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STUDIES ON INTESTINAL FLUID TRANSPORT

II. THE LOCATION OF FLUID ACCUMULATED IN THE WALL OF RAT JEJUNUM DURING INCUBATION *IN VITRO*

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SUMMARY

1. The quantity of fluid accumulated in the wall of jejunal sacs incubated *in vitro* was directly proportional to the rate of fluid transport.

2. The uptake of fluid was not reflected in an expansion of the serosal PEG 4000 space, but did expand the urea space.

3. The uptake of fluid could not be accounted for as an expansion of an extracellular space which could be demonstrated structurally, either as the development of sub-epithelial lakes, or as the distension of the lateral channels of the epithelial cells.

4. The dimensions of epithelial cells were shown to vary linearly with the gut fluid uptake.

5. It is suggested that the swelling of the epithelial cells indicates a specialized mechanism of fluid transport at the luminal cell border.

INTRODUCTION

A characteristic feature of the transport of fluid in the intestine is an increase in the degree of hydration of the gut wall. The magnitude of this tissue fluid accumulation is directly related to the rate of fluid transport, both *in vivo*[1], and *in vitro*[2], and it has been suggested that this fluid uptake occupies an extracellular compartment[3]. This distribution has not been demonstrated experimentally, although the phenomenon has been related to the appearance of large spaces in the sub-epithelial tissues on histological examination[4,5], and more recently to the distension of the lateral intercellular channels of the epithelial cells[6] which is a characteristic of fluid transport in several epithelia[7]. But several recent studies have suggested that the epithelial cells of the intestine[8–10], and of some other epithelia[11,12], may swell during transport, indicating a possibility that the fluid taken up by the tissue is not entirely extracellular. The present paper reports the results of an investigation of the distribution of the fluid taken up by the gut wall during incubation *in vitro*, and attempts to relate observed structural changes with a functional estimate of the extracellular space determined by the method described in the preceding paper[13]. Preliminary accounts of parts of this work have been presented[14,15].

METHODS

The *in vitro* preparation used in these experiments was the everted sac prepared from the mid-jejunum of the rat, and the techniques used for the estimation of fluid transport, uptake of fluid by the gut wall, and determination of the serosal PEG 4000 space were described in the preceding paper[13]. Experiments involving glycerol and urea were carried out with the aid of ^{14}C-labelled compounds obtained from the Amersham/Searle Corp., and preliminary experiments showed that these compounds were not significantly metabolized in the conditions used.

The gut fluid uptake is defined as the difference between the final and initial wet weights of the tissue, and the serosal fluid transfer is the increase in the volume of fluid within the sac during the incubation.

Experiments in vivo

Some experiments were performed in which the PEG 4000 space of intestine was determined with the intestine *in situ*. The animals were anesthetized with sodium pentobarbitone and the jugular cannulated with polythene tubing. The abdomen was opened and the intestine washed out with saline at 37°. The renal pedicles were ligated, and the abdomen closed. Heparin (200 units) was injected through the venous cannula, followed by 1 ml of saline containing 15 % PEG 4000. After 60 min a blood sample was taken by puncture of the abdominal vena cava. The intestine was removed and the middle fifth taken for estimation of PEG 4000. The PEG 4000 space was expressed as the ratio of the quantity of PEG 4000 in the tissue to the concentration of this solute in the plasma.

Microscopic observations

A series of experiments was performed with the objective of comparing the effects of fixation and embedment on the structure of incubated intestine. Everted sacs were incubated as described previously, and opened and drained at the end of the incubation. Each sac was then cut into two segments. One segment was immediately frozen in liquid N_2 and sectioned in a cryostat microtome. These sections were dried in air, fixed in formalin vapor, and stained with toluidine blue. The second segment was fixed in 10 % formol saline, dehydrated, and embedded in wax, before sectioning and staining with hematoxylin and eosin.

In some experiments the length and width of the epithelial cells were estimated by light microscopy. In these experiments sacs were incubated as described previously. At the end of the incubation, and after the sacs had been opened and drained, small pieces of tissue were fixed in osmium/dichromate fixative, embedded in epon and thick (2 μ) sections prepared. These sections were examined by phase contrast microscopy and estimates of the length and width across the nuclear region made on every third cell around a representative villus using a micrometer ocular. Thin sections (0.6 μ) were also cut from these specimens, double stained by conventional techniques, and examined in an electron microscope.

RESULTS

The relation between gut fluid uptake and fluid transfer

To confirm the relation between tissue fluid uptake and fluid transfer, the results of 67 individual experiments were plotted as shown in Fig. 1. The data were

taken from experiments involving several different incubation conditions in which glucose and/or mannitol were added to the incubation saline to alter the osmotic and metabolic characteristics. Some of these experiments are included in Table I. There was a linear relation between gut fluid uptake and serosal fluid transport over the range studied. A regression analysis of this relation gave the expression

$$GFU = 0.163\ (\pm 0.016)\ SFT + 0.240$$

where GFU is the gut fluid uptake, SFT is the serosal fluid transport, and the regression coefficient is given $\pm$ one S. E. Analysis of this correlation indicated a high degree of statistical significance ($P < 0.001$).

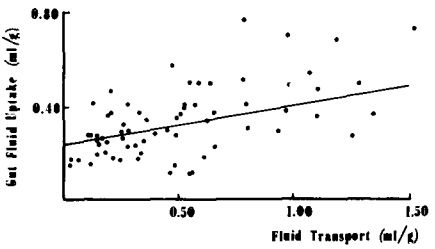

Fig. 1. Relation between gut fluid uptake and serosal fluid transfer. The points shown are taken from the data of several experiments in which fluid transfer and gut fluid uptake were altered by the addition of mannitol to the mucosal fluid or by the addition of metabolizable hexoses to the serosal fluid. The line drawn is that given by regression analysis of the 67 experiments plotted in the figure.

TABLE I

SEROSAL PEG 4000 SPACES AND GUT FLUID UPTAKE

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°. PEG 4000 (5 mg/ml) was present initially in serosal fluid. Other additions were made as indicated. Results are means $\pm$ S. E. and are expressed per g initial wet wt. The number of experiments is given in parentheses.

Addition		*Gut fluid uptake* (*ml/g*)	*Serosal PEG 4000 space* (*ml/g*)
Serosal fluid	*Mucosal fluid*		
None	None	0.37 $\pm$ 0.02 (11)	0.20 $\pm$ 0.01 (5)
222 mM glucose	0.5 mM phloridzin	0.65 $\pm$ 0.03 (5)	0.19 $\pm$ 0.03 (5)
None	100 mM mannitol	0.14 $\pm$ 0.02 (12)	0.18 $\pm$ 0.01 (6)
222 mM glucose	0.5 mM phloridzin + 150 mM mannitol	0.25 $\pm$ 0.02 (6)	0.17 $\pm$ 0.02 (6)

The relation between serosal PEG 4000 space and gut fluid uptake

A series of experiments was carried out to investigate the relation between the serosal PEG 4000 space and the gut fluid uptake. In these experiments the fluid transfer, and thus the gut fluid uptake, was varied either by the addition of mannitol to the mucosal fluid to act as an osmotic restraint to fluid transport[16], or by the addition of glucose to the serosal fluid to provide a metabolizable substrate and stimulate fluid transport[17,18]. In the experiments involving glucose, phloridzin (0.5 mM) was added to the mucosal fluid to inhibit the active transport of any glucose which diffused

across the gut wall. Thus none of these experiments involved a component of fluid transport associated with the transport of an organic solute. The results of these experiments are shown in Table I. Small variations were observed in the serosal PEG 4000 space in contrast to the large changes in gut fluid uptake which were produced by the different conditions. The value for PEG 4000 space determined *in vivo* was in close agreement with the serosal PEG 4000 space of the experiments *in vitro* shown in Table I. In a series of six experiments the PEG 4000 space of the middle fifth of the small intestine, estimated *in vivo*, was 0.20 ± 0.02 ml/g.

Glycerol and urea spaces

In a further series of *in vitro* experiments glycerol or urea were used to estimate the spaces occupied by these compounds. The intestine is permeable to these compounds and during the incubation measurable quantities of these solutes moved from the mucosal fluid, to which they were added initially, into the serosal fluid. However, during the 60-min incubation used in these experiments the mucosal and serosal fluids did not achieve diffusion equilibrium with respect to these solutes, and at the end of the incubation the serosal concentration was less than the mucosal concentration. Thus it was not possible to calculate a solute space directly and a different procedure was adopted. At the end of the incubation the concentrations of urea or glycerol in the mucosal and serosal fluids, and the quantity of the solute in the gut wall were estimated. The amount of glycerol or urea present in the mucosal fluid adhering to the tissue was calculated from the mucosal PEG 4000 space, and subtracted from the total amount present in the gut wall. The remaining quantity of glycerol or urea in the gut wall was assumed to be in equilibrium with the serosal fluid and a serosal solute space calculated. The results of these calculations are shown in Table II. The values for the serosal space obtained with glycerol or urea were much greater than the values obtained with PEG 4000, and are in good agreement with the values for total tissue water determined on sacs which had been incubated in the same conditions. The table also shows that the increase in gut water brought about by the addition of glucose to the incubation saline, gave a corresponding expansion of the urea space.

The effect of fixation and embedment on tissue structure

In view of the changes in sub-epithelial structure described by previous workers, the independence of the serosal PEG 4000 space and the gut fluid uptake required

TABLE II

GLYCEROL AND UREA SPACES OF INTESTINE *in vitro*

Conditions of incubation as in Table I. 5 mM glycerol or 5 mM urea were present initially in the mucosal fluid only. Serosal spaces were calculated as described in the text. Results are means ± S.E. and are expressed per g final wet wt. The number of experiments is given in parentheses.

Probe solute	*Other addition to mucosal fluid*	*Gut fluid uptake (ml/g)*	*Serosal solute space (ml/g)*	*Total gut water (ml/g)*
Glycerol	—	0.26 ± 0.01 (5)	0.79 ± 0.01 (5)	0.82 ± 0.02 (10)
Urea	—	0.26 ± 0.02 (5)	0.81 ± 0.01 (5)	
Urea	28 mM glucose	0.45 ± 0.01 (6)	0.88 ± 0.01 (6)	0.90 ± 0.01 (6)

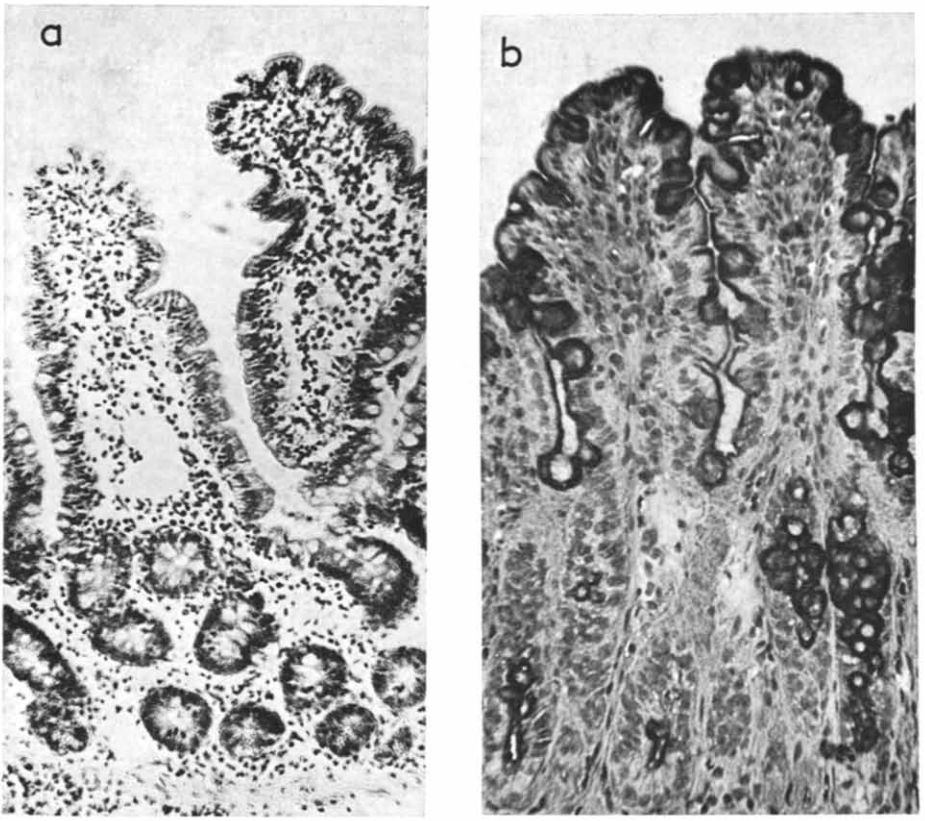

Fig. 2.

further investigation. Experiments were performed in which the structure of tissue frozen immediately after incubation was compared with the structure of tissue taken from the same everted sac, but fixed and sectioned by conventional histological techniques. Representative sections taken from two such experiments are shown in Fig. 2. Fig. 2a shows a wax-embedded section of tissue which had been incubated in the presence of glucose. Large spaces between the lamina propria and the epithelial layer and within the connective tissue can be seen. This section should be compared with Fig. 2b, which shows a frozen section of the same sac. The absence of the sub-epithelial spaces which characterized Fig. 2a is clear. Fig. 2c is a wax-embedded section taken from an experiment in which mannitol had been added to the mucosal fluid to inhibit fluid transport. In this experiment a gut fluid uptake of 0.12 ml/g was observed, and this should be compared with the value of 0.84 ml/g which was obtained in the experiment with glucose shown in Figs. 2a and 2b. As in the case of the glucose experiment, the experiment with mannitol showed sub-epithelial spaces in the wax-embedded section, but not in the frozen section which is shown in Fig. 2d. It is clear that the appearance of the large spaces in the sub-epithelial tissue can be related more readily to the procedure adopted in preparing the tissue for histology, than to the uptake of fluid by the gut wall during incubation.

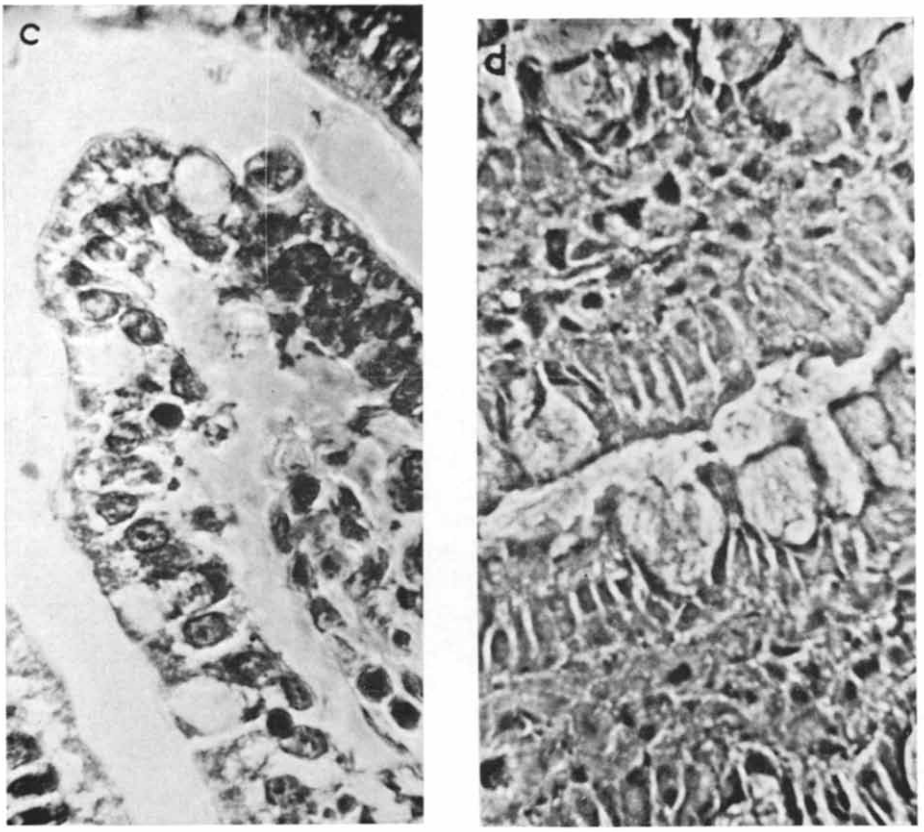

Fig. 2. Effect of fixation and embedment on structure of incubated intestine. a. Wax-embedded section of sac incubated in presence of glucose (× 150). b. Frozen section of the same sac as a (× 180). c. Wax-embedded section of sac incubated in presence of mannitol (× 945). d. Frozen section of the same sac as c (× 600).

The relation between gut fluid uptake and epithelial cell size

When the dimensions of epithelial cells of everted sacs were estimated, it was found that there was a significant relation between cell length and gut fluid uptake. The correlation coefficient for this relation was 0.8 indicating statistical significance at the $P < 0.05$ level. In contrast, cell width at the nuclear region did not show any significant variation with gut fluid uptake.

From the mean values for the cell lengths and widths in each condition of incubation, a figure for cell volume was calculated on the assumption that the epithelial cell could be represented as a right cylinder. Fig. 3 shows that this calculated cell volume is linearly related to gut fluid uptake. Regression analysis of this relation gave a correlation coefficient of 0.95, indicating a very high degree of statistical significance ($P < 0.001$).

The increase in cell length with gut fluid uptake was consistent with an observation which was made when the tissue from these experiments was examined by electron microscopy. Two representative sections are shown in Fig. 4. Fig. 4a is a section taken from an experiment in which mannitol had been present in the mucosal

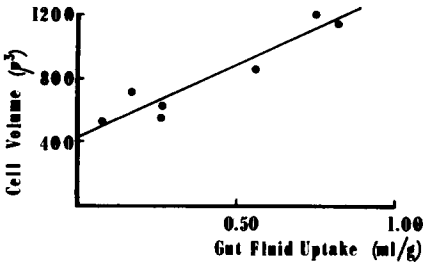

Fig. 3. Relations between cell volume and gut fluid uptake. The points shown are means of between 30 and 60 estimations of cell dimensions for each experiment plotted. The line drawn is that given by regression analysis of the mean points plotted in the figure.

fluid. It can be seen that the intercellular channel follows an extremely tortuous path. In contrast, the tissue shown in Fig. 4b, which was taken from an experiment in which glucose was used to stimulate gut fluid uptake, shows an almost straight intercellular channel, lacking almost completely the convolutions which characterize the channel shown in Fig. 4a.

The distension of the intercellular space, which has been described as characteristic of fluid transport in other epithelia, was not observed in these experiments. Occasionally, small lakes in the intercellular space were observed (see Fig. 4c).

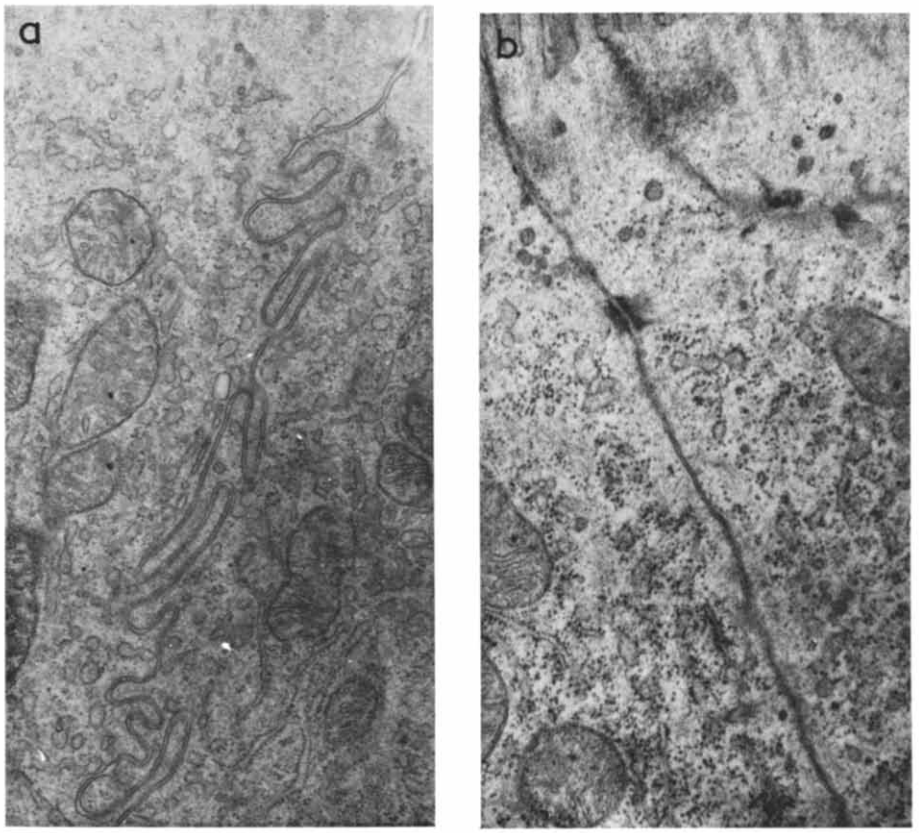

Fig. 4.

These were less than 0.5 μ wide, and their appearance could not be correlated with the fluid transport status of the tissue, since they were seen as frequently in tissue in which fluid transport had been inhibited with mannitol, as in those of tissue in which fluid transport had been stimulated with glucose. Usually the lateral membranes remained closely opposed along their entire length, as shown in Fig. 4d which is taken from tissue incubated in bicarbonate saline with no addition.

The relation of gut fluid uptake and metabolism

The results shown in Table III are taken from experiments designed to illustrate the metabolic dependence of the gut fluid uptake. In order to avoid the possibility of an involvement of active sugar transport in these experiments, the hexose was present initially only in the serosal fluid and phloridzin was added to the mucosal

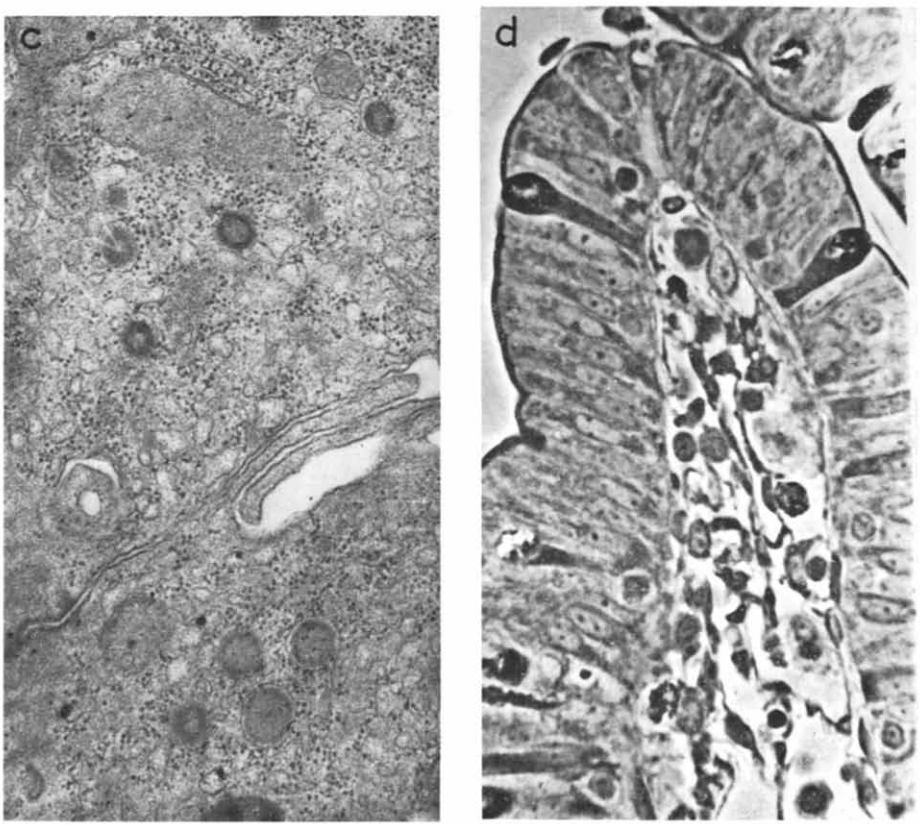

Fig. 4. Ultrastructural changes and fluid transport .a. Electron micrograph of intercellular channel in epithelium of tissue taken from experiment in which 100 mM mannitol was present in mucosal fluid. The intermediate junction region is shown at the upper right of the figure. Note the convolutions of the intercellular channel. (×20 100). b. Electron micrograph of intercellular channel in epithelium of tissue incubated in presence of glucose. Microvilli can be seen at upper left of figure. (×25 700). C. Intercellular lake. Separation of the lateral membranes, such as that shown in the supranuclear region, was occasionally observed, but the observation of this change was not related to the rate of fluid transport. (×25 800). d. Phase contrast micrograph of thick (2 μ) section of epon embedded tissue which had been incubated in bicarbonate saline with no addition. Note general lack of distension of the intercellular channels. (×600).

TABLE III

THE EFFECT OF HEXOSES ON GUT FLUID UPTAKE AND SEROSAL PEG 4000 SPACE

Conditions of incubation as in Table I, but the hexoses (222 mM) were added to the serosal fluid, and 0.5 mM phloridzin was present in mucosal fluid. Results are means ± S.E. and are expressed per g initial wet wt. The number of experiments is given in parentheses.

Hexose added to serosal fluid	*Gut fluid uptake (ml/g)*	*Serosal PEG 4000 space (ml/g)*
Sorbose	0.15 ± 0.01 (5)	0.22 ± 0.01 (5)
Mannose	0.37 ± 0.05 (9)	0.23 ± 0.03 (9)
Fructose	0.47 ± 0.04 (5)	0.21 ± 0.01 (5)
Glucose	0.65 ± 0.03 (5)	0.19 ± 0.03 (5)

fluid. The three hexoses which are metabolized by the intestine, glucose, fructose and mannose, gave greater gut fluid uptakes than did sorbose which is not readily utilized. No variation in serosal PEG 4000 space was observed.

DISCUSSION

Current working hypotheses for intestinal fluid transport are based on a three-compartment model[19] in which the transport of fluid into an intermediate compartment generates a hydrostatic pressure that sustains the flow of fluid across the tissue. The observation that the quantity of fluid accumulated in the gut wall is related to the rate of transport is consistent with the model, and it was clearly of interest to investigate the location of the fluid taken up by the tissue. Previous studies have suggested two possibilities for an extracellular location of the gut fluid uptake: spaces within the sub-epithelial connective tissue[4–6], and the distended lateral intercellular channels which characterize fluid transport in the gall bladder[7,20].

Sub-epithelial spaces

No relation between the serosal PEG 4000 space and the gut fluid uptake was observed in the present experiments. In these experiments, the gut fluid uptake was usually as large as the PEG 4000 space of intestine *in vivo*, and in some experiments considerable greater than this. While it is recognized that the serosal PEG 4000 space may underestimate the sub-epithelial extracellular space[13], it is improbable that PEG 4000 would be totally excluded from an extracellular compartment of the magnitude of the gut fluid uptake. Thus, the serosal PEG 4000 space would be expected to reflect a change in the extracellular space, if the gut fluid uptake occupied an extracellular compartment. The discrepancy between the previous histological observations suggesting the sub-epithelial connective tissue as a location of the gut fluid uptake, and the present functional extracellular space studies, was resolved by the finding that the appearance of the large spaces in the connective tissue was an artifact of the fixation and embedment procedures. Frozen sections of the same tissue showed none of the changes in sub-epithelial structure exhibited by

wax-embedded sections. It was concluded that the studies of serosal PEG 4000 space were substantiated by histological studies, and indicated that the sub-epithelial connective tissue was not the location of the gut fluid uptake.

Intercellular channels

The role of the long intercellular channels between the epithelial cells as the location of the intermediate compartment of the three-compartment model, appears to have been suggested first in the case of the mammalian gall bladder[20,21]. Subsequently, structural studies demonstrated a relation between channel width and the rate of fluid transport in this tissue[7,22,23]. In the case of the intestine less complete evidence is available for such a relation. Although distension of the lateral channels of intestinal epithelium has been noted by several workers[24–27], a detailed analysis relating channel dimensions to fluid transport status, such as that available for the gall bladder[7], appears to be lacking. It is noteworthy that in several cases a distension of the channels has been observed when the intestine contained distilled water[28,29], or an aqueous solution of glucose without salt[24], and in one study in which the comparison was made[28], no distension was observed if an isotonic saline solution was placed in the intestine in place of water.

In the present study distension of the intercellular channels was not observed in any condition. Except for a few cells at the tip of the villus immediately adjacent to the extrusion zone[30] where some separation between lateral membranes was observed, in most cases the lateral membranes of adjacent cells followed a parallel path over most of their length. Although small intercellular lakes were occasionally observed, the appearance of these was not related to the rate of fluid transport. Similar observations have been made on tissue depleted of alkali metal ions[31], and the appearance of these structures may represent deterioration of the tissue. One factor which may contribute to the discrepancy between the gall bladder and intestine, with respect to channel distension, is the distributing of desmosomes. These structures are believed to represent areas of firm binding between adjacent cells[32,33]. In the gall bladder epithelium, desmosomes are rarely seen below the level of the junctional complex at the luminal end of the cells (see, for example ref. 23), but in the case of the intestine the desmosomes appear to be distributed more extensively in the lateral regions of the epithelial cells (see ref. 34 for review). These observations do not indicate that the lateral channel hypothesis is inappropriate to intestinal fluid transport. There is no obvious reason why a compartment which does not expand should not serve as a region of local osmotic equilibration, and the appearance of Na^+ in the lateral channels of intestinal epithelium during fluid transport has been noted[35,36]. But the observations described above are not consistent with the lateral channels as the location of the gut fluid uptake. Even though the channels may not equilibrate with PEG 4000 in the serosal fluid[13], no structural evidence was obtained to suggest that the channels became distended.

Since both extracellular possibilities for the location of the gut fluid uptake could be excluded on the basis of these observations, it was concluded that the gut fluid uptake occupied an intracellular compartment. This conclusion was substantiated by the observation that the size of the epithelial cells increased in direct proportion to the gut fluid uptake over the range studied. Swelling of the epithelial cells was associated with the loss of the plications which characterize the lateral

surfaces when fluid transport was inhibited, and this observation confirmed the suggestion that the lateral folds may allow expansion of cell volume[34].

The mechanism of cell swelling

During incubation *in vitro* the intestine transports fluid from mucosal to serosal sides, and the fluid which is transported is iso-osmotic to that bathing the luminal surface of the tissue[37]. In order to sustain the continuous movement of fluid from mucosal to serosal sides of the tissue, fluid is required to enter the epithelial cell at the luminal surface, and leave at the serosal side. Currently it is considered that the entry process involves diffusion of solute followed by rapid osmotic equilibration of the intracellular compartment, and the exit process is associated with the generation of a standing osmotic gradient in the lateral spaces which provides the driving force for the sustained transport of fluid[6]. If this was the case, a straight line between the gut fluid uptake and the rate of fluid transport would not be expected. If the movement of water and solutes at the luminal side of the epithelial cells was governed by passive forces, a decrease in cell size when the osmolarity of the luminal fluid was increased would be expected and this was observed. But the stimulation of the rate of fluid transport by an increase in the rate of active solute pumping at the lateral membranes would also be expected to decrease cell size. This has been observed in the case of the gall bladder, where inhibition of the rate of solute pumping by cooling or by ouabain was associated with an increase in cell size[7,22]. In the intestine fluid transport can be stimulated by metabolizable substrates[18,38], and in these conditions both cell size (Fig. 3) and gut fluid uptake (Table III) increased. The increased cell size at increased fluid transport rates could be explained by a process of active solute accumulation at the luminal border of the epithelial cells. The restrictions required for this process to transport fluid which is isotonic to the luminal bulk phase are similar to those of isotonic fluid transport in general[21], and a similar geometrical arrangement allowing osmotic equilibration can be suggested. The microvilli of the brush border clearly might provide the narrow channels necessary for the generation of a standing osmotic gradient, and the possibility that these structures may serve this function has been considered previously[7,39]. Although the microvilli are short relative to the length predicted for such a system, the presence of structures within the microvillus core[40,41] may restrict solute diffusion and allow the generation of a standing osmotic gradient. In addition, the contribution of submicrovillus structures, such as the terminal web, to a resistance component restricting movement of solutes out of the microvillus, cannot be discounted.

The suggestion of an osmotically-active solute transfer process, located at the luminal border of the epithelial cells, but independent of organic solute transport, is consistent with the observations of CURRAN *et al.*[42]. This group have shown that organic-solute-independent Na^+ entry at the luminal border may not be explicable in terms of simple diffusion, and have suggested a process involving a specific interaction with a membrane component, which is subject to inhibition by Li^+, and which facilitates the downhill movement of Na^+ into the brush border. The nature of this interaction is not known, but it is clear that a specific process for Na^+ entry at this location would contribute to the generation of a standing osmotic gradient within the microvilli.

In summary, it is suggested that the primary site of osmotic coupling in intes-

tinal fluid transport is the brush border region. Transport of osmotically active solutes at the luminal cell membrane may lead to the generation of a standing osmotic gradient within the microvilli, and to the formation of an intracellular fluid which is isotonic to the luminal bulk phase.

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