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Active hexose transport across cultured human Caco-2 cells: characterisation and influence of culture conditions

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Human Caco-2 cells (passage 80 to 100) were seeded onto collagen-coated Millipore filter assemblies and these were maintained in culture either (a) floated on the surface of the medium or (b) submerged within the body of the medium. Structural and functional assessments were made over a 30-day period. After seeding, all cells assumed a flattened, squamous configuration and rapidly became confluent. Cells submerged within the medium formed polarised monolayers with well developed junctional complexes, abundant apical microvilli and increasing levels of alkaline phosphatase activity. Cells grown floated on the surface of the medium formed complex multilayers in which polarisation was confined to the surface layer. Junctional complexes and apical microvilli were similar to those seen in submerged monolayers but alkaline phosphatase activities were higher. Transepithelial electrical resistance increased rapidly from day 1, as the layers became confluent. Electrical resistance was higher and short-circuit current and potential differences were lower across monolayers than across multilayers. After 10 days in culture, the addition of D-glucose to the apical bathing solution, of all cell layers, caused a rapid rise in short-circuit current and potential difference. These changes were sodium-dependent and phlorizin-sensitive. Galactose and 3-*O*-methylglucose induced similar changes and the affinity constants for these hexoses ranked in the order reported for rat jejunum (K_m glucose 2.44 ± 0.52 mM; K_m galactose 8.05 ± 1.33 mM; K_m 3-*O*-methylglucose 22.0 ± 5.2 mM). Culture conditions had a marked effect on hexose maximum transport rates (glucose V_{max}: submerged 2.94 ± 0.20 $\mu A/cm^2$; floated 9.94 ± 0.82 $\mu A/cm^2$, $P < 0.05$) but affinity constants were unchanged. Apical to basolateral mannitol fluxes, used as an index of paracellular permeability, decreased from day 1 to day 5 and then remained steady. Fluxes across monolayers and multilayers were not significantly different. We conclude that sodium-dependent hexose transport occurs in cultured Caco-2 cell layers grown on permeable supports. Culture conditions, however, have a marked effect on both cell layer structure and function, and should be an important factor when considering Caco-2 cells as an in vitro model of enterocyte function.

Introduction

In recent years there has been considerable interest in the use of cultured epithelial cell lines as models of intestinal function [1,2]. The Caco-2 cell line is of particular interest for although it is derived from a human colonic cancer, in culture it develops characteristics of small intestinal epithelium. Brush border hydrolase activities are similar to those reported for normal villous enterocytes, suggesting a high level of apical membrane differentiation [3]. However, there are conflicting data about the presence of a fundamental apical membrane transport process, sodium-coupled glucose transport. While this transport process has been identified using biochemical techniques in cells grown on plastic [4], electrical measurements suggest it is not expressed in monolayers grown on permeable supports [5].

We wished to examine the transport characteristics of this cell line and found that while sodium-coupled hexose transport was readily identified, culture conditions had a profound effect on the morphological appearance and transport indices of these cells. Some of these data have been published in abstract form [6].

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Methods

Cell culture

Caco-2 cells were a kind gift from Dr. G. Wilson, Ciba-Geigy Pharmaceuticals. Cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 30 mM sodium bicarbonate, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were grown routinely in 75 cm^2 flasks, at 37°C, in a humidified atmosphere of 5% CO_2/95% air, and the medium was changed on alternate days. Monolayers became confluent 4 to 6 days after seeding and cells were passaged at a split ratio of 1 to 8 every 7 days. Cell detachment was facilitated by phosphate-buffered saline containing 0.2% EDTA and 0.25% trypsin. Cells from passage 80 to 100 were used in the present study.

For experimental purposes cells were seeded into specially constructed filter assemblies. Each assembly comprised a collagen-coated Millipore filter glued to the undersurface of a silicone washer, thereby creating a small apical reservoir [7,8]. Assemblies were floated on the surface of 25 ml of medium in Petri dishes, eight to ten assemblies per dish. Cells were seeded, at a density of 10^5 cells, in 200 μl of medium, into the apical reservoir (growing area 1.13 cm^2).

In previous studies, cultured epithelial cells have been maintained either in floated assemblies [7,8] or submerged within the body of the culture medium [4,9,10]. After 24 hours, therefore, filters were divided into two groups and the culture was continued under two different conditions (Fig. 1):

(a) Floated: in which assemblies remained floating on the surface of the medium throughout the whole period of culture. The medium bathing the basal surface of the filter was changed on alternate days while that within the small apical reservoir was left unchanged.

(b) Submerged: in which assemblies were submerged within the body of the medium allowing free access of medium to both apical and basal surfaces. Culture medium was again changed on alternate days.

Structural and functional assessments were made at 1, 3, 5, 8, 10, 12, 15, 20 and 30 days after seeding.

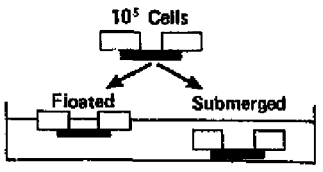

Fig. 1. Cells were seeded into the apical reservoir of specially constructed filter assemblies. After 24 h, half the assemblies were submerged within the body of the culture medium and half were left floating on the surface. Cells were maintained in culture for up to 30 days.

Morphological assessment

Filters were fixed for 60 min in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed for 30 min in 2% osmium tetraoxide. After dehydration, through a graded series of ethanol, filters were embedded in Araldite resin. For light microscopy and morphometric analysis 1 μm sections were stained with 1% aqueous toluidine blue. The surface area of epithelium relative to the corresponding underlying area of filter was quantitated by computerised image analysis (Kontron Electronics, Watford, Herts) [11]. For electron microscopy 60 to 70 nm sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H600 transmission electron microscope.

Alkaline phosphatase activity and protein assay

Cells were harvested, by gentle scraping, into 2 ml of 20 mM Hepes-Tris/300 mM mannitol (pH 7.4). The cell suspension was sonicated, twice for 10 s, on ice. Alkaline phosphatase activity was measured at 37°C using a kinetic colorimetric assay with *p*-nitrophenyl phosphate as substrate [12].

Protein was measured by the modified method of Lowry et al. [13]. The filter component of the assemblies were dissolved in 2 ml 1 M NaOH prior to analysis.

Transepithelial electrical measurements

Filter assemblies were mounted between Perspex half-chambers and apical and basolateral surfaces were bathed with 10 ml of glucose-free Ringer solution at pH 7.4. The solutions were maintained at 37°C by thermostated water jackets and were continuously circulated and gassed with 95% O_2/5% CO_2. Transepithelial potential difference (pd) was measured using calomel electrodes in series with thin 3 M KCl agar bridges. Filters were intermittently short-circuited using an automatic voltage clamp, that compensated for the resistance of the bathing solution and the filter itself. Current pulses were passed through Ag-AgCl electrodes in series with thin 1 M NaCl agar bridges. Electrical resistance was derived from Ohm's Law by measuring the pd change induced by an external current pulse of 100 μA. All electrical measurements were made after a 30 min period of stabilisation.

As short-circuit current (I_{sc}) represents net ion transport across the cellular layer, changes in I_{sc} following the addition of hexoses to the bathing solution were used as a simple index of sodium-coupled hexose transport. Michaelis constants were determined by measuring changes in I_{sc} following the incremental addition of hexoses to the apical bathing solution. Eadie-Hofstee plots of I_{sc} versus I_{sc}/[hexose] were constructed and the apparent maximum transport rate, V_{max} and the affinity constant, K_m (the concentration

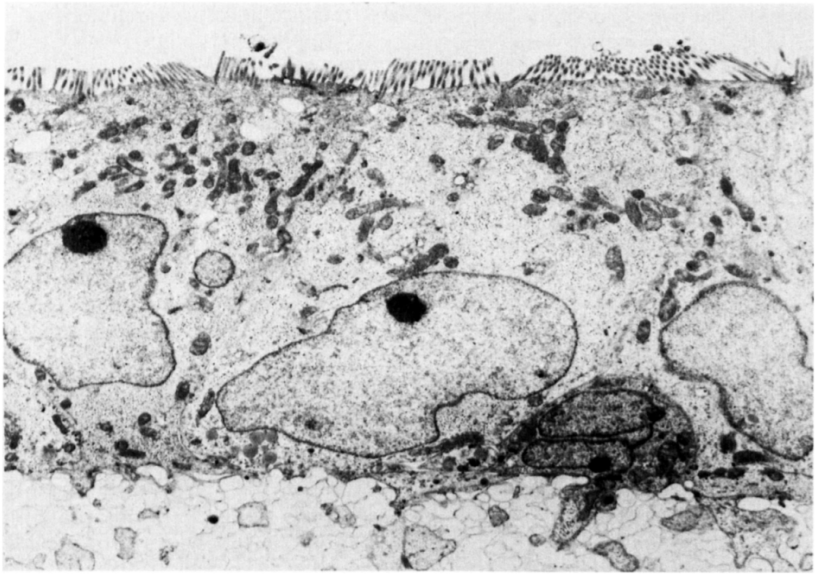

Fig. 2. Light microscopy of submerged Caco-2 cell layers 15 days after seeding. Magnification ×2500.

of hexose required for half-maximal I_{sc}), were calculated.

Mannitol flux measurements

The transepithelial unidirectional flux of mannitol was studied using filter-grown cells mounted in Perspex half-chambers as outlined above. Basal electrical activity was monitored throughout all flux studies as a guide to cell layer integrity. Following a 30 min period of stabilisation 5 μCi of ^{3}H-labelled mannitol in unlabelled mannitol (1.0 mM) was added to the apical compartment. An equivalent dose of unlabelled manni-

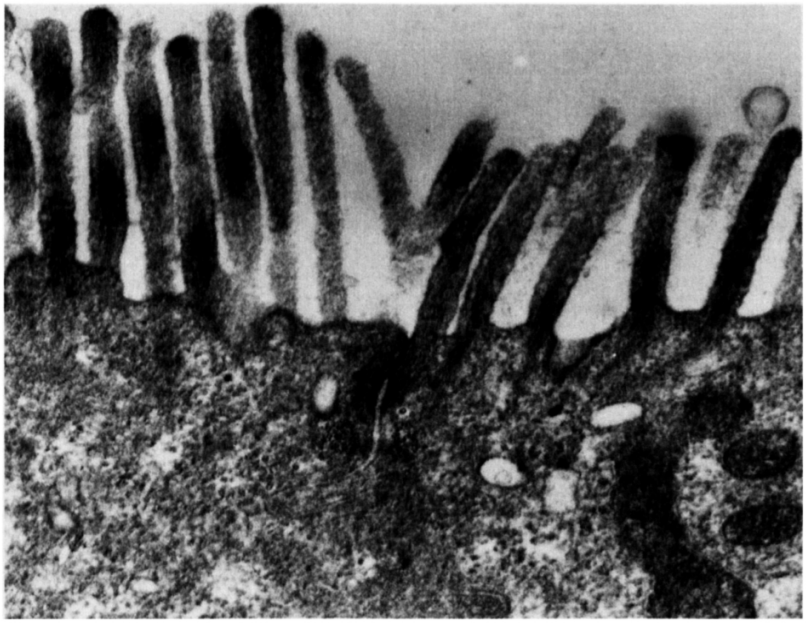

Fig. 3. Electron microscopy of the apical brush border and junctional complex of adjacent Caco-2 cells 15 days after seeding. Magnification ×25000.

tol was added to the basolateral compartment to maintain osmotic balance. Both compartments were sampled at 0, 1, 15, 30, 45 and 60 min and subject to liquid scintillation counting using Optiphase II scintillant and a LKB 1211 Minibeta counter. Results are expressed as percentage flux at 60 min.

All results are expressed as means ± S.E. unless otherwise stated. Comparisons were made using paired and unpaired Student's *t*-tests as appropriate.

Results

Morphology

Up to day 5, the morphological appearances of the cells grown under the two different culture conditions were identical. At 24 h, cells had taken on a flattened, squamous configuration forming an often incomplete monolayer on the surface of the filter. By day 3, the cells appeared cuboidal in shape, mitoses were common and the layer was invariably complete. By day 5, the cells had become columnar and began to show scanty apical microvilli. After day 5, cells grown on submerged assemblies continued to grow as columnar monolayers (Fig. 2). They developed abundant apical microvilli and occluding junctional complexes (Fig. 3) and intracellular accumulations of lipid and glycogen were common.

Cells grown on floated assemblies, however, exhibited strikingly different morphology. By day 10, the cells had formed a rather disorganised bilayer and by day 15 multilayering was a constant feature. Up to day 30 multilayers were most often four to six cells deep but occasionally examples up to ten cells deep were seen (Fig. 4). Polypoid protuberances were frequent and marked lateral space dilatation between the surface enterocytes was also common. The surface layer of cells, however, remained highly polarised and apical microvilli and junctional complexes were similar to those seen in cells grown on submerged filter assemblies.

The polypoidal nature of cell layers grown on floated assemblies was associated with an increase in apical surface area. This was quantified by morphometric analysis of the apical surface area to supporting filter area ratio. On day 5, this ratio was not significantly different in cell layers grown under the two culture conditions (floated 0.991 ± 0.009 versus submerged 0.996 ± 0.013). By day 15, however, a significant increase in apical surface area in cell layers grown on floated assemblies was evident (floated 1.547 ± 0.094,

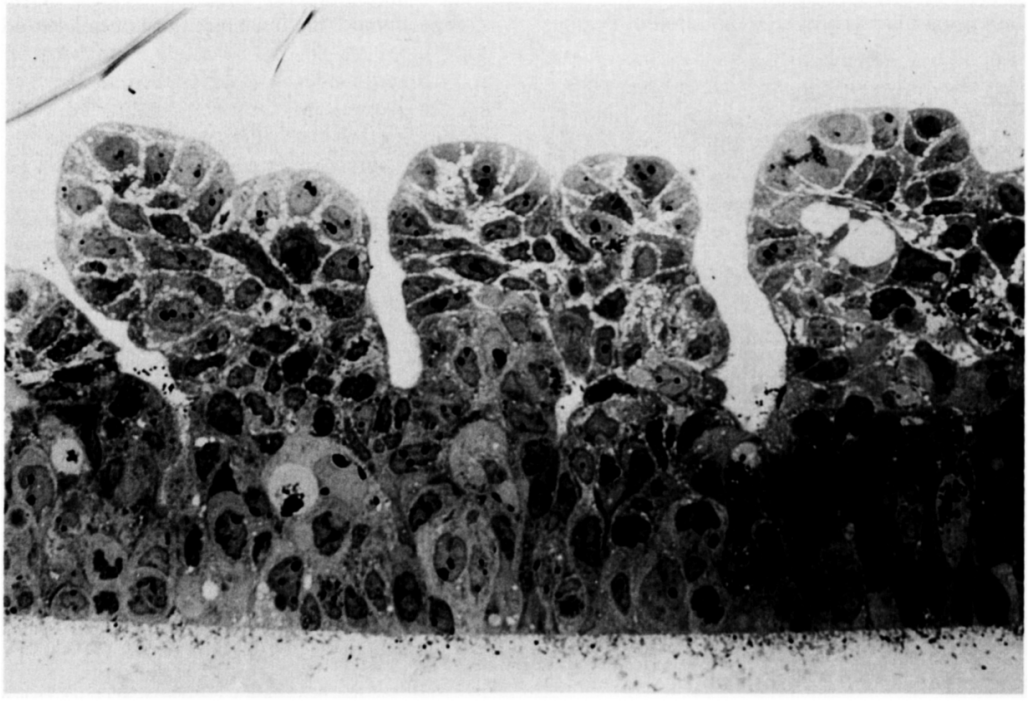

Fig. 4. Light microscopy of Caco-2 cells grown on floated filter assemblies 15 days after seeding. Note the multilayering, polypoidal surface and lateral space dilatation of the surface cell layers. Magnification ×200.

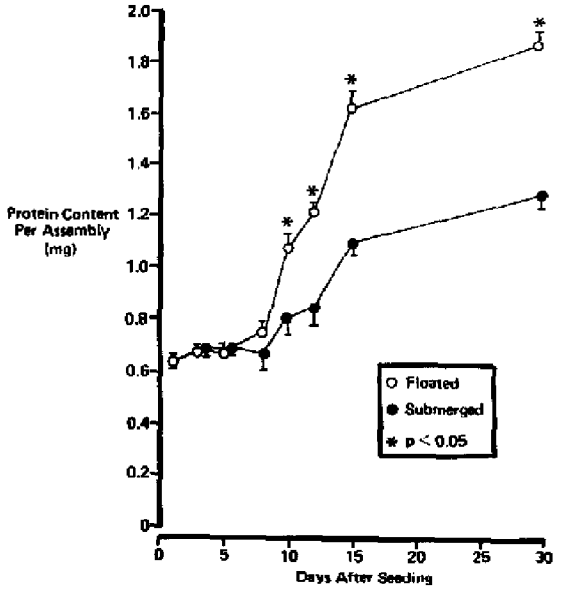

Fig. 5. Protein content (mg) of whole filter assemblies against time after seeding under both culture conditions. Mean ± S.E., $n = 8$.

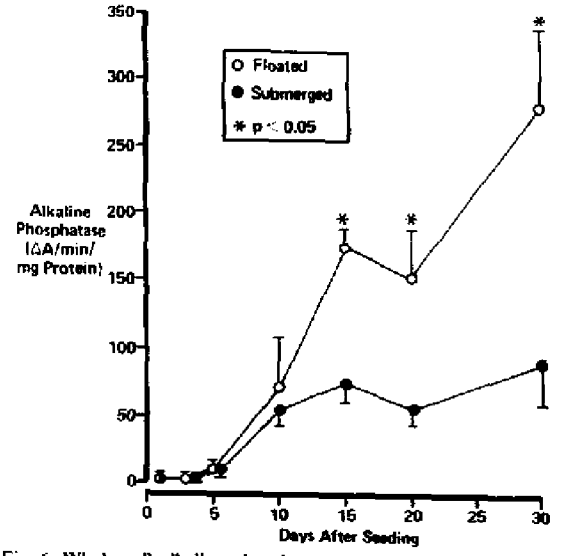

Fig. 6. Whole cell alkaline phosphatase activity (Δ absorbance/mg protein per min) against time after seeding under both culture conditions. Mean ± S.E., $n = 6$.

submerged 0.979 ± 0.011, $P < 0.0001$) and this persisted through to day 30 (floated 1.559 ± 0118 submerged 0.995 ± 0.010, $P < 0.0001$).

Protein and alkaline phosphatase levels

The change in protein content per filter assembly during culture is shown in Fig. 5. After an initial lag phase, protein content increased rapidly, from day 8, reaching a plateau around day 15. Values were significantly greater when cell layers were grown floating rather than submerged within the culture medium (day 15: floated 1.63 ± 0.07 mg, submerged 1.11 ± 0.05 mg, $P < 0.002$ and day 30: floated 1.88 ± 0.05 mg, submerged 1.33 ± 0.55, $P < 0.001$).

Alkaline phosphatase, an enzyme localised to the brush border has been used as an indicator of apical membrane differentiation [3]. This localisation was confirmed in Caco-2 cells by finding that alkaline phosphatase activity in brush border membrane preparations from cells grown on plastic was enriched 14-fold. Measurements of whole cell alkaline phosphatase activity in filter grown cells showed a progressive increase over the 30 day culture period (Fig. 6). Activity reached significantly higher values in cells grown on floated filter assemblies.

Transepithelial electrical results

The results of basal transepithelial resistance measurements are shown in Fig. 7. The resistance across unseeded collagen-coated cellulose filters was 17.4 ± 0.93 Ω cm^2. 24 h after seeding resistance values were low and variable (26.4 ± 12.2 Ω cm^2, $n = 12$). By day 3, however, values had increased considerably (137.7 ± 11.8 Ω cm^2) consistent with the morphological development of an intact monolayer. Thereafter, the electrical resistance of cell layers grown on floated assemblies increased only slightly (day 15: 158.2 ± 5.2 Ω cm^2 to day 30: 206.4 ± 16.6 Ω cm^2) whereas those grown submerged within the culture medium increased rapidly to day 15 (261.2 ± 6.2 Ω cm^2, $P < 0.0001$ compared with floated cells) and then remained steady to day 30 (283.5 ± 3.7 Ω cm^2, $P < 0.01$ compared with floated cells). Short-circuit current and potential difference were also influenced by culture conditions. Cells grown

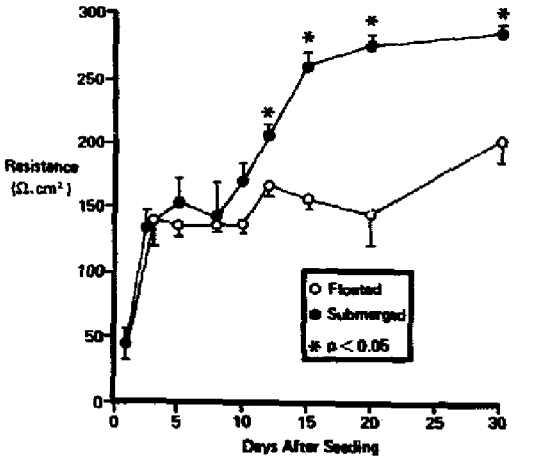

Fig. 7. Transepithelial electrical resistance ($\Omega \cdot$cm^2) against time after seeding under both culture conditions. Mean ± S.E., $n = 4$ to 12.

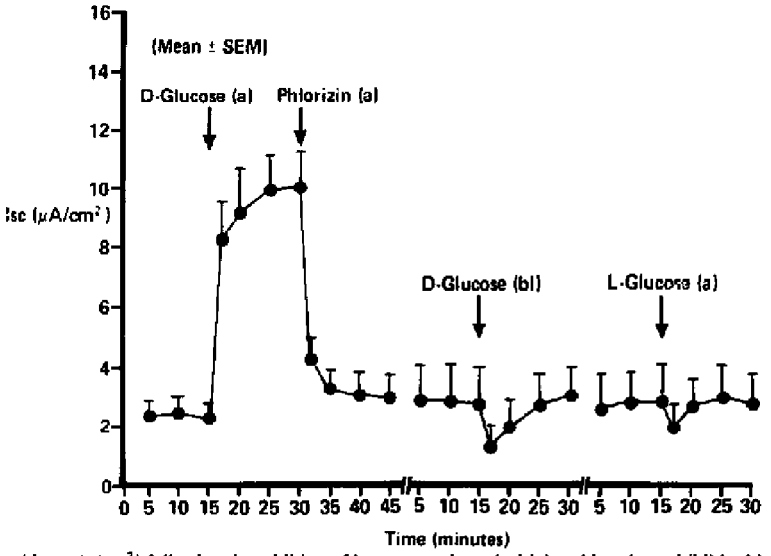

Fig. 8. Short-circuit current (I_{sc}, $\mu A/cm^2$) following the addition of hexoses to the apical (a) and basolateral (bl) bathing solutions of day 15 cell layers mounted in Perspex half-chambers. Mean ± S.E., $n = 8$.

submerged within the medium had consistently lower values of I_{sc} (1.15 ± 0.33 versus 3.39 ± 0.58 $\mu A/cm^2$, $P < 0.01$, day 15) and pd(0.25 ± 0.06 versus 0.46 ± 0.05 mV, $P < 0.01$, day 15).

Evidence of sodium-coupled glucose transport was demonstrated by the electrical responses of cell layers grown under both culture conditions. Fig. 8 shows the changes in I_{sc} associated with the addition of hexoses to the bathing solutions of day 15 cell layers grown on floated filter assemblies. The addition of 20 mM D-glucose to the apical bathing solution brought about a rapid rise in I_{sc} (2.39 ± 0.57 to 10.6 ± 1.28 $\mu A/cm^2$, $P < 0.0005$, $n = 8$). The apical addition of 0.5 mM phlorizin brought about an equally rapid reversal of glucose-stimulated I_{sc}, to near basal levels (86.2 ± 7.3% inhibition). Such changes were not seen when cells were bathed in sodium-free, choline-Ringers' solution. Furthermore, there were no significant changes in I_{sc} following the addition of 20 mM D-glucose to the basolateral solution or 20 mM L-glucose to the apical solution. Evidence of glucose transport was not seen before day 10 but was consistently present thereafter. The time of emergence of glucose transport was similar under the two culture conditions.

Glucose was not the only hexose transported by Caco-2 cell layers. The addition of either galactose or 3-*O*-methylglucose was associated with a rapid rise in I_{sc} which was reversed with phlorizin. These hexoses, however, had lower affinities for the transporter than did glucose (K_m glucose 2.44 ± 0.52 mM; K_m galactose 8.05 ± 1.33 mM, $P < 0.001$ versus glucose; K_m 3-*O*-methylglucose 22.0 ± 5.2 mM, $P < 0.05$ versus galactose).

The maximum transport rate, V_{max}, of hexoses under the two culture conditions are shown in Table I. V_{max} was consistently higher in multilayers than in monolay-

TABLE I

Maximum transport rates (V_{max}) of hexoses across Caco-2 cell layers under different culture conditions

Culture condition	V_{max} ($\mu A/cm^2$)			
	glucose		galactose	3-*O*-methyl-glucose
	day 15	day 30	day 15	day 15
Submerged	2.94 ± 0.20 ($n = 8$)	3.32 ± 0.28 ($n = 4$)	2.29 ± 0.36 ($n = 4$)	1.72 ± 0.64 ($n = 4$)
Floated	9.94 ± 0.82 * ($n = 8$)	15.12 ± 4.48 * ($n = 4$)	10.07 ± 0.30 * ($n = 4$)	6.62 ± 1.17 * ($n = 4$)

* $P < 0.05$.

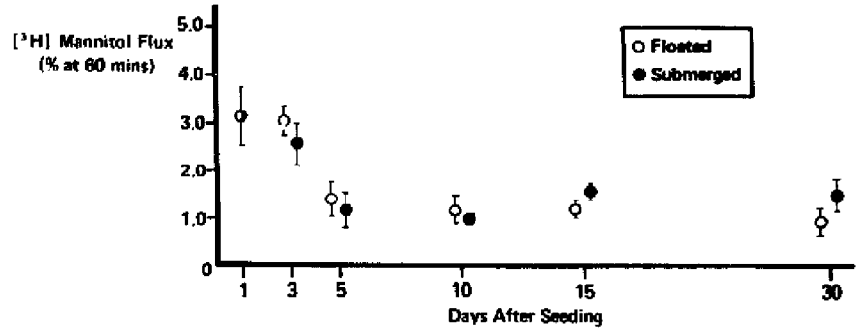

Fig. 9. Apical to basolateral [^{3}H]mannitol flux (% at 60 min) against time after seeding under both culture conditions. Mean ± S.E., n = 4–8.

ers and this was consistent for all three hexoses and on both day 15 and day 30 after seeding. As might be anticipated, culture conditions had no effect on hexose affinity constants (K_m glucose (submerged) 2.44 ± 0.52 mM, K_m glucose (floated) 1.94 ± 0.19 mM, n = 8, day 15).

Mannitol fluxes, at different times after seeding, are shown in Fig. 9. Without cells, the apical to basolateral flux of mannitol across collagen-coated filters was 3.83 ± 0.46% in 60 min. 24 h after seeding the percentage flux was unchanged (3.17 ± 0.68%) but thereafter decreased steadily to day 5 (floated 1.39 ±0.29%, $P <$ 0.005 versus day 1: submerged 1.18 ± 0.25%, $P < 0.002$ versus day 1) and then remained constant through to day 30. Fluxes were not significantly different across cell layers grown under the two culture conditions.

Discussion

The Caco-2 cell line was established in culture in 1974 from a moderately well-differentiated human colonic adenocarcinoma. Initially the cells were studied for their tumourogenic properties [14,15] but in 1983 Pinto et al. [3] reported spontaneous enterocytic differentiation of cells grown under 'standard culture conditions'. Others have also confirmed that cells form polarised monolayers with well developed tight junctions and abundant apical microvilli [16]. Brush-border hydrolases, normally absent from the colon, are increasingly produced during growth and activities approach those seen in the small intestine [3,17].

When grown on permeable supports, Caco-2 cell layers display a modest I_{sc} and serosa positive pd and a substantial transepithelial resistance. A cyclic-AMP-dependent apical chloride conductance is also expressed [18] but under basal conditions, sodium conductance is negligible [4]. Recent studies have demonstrated evidence of amino acid [19], cobalamin [20] and bile acid transport [21].

Despite this body of data, attesting to the enterocytic nature of Caco-2 cells, evidence for the presence of sodium-coupled glucose transport is conflicting. Using isotopic tracer studies, to monitor the intracellular accumulation of α-methylglucose into cells grown on plastic, Blais et al. [4] found evidence to support sodium-coupled hexose transport. Grasset et al. [5] on the other hand, could find no electrical evidence of glucose transport in filter-grown Caco-2 cell layers mounted in Ussing chambers. This failure to detect glucose transport is surprising and the reasons for this are not clear. It may be related to differences in seed density, passage number or culture conditions or to the relative insensitivity of the electrical measurements employed.

In the present study we found clear electrical evidence of active hexose transport across Caco-2 cell layers. The addition of D-glucose to the apical bathing solution brought about a rapid rise in I_{sc}, reflecting the coupled absorption of sodium ions [22]. Such changes were sodium-dependent, isomer specific and rapidly reversed by the specific inhibitor phlorizin. Other hexoses were similarly transported and the affinity constants for D-glucose, D-galactose and 3-*O*-methylglucose ranked in the same order as that reported for rat jejunum [23].

All previous studies have noted that the functional differentiation of cultured Caco-2 cells is a growth-related phenomenon. It was not surprising, then, that evidence of hexose transport was not present before day 10 and that once developed, V_{max} values changed little from day 15 through to day 30. This pattern of development paralleled the increasing levels of cellular protein and alkaline phosphatase activity.

It is well known that modifications to culture medium have profound effects on the growth and differentiation of HT-29 cells. This cell line, which is also derived from a human colonic adenocarcinoma, grows as an undifferentiated multilayer under standard culture conditions. When grown in a glucose-free medium or in the presence of sodium butyrate or suramin, however, these cells form polarised monolayers and undergo enterocytic differentiation [24–26].

In the present study, culture medium specifications were kept constant and the changes in cell layer struc-

ture and function were brought about by maintaining filter-grown cells at different depths within the culture medium. Why such a relatively minor modification to culture conditions was associated with such marked changes is unclear and deserves further study. Differences in oxygenation, nutrient supply or metabolite disposal, effects of hydrostatic pressure or the differential conditions imposed by the isolated apical reservoir are all possible explanations.

The multilayered appearances of cells grown in floated filter assemblies were striking. Caco-2 multilayers, however, were notably different from HT-29 multilayers, in that the surface cell layer became polarised, highly differentiated and polypoidal. Furthermore, this cellular arrangement may, in itself, account for some of the functional differences seen.

The apical surface area of Caco-2 multilayers clearly increased during culture. The size of individual surface cells, however, appeared similar in multilayers and monolayers. A greater surface cell number seems likely therefore, and there is also likely to be an increase in paracellular shunt pathways. As shunt pathways are the major determinant of electrical resistance in low resistance epithelia, this may explain the lower electrical resistances seen in Caco-2 multilayers. Furthermore, this increased surface cell number may account for the higher potential difference and short-circuit current seen in multilayers. It seems unlikely, however, that the 50% increase in surface area is sufficient to account for the 3-fold increase in alkaline phosphatase activity and 5-fold increase in glucose V_{max} seen at day 30. Upregulation of cellular transport systems and enzyme activities thus seems likely. The lack of an effect of morphological changes on mannitol flux was, perhaps, surprising. It may be that the greater barrier imposed by the basal multilayers was offset by the increase in surface paracellular pathways.

In conclusion, we have found evidence of sodium-coupled hexose transport in cultured Caco-2 cell layers. Minor variations in culture conditions, however, had a marked effect on cell layer structure and function. This suggests that investigators may be able to optimise the expression of specific transport mechanisms by modifying culture conditions. The finding that Caco-2 cells express a sodium-dependent glucose transporter when grown on permeable supports should enhance their value as an in vitro model of enterocyte function.

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