

STUDIES ON EPITHELIAL CELLS ISOLATED FROM GUINEA PIG SMALL INTESTINE

E. M. EVANS, J. M. WRIGGLESWORTH, K. BURDETT, and
W. F. R. POVER

From the Department of Biochemistry, The University of Birmingham, Birmingham 15, England. Drs. Evans and Burdett's present address is the Department of Medical Biochemistry, University of Manchester, Manchester 13, England. Dr. Wrigglesworth's present address is the Department of Biochemistry, Chelsea College of Science and Technology, London, S.W.3, England.

ABSTRACT

Sheets of mucosal epithelial cells were released from guinea pig small intestine after incubation with ethylenediaminetetraacetate. Cells in sheets retained their columnar shape for 24 hr at room temperature, and exclusion of nigrosine suggested they had intact plasma membranes. When sheets were disaggregated individual cells had normal morphology for at least 4 hr. During isolation 16% of the total protein and 24% of the total lactic dehydrogenase were lost from the cells, but subsequent enzyme leakage was low. Leakage increased with shaking, incubation at 37°C, or increasing the oxygen tension of the suspending medium, but was minimal when the Na⁺:K⁺ ratio in the medium was 8:1 and the osmolarity was high. Losses of particulate enzyme activities were negligible. Respiration was constant for up to 4 hr and was insensitive to calcium, bicarbonate, oxygen tension, and pH. It was inhibited by cyanide and iodoacetate and varied with the Na⁺:K⁺ ratio of the extracellular fluid and the structural integrity of the cells. All preparations concentrated potassium and excluded sodium, but lost this ability if ouabain was added or cells were broken. Potassium-42 uptake was also sensitive to temperature, ouabain, and structural integrity. The preparations are being used to study cell metabolism in the intestinal epithelium.

INTRODUCTION

If the epithelial cells lining the small intestine could be isolated intact from the underlying tissue their metabolic activities would be more amenable to study. It would then be possible to avoid the assumption that measurements made on mucosal scrapings necessarily represent the activity of the mucosal epithelium which may in fact constitute only a relatively small and variable proportion of the starting material. Various workers have isolated intestinal epithelial cells into suspension (1-7), but have made little or no attempt to show that the cells were functionally intact. With any method of tissue disaggregation there is the possi-

bility of cell damage, so it is essential to demonstrate that isolated cells to be used for metabolic studies have retained their structure and function. Clark and Porteous (8) did assess their preparation and concluded that it consisted of a suspension of epithelial cell ghosts. We (9) and, more recently, Kimmich (10) and Iemhoff et al. (11) have obtained preparations of intestinal epithelial cells which appear to be intact and therefore suitable for metabolic studies. In this report we present the evidence which supports this contention for cells isolated from guinea pig small intestine. As our ultimate aim was to study the metabolism of the

intestinal epithelium, properties which are unique to this tissue were not among the criteria of structure and function used to judge whether the isolated cells were intact.

MATERIALS AND METHODS

Animals

Adult guinea pigs of either sex, weighing between 400 g and 700 g were used. The animals were selected at random from available sources and were not starved before experiments.

Preparation of Cell Suspensions

Animals were killed by neck fracture, the small intestine was quickly excised, and mucus and food residues were washed from the lumen with 0.3 M sucrose. The epithelial cells were then removed from the mucosal surface by a method based on that of Stern and Jensen (5) in which 10 mM ethylenediaminetetraacetate (EDTA)¹ was substituted for citrate in the incubation fluid. The operations described were all performed at room temperature (20–23°C), plastic apparatus was used throughout (6), and each solution was pregassed with O₂:CO₂ (95%:5%) for 10 min. The whole length of the small intestine was used although it was usually divided into four lengths for ease of handling.

One end of each length of intestine was clamped with a pair of Spencer Wells artery forceps and the lumen was filled with a solution containing 96 mM NaCl, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 1.5 mM KCl, 10 mM EDTA; pH 6.8 (5). The free end of the intestine was then clamped and the whole length was incubated for 15 min in 0.3 M sucrose. After incubation, the EDTA solution was drained from the intestine and discarded. The mucosal surface was washed with 0.3 M sucrose and the lumen was then half-filled with sucrose phosphate buffer (see below). The epithelial cells were detached from the villi into the buffered sucrose solution by gently rubbing the intestine between the fingers, and collected by draining into a flask. The suspension was made up to 120 ml with buffered sucrose solution and centrifuged for 2 min at 80 *g*. The supernatant was discarded and the sedimented cells were resuspended in a small volume of the buffered sucrose solution by one slow withdrawal into a 20 ml syringe through a 2 mm-bore needle. The suspension was then diluted until it contained about 10 mg cell protein/ml.

¹ *Abbreviations:* EDTA, ethylenediaminetetraacetate; Q_{O_2} , the current output from the electrode calibrated by using solutions of known oxygen content and expressed as microliters of oxygen taken up per milligram cell protein per hour.

The final preparation of washed cells was usually obtained within 40 min of killing the animal and contained approximately 500 mg protein and 5×10^8 intact epithelial cells. Cells were counted in a standard blood-counting chamber ([Crista] Hawksley Ltd., London) with Neubauer rulings. To make an accurate estimate of cell numbers, large aggregates of cells were first disrupted by withdrawing preparations several times into a syringe through an 0.7 mm-bore needle until all the individual cells were dispersed. The columnar shape and microvillus "brush border" of the epithelial cells easily distinguished them from other types of cells and from cell fragments; only whole cells were counted. Preparations were studied by incident light-field illumination using a Leitz Orthomat microscope (E. Leitz, Inc., New York) and observations were recorded with an automatic camera attachment on Ilford Pan F film (Ilford Ltd., Ilford, England).

Cell suspensions were stained with nigrosine (final concentration 0.5% (12) and differential counts were made of stained and unstained cells. Protein was estimated by a modified biuret method (13).

Suspending Media

Preliminary work indicated that the isolated cells required a medium of comparable ionic strength to buffers such as Krebs, but of higher osmolarity. Of the various buffers used, the most satisfactory was 76 mM Na₂HPO₄, 19 mM KH₂PO₄ (pH 7.4) with the osmotic strength adjusted to 485 ideal mosmoles/liter with 200 mM sucrose. As invertase activity in the isolated cells was high (see below), an equivalent amount of mannitol was substituted for sucrose in those experiments in which no substrate was required. Other modifications were made as follows. Osmotic strength was varied by adjusting the sucrose concentration, and pH by altering the relative amounts of Na₂HPO₄ and KH₂PO₄. As the latter procedure also produced small changes in osmolarity and in the ratio of total Na⁺ to K⁺ ions, these were readjusted to the usual values by additions of sucrose and of NaCl and KCl.

Storage of Cells

Suspensions of isolated cells were maintained in equilibrium with air at room temperature in 500 ml Erlenmeyer flasks. The depth of liquid in the flasks was kept below 2 cm to retain a large surface area for diffusion of atmospheric oxygen. Continual agitation of suspensions was avoided as this was found to cause considerable cell damage. If cells were left without agitation they tended to clump together into large masses, but clumped cells could easily be dispersed before removing a sample, by gently shaking the

flask two or three times by hand. This technique kept the cell suspensions well oxygenated.

Cell Respiration

2-ml samples were withdrawn from the suspension at intervals of 10–15 min, and the rate of oxygen uptake from the extracellular fluid was measured at 37°C by using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The current output from the electrode was calibrated by using solutions of known oxygen content, and results were expressed as microliters of oxygen taken up per milligram cell protein per hour (Q_{O_2}).

Enzyme Assay

The activities of lactic dehydrogenase (EC1.1.1.27) and aldolase (EC4.1.2.7) were determined by using enzyme test combination kits from Boehringer & Soehne (Mannheim, Germany). These are based on the methods of Wróblewski and La Due (14) and Racker (15), respectively. 6-Phosphogluconate dehydrogenase (EC1.1.1.44) was determined by the method of Glock and McLean (16), β -glucuronidase (EC3.2.1.31) according to Gianetto and de Duve (17), and succinic dehydrogenase (EC1.3.99.1) by the method of Pennington (18). To measure invertase activity (EC3.2.1.26), cells were homogenized for 10 min at 4,000 rpm in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), and the homogenate was dialyzed for approximately 16 hr against 200 vol 10 mM Sörenson phosphate buffer, pH 7.0, to remove glucose. Invertase activity in the dialyzed fraction was assayed as described by Hübscher et al. (13).

Bacteriological Investigations

Samples of cells, serially diluted in 0.9% saline, were plated out on a range of nutrient media (Oxoid, Ltd., London, England) and incubated at 37°C for 72 hr. Organisms were identified and counted by usual bacteriological methods. The range of media and conditions of incubation were so chosen that all types of organism likely to occur were cultured.

Uptake of Radioactive Potassium

Potassium movement into the isolated cells was followed by labeling the extracellular fluid with potassium-42 (obtained as an isotonic potassium chloride solution, approximate activity 0.1 mCi/ml, from the Radiochemical Centre, Amersham, England). The labeled cell suspensions (specific activity 6 μ Ci/ml) were shaken very gently in air at room temperature, and 2-ml samples were withdrawn at various times. Cells were sedimented from each

sample by centrifugation at 80 *g* for 2 min, and rapidly washed once with 0.3 M sucrose. The radioactivity of washed cell pellets and of samples of the extracellular fluid was measured in uniform, thin-walled glass tubes by using a crystal scintillation counter (1/2 inch well type with sodium iodide crystal; Ekco Electronics Ltd., Southend-on-Sea, England).

Determination of Extra- and Intracellular Concentrations of Sodium and Potassium

Suspensions of cells in which there were about 25 mg cell protein/ml were incubated at 37°C in weighed tubes for 1 hr. The cells were then sedimented by centrifugation at 80 *g* for 2 min. The supernatant (extracellular fluid) was removed with a Pasteur pipette, taking care not to disturb the surface of the packed cell pellet, and was retained for determination of sodium and potassium. The sides of the tube above the pellet were wiped dry with tissues, and the tube plus contents was weighed. The cells were then homogenized with a small volume of 0.3 M sucrose by repeated rapid withdrawal into a syringe through a needle of bore size 0.3 mm. The homogenate (approximately 6 g) was reweighed and duplicate samples of 2.0 g were digested with 1 ml of concentrated nitric acid for 60 min at 95°C. The digest was cooled to room temperature and diluted for determination of sodium and potassium. Intracellular concentrations of sodium and potassium were calculated by deducting the contribution due to the extracellular Na^+ and K^+ trapped between the packed cells of the pellet from the total measurements for the digest. The intercellular volume of packed cells was measured by using an inulin dilution technique and found to be 0.72 ± 0.06 ml/g of packed cells (mean \pm SD, 7 determinations). Inulin was determined by the method of Schreiner (19), and Na^+ and K^+ were determined by using a flame photometer (Evans Electroelenium Ltd., Halstead, England).

RESULTS

During incubation with the EDTA solution, the epithelium appeared to separate from the mucosal surface (Fig. 1 *a*), but it was not completely detached as few epithelial cells were washed from the intestine with 0.3 M sucrose. When the intestine was filled with the buffered sucrose solution and gently rubbed between the fingers, the epithelium was released into the lumen as large sheets of cells (Fig. 1 *b*). The epithelium was completely stripped away from base to tip of the villi along the whole length of the intestine, so the total number of epithelial cells recovered in the final preparation

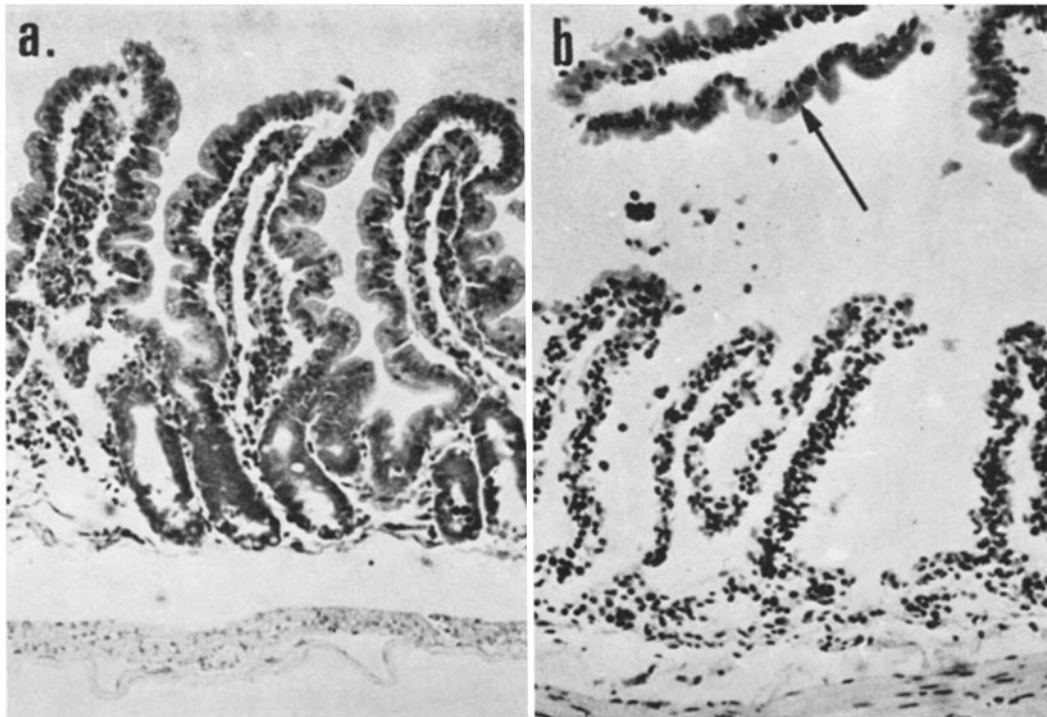


FIGURE 1

FIGURE 1 Guinea pig jejunum after 15 min of incubation with EDTA solution. $\times 40$. (a) Showing separation of epithelial layer from submucosal tissue; (b) after gentle rubbing, showing detachment of epithelial layer (arrow).

possibly represented 100% of those originally present on the villi. In the majority of preparations cells were not removed from the crypts of Lieberkühn.

The material removed from the intestine consisted mostly of large sheets of epithelium together with a few individual cells and some small cell clusters (Fig. 2). The isolated epithelial cells retained a columnar shape with easily identifiable nuclei and brush borders (Fig. 2, *inset*). Small groups and sheets of cells maintained normal morphology for up to 24 hr. In contrast, individual cells isolated from sheets by gently withdrawing the suspension several times into a syringe (see Materials and Methods) retained a normal appearance for up to 4 hr but thereafter began to deteriorate (Fig. 3).

Differential counts were made of stained and unstained cells after nigrosine was added to the suspension; nigrosine will penetrate and stain damaged cells but is excluded by cells with intact plasma membranes (12). Initially about 80% of

the isolated cells were unstained although after 2 hr at 22°C this value had fallen to 50%. Since these measurements could be made only after disaggregation of the cell sheets, they must give a minimum estimate of the number of intact cells in the preparation. Nigrosine did not enter the cells by normal absorptive processes because only about 1% of the cells were found to be stained after washed segments of intestine were incubated with nigrosine solution for 30 min at 37°C.

Respiration

Respiration varied from one preparation to another but there was no correlation between the initial Q_{O_2} value and the length of time for which it was maintained. The rate of respiration was constant for at least 2 hr (Fig. 4), and the results from 16 preparations of cells showed that 50% still maintained their original Q_{O_2} value after 200 min. When cells were suspended in the mannitol phosphate buffer their rate of respiration was slightly



FIGURE 2

FIGURE 2 A preparation of epithelial cells. Most material is in the form of large sheets of cells but several small clusters can be seen. $\times 100$. Inset ($\times 250$)—row of epithelial cells showing nuclei and surface microvilli.

lower than that in the sucrose phosphate buffer although it was maintained for the same length of time (Fig. 4). Respiration was completely inhibited by cyanide (1.5 mM) and was reduced by approximately 23% in the presence of iodoacetate (10^{-6} M).

The respiratory activity of cell suspensions was remarkably insensitive to several environmental changes. Cells were normally kept in equilibrium with air, though samples maintained anaerobically or at high oxygen tension (95% O_2) for 3 hr and then reequilibrated with air, respired at the same rate as controls. Respiration was independent of the extracellular pH within the range 4.5–10.0. Addition of bicarbonate (10 mM) did not change the respiration rate within 3 hr (20) and the Q_{O_2} values for cells suspended in the sucrose phosphate buffer were the same as those for cells suspended in Krebs-bicarbonate buffer (Fig. 4). Respiration was also unaffected by the addition of low concentrations of calcium to the suspensions (up to 0.6

mM calcium chloride), in contrast to cell homogenates or isolated mitochondria (21).

Cell respiration was, however, found to be sensitive to temperature, the ratio of Na^+ to K^+ ions in the extracellular fluid, and the structural integrity of the cells. A $Na^+ : K^+$ ratio of 8:1 not only gave high initial Q_{O_2} values, but these were maintained for at least 2 hr. Respiration was also optimal when the osmolarity of the extracellular fluid was about 485 ideal mosmoles/liter. In solutions of low osmolarity or when the cells were deliberately damaged by agitation or homogenization, Q_{O_2} values always decreased.

Invertase Activity

The activity of invertase in the cell suspensions was sufficient to produce many times more glucose from the only available exogenous substrate, sucrose, than would be required to support the observed rates of respiration. The specific activity

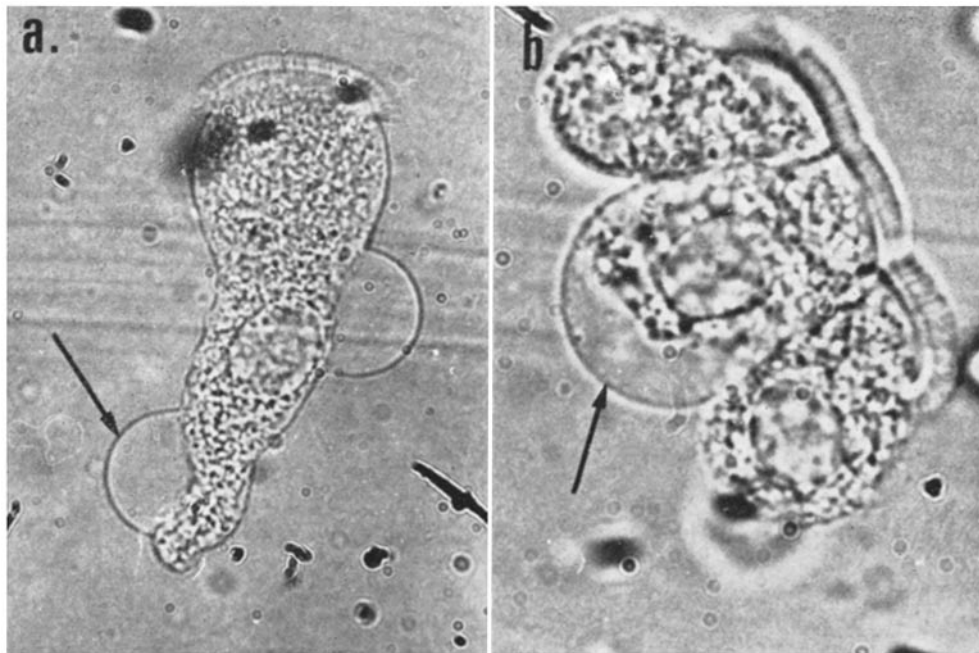


FIGURE 3

Individual epithelial cells 5 hr after isolation. Localized swellings (arrows) can be seen on the lateral (a) and basal edges (b) of the cells. Such swellings were only noted after cell sheets were disaggregated by passage through a syringe. $\times 500$.

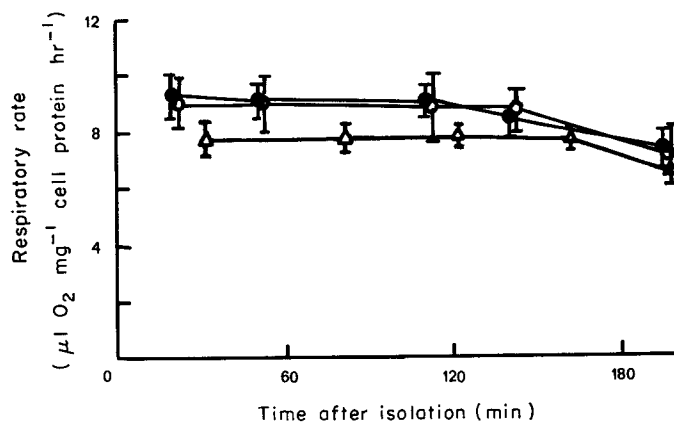


FIGURE 4 Change in respiratory activity of epithelial cell suspensions with time after isolation. Oxygen uptake was monitored at 37°C at an approximate cell protein concentration of 10 mg/ml . (●) cells suspended in phosphate-buffered sucrose solution ($76\text{ mM Na}_2\text{HPO}_4$; $19\text{ mM KH}_2\text{PO}_4$, 200 mM sucrose) at pH 7.4. (○) cells suspended in Krebs-Ringer bicarbonate solution (23) at pH 7.4. (△) cells suspended in phosphate-buffered mannitol solution ($76\text{ mM Na}_2\text{HPO}_4$, $19\text{ mM KH}_2\text{PO}_4$, 200 mM mannitol; pH 7.4).

was 3.29 ± 0.11 μmoles glucose produced/mg cell protein per 15 min (mean $\pm\text{SEM}$, five experiments) compared with a value of 1.93 ± 0.22 (mean $\pm\text{SEM}$, five experiments) for homogenates of mucosal scrapings.

Bacteriological Study

In addition to the 5×10^8 epithelial cells in a preparation from the whole length of small intestine there were usually about 3×10^7 viable

microorganisms. However, the following experiment showed that these did not contribute to the Q_{O_2} values. The total numbers of epithelial cells and viable microorganisms in a freshly prepared cell suspension were estimated, and the oxygen uptake rate was measured. One portion of the suspension was then withdrawn as a control and the remainder was homogenized in a Waring Blender until all the epithelial cells were completely degraded (45 min at 4,000 rpm); the total numbers of viable microorganisms in the homogenate remained the same as in the untreated sample. Respiration measurements showed that the untreated sample continued to take up oxygen whereas there was no oxygen uptake by the homogenate.

Loss of Intracellular Material

No protein or intracellular enzyme activity could be measured in the buffered EDTA solution used to release the epithelial cells from the villi. However, when cells were first detached into suspension there was some leakage of those enzymes found in the particle-free fraction of tissue homogenates. In Table I, the initial loss into the extracellular fluid of three of these enzymes is compared with that of cell protein and with that of the enzymes β -glucuronidase (lysosomal) and succinic dehydrogenase (mitochondrial). The extracellular fluid was discarded during the washing of the isolated cells (Materials and Methods).

The rate of appearance of lactic dehydrogenase activity in the extracellular fluid when washed cells were resuspended and maintained at room temperature is shown in Fig. 5. In suspensions which were not agitated, most of the enzyme that leaked from the cells did so during the first 2 hr; after 5 hr about 80% of the total initial activity was still retained. The rate of enzyme leakage increased if cells were continually shaken, even gently (Fig. 5), and when cells were incubated at 37°C or gassed continually with $O_2:CO_2$ (95%:5%) the rate was approximately double that for cells kept in equilibrium with air at room temperature. Although the enzyme loss varied with these experimental conditions, for different enzymes it was always in the order 6-phosphogluconate dehydrogenase > lactic dehydrogenase > aldolase.

The rate of leakage was affected by the ratio of Na^+ to K^+ ions in the extracellular fluid and was minimal when this was 8:1. The results in Table II show that the composition and osmolarity of the

TABLE I
Loss of Intracellular Material during Preparation of Epithelial Cells

Material	Loss* into extracellular fluid
Protein	16 ± 2
6-Phosphogluconate dehydrogenase	33 ± 12
Lactic dehydrogenase	24 ± 5
Aldolase	21 ± 7
β -Glucuronidase	2 ± 2
Succinic dehydrogenase	3 ± 1

Enzyme and protein were measured in the phosphate-buffered sucrose solution used to flush out the cells from the intestinal segments.

* Expressed as percentage of the total activity of the enzymes in freshly prepared suspensions of cells (mean \pm SD, three determinations).

cell suspending medium influenced the rate of appearance of lactic dehydrogenase activity in the extracellular fluid. In order to emphasize differences in leakage these measurements were made on suspensions which were gently agitated and continually gassed with $O_2:CO_2$ (95%:5%). After 1 hr of incubation most enzyme was retained by cells suspended in either the phosphate-buffered sucrose solution or Krebs-bicarbonate buffer (23) adjusted with sucrose to 485 ideal mosmoles/liter. If the osmolarity of either of these buffers was reduced, or if calcium ions were omitted from the Krebs buffer, enzyme loss greatly increased.

Ion Permeability Studies

After 1 hr at 37°C, the intracellular concentration of sodium was lower and that of potassium higher than their concentrations in the extracellular fluid (Table III). These ion gradients were not retained when ouabain (0.2 mg/ml) was added to suspensions before incubation. The ability to maintain Na^+ and K^+ ion gradients was also lost if cells were mechanically disrupted; there was no evidence that either ion was bound by cell fragments obtained by homogenization.

When sucrose in the extracellular fluid was replaced by mannitol, cells still maintained an intracellular sodium concentration lower than that of the solution but the ability to concentrate potassium appeared to be reduced (Table IV). Under these circumstances the energy required for ion

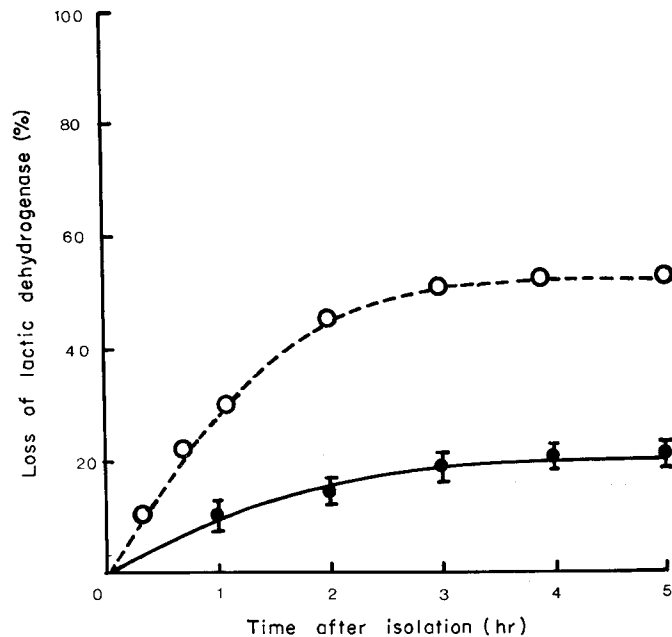


FIGURE 5 Loss of lactic dehydrogenase with time from epithelial cells. Cells were stored in phosphate-buffered sucrose solution at 20°C with (O) and without (●) agitation. Enzyme activity in the supernatant fluid, obtained by centrifuging the cells at 80 *g* for 2 min, is expressed as a percentage of the total activity of the suspension (mean \pm SEM, five experiments).

TABLE II
Leakage of Lactic Dehydrogenase from Cells Suspended in Different Extracellular Fluids

Extracellular fluid	Osmolarity	Enzyme leakage
	<i>ideal mosmoles/liter</i>	
(a) Phosphate-buffered sucrose, pH 7.4 (200 mM sucrose, 76 mM Na ₂ HPO ₄ , 19 mM KH ₂ PO ₄)	485	20 \pm 4
(b) Krebs bicarbonate buffer with added sucrose, pH 7.4	485	23 \pm 3
(c) Krebs bicarbonate buffer, pH 7.4	320	29 \pm 3
(d) Simms buffer, pH 7.3 (reference 22)	320	39 \pm 5
(e) 0.32 M sucrose	320	40 \pm 5
(f) Calcium-free Krebs bicarbonate buffer, pH 7.4	320	45 \pm 5
(g) Sucrose-free phosphate buffer, pH 7.4 (76 mM Na ₂ HPO ₄ , 19 mM KH ₂ PO ₄)	285	45 \pm 5
(h) 0.9% NaCl	300	54 \pm 8

Leakage was measured after 1 hr at room temperature (23°C) and is expressed as a percentage of the total activity in freshly prepared suspensions of cells. Values quoted are mean \pm SD (three experiments). Cells were gassed with O₂:CO₂ (95%:5%) and continually agitated in order to emphasize differences in leakage.

TABLE III
Distributions of Sodium and Potassium in Cell Suspensions

Experiment	Intracellular concentration		Extracellular concentration			
	(Na) _i *	(K) _i	(Na) _o *	(K) _o	(Na) _i /(Na) _o	(K) _i /(K) _o
	<i>mEq/liter</i>		<i>mEq/liter</i>			
1	106 ± 43	40 ± 2	195 ± 5	22 ± 1	0.55 ± 0.24	1.77 ± 0.10
2	77 ± 19	35 ± 1	183 ± 3	22 ± 1	0.42 ± 0.03	1.63 ± 0.03
3	79 ± 6	39 ± 1	177 ± 3	22 ± 1	0.45 ± 0.02	1.75 ± 0.04
+ ouabain (0.2 mg/ml)	184 ± 5	22 ± 1	182 ± 4	22 ± 1	1.01 ± 0.02	1.02 ± 0.01

Measurements were made after incubating cells for 1 hr at 37°C. The extracellular fluid was 200 mM sucrose, 76 mM Na₂HPO₄, 19 mM KH₂PO₄; pH 7.4. Values quoted are mean ± SEM for three samples of cells. Duplicated determinations were performed on each sample.

* Subscripts i and o refer to intracellular and extracellular, respectively.

TABLE IV
Distribution of Sodium and Potassium in Cell Suspensions with Mannitol Replacing Sucrose in the Extracellular Fluid

Experiment	Intracellular concentration		Extracellular concentration			
	(Na) _i *	(K) _i	(Na) _o *	(K) _o	(Na) _i /(Na) _o	(K) _i /(K) _o
	<i>mEq/liter</i>		<i>mEq/liter</i>			
1	90 ± 6	28 ± 5	185 ± 2	21 ± 1	0.49 ± 0.03	1.31 ± 0.09
2	111 ± 6	27 ± 1	190 ± 6	22 ± 1	0.59 ± 0.05	1.23 ± 0.03

Measurements were after 1 hr at 37°C. The composition of the extracellular fluid was 200 mM mannitol, 76 mM Na₂HPO₄, 19 mM KH₂PO₄; pH 7.4. Values quoted are mean ± SEM for three samples of cells. Duplicated determinations were performed on each sample.

* Subscripts i and o refer to intracellular and extracellular, respectively.

transport must have been derived from endogenous substrates (24).

Uptake of Potassium-42

Potassium-42 uptake was measured in untreated samples of cell suspensions, samples treated to 12 nonturbulent strokes in a Potter-Elvehjem homogenizer (gap spacing 200 μ), and samples homogenized for 2 min in a Waring Blendor at 3,000 rpm. The samples were gently shaken in conical flasks at 23°C in air, and 2 ml vol for assay were removed over the first hour after adding the labeled ion. The uptake of potassium-42 into untreated cell suspensions after 1 hr was taken as 100%. The material in the Potter-Elvehjem-treated samples contained 74% ± 10% (mean ± SD, four measurements) whole cells by direct count compared with untreated suspensions. No

whole cells were seen in samples homogenized in the Waring Blendor. The results (Fig. 6) show that the rate of exchange of potassium-42 during the first hour of incubation depended on the number of whole cells present in the preparation. Untreated cells exchanged the labeled ion at an initial rate (first 5 min) approximately 50 times greater than that of cells ruptured by Waring Blendor treatment.

The uptake of potassium-42 into the isolated cells was strongly dependent on temperature (Fig. 7) and was reduced by 84% in the presence of ouabain (0.1 mg/ml).

DISCUSSION

In order to study the integrated metabolism of the epithelial cell we needed a preparation in which there were large numbers of epithelial cells undamaged by the isolation procedure and uncon-

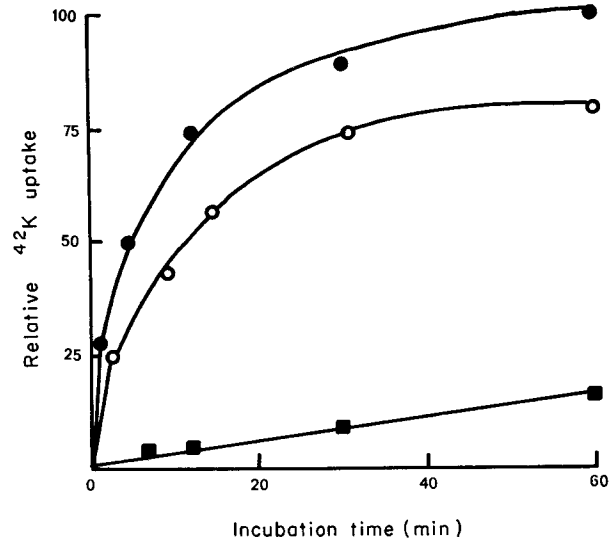


FIGURE 6 Uptake of potassium-42 into isolated epithelial cells. The cells were suspended in phosphate-buffered sucrose medium containing approximately $6 \mu\text{Ci/ml}$ of ^{42}K and incubated at 23°C in equilibrium with air. (●) untreated preparation; (○) preparation treated to 12 nonturbulent strokes in a Potter-Elvehjem homogenizer; (■) preparation treated to 2 min in a Waring Blender.

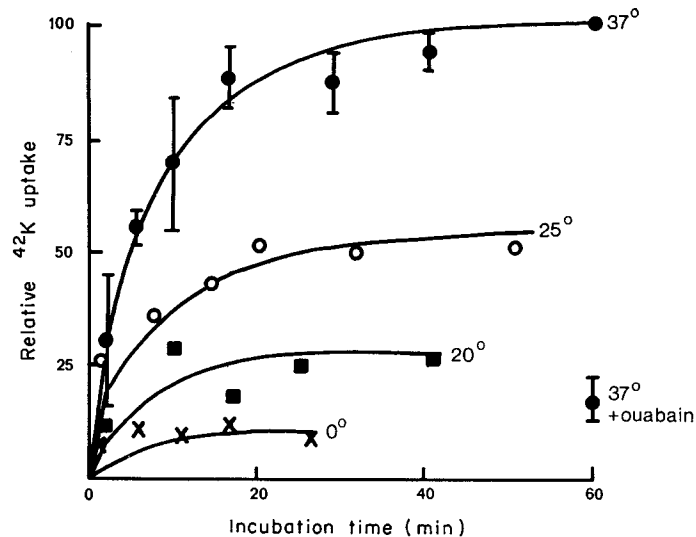


FIGURE 7 Temperature dependence of ^{42}K uptake into isolated epithelial cells. The cells were suspended in phosphate-buffered sucrose solution containing approximately $6 \mu\text{Ci/ml}$ of ^{42}K and incubated at the appropriate temperature in equilibrium with air. (●) 37°C ; (○) 25°C ; (■) 20°C ; (×) 0°C . The ^{42}K uptake after 1 hr at 37°C is expressed as 100%.

taminated with connective tissue cells or mucus. In the present study, guinea pig was chosen as the experimental animal because the intestinal mucosa of this species is robust, it secretes comparatively small amounts of mucus (25), and it seems to be

less susceptible to autolysis than that of the rat (26).

The integrity of isolated cells cannot be assessed merely by measurement of metabolic activity. Similarly, studies of morphology give no indica-

tion of the functional capacities of the cells. A comprehensive assessment of integrity can only be made by considering collectively the results of morphological and metabolic studies. In this investigation the yield of epithelial cells removed from guinea pig small intestine by the action of EDTA was large, and the cells maintained typical morphology for at least 4 hr as judged by light microscopy. Although studies with nigrosine stain suggested that initially about 20% of the isolated cells were damaged, it is likely that this resulted from the disaggregation of cell sheets. The cells retained lower sodium concentrations and higher potassium concentrations than those of the suspending medium, lost only small amounts of their soluble enzymes and had a respiratory activity characteristic of intact cells. Since the use of these preparations as a pure source of metabolically active epithelial cells depends on a detailed knowledge of their functional state, a closer consideration of some of these properties is given below.

Respiration

Every preparation of cells maintained considerable respiratory activity for 2–4 hr after isolation. Although respiration always decreased when cells were damaged, the eventual decrease shown by all preparations may not have been due to a breakdown of cell structure, but to depletion of some metabolic intermediate. If this was so, it may be possible to extend the period of respiratory activity by suitably supplementing the cell-suspending medium.

The insensitivity of the cells to the extracellular oxygen concentration was in marked contrast to the usually accepted view that it is essential to maintain the supply of oxygen to preparations of intestine *in vitro* (27). More recently, however, the respiration of a number of other tissue preparations has been found to remain unaffected by several hours of anaerobiosis (28). It may well be therefore that, at least for experiments of short duration, a continuous supply of oxygen is not critical. Furthermore, for normal storage purposes equilibration with air is sufficient to keep the cell suspensions oxygenated. The insensitivity of cell respiration to changes in pH and to the concentration of bicarbonate in the extracellular fluid provided additional evidence that the isolated cells could maintain their internal milieu despite environmental changes. These observations con-

trast with the findings that the respiratory activity of many isolated cells is critically dependent on the composition of the extracellular fluid (29–31), and may be an indication of structural integrity.

Respiratory activity varied with the ratio of $\text{Na}^+:\text{K}^+$ ions in the extracellular fluid and was maximal at a ratio of 8:1 (see also reference 31), a ratio that has been observed to give optimal rates for glucose absorption by perfused guinea pig intestine *in vitro* (32).

Bacteriological Study

As the intestinal lumen was washed thoroughly before isolating the epithelial cells, any bacteria remaining in the preparations must be those closely associated with the brush border (33–35). Animals were not routinely starved before experiments to reduce the bacterial count (5) as starvation is known to cause changes in metabolism. Bacterial contamination was not reduced by administration of antibiotics and therefore remained a feature of the cell suspensions. However, preparations of epithelial cells with associated bacteria probably approximate closely the state of the epithelial cell *in vivo* and, despite their presence, the contribution of bacteria to measurements of respiration was undetectable.

Leakage of Enzymes

Leakage of enzymes from the cells only began when the epithelium was completely detached from the villous cores, which suggested that the plasma membrane around the lateral and basal aspects of the cells is more fragile than that at the brush border. Leakage of lactic dehydrogenase from undisturbed cells was initially rapid but soon fell almost to zero. The loss of enzymes may have been due to the complete breakdown of a small number of fragile cells, such as those originally at the tips of the villi. Alternatively, all of the isolated cells may have lost a small proportion of their enzyme complement. It is also possible that passage of enzymes through the plasma membrane is a normal function in intestinal epithelial cells.

Extensive leakage of intracellular material would limit the usefulness of isolated cells for the study of metabolism. In the present investigation the leakage of soluble enzymes was very much lower than that from epithelial cells isolated from rat intestine (8). Kimmich (10) and Icmhoff

et al. (11) did not measure leakage of soluble enzymes, but the fact that their preparations showed considerable glycolytic activity implies that leakage was not great. In none of our preparations was the loss of enzymes from the cells sufficient to prevent the retention of considerable respiratory activity.

The consistent differences observed within any one experiment between the rates of leakage of individual enzymes may either reflect compartmentation of the enzymes within the cell cytoplasm, or be an indication of the size of "holes" in the plasma membrane. Differential enzyme leakage has also been observed from tumor cells (36).

Ion Permeability

In all the cell preparations studied the intracellular sodium concentration was considerably lower than the concentration of sodium in the extracellular fluid. The concentration gradients for sodium ions were similar to those described by Schultz et al. (24) for isolated sheets of rabbit intestinal mucosa. Although many of the cells also retained the ability to move potassium inwards against a concentration gradient, the gradients established for this ion were considerably lower than those reported for sheets of rabbit mucosa (24); and in a few preparations there was practically no potassium gradient. The observations suggest that in intestinal epithelial cells the systems responsible for maintaining high intracellular potassium concentrations may be more labile than those for sodium. In view of the large number of factors reported to be important in controlling potassium levels in intestinal mucosa (37), reasons for this cannot yet be given. The observations are interesting, however, in relation to the vectorial pumping of sodium and potassium ions by the Na^+/K^+ -stimulated adenosine triphosphatase of the cell surface (38). A site which is activated from the outside of the cell (K^+) would be more susceptible to chemical and physical damage during the cell isolation procedure than one which is activated from inside the cell (Na^+).

The studies with cell homogenates indicate that the measured ion gradients were functions of the intact cell. The temperature dependence and the sensitivity of the ion gradients to the presence of ouabain confirm that in the isolated cells there is integration of intracellular metabolism with

energy-requiring, membrane transport processes. The preparations are therefore suitable for studying both whole-cell metabolism and transport, particularly the transport of substances which are either accumulated, e.g. 3-O-methyl glucose (39), or chemically modified within the cell, e.g., the esterification of absorbed fatty acids. Ideally, metabolic experiments should be completed in as short a time as possible after removal of cells from the intestine, although our results indicate that cells isolated as described above could be expected to retain a normal functional state for at least 2 hr after isolation.

We wish to thank Mrs. B. Bartlett for the bacteriological investigations.

J. M. Wrigglesworth and K. Burdett were NATO/SRC and ICI Research Fellows, respectively. E. M. Evans held an MRC Research Studentship.

Received for publication 21 December 1970, and in revised form 7 June 1971.

REFERENCES

1. DALTON, A. J., and M. D. FELIX. 1953. Studies on the Golgi substance of the epithelial cells of the epididymis and duodenum of the mouse. *Amer. J. Anat.* **92**:277.
2. MATEYKO, G. M., and M. J. KOPAC. 1963. Cytophysical studies on living normal and neoplastic cells. Part I: separation into isolated populations. *Ann. N. Y. Acad. Sci.* **105**:183.
3. HUANG, K. C. 1965. Uptake of L-tyrosine and 3-O-methyl glucose by isolated intestinal epithelial cells. *Life Sci.* **4**:1201.
4. FERRIS, A. D. 1966. Isolation of the epithelial cells of the rat small intestine. *Can. J. Biochem.* **44**:687.
5. STERN, B. R., and W. E. JENSEN. 1966. Active transport of glucose by suspensions of isolated rat intestinal epithelial cells. *Nature (London)*. **209**:789.
6. SJÖSTRAND, F. S. 1968. A simple and rapid method to prepare dispersions of columnar epithelial cells from the rat intestine. *J. Ultrastruct. Res.* **22**:424.
7. HARRISON, D. D., and H. L. WEBSTER. 1969. The preparation of isolated intestinal crypt cells. *Exp. Cell Res.* **55**:257.
8. CLARK, B., and J. W. PORTEOUS. 1965. The isolation and properties of epithelial-cell "ghosts" from rat small intestine. *Biochem. J.* **96**:539.
9. EVANS, E. M. 1969. Studies on isolated intestinal epithelial cells. Ph.D. Thesis. University of Birmingham, Birmingham, England.

10. KIMMICH, G. A. 1970. Preparation and properties of mucosal epithelial cells isolated from small intestine of the chicken. *Biochemistry*. **9**:3659.
11. IEMHOFF, W. G. J., J. W. O. VAN DEN BERG, A. M. DE PIJPER, and W. C. HULSMANN. 1970. Metabolic aspects of isolated cells from rat small intestinal epithelium. *Biochim. Biophys. Acta*. **215**:229.
12. KALTENBACH, J. P., M. H. KALTENBACH, and W. P. LYONS. 1958. Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell Res.* **15**:112.
13. HÜBSCHER, G., G. R. WEST, and D. N. BRINDLEY. 1965. Studies on the fractionation of mucosal homogenates from the small intestine. *Biochem. J.* **97**:629.
14. WRÓBLEWSKI, F., and J. S. LA DUE. 1955. Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med.* **90**:210.
15. RACKER, E. 1947. Spectrophotometric measurement of hexokinase and phosphohexokinase activity. *J. Biol. Chem.* **167**:843.
16. GLOCK, G. E., and P. MCLEAN. 1953. Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. *Biochem. J.* **55**:400.
17. GIANETTO, R., and C. DE DUVE. 1955. Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β -glucuronidase and cathepsin by rat liver particles. *Biochem. J.* **59**:433.
18. PENNINGTON, R. J. 1961. Biochemistry of dystrophic muscle. Mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem. J.* **80**:649.
19. SCHREINER, G. E. 1950. Determination of inulin by means of resorcinol. *Proc. Soc. Exp. Biol. Med.* **74**:117.
20. WILLMER, E. N. 1965. *In Cells and Tissues in Culture*. Academic Press Inc., New York. **1**:261.
21. CHANGE, B. 1965. The energy-linked reaction of calcium with mitochondria. *J. Biol. Chem.* **240**:2729.
22. SIMMS, H. S., and M. SAUNDERS. 1942. Use of serum ultra-filtrate in tissue cultures for studying deposition of fat and for propagation of viruses. *Arch. Pathol.* **33**:619.
23. DAWSON, R. M. C., D. C. ELLIOTT, W. H. ELLIOTT, and K. M. JONES. 1962. *In Data for Biochemical Research*. Oxford University Press, Inc., London. **277**.
24. SCHULTZ, S. G., R. E. FUISZ, and P. F. CURRAN. 1966. Amino acid and sugar transport in rabbit ileum. *J. Gen. Physiol.* **49**:849.
25. WRIGGLESWORTH, J. M. 1965. Lysosomes and radiation damage to the small intestine. Ph.D. Thesis. University of Birmingham, Birmingham, England.
26. SHERRATT, H. S. A., and G. HÜBSCHER. 1963. Properties of mitochondrial preparations from the small intestinal mucosa of the guinea pig. *Biochim. Biophys. Acta*. **69**:403.
27. FISHER, R. B., and D. S. PARSONS. 1949. A preparation of surviving rat small intestine for the study of absorption. *J. Physiol. (London)*. **110**:36.
28. ROCHMAN, H., P. B. CLARK, H. G. LATHE, and F. M. PARSONS. 1967. The effect of temperature and anoxia of rat-kidney slices on their subsequent respiration. *Biochem. J.* **102**:44.
29. BERRY, M. N. 1962. Metabolic properties of cells isolated from adult mouse liver. *J. Cell Biol.* **15**:1.
30. EXTON, J. H. 1964. Metabolism of rat liver cell suspensions. 1. General properties of isolated cells and occurrence of the citric acid cycle. *Biochem. J.* **92**:457.
31. STERN, B. K., and R. W. REILLY. 1965. Some characteristics of the respiratory metabolism of suspensions of rat intestinal epithelial cells. *Nature (London)*. **205**:563.
32. RIKLIS, E., and J. H. QUASTEL. 1958. Effects of cations on sugar absorption by isolated surviving guinea pig intestine. *Can. J. Biochem. Physiol.* **36**:347.
33. REIMANN, H. A. 1965. Microbic phagocytosis by enteric epithelial cells. *J. Amer. Med. Ass.* **192**:1130.
34. HAMPTON, J. C., and B. ROSARIO. 1965. The attachment of microorganisms to epithelial cells in the distal ileum of the mouse. *Lab. Invest.* **14**:1464.
35. SCHAEGLER, R., R. DUBOS, and R. COSTELLO. 1965. Association of germfree mice with bacteria isolated from normal mice. *J. Exp. Med.* **122**:77.
36. WU, R., and E. RACKER. 1958. Leakage of enzymes from ascites tumour cells. *Fed. Proc.* **17**:339.
37. BROWN, M. M., and D. S. PARSONS. 1962. Observations on the changes in the potassium content of rat jejunal mucosa during absorption. *Biochim. Biophys. Acta*. **59**:249.
38. SKOU, J. C. 1965. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.* **45**:596.
39. KIMMICH, G. A. 1970. Active sugar accumulation by isolated intestinal epithelial cells. A new model for sodium-dependent metabolite transport. *Biochemistry*. **9**:3669.