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STUDIES ON INTESTINAL FLUID TRANSPORT

I. ESTIMATION OF THE EXTRACELLULAR SPACE OF EVERTED SACS OF RAT SMALL INTESTINE

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SUMMARY

1. The estimation of the extracellular space of intestine incubated *in vitro* has been investigated.

2. The extracellular space of intestine consists of two anatomically distinct compartments: adherent mucosal fluid and sub-epithelial (serosal) extracellular space.

3. The serosal solute spaces of high molecular weight compounds (PEG 1000, PEG 4000, inulin) were similar, and smaller than the serosal mannitol space. It was concluded that, since the possibility that mannitol penetrates an intracellular compartment cannot be excluded, the high molecular weight solutes are preferable in investigation of the dynamic extracellular compartment involved in fluid transport.

4. Penetration of the serosal extracellular space by PEG 4000 was not influenced by fluid transport, and it was concluded that PEG 4000 equilibrates with the space which it penetrates.

INTRODUCTION

The estimation of extracellular space by the use of water-soluble solutes which do not penetrate cells, is a well established technique which has been applied to tissues both *in situ*[1,2], and *in vitro*[3,4]. The procedure is open to the criticism that the extracellular space is not a single homogeneous compartment, and results obtained with any particular solute are not readily identifiable with a distinct anatomical space[3,5]. But application of this technique has yielded useful information concerning relations between intracellular and extracellular compartments[6–8].

Due to the functional and structural peculiarities of the tissue, the estimation of the extracellular space of the intestine by the probe-solute technique, involves additional considerations in the interpretation of results. For example, the continuous net transport of fluid from one side of the tissue to the other, might impose a restriction on the movement of solute used to probe the compartment. This paper reports the results of experiments intended to investigate some of the considerations involved in the estimation of extracellular space of intestine incubated *in vitro*.

METHODS

In vitro preparations

The preparation used in most experiments was the everted sac of rat small intestine[9]. Male albino rats, weighing 180–200 g, were allowed food and water *ad libidum* to the day of experiment. The general technique used in preparation of the sacs was similar to that described by BARRY *et al.*[10], but since the procedure was varied in some experiments relevant details will be given.

The animals were anesthetized with sodium pentobarbitone (70 mg/kg, intraperitoneal) and the intestine washed out with 0.9% saline at room temperature. The intestine was stripped from the mesentery and rinsed free of blood in ice-cold saline before eversion over a glass rod. A sac, weighing about 1 g, was prepared from the middle of the gut, blotted between filter papers dampened with ice-cold saline, and drained before weighing. The sac was filled with 1 ml of bicarbonate saline[11], weighed again and placed in a flask containing 20 ml of the same saline. The flask was shaken for 1 h at 37°. At the end of the incubation period the sac was blotted between damp filters papers, weighed, cut open and drained, and weighed again. The tissue was homogenized and solute determinations made on samples of the homogenate and the mucosal and/or serosal fluids. Solutes were added to the mucosal or serosal fluids as described in the text.

In determining the extracellular space of the intestine incubated *in vitro* the probing solute was present initially in either the mucosal fluid or the serosal fluid. At the end of the incubation the amount of the probing solute present in the gut wall was estimated, and the space occupied calculated as the ratio of this amount to the final concentration of the solute in the mucosal or serosal fluid. When the solute was present initially in the mucosal fluid the space estimated is called the mucosal solute space, and when the solute was present initially in the serosal fluid the space is called the serosal solute space.

Significant changes in tissue wet weight were observed during incubation *in vitro*, in agreement with previous workers[12], and these changes in tissue weight indicated that consideration must be given to the basis on which the results are expressed, *i.e.*, either per g initial tissue weight or per g final tissue weight. Calculation shows that expression of the results per g final tissue weight would underestimate the extracellular compartment, if a fraction of the fluid uptake was intracellular. Results expressed per g initial weight are not subject to this discrepancy.

In some experiments the tissue was cut into short rings after eversion[13], and incubated as described above. At the end of the incubation the tissue was collected by filtration through cotton gauze, blotted between dry filter papers, weighed and homogenized. Other experiments were carried out using mucosal tissue scraped free of the underlying muscle layers as described by DICKENS AND WEIL-MALHERBE[14]. Due to the dissolution of a small part of the tissue during the incubation of these two preparations, it was not possible to assess accurately the initial weight of the tissue used for analysis. Accordingly the results of these experiments are expressed in terms of the final weight of the tissue recovered after blotting, and where results of experiments with rings or scraped mucosa are compared with those of sac experiments, the results of the sac experiments are expressed on the same basis. Since

these experiments were performed in similar conditions of incubation, it is considered that this procedure for comparison is justified.

Electron-microscopic observations

Experiments were performed to investigate the distribution of colloidal thorium dioxide in the extracellular space. In these experiments sacs were incubated as described above, but with the addition of 2.5 % colloidal thorium dioxide to the serosal fluid. At the end of the incubation the sacs were opened and drained, and small pieces fixed in osmium/dichromate before dehydration and embedment in epon. Thin sections (0.6 μ) were cut, double stained by conventional techniques, and examined in an RCA EMU-3H electron microscope.

Chemicals

PEG 1000 and PEG 4000 were estimated by the method of HYDEN[15]. ^{14}C-labelled mannitol was obtained from Amersham/Searle Corp. and was estimated by liquid scintillation counting of the supernatant obtained (deproteinized with 0.17 M $Ba(OH)_2$ and 5 % (w/v) $ZnSO_4 \cdot 7H_2O$) in the scintillator described by BRAY[16]. Inulin was estimated by a colorimetric method[17] with appropriate correction for tissue blank values.

Statistics

Results of experiments are expressed as mean ±S.E. Where results of different experiments are compared the Student t test was used[18].

RESULTS

Solute spaces of intestine incubated in vitro

A series of experiments was carried out to determine the solute spaces of rat small intestine using several probing solutes. Table I shows the results of experiments in which PEG 1000, PEG 4000, inulin and mannitol were used. In these experiments the solute was present initially either in the mucosal fluid only or in the serosal fluid only.

TABLE I

SOLUTE SPACES OF SMALL INTESTINE

Everted sacs of rat small intestine prepared from the middle fifth of the combined jejunum and ileum were filled with 1 ml of bicarbonate saline and incubated in 20 ml of the same saline for 1 h at 37°. Probing solutes were present initially either in the mucosal fluid or in the serosal fluid, *i. e.* mucosal and serosal spaces were calculated in different experiments. Results are means ± S.E. and are expressed per g initial wet wt. The number of experiments is given in parentheses.

Probe solute	*Mucosal space (ml/g)*	*Serosal space (ml/g)*
Inulin (5 mg/ml)	0.26 ± 0.01 (6)	0.21 ± 0.01 (5)
PEG 1000 (5 mg/ml)	0.24 ± 0.01 (5)	0.23 ± 0.01 (5)
PEG 4000 (5 mg/ml)	0.24 ± 0.04 (6)	0.20 ± 0.01 (6)
Mannitol (5 mM)	0.30 ± 0.01 (5)	0.50 ± 0.01 (5)

Using inulin, PEG 1000 or PEG 4000, no significant differences were found between the mucosal solute spaces, or between the serosal solute spaces estimated with these solutes. When mannitol was used the estimated mucosal space was significantly greater than the mucosal inulin space ($P<0.05$), and the serosal mannitol space was significantly greater than the serosal inulin space ($P<0.01$). For reasons which are discussed below PEG 4000 was selected for further study.

Table I also shows that the mucosal and serosal PEG 4000 spaces were not significantly different, and the possibility was considered that the same anatomical space was being estimated by the two procedures. A series of experiments was performed in which PEG 4000 was placed on both sides of the intestine and the amount of PEG 4000 taken up by the gut wall during incubation compared with the amounts taken up when the solute was present initially in either the mucosal or serosal fluid only. If the two PEG 4000 spaces were anatomically distinct the amount of solute taken up by the gut should be equal to the sum of the amounts taken up when PEG 4000 was added to either mucosal or serosal fluids in separate experiments. If the mucosal and serosal spaces were identical the sum of the amounts of the solute taken up in the separate experiments would be greater than the amount taken up when PEG 4000 was present initially on both sides of the gut. The results of these experiments are shown in Table II. The amount of PEG 4000 taken up by the gut when the solute was present initially in both mucosal and serosal fluids was not significantly different from the sum of the amounts taken up when the solute was present in either the mucosal or serosal fluids in separate experiments, indicating that the mucosal and serosal PEG 4000 spaces were anatomically distinct.

TABLE II

UPTAKE OF PEG 4000 BY INTESTINE

Conditions of incubation as in Table I. Results are expressed as mg PEG 4000 taken up per g initial wet wt. and the number of experiments is given in parentheses.

Initial location of PEG 4000	*Mucosal fluid*	*Serosal fluid*	*Mucosal and serosal fluids*	*Sum of columns 1 and 2 (for explanation, see text)*
Amount of PEG 4000 taken up by gut (mg/g)	1.25 ± 0.19 (6)	0.48 ± 0.04 (6)	1.77 ± 0.12 (6)	1.73 ± 0.19

To investigate further the distribution of PEG 4000 taken up by the gut during incubation a series of experiments was performed in which alterations were made in the technique of handling the sacs. The results of these experiments are shown in Table III. In the experiments shown in the first line of the table the sacs were handled as described in METHODS. The experiments in the second line were treated similarly up to the point at which the serosal fluid was collected at the end of the incubation. After the sac had been drained, it was opened and firmly blotted between dry filter papers before weighing. This final dry blot removed most of the PEG 4000 taken up from the musosal fluid, but had no significant effect on the uptake of PEG 4000 from the serosal fluid. These experiments indicate that little of the PEG

TABLE III

EFFECT OF BLOTTING PROCEDURE ON UPTAKE OF PEG 4000

Conditions of incubation as in Table I, but different techniques were used for handling the sacs. For details see text. Results are expressed per g initial wet wt. and the number of experiments is given in parentheses.

Blot after incubation	*PEG 4000 uptake (mg/g) from*	
	Mucosal fluid	*Serosal fluid*
Wet	1.25 ± 0.18 (6)	0.48 ± 0.08 (6)
Dry	0.03 ± 0.01 (6)	0.50 ± 0.04 (5)

4000 taken up by the gut wall from the serosal fluid is extractable by blotting, and suggest that a negligible fraction of the serosal PEG 4000 uptake is due to adherent serosal fluid. To confirm this suggestion a series of experiments was performed in which sacs were prepared as described in METHODS, filled with 1 ml of serosal fluid, weighed and then immediately opened, drained and weighed again. In a series of five experiments the mean change in weight of the sac was +0.010 ± 0.005 g, *i.e.* the volume of serosal fluid retained in the sac after draining represented about 5 % of the serosal PEG 4000 space.

Time-course of uptake of PEG 4000

Fig. 1 shows the results of experiments in which the period of incubation was varied and the apparent serosal PEG 4000 space estimated. The values obtained at 30, 60 and 90 min (0.21 ± 0.01, 0.20 ± 0.01 and 0.21 ± 0.02 ml/g, respectively) were not significantly different.

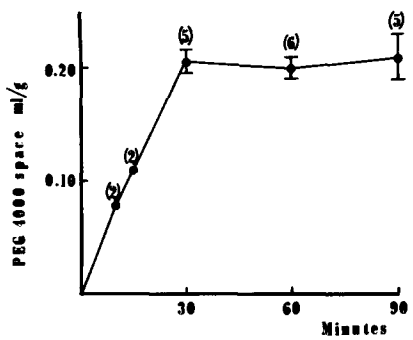

Fig. 1. Effect of length of incubation period on apparent serosal PEG 4000 space. Conditions of incubation as in Table I, but length of incubation period varied. Numbers in parentheses are the number of observations.

Equilibration of serosal PEG 4000 space with serosal fluid

In Table IV are shown the results of experiments in which conditions were modified to test the completeness of equilibration between the serosal PEG 4000 space and PEG 4000 in the serosal fluid. The objective of these experiments was to test the possibility that the flow of fluid associated with trans-intestinal fluid transport, might impede the entrance of PEG 4000 into the serosal PEG 4000 space

TABLE IV

EFFECT OF FLUID TRANSFER ON SEROSAL PEG 4000 SPACE

Conditions of incubation as in Table I, but in some experiments length of incubation increased to 90 min and in other experiments a 60-min incubation was followed by a further 30 min in which mannitol was added to the mucosal fluid to inhibit further fluid transfer. Results are expressed per g initial wet wt. and number of experiments is given in parentheses.

Incubation time (min)	*Fluid transfer (ml/g)*	*Serosal PEG 4000 space (ml/g)*
90	0.98 ± 0.10 (12)	0.22 ± 0.01 (6)
60 + 30 (with mannitol)	0.68 ± 0.18 (10)	0.20 ± 0.02 (5)

from the serosal fluid. In these experiments a 60-min incubation was followed by a further 30 min in which the sac was suspended in fresh incubation saline, and in some experiments the saline of the second incubation contained 50 mM mannitol to act as an osmotic restraint to fluid transport. The addition of mannitol to the mucosal fluid in the second incubation period reversed the direction of fluid flow during this period as shown by the apparent decrease in net fluid transfer. But no change was observed in the serosal PEG 4000 space.

PEG 4000 space of sacs, rings and mucosal scrapes

Table V compares the serosal PEG 4000 space of everted sacs with the PEG 4000 space of the other *in vitro* preparations described in METHODS. It should be emphasized that all the preparations were blotted between dry filter papers to remove adherent incubation saline, and the results are expressed in terms of the final weight of the tissue. Thus the conditions of the experiments correspond closely for the three types of preparation, and the PEG 4000 spaces were not significantly different.

TABLE V

PEG 4000 SPACE OF SEVERAL PREPARATIONS OF INTESTINE *in vitro*

Conditions of incubation as in Table I, but all preparations were dry blotted at the end of the incubation and results are expressed as ml per g final wet wt. of tissue. The number of experiments is given in parentheses.

Serosal PEG 4000 space of everted sacs (ml/g)	*PEG 4000 space of everted rings (ml/g)*	*PEG 4000 space of mucosal scrapes (ml/g)*
0.18 ± 0.02 (6)	0.19 ± 0.01 (9)	0.20 ± 0.02 (5)

Distribution of colloidal thorium dioxide in the extracellular space

In order to investigate the extent of penetration of a marker into the extracellular space, everted sacs were incubated with 2.5% thorium dioxide added to the serosal fluid. This compound is electron-opaque and is revealed as a dense deposit in the electron microscope. Fig. 2 shows part of an intercellular channel between the epithelial cells of tissue taken from such an experiment. A deposit of thorium dioxide can clearly be seen within the channel. In this experiment glucose

(28 mM) had been added to the mucosal fluid to maximize fluid transport, but it is clear that even in this condition the marker was able to traverse the space between the serosal fluid and the epithelial cells.

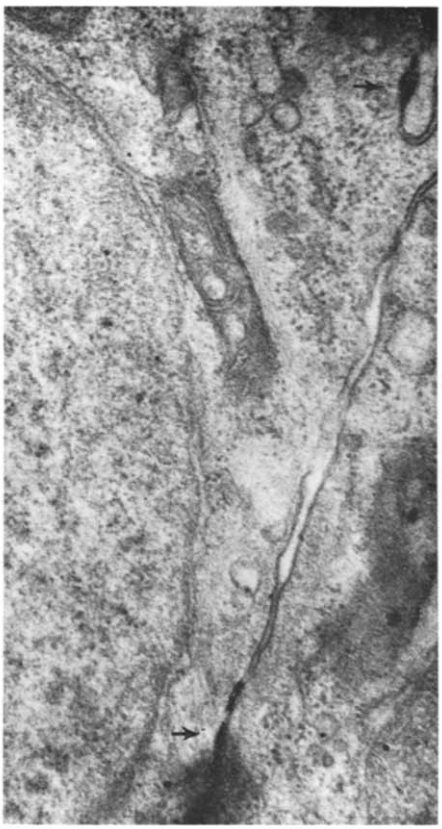

Fig. 2. Distribution of colloidal thorium dioxide in serosal extracellular space. Everted sacs were incubated in the conditions shown in Table I, but with 2.5% thorium dioxide added to the serosal fluid. The figure shows an electronmicrograph of an intercellular channel in the epithelial layer at the nuclear level. The black deposits (→) which can be seen in the channel are thorium dioxide (× 38800).

DISCUSSION

The application of the probe-solute procedure to the determination of the extracellular space, of a fluid-transporting epithelium, such as the intestine, is complicated by the need to consider two factors related to the structural and functional peculiarities of the tissue: the orientation of the tissue with respect to solute penetration, and the flow of fluid through the extracellular space.

Structural considerations

In order to account for many of the characteristic features of transintestinal transport of fluid and solutes, it is necessary to postulate a barrier to free diffusion associated with the epithelium. The properties of this layer have been well characterized[19,20],

and it is generally accepted to have very low permeability to water-soluble compounds of high molecular weight[21]. Thus a model of intestinal structure based on these observations would predict that the extracellular space of the intestine is accessible to probing solutes only from the serosal side of the tissue, and several previous studies have accepted this assumption[22–24]. In the present study the initial experiments suggested that this model for the intestine may be incorrect. The similarity in magnitude of the mucosal and serosal solute spaces determined with solutes of high molecular weight, opened the possibility of a single extracellular space which could be penetrated from either mucosal or serosal sides. Direct experimentation showed that the assumption of the model was justified, and that the agreement between mucosal and serosal solute spaces was fortuitous. The extracellular space of everted sacs of rat small intestine is composed of two anatomically distinct compartments. One, accessible to probe solutes present in the mucosal fluid, could be extracted by blotting the intestine with dry filter papers, and probably represents fluid trapped between the villi and in the crypts. The second compartment, accessible to probing solutes from the serosal side, could not be extracted by blotting and is associated with the sub-epithelial extracellular space. Accordingly, the mucosal solute space can be dismissed from further discussion, although it should be pointed out that, in considering total tissue water, the quantity of adherent mucosal fluid may be as large as the sub-epithelial extracellular space, and is markedly dependent upon the technique of tissue handling.

Equilibration problems

If a probing solute is to reflect the magnitude of the extracellular space accurately, it must achieve diffusion equilibrium with the whole of the extracellular compartment. In a fluid-transporting tissue, there are three factors which may influence the process of equilibration: time, accessibility and fluid flow.

(*i*) *Time.* The extent of penetration of the extracellular space by the probing solute is time dependent. In present experiments, the apparent serosal PEG 4000 space increased during the first 30 min of incubation, but no significant change was observed during a further 60 min. It was concluded that, in sacs incubated for 60 min, the serosal PEG 4000 space represented a steady state value, and a 60-min incubation was used in subsequent studies.

(*ii*) *Accessibility.* Previous studies with non-epithelial tissues *in vitro*[3,5,25] and *in vivo*[1], have suggested that the extracellular space is not a homogeneous compartment with respect to penetration by probing solutes. Several studies have indicated that penetration of some areas of the extracellular space is slower for larger molecules such as inulin, than for compounds of smaller molecular size such as sucrose[1,3]. It has been suggested that the equilibrium inulin space is the same as the equilibrium sucrose space, but that inulin penetrates the finer interstices of the extracellular compartment with difficulty, and requires a considerably longer period for full equilibration. In the present study, no increase in the serosal PEG 4000 space was observed following the establishment of the apparent steady state after 30 min of incubation. Accordingly, it is suggested that a component of the intestinal extracellular space which is poorly penetrated by PEG 4000 is either small in total volume, or so closely confined as to be effectively impermeable to this solute. The latter suggestion has been considered previously in the case of smooth muscle preparations.

It is suggested that there is a space corresponding to each extracellular solute, and the magnitude of any particular solute space is dependent upon the ease with which solute penetrates mucopolysaccharide[5]. The experiments described above showed that mannitol gave a larger serosal solute space than did PEG 4000, and this is consistent with the concept of a number of unique solute spaces. But it should be pointed out that mannitol has been shown to cross the intestinal wall at a rate which was sufficient to cause a detectable fluid movement[26], suggesting that mannitol may penetrate the epithelial cells. In this context, it is of interest to note that the serosal mannitol space was significantly greater than the mucosal mannitol space, and LIPPE *et al.*[27] have shown that the serosal aspect of intestinal epithelial cells is more permeable to water-soluble solutes than the mucosal surface. A similar discrepancy between mannitol and inulin spaces in studies with intestine *in vitro* has been observed previously[28]. In this study it was also concluded that the possibility of penetration by mannitol into the intracellular compartment could not be excluded. Some evidence for the possibility that the smaller extracellular space markers may penetrate the intracellular compartment was presented by BOZLER[4], who found that, in relatively acellular connective tissue, sucrose and inulin gave the same value for extracellular space, and that the value for this space was equivalent to the total tissue water. However, PAGE[3] using the papillary muscle of cat heart, concluded that mannitol remained extracellular, and accounted for the differences between the mannitol and inulin spaces by suggesting that mannitol could penetrate regions of the extracellular space, such as invaginations of the sarcolemma, which are inaccessible to inulin and conventionally considered to be intracellular.

In view of the uncertainty concerning the distribution of smaller solutes, it was considered that a larger molecule, such as PEG 4000, was more appropriate to the determination of intestinal extracellular space. In this tissue the component of the extracellular space which is of major interest is that which is concerned in transport. Relatively static components of the extracellular fluid trapped in the fine interstices of connective tissue, sequestered in mucopolysaccharide, or structured in small compartments, probably exchange more slowly with the solute and fluid in the transport 'stream' than does the bulk of the extracellular fluid.

A localized compartment of the interstitial fluid which is believed to be of considerable importance in transport, is that occupying the intercellular channels of the epithelial cells[29,30]. It has been suggested that these channels form a unique compartment for the establishment of a local osmotic gradient[31], which does not readily equilibrate with the sub-epithelial extracellular space when fluid transport is occurring. It is clear that PEG 4000 would not equilibrate with the fluid in this compartment. An analysis of the situation[32] has shown that the discrepancy may be as large as 60 % of the volume of the confined compartment in the stomach, but less than this in the intestine where the channels concerned in fluid and solute transport are smaller. It is shown in the subsequent paper[33] that the lateral intercellular channels of intestine do not achieve the expansion during fluid transport which is observed in the gall bladder[30,34]. Semi-quantitative assessment of the channel dimensions, based on electron micrographs of tissue transporting at a maximum rate, showed that the channels contribute less than 10 % of the total serosal extracellular space (M. J. JACKSON AND M. M. CASSIDY, unpublished observations). Further, the observation that colloidal thorium dioxide was capable of penetrating

the channels during maximal fluid transport, indicated that the serosal PEG 4000 space did include a component from this region, although it was recognized that this may not be fully equilibrated, and therefore underestimated.

(*iii*) *Fluid flow*. With the preparation under consideration, a continuous flow of fluid occurs from mucosal to serosal sides during the course of the incubation. This fluid stream must pass through part of the sub-epithelial extracellular space, and the possibility was considered that this may displace the equilibrium for the probing solute, such that the extracellular space was more dilute with respect to PEG 4000 than the serosal fluid within the sac. The experiments in which mannitol was used as an osmotic restraint to fluid transport showed that there was no expansion of the apparent extracellular space, even when the direction of fluid movement was reversed during the latter part of the incubation. This observation indicates that the position of the equilibrium between the extracellular space and the serosal fluid with respect to PEG 4000, was not influenced by fluid transport. This finding may be explicable by recent observations[35–37] that most of the fluid transported *in vitro* is moved to the serosal side *via* the blood and lymph vessels. Thus if PEG 4000 penetrated the extracellular space by diffusion through the interstitial space, this diffusion would not be opposed by fluid flowing in the opposite direction through the vessels. In addition, PEG 4000 in the interstitial space would equilibrate with fluid in the blood and lymph vessels and become distributed throughout the entire extracellular space of the tissue. Although it has been suggested, in the general case, that the relative impermeability of lymph vessels to large molecules is responsible for the discrepancy between inulin and sucrose space[38], the observation that the penetration of particles as large as chylomicrons into the intestinal lacteals occurs by an extracellular route[39], indicates that PEG 4000 could pass readily into the intestinal lymphatics.

Another complication introduced by the phenomenon of fluid transport is concerned with the continuous dilution of the serosal fluid within the sac. Thus the serosal bulk phase does not represent a constant concentration with respect to the probing solute. Further, since the sac is a closed compartment the serosal fluid is unstirred, and the possibility was considered that these factors may influence equilibration. In the cases of rings and scrapes of mucosal tissue the incubation medium is well stirred and maintains a constant concentration with respect to the probing solute. These preparations transport solutes[13,40] and presumably fluid, although the latter property cannot be demonstrated. When these preparations were handled in the same way as sacs, *i. e.* dry blotted to remove adherent mucosal fluid, the estimated PEG 4000 spaces were not significantly different from the serosal PEG 4000 spaces of sacs incubated in the same conditions.

On the basis of these observations it was concluded that, while the structural and functional peculiarities of intestine incubated *in vitro* intuitively appear to preclude the determination of extracellular space by the dilution technique, no evidence could be obtained to suggest that the application of the technique to the intestine was less valid than in the case of non-epithelial tissues.

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