Preparation and Properties of Mucosal Epithelial Cells Isolated from Small Intestine of the Chicken*

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ABSTRACT: A procedure has been devised for preparing suspensions of isolated intestinal epithelial cells in high yield and with good retention of morphological, metabolic, and transport properties. Photomicrographs show the cells are structurally intact and retain the brush borders typical of intestinal cells *in vivo*. The isolated cells produce lactic acid and carbon dioxide from glucose at a linear rate for at least a 2-hr interval, and retain sensitivity to oligomycin and dinitrophenol indicating normal energy conservation processes have been maintained. Galactose and valine are accumulated against concentration gradients by a process which is inhibited by dinitrophenol, ouabain, and oligomycin. Galactose transport is completely inhibited by phloridzin, but valine accumulation is unchanged or slightly stimulated. Once metabolite has been accumulated, any of the inhibitors cause a discharge by the cells which proceeds until concentration in the intracellular space is equal to that in the bathing medium. At substrate concentrations of 1.25 mM, gradients from 4- to 8-fold can normally be generated. Gradients of 30-fold have been produced at lower substrate levels. K_m for galactose and valine are 2.5 and 4.2 mM, respectively, in good agreement with values reported for everted sacs. V_{max}, however, are significantly higher than data reported for sacs. Reasons for these differences are discussed.

The multiple transport processes carried out by the mucosal epithelial cells of the small intestine have long been recognized and appreciated by biochemists and physiologists alike. This awareness has led to an enormous volume of research and commentary on various aspects of the intestinal transport machinery, and many of these investigations have been most fruitful in revealing new facets of biological transport (for reviews, see Wilson, 1962, Crane, 1960b, 1965, 1968, and Wiseman, 1968). Furthermore, it is becoming increasingly apparent that active intestinal transport may be quite similar to that in cells originating from tissues other than intestine and much recent work focuses on that possibility (Schultz and Curran, 1969; Koser and Christensen, 1968; Kleinzeller *et al.*, 1967; Eddy, 1968; Vidaver, 1964). One might expect, then, that the methods available for the study of biochemical transport phenomena might be best developed for intestine, the tissue which has generated the majority of mammalian active transport studies.

In this regard, it is somewhat surprising that most of these studies have been performed with systems which yield rather indirect information about the transfer step in which metabolite moves from a compartment of low concentration to one with high concentration, *i.e.*, movement across the plasma membrane barrier of the epithelial cell. Instead, transport is normally evaluated in tissue preparations with multiple cell types in which metabolite accumulation is a composite of active transfer and subsequent diffusion to underlying cell layers (Wilson and Wiseman, 1954; Fisher and Parsons, 1949; Crane and Wilson, 1958; Crane and Mandelstam, 1960). Diffusional entry and diffusion following active entry cause considerable ambiguity in interpreting the nature of the active transport process. In addition, intracellular volume of only the epithelial cell population is impossible to accurately determine in intact tissue preparations. As a result, intracellular metabolite concentrations and concentration gradients established by the epithelial cells are frequently poorly estimated.

Because of these limitations an alternative system for more direct evaluation of the transport mechanism is desirable. One especially attractive approach is through the use of isolated epithelial cell preparations which can be handled as homogeneous suspensions. This paper is a report of one method which produces cells in high yield which maintain their ability to actively transport sugars, amino acids, and monovalent ions. The succeeding paper is a more detailed consideration of active sugar accumulation by the cells and the role of sodium in that process.

Methods

Materials. Male white Leghorn chickens from 1- to 6-weeks-old and obtained from Babcock Poultry Farms, Ithaca, N. Y., were used for all cell preparations. Hyaluronidase (type I) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. All other reagents and salts were obtained from commercial suppliers and were of reagent grade quality. The standard incubation medium contained 120 mM NaCl, 20 mM Tris-Cl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mg/ml of BSA.[1] Isolation media contained 1 mg/ml of hyaluronidase in addition to the above components. In some cases, noted in the

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[1] Abbreviations used are: BSA, bovine serum albumin; DNP, dinitrophenol; PPO, 2,5-diphenyloxazole; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; 3-OMG, 3-*O*-methylglucose; Gal, galactose.

text, 10 mg/ml of BSA was included instead of the usual amount, or 240 mM mannitol was used in place of NaCl. All experiments were performed in siliconized glass vessels or in plastic lab ware to prevent damage to the isolated cells.

Preparation of Epithelial Cell Suspensions. Epithelial cells were prepared from the small intestine of 1–6-week-old chickens by a method similar to that used by Perris (1966) on rat intestine. The chickens were sacrificed by decapitation, the abdominal cavity was opened, and the entire small intestine removed as quickly as possible by carefully cutting the supporting mesentery. The excised intestine was carefully trimmed of adhering pancreatic tissue and the luminal contents flushed out with 10 ml of ice-cold 150 mM NaCl with the aid of a syringe. The intestine was then cut into segments about 1.5 in. in length and each segment slit lengthwise with fine scissors so that flat sheets of intestinal tissue were produced. These sheets were placed in a 50-ml polyethylene beaker containing 20.0 ml of isolation media. The beakers were then incubated at 37° for 30 min with moderate shaking (100 cycles/min) in a Dubnoff-type thermostated water bath. At the end of this time, some cells normally have sloughed off spontaneously, but large numbers can then be removed by agitation of the segments with the tip of a plastic pipet. The suspension produced by the mechanical agitation is then poured through nylon stocking material to remove intact intestinal tissue and further disperse the cell population. This cell filtrate is poured into 15 ml of polyethylene centrifuge tubes and centrifuged at 100*g* for 1 min to sediment the intact cells. After removing the supernatant the pellet is resuspended in 5 ml of incubation media by sucking media into a 1-ml plastic pipet and gently blowing onto the surface of the pellet. After the pellet is completely dispersed, more media is added to bring the total volume to 15 ml. The suspension is again centrifuged for 1 min at 100*g*. This washing procedure is normally repeated once or twice to completely remove hyaluronidase and until the cells sediment rapidly and uniformly, leaving a minimum of debris in the supernatant. After the final wash, the cell pellet volume is measured and the cells are suspended in 5–10 volumes of fresh media. This suspension is ready for experimental use. All steps following the incubation with hyaluronidase are performed at ice temperature.

Normally a 4–6-week-old chicken will yield 2–3 ml of packed epithelial cells. Younger chickens produce somewhat smaller yields, but the cell properties are identical with those described in the text. Often, only two-thirds of the small intestine proximal to the stomach was used. Dry weights were determined by drying 1.0 ml of cell suspension overnight at 65° and substracting the weight of the salts in an identical quantity of medium. Cell protein was determined by the biuret method (Layne, 1957).

Some experiments were performed with intact rings of intestinal tissue in order to compare properties of cells before and after isolation. In these cases a segment of intestine which had been flushed of its luminal contents was laid on a glass plate and cut in transverse sections 1–2 mm in width with a fine scissor or razor blade. The sections were randomized by swirling in a large beaker at 0° and the experiments performed in the usual incubation medium using four sections per vessel.

INTRACELLULAR SPACE. Measurements of intracellular space were performed by classical techniques in which radioactively labeled solute was added to a cell suspension in a fine bore graduated centrifuge tube and the suspension was centrifuged to sediment the cells. An aliquot of supernatant was counted to determine counts per minute per microliter of media. The supernatant was then withdrawn as completely as possible and the walls of the tubes were wiped free of adhering media with tissue paper. The pellet volume was read, and the pellet suspended in a known volume of nonradioactive media. An aliquot of the resuspended pellet was then counted and the total radioactivity trapped by the pellet was calculated. From this value the microliters of media trapped by the pellet is calculated, and by subtracting that volume from the total pellet volume, the cell volume may be determined.

Cell volume determined in this manner can have different meanings depending on the nature of the labeled solute used. A solute which is totally excluded such as polyethylene glycol (av mol wt 4000) will provide an estimate of total intracellular water volume *plus* the volume of cell solids, plasma membrane, and membranes of cellular organelles. On the other hand, a solute which readily penetrates the cell (but which is not actively accumulated) will provide an estimate only of plasma and organelle membrane volume. By taking the difference in volumes obtained with a penetrating solute as compared with a nonpenetrant, one can calculate the volume of intracellular space (IS). In the experiments reported in this paper, [^{14}C]galactose in the presence of 1 mM DNP is used to provide a penetrating but nonaccumulated solute. The values obtained by this procedure are in good agreement with those calculated by other methods as discussed in the Results and Discussion sections of this and the succeeding paper. Intracellular concentration can readily be calculated using the value obtained for intracellular volume, the value for specific activity of metabolite in the medium, and the number of counts per minute of metabolite taken up by the cells. The following formula was used: mM intracellular concn = (cpm in cells/cpm/mμmole)/μl of IS. Concentration ratios are expressed as concn in cells/concn in media.

METABOLIC AND TRANSPORT ACTIVITY. Measurement of CO_2 production from labeled substrate was performed by methods similar to those used with isolated mitochondria (Kimmich and Rasmussen, 1969). Cell suspension (1 ml) was added to 1.0 ml of the standard incubation media with 2 mM substrate and 0.1 μCi of the corresponding [^{14}C]substrate in a siliconized 25-ml erlenmyer flask containing a center well. A small glass vial containing 0.2 ml of hydroxide of Hyamine was placed on the center well, the flask top was closed with a serum stopper, and the flask was incubated at 37° with gentle shaking (80 cycles/min) in a thermostated water bath. After the proper incubation interval a small amount of 2 N H_2SO_4 was injected into the flask to stop metabolic activity, and the vessels were shaken an additional hour to allow time for complete diffusion of liberated CO_2 to the Hyamine. After this time the Hyamine vial was removed from each flask, dropped directly into toluene-based POPOP–PPO scintillation fluid, and counted for radioactivity in a Picker liquid scintillation spectrometer.

Glycolytic activity was assessed by measuring lactate formation from glucose. One milliliter of cells was incubated in 3.0 ml of standard incubation medium supplemented with 5 mM glucose in 50-ml plastic beakers. Temperature was maintained at 37° and the vessels were shaken at 80

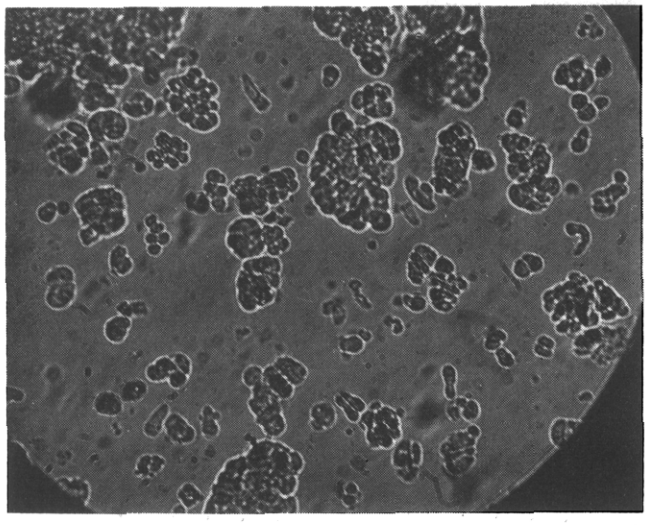

FIGURE 1: Light micrograph (40×) of isolated intestinal epithelial cells suspended in mannitol medium. Use of mannitol helped preserve cell integrity in the relatively anoxic environment beneath the cover slip. See text for details.

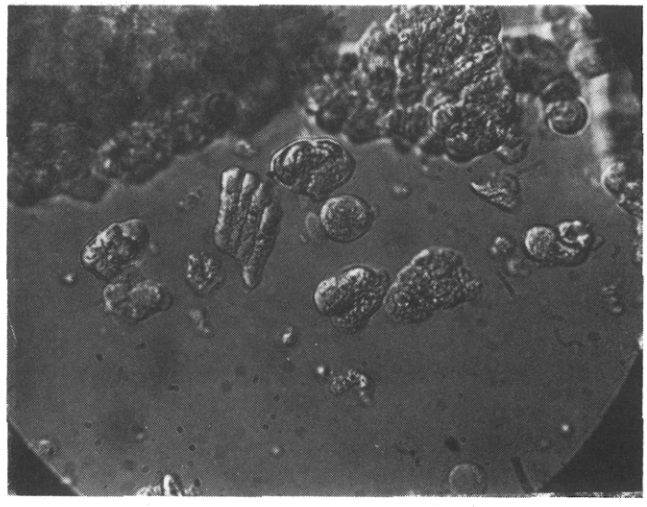

FIGURE 2: Light micrograph (200×) of isolated epithelial cell suspension prepared from chick intestine. The cells were in mannitol medium. Note the columnar shape of cells incompletely freed in contrast to individual cells. Clumping of cells is extensive in the quiescent environment beneath the cover slip.

cycles/min. After the incubation interval the beaker contents were poured into centrifuge tubes containing 1.0 ml of 25% trichloroacetic acid to stop metabolic activity. The protein was sedimented by centrifugation and lactate was determined on an aliquot of the supernatant by the method of Barker and Summerson (1941).

When accumulation of sugars and amino acids was determined, 1.0 ml of cells was added to 3.0 ml of medium containing substrate at the appropriate concentration and 0.5 μCi of [^{14}C]substrate in 50-ml plastic beakers. At intervals, 200-μl samples were removed with the aid of a micropipet and the sample was released on the surface of a Millipore filter (0.65 μ pore size) held on a fritted-glass disk under negative pressure. The cell pellet retained by the filter was washed with 5.0 ml of ice-cold isotonic saline to remove radioactivity adhering to cell surfaces. The initial filtration was complete within 10 sec and the entire process including the wash could be completed in less than 20 sec. Incubation temperature was 37°, and shaking at 100 cycles/min was required in order to maintain adequate oxygenation and uniform suspension and sampling. The filters were placed in an oven at 65° for 10–15 min until completely dried, and then dropped directly into toluene-based scintillation fluid and counted for radioactivity. Counts retained by the filters represent material accumulated by the cells. The white Millipore filter becomes transparent in toluene and quenching is negligible.

Cell viability was estimated by determining the fraction of the population able to exclude 0.2% trypan blue (Girardi *et al.*, 1956). Photomicrographs were made using a Zeiss microscope equipped with interference optics and a Polaroid camera supplied with type 55 positive–negative film packets. No stain was used for any of the micrographs illustrated.

Results

Figures 1–3 are photomicrographs of a typical suspension of isolated cells prepared by the method described earlier. In the presence of calcium there is considerable clumping of small groups of cells as shown (Figure 1, 40×), but even under these conditions the suspension remains easily handled by micropipet with a high degree of accuracy. Multiple samples show a deviation in dry weight of less than 5%. The cells are easily damaged by pressure, so a spacer must be used between the glass slide and cover slip. This prevents obtaining micrographs with large numbers of cells all sharply in focus. In more restricted fields of view possible at higher magnification, sharpness can be considerably improved as shown in Figure 2 (200×). At this magnification it is possible to see the cell brush border which appears as a fan-shaped fibrous structure localized over a small segment of the cell periphery. Cells once freed from the restraints of the intestinal wall in most cases lose their characteristic columnar shape and assume a spherical shape. Cells still joined to neighboring

FIGURE 3: Light micrograph (200×) of an isolated intestinal epithelial cell in mannitol medium. The brush border can be readily seen at the upper edge of the cell. Details are given in the text.

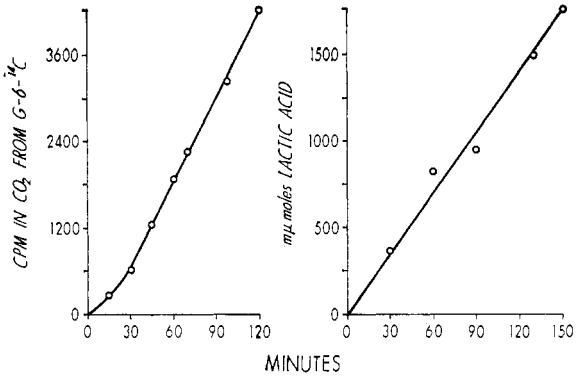

FIGURE 4: Carbon dioxide and lactic acid production from glucose by isolated intestinal cells. An aliquot of cells equal to 10 mg of protein was used in each vessel for CO_2 production, and 2–3 mg of protein/vessel for lactate production. Standard incubation medium was used, but with 10 mg/ml of BSA. Incubation volume, 2.0 ml; temperature, 37°.

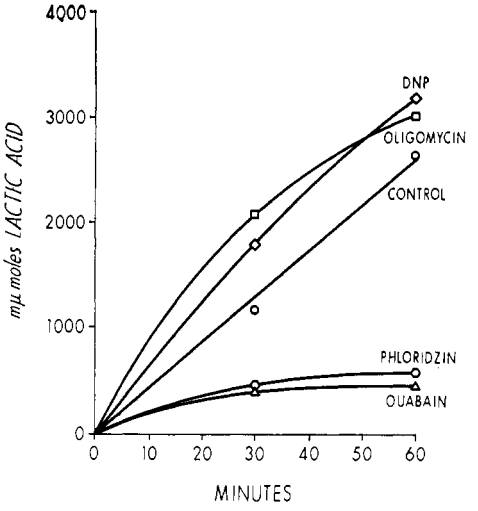

FIGURE 6: Effect of various agents on lactic acid production from glucose by isolated intestinal cells. Each vessel contained cells equivalent to 4.5 mg of protein in a total volume of 2.0 ml. The standard incubation medium was supplemented with 10 mg/ml of BSA. Temperature, 37°.

cells tend to remain columnar, however, as can be seen from the micrograph.

Figure 3 shows a single cell at 200× magnification, in which the striated nature of the brush border is more clearly defined. The morphology of this structure is readily apparent visually under the microscope by focusing on different planes, but somewhat difficult to capture on film due to its low contrast and the necessity of photographing at a single plane of focus. Nevertheless the size and shape of the brush border are readily seen from the photographs. Only those cells oriented so the brush border is at the cell periphery and in the plane of focus for a given aspect of observation appear to possess the structure, so typical preparations have a large number of cells in which none is visible. Figure 3 also indicates that the plasma membrane of the cell is still totally intact following the enzymatic and mechanical treatment which is necessary to free the cells from the mucosal surface of the intestinal wall. Extensive examination of these suspensions reveals that a high percentage of the total population is likewise structurally intact. This fact is borne out by the observation that trypan blue will typically stain only about 20% of the total cell population indicating approximately 80% viability. Typical yields are about 100 mg of cell protein from one small intestine for a 4–6-week-old chicken.

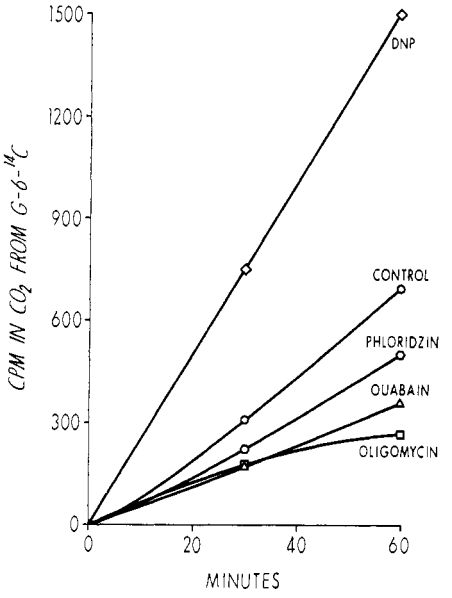

FIGURE 5: Effect of various agents on CO_2 production from glucose by isolated intestinal cells. Each vessel contained cells equivalent to 3.5 mg of protein in a total volume of 2.0 ml. The standard incubation medium was employed supplemented with 10 mg/ml of BSA. Temperature, 37°.

By direct observation then, the isolated cells appear to be structurally intact and possess at least some of the morphological structure characteristic of intestinal epithelia before isolation. In order for such a preparation to be experimentally useful, however, it is necessary to make certain that the metabolic and transport capabilities are the same after isolation as before. This information would add further support to the idea that little damage is incurred by the cells during isolation and that they might be of use for detailed study of the transport mechanism.

Figures 4–6 show the rates of lactate and carbon dioxide production from glucose by the isolated cells and the effects of several agents known to modify metabolic and transport activity in everted intestinal sacs. Note especially that under control conditions both lactate and CO_2 production are linear functions for at least a 2-hr interval. This represents a period of linear metablic activity about three times longer than the best preparations reported elsewhere in the literature (Perris, 1966; Stern and Reilly, 1965). Furthermore stimulation of both processes by DNP indicates that the cells have retained at least a portion of their ability to couple metabolic activity to energy production. The short lag of 5–8 min before CO_2 production becomes linear with time probably represents the time required for the metabolite pool to become uniformly labeled with ^{14}C. When labeled Krebs cycle intermediates are used, which produce CO_2 more directly,

TABLE I: Effect of Various Inhibitors on [^{14}C]CO_2 Production from [1-^{14}C]Glucose by Isolated Intestinal Cells as Compared to Intact Rings of Intestinal Tissue.

Agent Added	Rings		Cells			
			−DNP		+DNP	
	mμmoles of CO_2[a]	Change	mμmoles of CO_2[b]	% Change	mμmoles of CO_2[b]	% Change
	68		52		84	[c]
DNP (200 μM)	130	+94	84	+62		
Oligomycin (5 μg/ml)	47	−31	12	−77	36	−57
Ouabain (100 μM)	40	−41	17	−67	24	−71
Phloridzin (100 μM)	44.5	−35	35	−33	52	−38

[a] Values given are for three rings of tissue approximately 2 mm in width, 1-hr incubation, 37°. [b] Values given are for 1 mg of cell protein, 1-hr incubation, 37°. [c] All per cent changes for a given column refer to changes relative to the first value in that column.

no lag is observed, although again linear rates of CO_2 evolution are maintained for 2 hr or more.

[6-^{14}C]Glucose was used as substrate in the experiment shown for carbon dioxide formation, but analogous results have been obtained when the label is uniformly distributed or in C-1. When [1-^{14}C]glucose is used, [^{14}C]carbon dioxide production is only 5–10% higher indicating that hexose monophosphate shunt activity is lacking or minimal. Those tissues with rapid shunt metabolism produce as much as 20 times more labeled CO_2 from [1-^{14}C]glucose than from glucose labeled in the 6 position (Rognstad and Katz, 1969).

Finally, the severe inhibitory effects of ouabain, oligomycin, and phloridzin on carbon dioxide production are all noteworthy for they indicate again that the cells as isolated retain sensitivity to those agents which inhibit before isolation (Csaky and Hara, 1965; Parsons *et al.*, 1958). It is interesting that lactate production was significantly stimulated by oligomycin. This represents a dissociation of effects between ouabain and oligomycin, both of which inhibit monovalent ion transport completely (Csaky and Hara, 1965; Whittam *et al.*, 1964; Blake *et al.*, 1967).

Normal response of the isolated cells toward inhibitors is further emphasized in Table I which shows that the degree of inhibition of carbon dioxide production is as good for each agent when isolated cells are used as when transverse sections of intestinal tissue were employed. Furthermore, low doses of uncoupler (DNP) were partially effective in overcoming inhibition due to oligomycin as has been observed with isolated mitochondria (Huijing and Slater, 1961). On the other hand, inhibition due to ouabain or phloridzin was unaffected by the uncoupler.

Another attribute which intact cells normally possess is the ability to exclude phosphorylated metabolites from crossing the plasma membrane. This fact offers another way of testing for the integrity of the cell membrane after isolation. If [1-^{14}C]glucose 6-phosphate is unable to penetrate the isolated cells, it should be much less effective in supporting [^{14}C]CO_2 production than [1-^{14}C]glucose, but support it equally well if the membranes are damaged and allow unrestricted entry. Table II shows the cells behave in exactly that way. A suspension of cells formed [^{14}C]CO_2 from the phosphorylated hexose at a rate only 22% as fast as from the free sugar, yet both substrates are metabolized by identical routes with the exception that glucose must undergo an initial phosphorylation to generate glucose 6-phosphate. In fact, to the extent that phosphorylation is the rate-limiting metabolic step, one might expect even more CO_2 from glucose-6-P than from free glucose if both penetrated to the same degree. Furthermore, it is quite possible that some glucose 6-phosphate is hydrolyzed to free glucose by extracellular phosphatases and then enters the cells and is metabolized. The carbon dioxide production from glucose 6-phosphate by direct metabolism may thus be considerably smaller than the rate determined experimentally (22% of the rate from free glucose). Therefore the data again indicate a cell population with 80% or more of the cells retaining normal properties.

Table II also shows that phloridzin, an inhibitor of active intestinal sugar accumulation, inhibits metabolism of both substrates, but that of free glucose to a greater extent. This probably reflects the fact that glucose 6-phosphate does not utilize an active entry system as does glucose. Low concen-

TABLE II: Production of [^{14}C]CO_2 from [1-^{14}C]Glucose and [1-^{14}C]Glucose-6-P by Isolated Intestinal Cells.

	mμmoles of [^{14}C]CO_2/mg of Protein	
	[1-^{14}C]Glu	[1-^{14}C]Glu-6-P
Control	59.2	13.1
100 μM phloridzin	26.2	9.2
200 μM DNP	92.2	23.0
Inhibited by phloridzin (%)	56	30
Stimulated by DNP (%)	56	76
CO_2 from Glu-6-P compared to glucose (%)		22.1

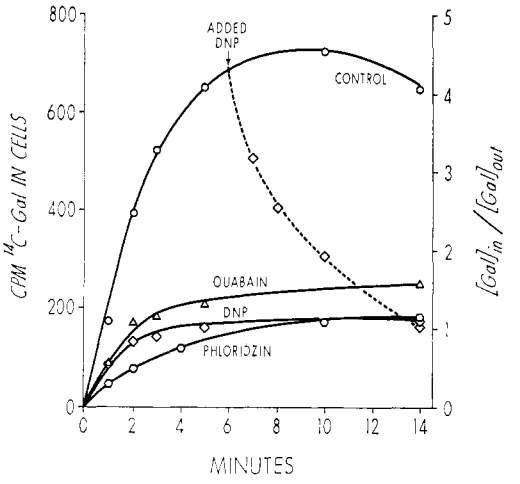

FIGURE 7: Accumulation of [^{14}C]galactose by isolated intestinal epithelia cells, and the effect of several inhibitors. Cells (10 mg of protein) were incubated in the standard medium and 200-μl samples taken at the indicated points. Total incubation volume, 4.0 ml; temperature, 37°.

TABLE III: Metabolism of Various Substrates by Isolated Intestinal Cells.

Substrate	mμmoles of Product Formed/ mg of Protein per Hr	
	Lactate	CO_2
Glucose	277	315
Galactose	10[a]	5.0
3-OMG	10[a]	4.0
Mannose	125	ND
Sucrose	85	ND
Fructose	123	ND
Sorbose	10[a]	ND
Ribose	28.7	ND
Glucose + NaF	110	ND
Pyruvate	ND	8.4
Citrate	ND	300

[a] 10 mμmoles of lactate represents the lower limit of sensitivity for the assay procedure. ND, not determined.

trations of uncoupler, on the other hand, stimulate glucose 6-phosphate metabolism to an even greater extent than that of free glucose. Inhibition of an active entry system is again not a factor for the phosphorylated compound and dinitrophenol probably stimulates metabolism with no effect on entry.

Both morphological and biochemical data are thus indicative that intestinal cells prepared by this procedure are similar to those cells before isolation. It can also be shown that they retain the ability to actively transport sugars and amino acids by a mechanism characteristic of those cells *in situ*. The next series of experiments was designed for that purpose.

Table III shows the rate of lactate and [^{14}C]CO_2 production for a number of different substrates. It is apparent from these data that in contrast to glucose there is little if any metabolic utilization of galactose or 3-OMG and these sugars therefore seem ideal for use in transport experiments where metabolic transformation must be absent or minimal.

Since intestine from a number of species has been shown to actively accumulate galactose this sugar was chosen as substrate for experiments in which that ability in the isolated cells was monitored. Figure 7 shows the uptake of [1-^{14}C]galactose by the cells as a function of time and the effects of several agents known to inhibit active sugar transport in everted intestinal sacs. The data clearly show that the cells are capable of generating a concentration gradient of galactose in a period of time less than 1 min, that near-maximal gradients are established within 5 min, and after that time a steady state is maintained during which little net transfer of galactose occurs. Concentration ratios were calculated as outlined under Methods, and it is noteworthy that the value obtained by that procedure can be used to show that only equilibration between cell interior and the medium occurs when dinitrophenol is present. This has been observed in a large number of experiments, and it is becoming increasingly apparent that the intracellular space can also be accurately estimated by the following calculation: μl of IS = cpm in cells with DNP/cpm/μl of medium. The speed and simplicity of the latter method make it preferable to the more classical technique. Further proof of the validity of the method is indicated in Figure 7. In one case, the energy conserving mechanism of the cell population was interrupted with dinitrophenol after a period of sugar accumulation had been allowed. Following that time active movement of sugar is precluded and only diffusion down a concentration gradient is possible. The fact that [^{14}C]galactose is lost from the cells constitutes good evidence that a concentration gradient of galactose had been established prior to DNP addition. Even more important, sugar is lost to the same level as was obtained when DNP was present from time zero indicating that under these conditions the cells load only to a point where intracellular sugar concentration is equal to that in the medium. The counts per minute of [^{14}C]galactose expected in the cells for a concentration ratio of 1.0 was calculated using a value for intracellular space determined by the procedure cited in the Methods and found to be 160 cpm. Note the excellent agreement between this value and that obtained from the plateau region of the curve for galactose uptake in the presence of DNP.

The remainder of the data in Figure 7 is again consistent with information obtained from other systems regarding the nature of the sugar transport process. Phloridzin, which is thought to be a rather specific inhibitor of intestinal sugar transport, caused complete inhibition of active galactose accumulation by the isolated cells. Ouabain, an inhibitor of active Na^+ and K^+ transport, also inhibited severely. This too has been noted in other types of intestinal preparations (Csaky and Hara, 1965; Csaky *et al.*, 1961) and is a key observation in the development of at least one highly regarded model for sodium-dependent active metabolite accumulation (Crane, 1965, 1968). Some aspects of this model are treated in detail in the following paper.

Figure 8 shows accumulation of galactose by isolated cells at several different galactose concentrations. The specific activity of galactose was the same at all concentrations so

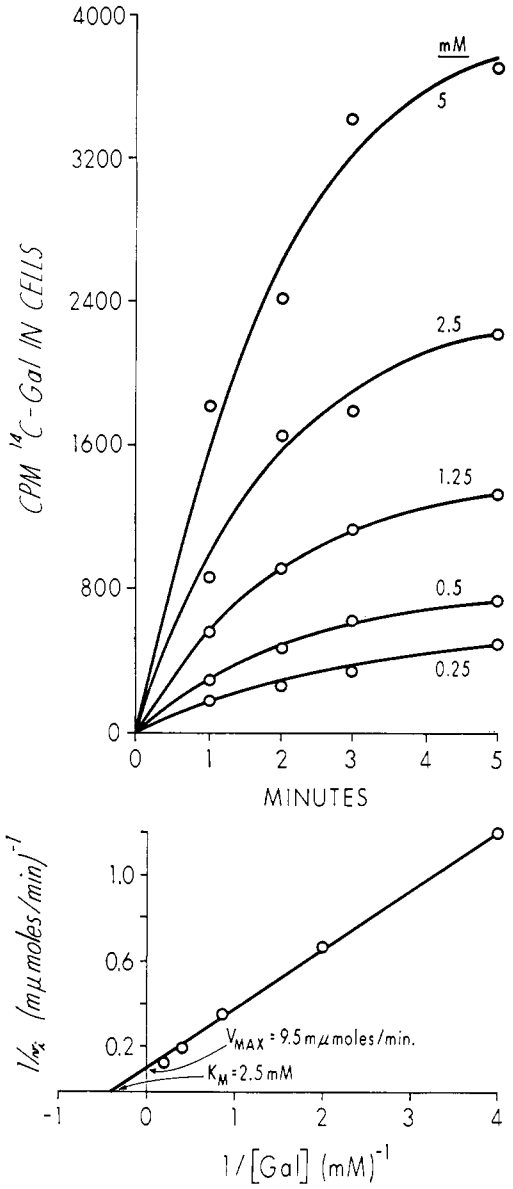

FIGURE 8: Upper: accumulation of [^{14}C]galactose by isolated intestinal cells at various concentrations of galactose. Technique is described in the text. Cells (20 mg of protein) were incubated in 4.0-ml total volume; temperature, 37°. Lower: reciprocal plot of initial galactose transport velocity *vs.* substrate concentration. The indicated V_{max} refers to the rate for only those cells in a 200-μl aliquot. V_{max} are expressed in standard units in the text.

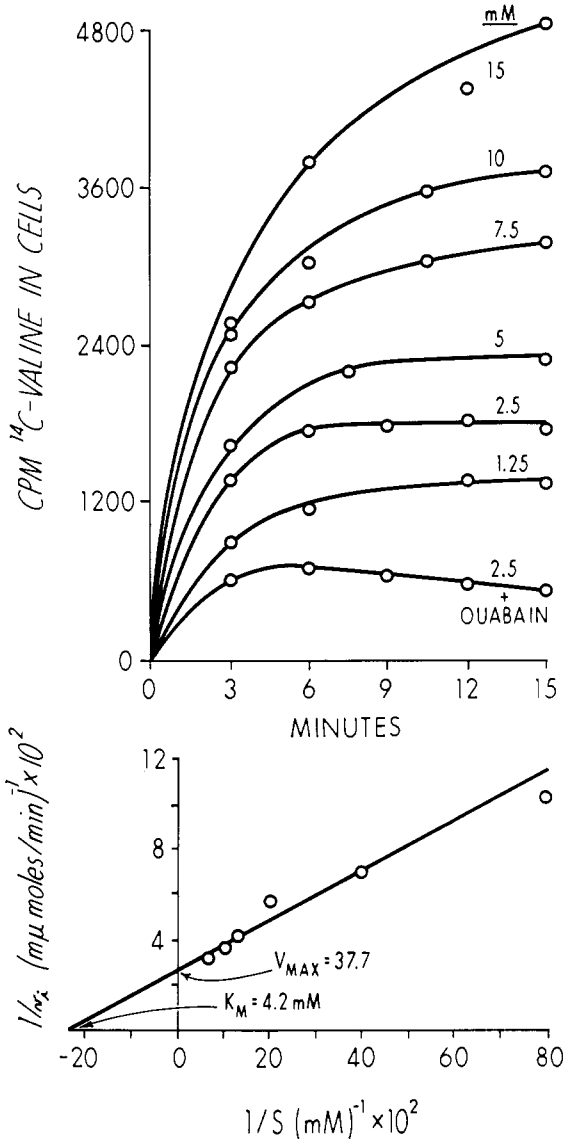

FIGURE 9: Upper: accumulation of [^{14}C]valine by isolated intestinal cells at various concentrations of valine. Cells (9.5 mg of protein) were incubated in a total volume of 4.0 ml; temperature, 37°. Lower: reciprocal plot of initial valine transport velocity *vs.* substrate concentration. The indicated V_{max} again refers only to the rate for cells in a 200-μl aliquot of the suspension.

counts per minute directly reflect the amount of sugar retained by the cells. The counts per minute in the cells equivalent to equilibration of galactose between cells and medium varies with sugar concentration, however. At 0.25 mM this value is equal to 117 cpm, and is proportionately greater for the higher concentrations. From this value it can be calculated that after 5 min the concentration ratios are 4.2, 3.1, 2.3, 1.9, and 1.6 for 0.25, 0.5, 1.0, 2.0, and 5.0 mM galactose, respectively. Decreasing gradients with increasing sugar concentrations are expected for carrier mediated active transport processes (Wilbrandt and Rosenberg, 1961) and have been described by other groups using various preparations of intact intestine (Crane and Mandelstam, 1960).

The lower half of Figure 8 shows a Lineweaver–Burk type plot for the galactose transport data. Rates of sugar accumulation over the first 3-min interval were used to calculate the initial velocity of transport at each sugar concentration. A K_m of 2.5 mM is indicated for galactose, in excellent agreement with literature values obtained with everted gut sacs and transverse intestinal slices (Crane, 1960a). V_{max}, on the other hand, is 250 mmoles/l. of cell H_2O per hr which is slightly higher than comparable literature values.

Figure 9 illustrates similar data for valine accumulation by a suspension of isolated intestinal cells. In this case also the magnitude of the gradient generated is proportional to valine concentration. The K_m for valine uptake is about 4.2 mM as reported in other studies (Wiseman, 1968; Curran *et al.*, 1967; Reisen and Christensen, 1969), but the V_{max}

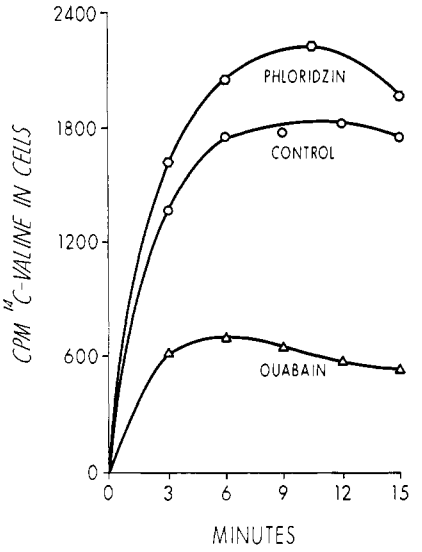

FIGURE 10: Effect of phloridzin and ouabain on the accumulation [^{14}C]valine by isolated intestinal cells. Cells (9.5 mg of protein) were incubated in a total volume of 4.0 ml at a temperature of 37°.

of 500 mmoles/l. of cell H_2O per hr is once again significantly higher than that obtained with other techniques (Wiseman, 1968; Curran *et al.*, 1967; Reisen and Christensen, 1969).

Figure 10 shows that valine accumulation is insensitive to inhibition by phloridzin in contrast to galactose transport. Active valine entry is completely inhibited by DNP and severely inhibited by ouabain, however. Data shown for this experiment was obtained with the same cell preparation used for Figure 9. The extent of accumulation indicated for the case with ouabain represents a concentration gradient of only 1.5-fold. By contrast, in the absence of ouabain, concentration gradients of 7.9, 5.1, 3.3, 3.0, 2.7, and 2.3 were established at 1.25, 2.5, 5.0, 7.5, 10, and 15 mM valine, respectively.

Discussion

The ability to prepare suspensions of isolated intestinal epithelial cells which retain their metabolic and transport capability should offer a productive new avenue of approach to the study of metabolite transport. The ease of manipulation and reproducible sampling offer significant advantages over those systems using more intact tissue preparations. More important, use of cell suspensions allows a degree of homogeneity in cell populations unable to be attained with more classical techniques. It is a well-known fact that the capability for sugar and amino acid transport exhibited by intestinal segments is a function of the position along the intestine from which they are taken (Crane and Mandelstam, 1960; Fisher and Parsons, 1953). Sacs prepared from jejunum are more active than those from other areas, for example. This makes comparisons of transport activity difficult for sacs taken from a single animal, and normal biological variation makes comparisons between different animals a major task, also. The utility of a homogeneous easily sampled cell population is obvious. Furthermore, the technique of Millipore filtration allows collection of cell samples in which contamination by extracellular fluid is minimal. Typically 200-μl samples may be filtered containing as much as 2 mg of cell dry weight, and after the usual 5-ml wash only 0.2–0.3 μl of extracellular fluid will be trapped by the cells. This value can be estimated by extrapolating metabolite accumulation curves to time zero and noting the intercept with the y axis. Cpm at intercept/cpm/μl of medium = μl of medium trapped by the filter. The extrapolation is best made for experiments in which uptake of metabolite by the cells is slow, *e.g.*, [^{14}C]galactose uptake in the presence of DNP or phloridzin (see Figure 7). Estimates of intracellular space determined by the counts per minute of metabolite taken up in the presence of DNP will usually be slightly overestimated due to the error introduced by this "trapping" of extracellular fluid. Nevertheless for most purposes, the rapidity and ease of this procedure for calculating intracellular volume makes it the method of choice. For extremely critical space measurements such as are necessary in the following paper, one can make the required correction for trapping of medium from the same set of data used to estimate intracellular space.

Both of the procedures cited for calculating intracellular space are worthy of additional comment. By using labeled galactose in the absence of transport ability (DNP present) one obtains an estimate for only that space in which sugar ordinarily distributes. This is exactly the space which one should consider for an accurate calculation of the sugar concentration gradient which the cell is capable of establishing, for it excludes that portion of the cell interior which is impermeable to sugar entry (possibly such organelles as the nucleus and mitochondria, for example). This is another reason why space measurements on an isolated cell population are a better reflection of true volume of the cell compartment to which transport occurs than are the usual methods used for intact tissue. In fact, we have found that mannitol, which has frequently been used to estimate extracellular space in everted sacs (Bihler, 1969; Bihler and Crane, 1962), penetrates isolated intestinal cells fully. Thus it seems quite possible that space measurements made with mannitol are estimating volume of an entirely different cell type than those responsible for sugar transport (Bihler and Crane, 1962). Similar doubts may be raised whatever labeled solute is employed to measure volume of a specific compartment in intact tissue preparations containing several cell types. Such ambiguities cause considerable uncertainty in the accuracy and meaning of concentration gradients calculated from the space measurements in such preparations.

The degree of retention of metabolic and transport capability by the isolated cells is perhaps even more exciting. At 1 mM galactose and 80 mM NaCl, typical concentration gradients established by the cells are 4–6-fold. At lower substrate concentrations (100 μM) gradients of 30-fold have been noted, which are as large as any reported for other intestinal systems (Crane and Mandelstam, 1960). Phloridizin (200 μM) destroys production of these gradients rather completely, but does not seem to prevent loading of the cells to equilibrium with the medium. This is in contrast to data reported for everted sacs where phloridzin has been shown to inhibit even nonactive sugar entry (Bihler and Crane, 1962). It is important to remember, however, that isolated cells lose their normal fixed orientation, and that lateral and serosal as well as mucosal surfaces become

exposed to the incubation medium. Since studies with everted sacs have shown serosal sugar entry to be less restricted and specific than mucosal uptake (Wilson and Vincent, 1955) it is possible that this fact accounts for phloridzin insensitive entry. On the other hand, error inherent in space estimates obtained with sacs would tend to overestimate epithelial cell volume and hence underestimate metabolite concentration in those cells, and this may be a contributory factor to the apparent discrepancy in the two systems.

Over the past few years several attempts at preparing cell suspensions from intestinal mucosa have been made (Perris, 1966; Stern and Reilly, 1965; Sognen, 1967; Harrer *et al.*, 1964; Stern, 1966; Huang, 1965). In every case, however, the cells once isolated rapidly lost their metabolic activity as assessed by rate of oxygen consumption. The best preparation in this regard seems to be that of Perris (1966) who reported linear respiratory activity for a period up to 40 min with cessation after that time. By contrast our cell preparation produces CO_2 and lactate from glucose at a constant rate for more than 2 hr. Furthermore, DNP and oligomycin exert their characteristic effects indicating the cellular energy transducing system is still functioning. This fact is further emphasized by the observation that concentration gradients of both sugars and amino acids can be generated, and the formation of such gradients is prevented by inhibitors of the energy conservation process. To our knowledge, only three other intestinal cell preparations were tested for sugar transport ability (Sognen, 1967; Stern, 1966; Huang, 1965). In one instance no concentration gradients could be established (Sognen, 1967), while in another gradients as large as 4-fold were established, but their formation was only partially prevented by concentrations of DNP as large as 3 mM (Huang, 1965). In the third case, gradients of variable magnitude were observed, but their formation was not sodium dependent (Stern, 1966). The latter fact casts some doubt on the idea that a sugar gradient had been generated since the sodium requirement for active sugar accumulation by the intestine is a well-established fact which has been demonstrated in a wide variety of systems. Sodium ion is required for sugar transport by cells isolated by the method described here and the nature of this requirement is the subject of the following paper. A role for sodium is implied by the data cited in Figures 4 and 10 in which ouabain severely inhibits active galactose and valine transport similar to its well-known inhibitory effect on sodium transport.

The comparative effects of ouabain and oligomycin on CO_2 and lactic acid production require further comment. Ouabain has been shown to markedly inhibit lactate production in a number of cell types, presumably by preventing energy turnover normally required for monovalent ion transport (Whittam *et al.*, 1964). A similar effect is shown in the present data (Figure 6). Oligomycin has recently been shown to have some ouabain-like effects in that it inhibits ($Na^+ + K^+$) activated ATPase and active monovalent ion transport (Whittam *et al.*, 1964; Skou, 1965; Blake *et al.*, 1967; Glynn and Chir, 1968). We have shown similar effects of oligomycin on isolated intestinal cells (G. A. Kimmich, unpublished results). Therefore one might expect oligomycin to have the same inhibitory effect on lactate production as does ouabain. Instead, lactate production is markedly stimulated by oligomycin. Of course it is important to remember that oligomycin also blocks a terminal step of oxidative phosphorylation (Lardy *et al.*, 1958; Chance and Hollunger, 1963). In tightly coupled cells one would thus expect less pyruvate to be utilized by the Krebs cycle and more to appear as lactate. That less Krebs cycle activity actually occurs is suggested by the large decrease in CO_2 production observed in the presence of oligomycin (Figure 5). However, we have calculated that the potential increase in lactate production represented by the decrease in CO_2 production is far from sufficient to account for the observed 7-fold greater production of lactate in the presence of oligomycin as compared to the case with ouabain (Figure 6). Perhaps a change in adenine nucleotide ratios is induced by oligomycin which leads to more rapid glycolytic activity. On the other hand, it is possible that ouabain has inhibitory effects in addition to those described for ion transport. These possibilities are being considered further in our laboratory.

Finally, a word should be mentioned about the determination of the kinetic constants for sugar and amino acid transport by the isolated cells. It is noteworthy that these values can be obtained with one cell suspension prepared from a single intestine. Furthermore estimates of true initial velocity are obtained. Samples can be collected as rapidly as every 30 sec over the entire duration of metabolite uptake using the Millipore technique. With everted sacs only one sample is commonly obtained and transport assumed to be uniform during the entire period of exposure to metabolite (Crane, 1960a). Because the samples are often collected only after 10–30 min and the present data show maximal epithelial accumulation occurs as early as 5 min the calculated values for initial velocity are probably grossly underestimated when sacs are employed. This is reflected by the fact that our values for V_{max} of the transport system are significantly higher than those obtained in other systems. The more precise estimate of epithelial cell intracellular volume achieved in the present case also contributes to this difference. On the other hand, K_m for transport, which are less affected by these parameters, are in good agreement with reported literature values.

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