 1 per cent unlabelled PEG 4000.

After extraction, aliquots were taken from medium and from homogenates exposed to isotope for counting as described above. Immediately before aliquots were obtained from homogenates without isotope, 25 μl. isotope was added to the extraction fluid. After vigorous shaking aliquots were taken for counting. These homogenates therefore served as controls to ensure that any disappearance of counts from the homogenates dried with isotopes could not be the result of non-specific quenching by substances extracted from the tissue.

Presentation of results. The space occupied by any substance was calculated as under:

$$\text{space} = \frac{\text{concentration of substance in tissue wet weight}}{\text{concentration of substance in medium}} \times 100 \text{ per cent.}$$

The spaces are expressed as a percentage of the wet weight of tissue rather than as a percentage of tissue water. Though somewhat less satisfactory this method of expression is common in the literature and was unavoidable here since some results were obtained after extraction of undried tissues for which no values for tissue water could be obtained.

The values quoted in the text, tables and figures are the mean $\pm$ S.E. of mean of the number of observations shown in parentheses. The statistical significance of differences between groups have been determined using Student's *t* test.

RESULTS

Inulin space in isolated tissues

Table 1 allows a comparison between inulin spaces in undried or dried tissues for kidney and liver slices and for toad bladder epithelial cells. Drying rat kidney or liver slices prevented subsequent extraction of a

considerable amount of the inulin which had entered the tissue during incubation. This effect of drying was also seen in guinea-pig and rabbit renal cortical slices, though here the differences were less marked. In contrast, drying of toad bladder epithelial cells had little effect on the full extraction of inulin.

TABLE 1. Inulin spaces of tissues incubated 1–2 hr and then extracted either without or after drying, and measured using either [^{3}H] or [^{14}C]inulin

	Undried tissue	Dried tissue	
	space (per cent tissue wet wt.)		P
Kidney slices			
Rat	$40{\cdot}2 \pm 0{\cdot}4$ (137)	$26{\cdot}9 \pm 0{\cdot}4$ (124)	$< 0{\cdot}001$
Rabbit	$32{\cdot}7 \pm 0{\cdot}9$ (8)	$28{\cdot}3 \pm 1{\cdot}1$ (8)	$< 0{\cdot}01$
Guinea-pig	$31{\cdot}1 \pm 0{\cdot}9$ (8)	$27{\cdot}7 \pm 0{\cdot}6$ (8)	$< 0{\cdot}01$
Liver slices			
Rat	$30{\cdot}3 \pm 1{\cdot}6$ (16)	$17{\cdot}9 \pm 1{\cdot}2$ (15)	$< 0{\cdot}001$
Toad bladder epithelial cells	$43{\cdot}6 \pm 1{\cdot}9$ (10)	$41{\cdot}7 \pm 2{\cdot}1$ (10)	$> 0{\cdot}50$

Most experiments were performed using slices of rat renal cortex, since this tissue was readily available and had been used in the studies involving PEG 6000 which we wished to examine. This discrepancy in inulin spaces was found in every experiment with this tissue and the difference was always statistically significant. However, the magnitude of the difference varied from experiment to experiment. The reason or reasons for this remain unclear. One possible explanation may be that there was some variation in ages between animals used over the 9 months during which these experiments were performed. Earlier experiments, where the largest differences were found, employed rats between 9 and 12 months of age. Later experiments with younger adults (4–6 months) showed somewhat less marked differences. Unfortunately a shortage of older rats has prevented a full investigation of this possibility. However, one experiment, where spaces in renal cortical slices from either 4 month or 12 month animals were directly compared, showed a significant, though small, difference in spaces measured on undried slices. (Δ 4·5 per cent tissue wet wt., $P < 0{\cdot}001$, $n = 23$) with no difference between spaces of dried slices ($P > 0{\cdot}99$), these spaces in the dried slices being in both cases significantly less ($P < 0{\cdot}001$) than those in the undried tissue.

The results provided in Table 1 therefore clearly establish that significantly more inulin could be extracted from mammalian tissue slices if the tissue were not dried. This unexpected result led us to investigate this finding in more detail.

(i) *Validity of methods used for the measurement of inulin spaces*

Though some slices were analysed after drying, medium samples were normally simply diluted and analysed without prior drying, the procedure which is commonly followed. If drying of samples containing inulin caused a loss of radioactivity, this could explain the discrepancy between inulin spaces of dried and undried tissue. This possibility, however, was excluded by comparing the inulin counts in samples of medium before or after drying under the same conditions as tissues were dried. There was no loss of counts upon drying (Table 9). This was also true for both [^{14}C]PEG 4000 (Table 9) and [^{3}H]PEG 1000 (c.p.m. undried medium 1858 ± 41, c.p.m. dried medium 1908 ± 20, $n = 20$ for each), but not, as will be mentioned later, for either [^{14}C] or [^{3}H] sucrose. The conclusion that drying did not lead to loss of inulin from tissues was supported by the observations that drying of tissue slices for periods up to 48 hr, compared with the more usual 2 hr, produced no change in the space in dried tissue, and by the fact that in toad bladder epithelial cells there was no difference in space between dried and undried tissue.

Normally, inulin was extracted from the tissue for at least 14 hr using either 0·1 M nitric acid or distilled water. As Table 2*a* shows, extraction for periods substantially in excess of 14 hr yielded little more inulin and therefore it is probable that nearly all the extractable inulin had been recovered after 14 hr from both undried and dried tissue. Results in this Table include both extraction with nitric acid or water, there being no significant differences between the values for undried or dried slices with either procedure.

The possibility that inulin was 'bound' or adsorbed in a highly specific way to some tissue component during incubation was rendered unlikely by a consideration of the results of experiments (Table 2*b*) in which slices were incubated with either labelled inulin alone or labelled inulin with a large excess (about 250 times more) of unlabelled inulin. The presence of the unlabelled inulin had no detectable effect on the inulin space in either undried or dried tissue.

Table 2*c* shows that the results obtained did not reflect any differences in behaviour between [^{14}C] and [^{3}H]inulin. When both isotopes were present together the spaces they occupied in rat renal cortical slices extracted without drying were similar. This was true also in toad bladder epithelial cells extracted after drying.

We also examined the possibility that the present results were in some way associated with the use of radioactive inulin. The results shown in Table 2*d* demonstrate that in the same slices both chemical and radioactive determination of inulin gave a similar result both for undried and dried

TABLE 2

(*a*) Time required for extraction of inulin from rat renal cortical slices

Extraction time (hr)		Undried tissue	Dried tissue	*P*
		space (per cent tissue wet wt.)		
14		40·2 ± 0·9 (24)	26·7 ± 0·7 (35)	< 0·001
60		40·8 ± 1·1 (24)	27·9 ± 0·7 (35)	< 0·001
	P	> 0·60	> 0·20	
14		40·5 ± 1·0 (24)	25·3 ± 1·0 (23)	< 0·001
240		42·1 ± 1·0 (24)	27·6 ± 1·0 (23)	< 0·001
	P	> 0·20	> 0·05	

(*b*) Effect of unlabelled inulin (0·5 % w/v) in the incubation medium on the inulin space of rat renal cortical slices

[^{3}H]inulin alone	41·3 ± 2·0 (6)	24·8 ± 1·2 (12)	< 0·001
[^{3}H]inulin with unlabelled inulin	42·6 ± 3·3 (6)	22·5 ± 1·2 (11)	< 0·001
P	> 0·70	> 0·10	

(*c*) Inulin space measured simultaneously with both [^{3}H] and [^{14}C]inulin

	[^{3}H]inulin	[^{14}C]inulin	*P*
Rat kidney slices (undried) (11)	40·9 ± 1·0	39·0 ± 0·8	> 0·10
Toad bladder epithelial cells (dried) (9)	38·0 ± 2·3	37·8 ± 2·7	> 0·90

(*d*) Inulin space of rat renal cortical slices measured simultaneously with both [^{3}H]inulin and unlabelled inulin (0·5 % w/v)

	Undried tissue	Dried tissue	*P*
	space (per cent tissue wet wt.)		
Chemical analysis	33·5 ± 0·9 (9)	29·2 ± 0·9 (8)	< 0·01
Isotopic analysis	35·4 ± 0·6 (9)	29·9 ± 0·8 (8)	< 0·001
P	> 0·10 (paired analysis)	> 0·50 (paired analysis)	

(*e*) Effect of unlabelled inulin (0·025 % w/v) in the extraction fluid on the inulin space of rat renal cortical slices

Dist. H_2O	33·3 ± 0·9 (11)	20·8 ± 0·9 (13)	< 0·001
Dist. H_2O with unlabelled inulin	34·9 ± 1·6 (8)	20·7 ± 2·6 (10)	< 0·001
P	> 0·30	> 0·95	

In all experiments tissues were incubated at 25° C in oxygenated media for 2 hr.

tissue and confirm the observation that less inulin can be extracted from tissue which has been dried (since the medium and slices contained a large excess of chemical inulin compared with labelled inulin – about 250 times more – results of chemical analysis would not have been measurably

affected by the contribution to the chemical analysis of inulin in the radioactive form).

From the above considerations we concluded that the discrepancy between the inulin spaces in dried and undried tissues was not an artifact caused by some methodological problem but instead, with the exception of the toad bladder epithelial cells, truly reflected a failure to extract from the dried tissues all the inulin which had entered them during incubation. It was clearly difficult to recover this inulin from the dried tissue, since extraction for as long as 10 days (Table 2*a*) led to little increase in inulin space in dried tissue. Neither, as Table 2*e* shows, did the addition of relatively enormous quantities of inulin to the fluid used for extraction. Slices were extracted, both without or after drying, in distilled water containing 0·025 g/100 ml. unlabelled inulin. This seemed to be the highest concentration of inulin which would remain in solution in the scintillation fluid and was about 300 times greater than the final concentration of radioactive inulin in the medium samples and several thousand times greater than the final concentration of radioactive inulin in most tissue slices. This very large excess of unlabelled inulin did not affect the amount of inulin which could be extracted from dried or undried tissue. These results support the conclusion that all the inulin which can be extracted is lost from undried tissue and indicate that the inulin remaining in the dried tissue is unable to exchange with inulin present in the extraction fluid.

(ii) *Rate of equilibration of inulin in tissue*

Since inulin appeared to penetrate a larger fraction of the tissue water than had previously been reported, a study was made of the rate at which this substance entered the tissue. Table 3 shows the results of these experiments. There was an initial, relatively rapid penetration of inulin into about 25 per cent of the tissue wet weight. This was followed by a slower, continuous penetration into a larger fraction of the tissue water, from which, in the dried tissue, the inulin could not be extracted. The inulin space in the dried tissue was always significantly less than the space at the same time in the undried tissue. It remained constant between 1 and 4 hr in dried tissue ($P > 0{\cdot}90$) whereas the space in the undried tissue increased significantly over the same time ($P < 0{\cdot}01$). Detailed analysis of this type of experiment is made difficult by problems associated with slice thickness, and by the possibility that at least some of the rapid penetration may represent entry of inulin into damaged cells at the periphery of the slice. For example, though the slices are only 0·2–0·3 mm thick, over the early minutes of incubation entry into surface cells and entry into more central portions of extracellular water could both be occurring together and could thereby distort the relationships between spaces in undried and dried

slices. Nevertheless, it would seem reasonable to conclude that inulin rapidly equilibrates in at least one compartment, from which it can be readily extracted, and then more slowly enters one or more compartments from which, in dried tissue, it cannot be recovered.

The results which we had obtained using inulin as a marker for the extracellular space led us to examine the spaces occupied in the tissues by sucrose, by PEG 1000, and by PEG 4000, in the hope that the results obtained would enable a more accurate delineation of the extracellular compartment. Sucrose, a much smaller molecule, readily soluble in water, might be expected to behave like inulin though it might be more readily extracted from the tissue. PEG 1000 and PEG 4000, both of which have only recently become available in isotopically labelled forms, are inert uncharged molecules which might be expected to be large enough to be excluded from cells yet small enough (PEG 4000 has an average molecular weight close to that of inulin) to equilibrate relatively rapidly in the extracellular fluid.

TABLE 3. Rate of equilibration of inulin in slices of rat renal cortex ($n = 6$)

Incubation time (min)	Undried tissue space (per cent tissue wet wt.)	Dried tissue space (per cent tissue wet wt.)	P
7·5	22·4 ± 1·0	14·6 ± 1·6	< 0·005
15	26·2 ± 0·7	19·3 ± 1·7	< 0·005
30	26·2 ± 0·7	21·4 ± 1·7	< 0·05
60	31·4 ± 0·8	25·5 ± 1·1	< 0·01
120	33·6 ± 0·5	27·2 ± 1·3	< 0·005
240	36·1 ± 1·1	25·7 ± 0·9	< 0·001
Space at 1 hr compared with space at 4 hr	$P < 0.01$	$P > 0.90$	

Sucrose space in isolated tissues

Sucrose has been quite widely used as an extracellular marker for a variety of tissues, including renal cortical slices, liver slices and toad bladder epithelial cells. In a series of experiments with rat renal cortical slices (Table 4) it was found that sucrose equilibrated rapidly (within 10 min) in about 26 per cent of tissue wet weight but then continued to enter tissue and after 4 hr incubation had still not reached an equilibrium distribution though by then it occupied about 60 per cent of tissue wet weight. Since this space in a normal rat renal cortical slice with water content about 2·50 kg water/kg dry wt. represents nearly 80 per cent tissue water, sucrose must have entered cellular water in these experiments.

The possibility has been raised that sucrose may be a more suitable

marker for extracellular space in toad bladder epithelial cells than inulin (Al-Awqati, Leaf, Macknight & Civan, 1972). The present results confirmed that the sucrose space was greater than the inulin space in dried tissue (sucrose, $44\cdot3 \pm 3\cdot1$; inulin, $37\cdot8 \pm 3\cdot8$; Δ $6\cdot8 \pm 1$; $P < 0\cdot001$ from paired analysis) in this preparation. However, experiments in which $^{14}CO_2$ production was measured during incubation of toad bladders in medium containing [^{14}C]sucrose revealed that the tissue was metabolizing sucrose (Q. Al-Awqati, A. Leaf, A. D. C. Macknight & M. M. Civan, unpublished observations).

TABLE 4. Rate of equilibration of sucrose in slices of rat renal cortex ($n = 6$)

Incubation time (min)	Undried tissue space (per cent tissue wet wt.)	Dried tissue space (per cent tissue wet wt.)	P
7·5	$26\cdot7 \pm 1\cdot0$	$26\cdot4 \pm 2\cdot0$	$> 0\cdot90$
15	$30\cdot5 \pm 1\cdot1$	$31\cdot5 \pm 2\cdot0$	$> 0\cdot60$
30	$31\cdot7 \pm 0\cdot9$	$32\cdot7 \pm 1\cdot1$	$> 0\cdot40$
60	$38\cdot6 \pm 0\cdot5$	$40\cdot1 \pm 1\cdot7$	$> 0\cdot40$
120	$48\cdot5 \pm 0\cdot7$	$48\cdot5 \pm 2\cdot4$	$> 0\cdot98$
240	$62\cdot8 \pm 3\cdot2$	$56\cdot4 \pm 1\cdot4$	$> 0\cdot05$
Space at 1 hr compared with space at 4 hr	$P < 0\cdot001$	$P < 0\cdot001$	

Interpretation of results with sucrose as extracellular marker may also be complicated by methodological problems apart from the possibility of cellular metabolism of the marker. For example, a comparison of the ^{3}H and ^{14}C counts obtained from medium samples containing both isotopes of sucrose revealed that drying the medium sample at 105° C before dilution and analysis resulted in the loss of 44 per cent of ^{3}H counts and 11 per cent of ^{14}C counts over 48 hr. Samples simply allowed to evaporate to dryness at room temperature did not lose counts though subsequent drying of these samples at 105° C caused a similar loss of both ^{3}H and ^{14}C counts. Counts may also be lost from samples extracted with distilled water. For example, after standing at room temperature for 48 hr 40 per cent of the ^{14}C and 9 per cent of the ^{3}H counts originally present in the samples had disappeared. Since sucrose is an unsuitable extracellular marker for both tissue slices and toad bladder epithelial cells, we have not investigated in any detail the reasons for these losses of radioactivity from sucrose samples but mention them here only to stress the problems which can be associated with the use of labelled sucrose as an extracellular marker in isolated tissues.

PEG 1000 space in isolated tissues

Table 5 shows spaces measured with [^{3}H]PEG 1000 in metabolizing tissues, together with [^{14}C]inulin spaces measured simultaneously. Unlike PEG 4000 (see below) there was no difficulty in extracting PEG 1000 from either wet or dried tissue with distilled water or 0·1 M nitric acid. It did not prove necessary to add 1 per cent PEG 1000 to the extraction fluid. (Because PEG 1000 occupied such a large space in dried liver slices and had been found to be freely extracted from dried renal cortical slices and toad bladder epithelial cells no experiments were performed with undried

TABLE 5. [^{3}H]PEG 1000 and [^{14}C]inulin spaces measured simultaneously in metabolizing tissues incubated 90–120 min

	Undried tissue	Dried tissue	P
	(space per cent tissue wet wt.)		
Rat kidney slices			
[^{3}H]PEG 1000	46·8 ± 1·2 (10)	45·2 ± 1·0 (10)	> 0·30
[^{14}C]inulin	36·0 ± 0·8 (10)	28·3 ± 0·9 (10)	< 0·001
P	< 0·001	< 0·001	
Rat liver slices			
[^{3}H]PEG 1000		60·5 ± 1·1 (18)	
[^{14}C]inulin		25·8 ± 1·5 (18)	
P		< 0·001	
Toad bladder epithelial cells			
[^{3}H]PEG 1000	41·5 ± 1·2 (16)	48·9 ± 1·6 (8)	
[^{14}C]inulin	40·6 ± 2·0 (16)	49·6 ± 1·6 (8)	
P	> 0·40	> 0·40	

(Experiments with rat liver slices performed in collaboration with J. Pilgrim and B. Robinson; experiments on undried toad bladder epithelial cells performed in collaboration with Q. Al-Awqati, A. Leaf & M. M. Civan. Values for dried toad bladder epithelial cells were obtained in a different experiment.)

liver slices.) It can be seen that PEG 1000 had a volume of distribution in both liver and kidney which was much greater than that of inulin. Indeed, like sucrose, PEG 1000 must have entered cells in these tissues, for it occupied 66 per cent of the tissue water in kidney slices (mean tissue water content 2·28 ± 0·03 kg water/kg dry wt.) and 83 per cent in liver slices (mean tissue water content 2·59 ± 0·03 kg water/kg dry wt.) giving derived non-PEG 1000 spaces or 'intracellular water' of 0·76 kg/kg dry wt. and 0·43 ± 0·4 kg/kg dry wt. respectively, impossibly low values.

In contrast, however, the distribution of PEG 1000 in both undried and dried toad bladder epithelial cells was close to that of inulin.

PEG 4000 space in isolated tissues

In recent years PEG 6000 has been used to control cellular swelling in low ion media and in experiments where metabolism has been inhibited. The underlying assumption has been that the PEG remains extracellular and thus balances the colloid osmotic effect of intracellular macromolecules. Though there is evidence to support this assumption (Wiggins, 1965) it remains to be confirmed. Unfortunately labelled PEG 6000 is not available commercially but [^{14}C]PEG 4000 has recently been introduced. Having an average mol. wt. lower than PEG 6000 it should, if anything, tend to over-estimate rather than underestimate the volume of distribution of PEG 6000.

Preliminary experiments were required to establish an effective method for the extraction of PEG 4000 from tissue. Extraction from dried tissue yielded variable results and extraction from undried tissue was only rapid if unlabelled PEG 4000 was present in the extraction fluid. The presence of unlabelled PEG 4000 in the incubation medium did not appear to affect subsequent PEG extraction suggesting that binding or adsorption of PEG 4000 to specific tissue sites was not a factor preventing subsequent extraction. In some early experiments it appeared that extraction might be more effective if distilled water rather than 0·1 M nitric acid was used. Table 6 allows comparison of extraction of [^{14}C]PEG 4000 from both undried and dried rat renal cortical slices with either distilled water or distilled water containing 1 per cent PEG 4000. Only in undried tissue extracted with distilled water containing 1 per cent PEG 4000 was 14 hr an adequate time for extraction. In this case a further 46 hr extraction produced little further recovery [^{14}C]PEG 4000 from the tissue. For this reason undried tissue was extracted with distilled water containing 1 per cent PEG 4000 in all experiments where PEG 4000 spaces were determined.

Table 7 summarizes results in which [^{14}C]PEG 4000 spaces of metabolizing tissues were measured. The important observation which emerges from these experiments is that, for rat renal cortical slices, and rat liver slices, the PEG 4000 space was comparable with the inulin space obtained in tissue extracted after drying as shown in Table 1. Spaces in toad bladder epithelial cells are much more variable from preparation to preparation reflecting the fact that the whole hemibladder is blotted before cells are scraped for analysis. Since the epithelial cells cannot be blotted on their serosal surfaces this leads to a much greater scatter in the individual measurements. For this reason the only valid comparison between PEG and inulin spaces is provided by paired analyses of simultaneously measured spaces. As Table 7 shows, the inulin space in the toad bladder epithelial cells was somewhat higher than the PEG 4000 space ($P < 0{\cdot}001$).

TABLE 6. Conditions required for adequate extraction of [^{14}C]PEG 4000 from rat renal cortical slices incubated 2 hr at 25° C in oxygenated medium

	Undried tissue	Dried tissue	
	Space (per cent tissue wet wt.)		P
Extraction with dist. H_2O			
14 hr	10·6 ± 0·6 (6)	8·7 ± 0·4 (6)	< 0·05
60 hr	19·3 ± 0·9 (6)	15·8 ± 0·7 (6)	< 0·02
P	< 0·001*	< 0·001*	
Extraction with dist. H_2O + 1 per cent PEG			
14 hr	20·8 ± 0·8 (6)	17·2 ± 1·2 (6)	< 0·05
60 hr	21·6 ± 0·7 (6)	20·2 ± 1·7 (6)	> 0·40
P	> 0·10*	< 0·05*	

* Test of paired data.

TABLE 7. [^{14}C]PEG 4000 spaces in metabolizing tissues incubated for 2 hr at 25° C

Rat renal cortical slices	23·5 ± 0·9 (35)
Rat liver slices	18·3 ± 0·7 (8)
Toad bladder epithelial cells*	53·3 ± 1·4 (10)

Tissues extracted without drying with dist. H_2O containing 1 per cent PEG 4000.
* Simultaneously measured [^{3}H]inulin space 59·2 ± 1·2 (10).

TABLE 8. Rate of equilibration of PEG 4000 in slices of rat renal cortex ($n = 8$)

Incubation time (min)	Dried tissue space (per cent tissue wet wt.)
7·5	11·9 ± 0·8
15	13·5 ± 0·5
30	15·7 ± 0·6
60	16·6 ± 0·9
120	17·9 ± 1·0
180	17·5 ± 0·5
240	18·6 ± 0·6

Table 8 presents the results of a kinetic study in which the rate of equilibration of PEG in tissue water in metabolizing rat renal cortical slices was examined. Between 30 and 60 min PEG had equilibrated in 16·6 ± 0·9 per cent tissue wet wt. Over the next 3 hr this space increased only slightly.

DISCUSSION

The results demonstrate that drying slices of kidney and liver prevents complete extraction of inulin from the tissues. This result, which was most unexpected, does not seem to have been recorded previously. The inulin space measured in dried renal cortical slices was exactly comparable to

that reported by a number of workers using renal slices from a variety of mammalian species (Fox, Thier, Rosenberg & Segal, 1964; Gans, Baillie & Biggs, 1966; Kleinzeller & Cort, 1960; Macknight, 1968; Maude, 1969; Robinson, 1950; Swan, Ellington & Miller, 1960; Whittam, 1956; Whittembury, 1965). However, since the methods employed for incubation of slices, for extraction of inulin and for subsequent analysis differ so greatly from study to study, it is not possible to decide why this discrepancy in inulin spaces in dried and undried slices has not previously been detected.

The problem then is to decide whether one should regard inulin as an appropriate marker for the determination of tissue extracellular space. The results of the kinetic experiment (Table 3) reveal that inulin entered the tissue at at least two different rates. This must imply either that inulin diffused into most of the extracellular water rapidly and then much more slowly equilibrated in a relatively less accessible extracellular compartment or alternatively, that inulin equilibrated relatively rapidly in the extracellular water and then much more slowly entered either damaged or normal cells. Whichever hypothesis is correct it would seem that after drying of mammalian tissue slices inulin could only be readily extracted from the compartment in which it rapidly equilibrated.

Inulin may have entered either damaged or normal cells during incubation, and subsequent drying of the tissue could then have resulted in a combination of inulin with intracellular constituents. If this were so then inulin dried with homogenized tissue should not be completely recovered during subsequent extraction. This was tested using the procedure described in the methods section with homogenized rat liver. Liver was used rather than kidney as renal tissue consists of medulla as well as cortex which would have needed to be removed before homogenization. In addition, a much greater mass of liver tissue for homogenization was available from each animal. Table 9 contains the pooled results of three separate experiments performed with homogenized liver tissue from a total of six rats. Both [^{3}H]inulin and [^{14}C]PEG 4000 were added together to some samples of homogenate as described in the Table. Thus these samples were exposed to the same concentrations of both isotopes. In addition, extracts from some samples of homogenate which had not been exposed to isotopes were counted to confirm that extraction of homogenate alone did not affect the background counts.

There are a number of minor differences apparent in Table 9 for which no explanation is forthcoming but the major difference is the failure to recover about half the inulin and PEG 4000 added to tissues which were subsequently dried, even though dried tissues were homogenized and extracted for up to 10 days. Indeed, even after extraction for 10 days,

about 48 per cent of the inulin and PEG 4000 counts had not been recovered. In contrast, similar experiments performed with liver homogenate and [^{3}H]PEG 1000 confirmed that this isotope was fully recoverable when dried with tissue (dried medium, c.p.m. 1858 ± 41, $n = 20$; undried medium, c.p.m. 1908 ± 19, $n = 20$; tissue dried with isotope, c.p.m. 1881 ± 57, $n = 20$; isotope added after drying of tissue, c.p.m. 2125 ± 17, $n = 10$).

The results obtained with homogenized tissue were therefore completely consistent with the hypothesis that inulin entered cells from which after drying it could not be extracted. Inulin is a fructosan and preparations show some polydispersity (Phelps, 1965). Though paper chromatography confirmed the relative homogeneity of the inulins used in these experiments it is possible that it was predominantly lower molecular weight inulin molecules which entered the kidney and liver cells during incubation and could not subsequently be recovered from dried slices. As normally used, however, the term 'inulin space' must include the distribution of such lower molecular weight inulin molecules, and it is in this sense that the term is used in the present paper.

Two considerations favour the possibility that this inulin had entered normal cells rather than damaged cells on the outside of the slices. One would anticipate that grossly damaged cells would allow entry of both inulin and PEG 4000, yet, though neither could be extracted from dried tissue homogenate, only with inulin was there a smaller space in dried slices. This would imply that only inulin had the opportunity of interacting with intracellular constituents in the slice.

A more direct test of the possibility that inulin entered damaged cells rather than more normal cells in slices was provided by a comparison of inulin spaces in outer and inner slices of rat renal cortex. Outer slices were incubated separately from the inner slices throughout the 10 min equilibration in balanced saline medium and the subsequent 2 hr incubation in similar medium containing [^{3}H]inulin. They were then extracted overnight in distilled water without drying. Outer slices from rat renal cortex have only their inner surface sliced and therefore have only approximately half the number of damaged cells when compared with the next or inner slice which is cut on both surfaces. If inulin were entering only damaged cells the inulin space in the undried outer slice should be significantly less than the space in the undried inner slice. Yet, with twelve slices from the same kidneys in each group, the inulin space as a percentage of tissue wet weight was $38{\cdot}8 \pm 0{\cdot}3$ for the outer slice and $37{\cdot}8 \pm 0{\cdot}6$ for the inner slice, values which do not differ ($P > 0{\cdot}10$). This observation would seem to exclude the possibility that the greater inulin space in undried slices was a consequence of inulin entry only to damaged cells.

The fact that PEG 4000 equilibrated in a space similar in magnitude to that from which inulin could be extracted from dried renal cortical slices (Tables 1 and 7), together with the observation that sucrose rapidly equilibrated in a similar percentage of tissue wet weight before its concentration in tissue increased much more slowly (Table 4) suggests that in

TABLE 9. Extraction of [^{3}H]inulin and [^{14}C]PEG 4000 from homogenized rat liver. Aliquots (25 μl.) of medium with isotopes added to homogenized tissue before or after drying and extraction (dried tissues) or before or after extraction and centrifugation (wet tissues). Aliquots of medium (25 μl.) without tissue were also extracted without or after drying. All samples extracted in 10 ml. dist. water containing 1 per cent (w/v) unlabelled PEG 4000 for 10 days, and 2 ml. of extract counted. Each value represents mean ± S.E. of mean of number of individual samples shown in brackets

	Counts per min	
	[^{3}H]inulin	[^{14}C]PEG 4000
Medium		
Undried	1362 ± 23 (30)	846 ± 7 (30)
Dried	1444 ± 36 (20)	852 ± 7 (20)
	$P > 0.05$	$P > 0.60$
Wet tissues		
Isotope added before centrifugation	1235 ± 6 (10)	834 ± 4 (10)
Isotope added after centrifugation	1312 ± 11 (10)	886 ± 6 (10)
	$P > 0.30$	$P < 0.001$
Dried tissues		
Isotope added before drying	772 ± 40 (27)	455 ± 11 (27)
Isotope added after drying	1517 ± 13 (10)	804 ± 10 (10)
	$P < 0.001$	$P < 0.001$

renal cortical tissue the true extracellular space lies somewhere between 20 and 30 per cent of tissue wet weight. In liver slices the PEG 4000 space and inulin space of dried tissue were identical (Tables 1 and 7). This suggests that the true extracellular space in these slices incubated *in vitro* was approximately 18 per cent tissue wet weight. It is very important however to stress that extracellular spaces expressed as per cent tissue wet weight may vary from one experiment to another and in particular from one experimenter to another. Total tissue water is calculated by loss of weight of wet tissue upon drying. But the initial wet weight of tissue is entirely dependent upon the adequacy and reproducibility of the blotting technique. Liver slices are much more fragile than renal cortical slices and their handling and blotting therefore more delicate and difficult. As an illustration of the variability in tissue water which may be found, Table 1 contains values for the inulin spaces in liver slices handled by the authors and Table 5 values for inulin spaces in liver slices incubated at the same temperature under identical conditions in this laboratory but handled by

J. Pilgrim and B. Robinson. The inulin space in Table 5 is significantly higher than that in Table 1. However the mean tissue water contents of slices used for Table 5 was 2.59 ± 0.03 ($n = 18$) kg water/kg dry wt. whereas the mean tissue water of slices used for Table 1 was 2.09 ± 0.04 ($n = 15$) kg water/kg dry wt. Using the inulin spaces, the derived intracellular water contents are 1.66 ± 0.05 kg water/kg dry wt. and 1.54 ± 0.05 kg water/kg dry wt. respectively, values which do not differ significantly ($P > 0.10$). Results such as these demonstrate the dangers of deriving 'intracellular' water contents in experiments with tissue slices using for this purpose values for extracellular space taken from the literature. If it had been assumed that the space in slices with tissue water content of 2·59 kg/kg dry wt. was 17·9 % tissue wet weight the derived 'intracellular' water content would have been 1·95 kg/kg dry wt. not the 1·66 kg/kg dry wt. actually measured.

While it seemed that inulin rather fortuitously provides a reasonable measure of extracellular water when it is extracted from dried kidney and liver slices, there was no evidence that inulin had entered cells in toad bladder epithelial cell preparations for the spaces in dried and undried samples were similar (Table 1). The recent finding that in those preparations sucrose can occupy a greater space than inulin had raised the possibility that sucrose might prove the more accurate extracellular marker (Al-Awqati *et al.* 1972). However, the demonstration that sucrose can be metabolized by these cells shows that it must enter the cellular water (as it does in renal cortical slices) and it is therefore clearly not a satisfactory extracellular marker in this tissue. The finding (Table 5) that PEG 1000, which entered cells in kidney and liver slices, occupied the same space as inulin in toad bladder epithelial cell preparations lends strong support to the suitability of both substances as extracellular markers here. As Table 7 shows PEG 4000 occupied a similar though significantly lower space than inulin in toad bladder epithelial cells.

The results presented in this paper illustrate the difficulties of determining accurately the extracellular water in isolated tissues. Clearly when tissues are incubated in a medium similar in ion concentrations to their *in vivo* interstitial fluid the derived intracellular potassium contents will be virtually unaffected by the size of the extracellular space, though derived intracellular water, sodium and chloride contents will all be markedly dependent upon the value used for the extracellular space. In contrast however, it can be calculated that derived intracellular sodium, chloride and potassium concentrations may be much less dependent upon the measured extracellular space over the range in which it is likely that the true extracellular space lies.

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