The Isolation and Properties of Epithelial-Cell 'Ghosts' from Rat Small Intestine

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(*Received* 26 *November* 1964)

1. The preparation of gram quantities of isolated epithelial-cell 'ghosts' from mucosal scrapings of rat small intestine is described. The method involves dispersing the tissue by gentle homogenization in 6% dextran in Krebs–Ringer phosphate, pH 7·4, followed by filtration through nylon cloth and sedimentation by low-speed centrifuging. 2. The isolated epithelial-cell 'ghosts' contained all of the DNA, but only 52% of the protein and 53–57% of the RNA of the original homogenate. They contained most of the activity of the following enzymes found in the homogenate: aminopeptidase (71%); alkaline β-glycerophosphatase (82%); invertase (92%); adenosine triphosphatase (93–116%); acid β-glycerophosphatase (83%); non-specific esterase (76%); succinate dehydrogenase (96%). Only small proportions of the total lactate-dehydrogenase (10%) and phosphoglucose-isomerase (2%) activities found in the homogenate were recovered in the isolated cell 'ghosts'. 3. The epithelial-cell 'ghost' preparation did not respire unless cofactors and substrates were added, and did not consume glucose or produce lactic acid from glucose. 4. The effect of varying the composition of the homogenization medium was studied. Concentrations of dextran (mol.wt. 15×10^4) from 1 to 12%, solutions of dextrans (all at 6%) with mol.wt. varying between $3{\cdot}6 \times 10^4$ and 2×10^6, and a solution of 8% polyethylene glycol (mol.wt. 4000) served equally well for the production of epithelial-cell 'ghosts'. Two of these solutions, however, 12% dextran (mol. wt. 15×10^4) and 6% dextran (mol.wt. 2×10^6), were too viscous to allow the complete sedimentation of the cell 'ghosts' at low relative centrifugal forces. Omission of either Krebs–Ringer phosphate or dextran from the medium resulted in almost complete cell breakage during the homogenization. 5. The isolated cell 'ghosts' were used as a starting material for subcellular fractionation of rat intestinal mucosa by differential centrifugation. The distributions of protein and succinate-dehydrogenase activity among the fractions were compared with corresponding values in fractions isolated by differential centrifugation of mucosa homogenized in 0·3M-sucrose–5mM-EDTA, pH 7·4. The method in which cell 'ghosts' were used as starting material gave a better separation and cleaner fractions than the method in which untreated mucosal scrapings were used.

Numerous techniques *in vitro* have been devised for the study of transport and metabolism in intestinal tissue. These include the use of whole perfused intestine (Öhnell, 1939), segments through which a suitable medium is circulated (Fisher & Parsons, 1949; Wiseman, 1953; Darlington & Quastel, 1953), everted sacs (Wilson & Wiseman, 1954; Crane & Wilson, 1958), small rings and segments (Agar, Hird & Sidhu, 1954; Crane & Mandelstam, 1960), isolated villi and mucosal or serosal sheets (Crane & Mandelstam, 1960) and mucosal scrapings (Kay & Entenman, 1959). In addition, numerous studies have been carried out with mucosal homogenates (Dickens & Weil-Malherbe, 1941; Dawson & Isselbacher, 1960; Saunders & Dawson, 1962; Taylor, 1963) and isolated subcellular particles (Hers, Berthet, Berthet & de Duve, 1951; Glover & Stainer, 1959; Clark & Hübscher, 1961; Carnie & Porteous, 1962; Sherratt & Hübscher, 1963; Robinson, 1963; Porteous & Clark, 1965). Recently methods have been described for the isolation of intact microvillus membranes (Miller & Crane, 1961; Gallo & Treadwell, 1963). Dalton & Felix (1953) have described the preparation of dispersions of epithelial cells of mouse duodenum for light-microscopy.

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However, as far as we are aware, the isolation of gram quantities of segregated intestinal epithelial cells and their use in metabolic studies have not been described. Wilson (1962) has stressed the value such a preparation would have in the study of transport and metabolism and has pointed out that the use of suspensions of isolated single epithelial cells would be the logical development of the methodology of studying the intestine *in vitro*.

During tests of the suitability of a number of solutions for the homogenization and subsequent fractionation of rabbit intestinal epithelial cells, it was observed that the homogenate prepared in the dextran medium of Birbeck & Reid (1956) consisted almost entirely of apparently intact epithelial cells, as seen by light-microscopy. Further investigations with this medium and modifications of it led to the work described in the present paper. The term 'ghost' has been used throughout in preference to the term 'cell' because of the loss of cell-sap protein and the lack of endogenous respiration of the preparation.

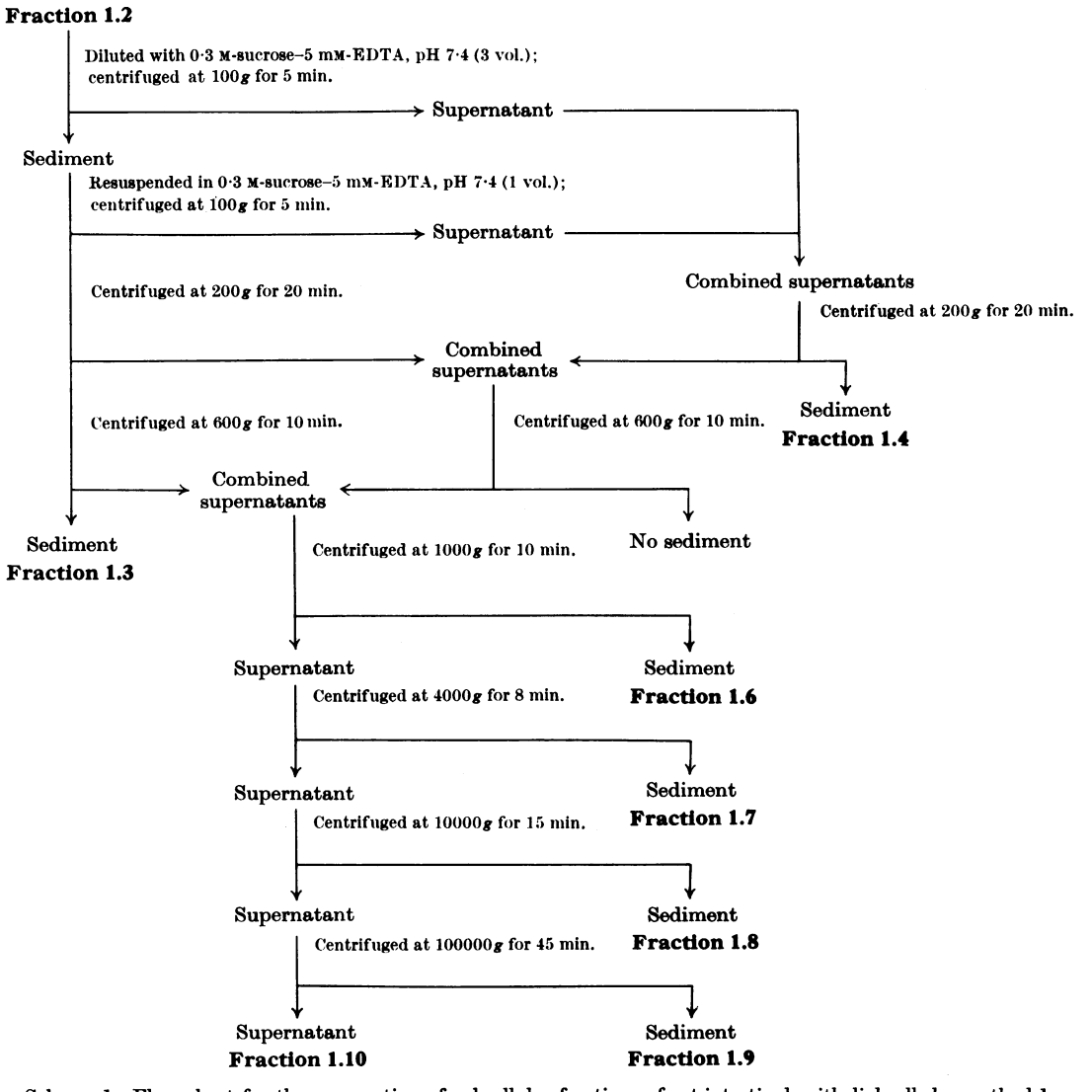
EXPERIMENTAL

Preparation of intestinal mucosal scrapings. Rats of the Wistar strain were used throughout and were not starved before use. The rat was killed by neck fracture and the small intestine cut close to the stomach and appendix. The intestine was washed out under pressure *in situ* with cold Krebs–Ringer phosphate solution, pH 7·4, delivered from a wash bottle inserted into the proximal end of the intestine. The whole small intestine was removed and placed on a glass plate. Mucin and any remaining food particles were first removed by gently compressing and stroking the intestine with a glass slide. The mucosa was then obtained by vigorously compressing and stroking the intestine with a glass slide.

Preparation of intestinal epithelial-cell 'ghosts' (preparations A and B). The mucosal scrapings were homogenized in 10 vol. of cold 6% dextran (mol.wt. 15×10^4) (Glaxo Laboratories Ltd., Greenford, Middlesex) dissolved in Krebs–Ringer phosphate, pH 7·4, by using the loose-fitting homogenizer (radial clearance 0·2 mm., internal diam. 20 mm.) described by Carnie & Porteous (1962). The suspension (30 ml.) was homogenized for 1 min. at 2000 rev./min. with very slow vertical movements of the pestle (five complete strokes). The homogenate was filtered through nylon cloth as described by Porteous & Clark (1965). A portion of the filtrate (preparation A) was retained. The remainder was centrifuged at 1700*g* for 5 min., when a well-packed sediment and slightly turbid supernatant were obtained. The supernatant was retained (supernatant b_1) and the buff flocculent sediment was resuspended by stirring with a glass rod in 20 vol. of Krebs–Ringer phosphate solution, pH 7·4, and again centrifuged at 1700*g* for 5 min. The second slightly turbid supernatant was retained (supernatant b_2), and the sediment (almost white and well packed) was resuspended as before in Krebs–Ringer phosphate. Normally three resuspensions in Krebs–Ringer phosphate were carried out, thus giving supernatants b_1, b_2, b_3 and b_4, but in some experiments as many as five such washes were performed (giving supernatants b_1–b_6). The final well-packed almost-white sediment was resuspended in Krebs–Ringer phosphate, 6% dextran in Krebs–Ringer phosphate or 0·3 M-sucrose–5 mM-EDTA, pH 7·4, depending on which determination or further procedures were to be carried out. The resuspended final sediment of epithelial-cell 'ghosts' was called preparation B.

Subcellular fractionation of rat intestinal mucosa. Method 1. Mucosal scrapings were obtained as described above. Attempts to fractionate the epithelial cells by the method described by Porteous & Clark (1965) for rabbit intestinal epithelium failed. The filtrate (fraction 1.2; cf. fraction II described by Porteous & Clark, 1965) obtained after passing the 0·3 M-sucrose–5 mM-EDTA homogenate through nylon cloth was a gel-like mass from which, on three separate occasions, it was impossible to obtain any sediment at the low centrifugal forces required for the preparation of nuclei. The method was modified as follows and is outlined in Scheme 1: the filtrate (fraction 1.2) was diluted with 0·3 M-sucrose–5 mM-EDTA, pH 7·4 (3 vol.), and then centrifuged at 100*g* for 5 min. The supernatant was retained. The very poorly packed sediment was resuspended in sucrose–EDTA (1 vol.) and centrifuged at 100*g* for 5 min. The supernatant was retained. The sediment was again very poorly packed and was centrifuged without further dilution at 200*g* for 20 min. The supernatant was retained. The sediment was still not very well packed and was centrifuged at 600*g* for 10 min. without further dilution. The supernatant was retained. The sediment was retained and called fraction 1.3. The supernatants after the two centrifugations at 100*g* for 5 min. were combined and centrifuged at 200*g* for 20 min. A small poorly packed gel-like sediment was obtained and called fraction 1.4. The supernatant was combined with the supernatant obtained after the first centrifugation at 200*g* for 20 min. and centrifuged at 600*g* for 10 min. There was a loose flocculent precipitate, but because it was impossible to obtain a clean supernatant the whole suspension was combined with the supernatant obtained after the first centrifugation at 600*g* for 10 min. and centrifuged at 1000*g* for 10 min. The loose gel-like sediment obtained was retained and called fraction 1.6. There was no fraction 1.5 corresponding to fraction V of Porteous & Clark (1965), because no sediment could be obtained by centrifuging at 600*g* for 10 min. Fractions 1.7, 1.8 and 1.9 were obtained by centrifuging successive supernatant suspensions at 4000*g* for 8 min., 10000*g* for 15 min. and 100000*g* for 45 min. The final clear supernatant was retained and called fraction 1.10. Each fraction was analysed for protein and succinate-dehydrogenase activity.

Method 2. Mucosal scrapings were homogenized in 6% dextran in Krebs–Ringer phosphate solution and isolated cell 'ghosts' prepared from the homogenate as described above. The supernatants obtained from the several resuspensions of the 'ghosts' were combined and retained. The final sediment (preparation B) obtained after the last wash with Krebs–Ringer phosphate was resuspended in 0·3 M-sucrose–5 mM-EDTA, pH 7·4 (10 vol.). The suspension was rehomogenized with a loose-fitting Teflon-tipped homogenizer (Carnie & Porteous, 1962) at 2000 rev./min. for 1 min. as described by Porteous & Clark (1965). The resulting homogenate (fraction 2.2) was then fractionated by differential centrifugation. The scheme outlined by



Scheme 1. Flow chart for the preparation of subcellular fractions of rat intestinal epithelial cells by method 1. Details are given in the text.

Porteous & Clark (1965) for the fractionation of rabbit mucosa was followed exactly to give subcellular fractions 2.3–2.10, corresponding to fractions III–X of Porteous & Clark (1965). None of the difficulties experienced in method 1 were encountered. Each of the fractions was analysed for protein and succinate-dehydrogenase activity.

Chemicals. Intradex (consisting of 6% dextran in 0·9% NaCl) was purchased from Glaxo Laboratories Ltd., who also gave a sample of dextran powder (average mol.wt. 15×10^4). Pharmacia (G.B.) Ltd. (London, W.13) supplied dextran 40 (average mol.wt. $3{\cdot}6 \times 10^4$), dextran 500 (average mol.wt. $37{\cdot}5 \times 10^4$) and dextran 2000 (average mol.wt. 200×10^4). British Drug Houses Ltd. (Poole, Dorset) supplied polyethylene glycol (average mol.wt. 4000). Disodium ATP was obtained from the Sigma Chemical Co. (London), and tris, NAD and the materials for the lactic acid determinations from C. F. Boehringer und Soehne G.m.b.H. (through Courtin and Warner, Lewes, Sussex). ATP and NAD were neutralized with NaOH before use. Hydrazine hydrochloride, *p*-nitrophenyl acetate, glycine, glucose 6-phosphate (barium salt) and nicotinamide were laboratory reagents from British Drug Houses Ltd. Barium glucose 6-phosphate was converted into the free acid by treatment with Dowex 50W (X4; H^+ form) and neutralized with KOH. Nicotinamide was recrystallized from benzene before use. The Distillers Co. Ltd. (Speke, Liverpool) supplied glutathione, which was neutralized before use. Active charcoal (quality no. 130) from Sutcliffe, Speakman

and Co. Ltd. (Leigh, Lancs.) was washed with 5% (w/v) trichloroacetic acid until no more inorganic orthophosphate could be extracted. Other chemicals used were of A.R. grade. Glass-distilled water was used throughout.

Buffers. Krebs–Ringer phosphate was prepared as described by Cohen (1949). Phosphate buffers were prepared by mixing the correct proportions of $Na_2HPO_4,12H_2O$ and KH_2PO_4 of the required molarity. Tris buffers were prepared by titrating tris solution with HCl to the required pH value and diluting to give the desired molarity with respect to tris. Glycine buffer was prepared by adjusting a solution of glycine and hydrazine hydrochloride to pH 9 with KOH, and diluting so that the final concentration of glycine was 0·5 M and that of hydrazine 0·4 M. Measurements of pH were made at room temperature with a direct-reading model 23A (Electronic Industries Ltd., Richmond, Surrey) pH-meter.

Determinations. The materials used and the method of assay of protein, DNA, RNA, aminopeptidase, alkaline β-glycerophosphatase, invertase, succinate dehydrogenase and acid β-glycerophosphatase were as described previously (Porteous & Clark, 1965; Clark & Porteous, 1965). Light-microscopy was carried out as described by Carnie & Porteous (1962).

For the assay of adenosine triphosphatase, invertase and alkaline β-glycerophosphatase the fractions were dialysed for 16 hr. against glass-distilled water initially adjusted to pH 7 with $NaHCO_3$.

Assay of adenosine-triphosphatase activity. A sample of the dialysed fraction was added to tris–HCl buffer, pH 7·4 (50 μmoles), KCl (38 μmoles), $MgSO_4$ (20 μmoles) and ATP (20 μmoles), in a final volume of 1 ml. After incubating at 37° for 15 min. the reaction was terminated by the addition of 10% (w/v) trichloroacetic acid (1 ml.) followed by 2 ml. of a 12% (w/v) charcoal suspension in cold 5% (w/v) trichloroacetic acid. After filtering through Whatman no. 5 paper, inorganic orthophosphate was measured on a portion (2 ml.) of the filtrate by the method of King (1932). One unit is that amount of enzyme which will bring about the liberation of 1 μmole of inorganic orthophosphate/15 min. at 37°.

Assay of non-specific esterase activity. To 1·8 ml. of 66 mM-phosphate buffer, pH 7, in a 1 cm. glass cuvette, was added a portion of the cell preparation to be assayed and water to 2 ml. Reaction was started by adding 1 ml. of substrate solution (6·3 mg. of *p*-nitrophenyl acetate dissolved in 1 ml. of methanol and diluted to 100 ml. with water). The extinction at 400 mμ was measured after 1, 2, 3, 4 and 5 min. against a blank solution containing the appropriate concentrations of buffer and enzyme but no substrate. The readings were corrected for the liberation of *p*-nitrophenol in the absence of enzyme. Enzyme activity was related to the rate of change in extinction value ($\Delta E_{400m\mu}$/min.) over the first 5 min. One unit is that amount of enzyme which will bring about the liberation of 1 μmole of *p*-nitrophenol/min. at 17°.

Assay of lactate-dehydrogenase activity. To 2 ml. of glycine–KOH buffer, pH 9, in a 1 cm. silica cuvette was added 5 mM-lactic acid (0·8 ml.) and 0·1 ml. of the cell preparation. Reaction was started by adding 10 mM-NAD (0·1 ml.). The extinction at 340 mμ was measured at time-intervals up to 20 min. against a control solution lacking NAD. Enzyme activity was determined from the $\Delta E_{340m\mu}$/min. over the initial linear part of the curve, after correcting for the change in $E_{340m\mu}$ in the absence of added substrate. One unit is that amount of enzyme which will oxidize 1 μmole of lactate/min. at 17°.

Assay of glucose 6-phosphate catabolism. A portion of the cell preparation in Krebs–Ringer phosphate (0·2 ml.) was added to glucose 6-phosphate (20 μmoles), glutathione (10 μmoles), NAD (1 μmole), ATP (1 μmole), nicotinamide (33 μmoles) and $MgSO_4$ (7 μmoles), in a final volume of 0·8 ml. The final pH was 7·4. Incubation was carried out for 0 and 60 min. at 37°. Reaction was terminated by the addition of cold 72% (v/v) perchloric acid (0·05 ml.) and the reaction tubes were cooled in ice. After centrifuging, the lactic acid concentration of the supernatant solution was measured by following the change in extinction after adding lactate dehydrogenase and NAD.

Assay of phosphoglucose-isomerase activity. To 0·2 M-phosphate buffer, pH 7·4 (1 ml.), was added 0·1 M-glucose 6-phosphate (1 ml.), a portion of the cell fraction to be analysed and water to 5 ml. The tubes were incubated at 37° and samples (1 ml.) were withdrawn at 0, 10, 20 and 40 min. into tubes containing 6% (v/v) perchloric acid (1 ml.). After cooling and centrifuging, the total ketose concentration in the clear supernatant solution was measured by the method of Roe, Epstein & Goldstein (1949). One unit is that amount of enzyme which will bring about the production of 1 μmole of ketose/20 min. at 37°.

Assay of glucose utilization. Portions (3 ml.) of a suspension of isolated cell 'ghosts' in Krebs–Ringer phosphate, pH 7·4, were incubated with 0·1 M-glucose (0·1 ml.) at 37° for 0, 5, 10, 20, 30 and 45 min. At the end of the incubation period the samples were deproteinized with $Ba(OH)_2$ and $ZnSO_4$ solutions (Somogyi, 1945), and the glucose in the filtrate was measured by the glucose-oxidase method described by Porteous & Clark (1965).

Other analytical methods. The viscosities of dextran and polyethylene glycol solutions were related to that of Krebs–Ringer phosphate solution by measuring the times taken for equal volumes of the solutions to flow through a capillary tube under the same head of liquid. The determinations were carried out on solutions precooled in ice. Any effect on the hydrostatic pressure of the small variations in specific gravity of the solutions was ignored.

Relative turbidity measurements were made with a nephelometer (Evans Electroselenium Ltd., Halstead, Essex).

Oxygen consumption by cell-'ghost' suspensions was measured in a conventional Warburg manometric apparatus at 37° with air as the gas phase. The 'ghosts' were suspended in Krebs–Ringer phosphate, pH 7·4.

RESULTS

Preliminary experiments with the Birbeck & Reid (1956) medium (0·23 M-raffinose hydrate–1 mM-EDTA–0·035% heparin, in Intradex) and modifications of it showed that homogenization of rat intestinal mucosal scrapings in Intradex alone was sufficient to produce apparently intact epithelial cells. A 6% solution of Glaxo dextran (mol.wt. 15×10^4) in Krebs–Ringer phosphate, pH 7·4, gave the same results and was substituted for Intradex (a commercial solution of dextran in saline).

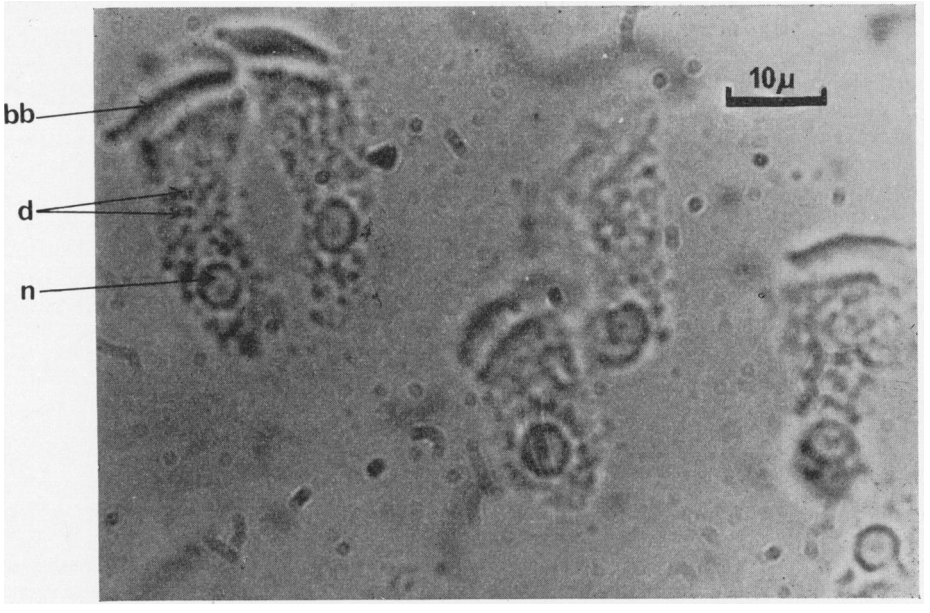


Fig. 1. Photomicrograph of a group of cell 'ghosts' isolated from rat intestinal mucosa. Unstained preparation photographed with bright-field illumination: d, dark granules; bb, brush border; n, nucleus.

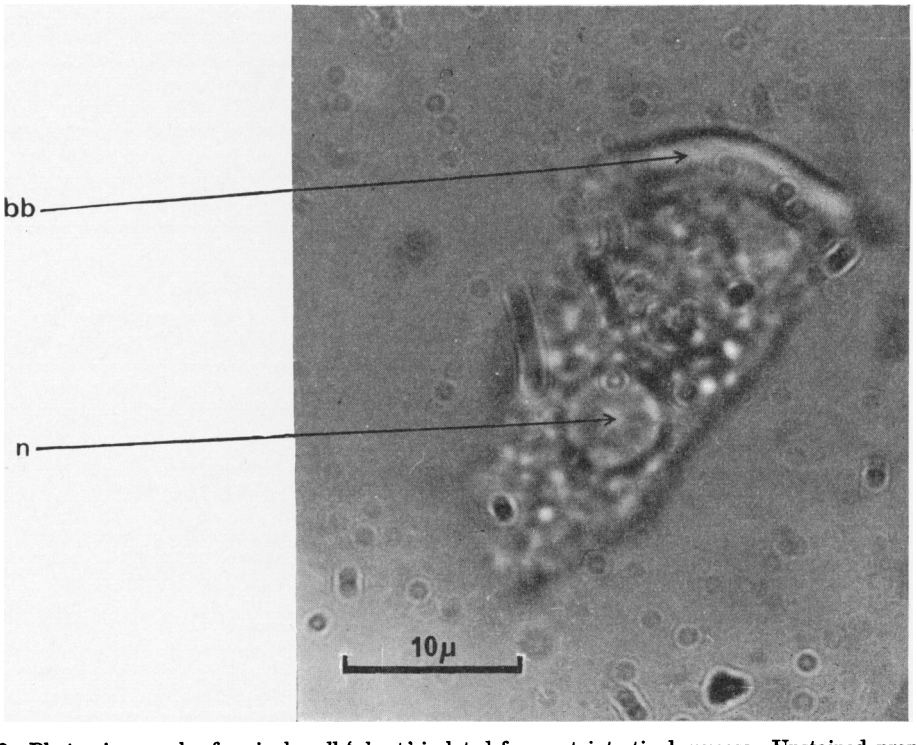


Fig. 2. Photomicrograph of a single cell 'ghost' isolated from rat intestinal mucosa. Unstained preparation photographed with bright-field illumination: bb, brush border; n, nucleus.

Light-microscopy of cell 'ghosts'. Preparation B consisted of apparently intact epithelial cells (Figs. 1 and 2). The brush border and nucleus were clearly recognizable in each cell even in an unstained preparation. The nucleus was always located in the basal part of the cell and did not appear to be distorted in any way. The remainder of the cell was very granular in appearance and large dark granules were often visible immediately above the nucleus. There were few extracellular granules, no extracellular nuclei and no free brush borders. Large masses of clumped cells were often visible. The combined supernatants (b_1–b_4) fraction was virtually optically clear except for some granules under Brownian movement. Preparation A was similar in appearance to preparation B except that preparation A contained more free granules and masses of unrecognizable tissue (possibly of subepithelial origin).

Chemical determinations and enzyme assays on cell 'ghosts' (Table 1). Preparation B contained only about half (52%) of the protein of preparation A, the remainder (51%) being found in the combined supernatants obtained during the isolation of preparation B from preparation A. The amount of protein extracted from the cells into the combined supernatants varied from experiment to experiment, but values lay between 35 and 69% of the total. In an individual experiment successive supernatants b_1–b_6 contained 35·5, 13·0, 4·4, 1·8, 0·2 and 0% of the protein found in preparation A.

The combined supernatants (b_1–b_4) in one experiment were subjected to differential centrifugation. A small amount (3%) of the protein of the combined supernatants was recovered in a very small sediment after applying 1000*g* for 10min., a further 4% in a small sediment after 4000*g* for 8min., another 6% in a small sediment after 10000*g* for 15min. and another 5% in a small sediment after 100000*g* for 45min. The final clear supernatant after applying 100000*g* for 45min. contained 73% of the protein of the combined supernatants.

Preparation B contained all of the DNA (112–113%) of preparation A but only 53–57% of the RNA. Preparation B also contained most of the activity of the brush-border enzymes measured in

Table 1. *Protein, DNA and RNA contents and nine enzyme activities in isolated cell 'ghosts' (preparation B) and in the combined supernatants (b_1–b_4) obtained during the isolation of the cell 'ghosts' from preparation A*

Determinations were carried out as described in the text. Alkaline-β-glycerophosphatase, adenosine-triphosphatase and invertase activities were determined on dialysed samples. Preparation B was suspended: in Krebs–Ringer phosphate, pH7·4, for the determination of DNA, RNA, aminopeptidase activity, acid-β-glycerophosphatase activity and non-specific esterase activity; in 6% dextran in Krebs–Ringer phosphate for the determination of lactate-dehydrogenase and phosphoglucose-isomerase activities; and in Krebs–Ringer phosphate, dextran in Krebs–Ringer phosphate or 0·3M-sucrose–5mM-EDTA, pH7·4, for the determination of protein and succinate-dehydrogenase activity. The second column gives the absolute content or activity of protein, DNA, RNA and nine enzymes in preparation A. The definition of enzyme units is given in the text or by Porteous & Clark (1965). The results in the last two columns are expressed as percentages of the content or activity of preparation A. Where a number of results are available the mean ± S.E.M. is given (values in parentheses are the numbers of experiments performed). N.M., Not measured.

Component	Absolute content or activity of preparation A (protein: mg./g. wet wt. of tissue; DNA and RNA: μg./mg. of protein; enzymes: units/mg. of protein)	Content or activity (% of content or activity of preparation A)	
		Preparation B	Combined 8500*g*-min. supernatants (b_1–b_4)
Protein	98±5 (7)	52±4 (10)	51±4 (10)
DNA	59	112	1·7
	47	113	N.M.
RNA	70	57	N.M.
	61	53	N.M.
Aminopeptidase	3·0	71	45
Alkaline β-glycerophosphatase	4·5	82	20
Invertase	14	92	35
Adenosine triposphatase	0·6	116	N.M.
	1·4	93	19
Acid β-glycerophosphatase	0·7	83	9
Non-specific esterase	0·085	76	N.M.
Lactate dehydrogenase	0·01	10	98
Phosphoglucose isomerase	13	2	89
Succinate dehydrogenase	0·11±·03 (4)	96±8 (4)	10±4 (4)

preparation A (Porteous & Clark, 1965), namely aminopeptidase (71%), alkaline phosphatase (82%) and invertase (92%), as well as adenosine triphosphatase (93–116%), which is also probably located predominantly in the brush border (Padykula, Strauss, Ladman & Gardner, 1961; Ashworth, Luibel & Stewart, 1963). The mitochondrial succinate-dehydrogenase activity measured in preparation A was also recovered mainly in preparation B (96%), as was the acid-phosphatase activity (83%) and the non-specific esterase activity (76%). Of the small amount of succinate-dehydrogenase activity that appeared in the combined supernatants, 60% was sedimented on centrifuging at 10000*g* for 15min., 6% on centrifuging at 100000*g* for 45min. and 24% remained in the final supernatant after applying 100000*g* for 45min.

The isolated cell 'ghosts' did not catabolize glucose 6-phosphate (see the Experimental section for details), and it was subsequently found that the combined supernatants obtained from preparation B contained most of the activity of the two glycolytic enzymes assayed (Table 1), namely lactate dehydrogenase (98%) and phosphoglucose isomerase (89%). The addition of this supernatant to the cell 'ghosts' permitted the 'ghosts' to catabolize glucose 6-phosphate to lactic acid at the same rate as preparation A (0·66 μmole of lactate formed/hr./mg. of protein).

The cell 'ghosts' (preparation B) did not consume glucose, and no measurable lactate was produced from added glucose. Further, the isolated cell 'ghosts' did not consume oxygen in the absence of added cofactors, but a preliminary result has indicated that the cells oxidize succinate, pyruvate and oxaloacetate when the medium is fortified with various cofactors. The medium used was the fortified medium described by Sherratt & Hübscher (1963) except that AMP and hexokinase were not included. There was a small but significant oxygen uptake in the absence of added succinate, pyruvate or oxaloacetate when the 'ghost' cells were suspended in the fortified medium (less AMP and hexokinase) of Sherratt & Hübscher (1963).

Effect of varying the concentration of dextran or the type of dextran in the homogenization medium used to prepare cell 'ghosts'. Two experiments were carried out. In the first, weighed portions (800mg.) of mucosal scrapings were suspended in 10vol. of either Krebs–Ringer phosphate, pH 7·4, or Krebs–Ringer phosphate, pH 7·4, containing 1, 2, 3, 4, 6 or 12% of Glaxo dextran (mol.wt. 15×10^4). In the second experiment weighed portions of mucosal scrapings were suspended in 10vol. of either Krebs–Ringer phosphate, pH 7·4, or Krebs–Ringer phosphate, pH 7·4 containing 6% of dextran 40 (mol.wt. $3{\cdot}6 \times 10^4$), 6% of dextran 500 (mol.wt. $37{\cdot}5 \times 10^4$), 6% of dextran 2000 (mol.wt. 200×10^4) or 6% of Glaxo dextran (mol.wt. 15×10^4). Each suspension was homogenized as described above, by using the same equipment and duration and number of vertical strokes of the pestle each time. Each homogenate was filtered as described above and the filtrate centrifuged at 1700*g* for 5min. The supernatants were carefully removed and analysed for succinate-dehydrogenase activity and the turbidity of the supernatant was measured. The protein concentration in the supernatant was also measured in some cases. The relative viscosities of each of the suspending media were also measured. The results of these two experiments are shown in Table 2.

There was a marked fall in the succinate-dehydrogenase activity of the supernatant as the dextran (mol.wt. 15×10^4) concentration was raised from 0 to 1, 2 or 3%. With 12% dextran, however, the activity in the supernatant was high. The turbidity measurements showed an almost identical pattern. Light-microscopy of the 8500*g*-min. sediments showed that there was almost complete cell breakage in the absence of dextran. But with 1–12% of dextran in the homogenization medium the cells appeared to be intact. Varying the molecular weight of the dextran but keeping the concentration constant in the homogenization medium seemed to have little effect on the amount of succinate-dehydrogenase activity that appeared in the supernatant, except with the dextran of mol.wt. 200×10^4, when the activity of the supernatant was high. The succinate-dehydrogenase activity of the supernatants was paralleled by the turbidity of the supernatants in Expt. 2 (Table 2). The amount of protein found in the supernatant was not affected by the molecular weight of the dextran in the homogenization medium, but significantly more protein appeared in the supernatant when dextran was omitted from the medium. Light-microscopy of the 8500*g*-min. sediments of Expt. 2 (Table 2) showed that each of the four dextrans used served equally well for the preparation of apparently intact cells. Again, complete cell breakage was observed in the absence of dextran. As expected, the relative viscosity of the homogenization media increased both with the molecular weight of the dextran used and also with the concentration incorporated in the medium. The specific gravities of the media varied between 1·00g./ml. for Krebs–Ringer phosphate and 1·02g./ml. for 12% Glaxo dextran (mol.wt. 15×10^4) in Krebs–Ringer phosphate and 6% dextran 2000 in Krebs–Ringer phosphate.

Homogenization of rat intestinal epithelial cells in 6% Glaxo dextran (mol.wt. 15×10^4) dissolved in water instead of Krebs–Ringer phosphate again brought about almost complete cell breakage as judged by light-microscopy, and

Table 2. *Effect of varying the concentration and type of dextran on the turbidity, succinate-dehydrogenase activity and protein content of the supernatant obtained after low-speed centrifugation of homogenates (preparation A) of intestinal epithelial cells in solutions of dextran in Krebs–Ringer phosphate*

Weighed portions of tissue were homogenized in Krebs–Ringer phosphate, pH 7·4, containing dextran as shown. After centrifuging at 1700*g* for 5 min., turbidity, succinate-dehydrogenase activity and protein were determined in the supernatants. The determination of the relative viscosity of the medium (without tissue) and other methods are described in the text. The arbitrary values of 100 assigned to the succinate-dehydrogenase activity of the 8500*g*-min. supernatants obtained from the homogenates prepared in Krebs–Ringer phosphate (without dextran) correspond to approximately half of the activity of the original homogenates. N.M., Not measured.

	Homogenization medium			8500*g*-min. supernatant (b_1)		
Expt. no.	Concn. of dextran (%, w/v)	Average mol.wt.	Relative viscosity	Relative turbidity	Relative succinate-dehydrogenase activity	Relative protein concentration
1	0	—	1·0	100	100	N.M.
	1	15×10^4 (Glaxo)	1·3	60	44	N.M.
	2	15×10^4 (Glaxo)	1·7	45	37	N.M.
	3	15×10^4 (Glaxo)	2·0	39	31	N.M.
	4	15×10^4 (Glaxo)	2·7	40	33	N.M.
	6	15×10^4 (Glaxo)	3·6	39	34	N.M.
	12	15×10^4 (Glaxo)	10·2	102	143	N.M.
2	0	—	1·0	100	100	100
	6	$3·6 \times 10^4$ (40)	2·5	46	38	64
	6	15×10^4 (Glaxo)	3·6	40	34	N.M.
	6	$37·5 \times 10^4$ (500)	7·1	49	46	64
	6	200×10^4 (2000)	16·2	99	89	65

the sediment after centrifuging at 1700*g* for 5 min. contained only 53% of the total succinate-dehydrogenase activity as compared with 96% when the Krebs–Ringer phosphate medium containing dextran was used (Table 1).

To test whether the apparent integrity of the cells after homogenization in Krebs–Ringer phosphate containing dextran could be attributed to the increased viscosity of the medium or to some other non-specific effect of high-molecular-weight compounds, homogenization in an 8% solution of polyethylene glycol in Krebs–Ringer phosphate, pH 7·4, was tried. The average molecular weight of the polyethylene glycol was 4000 and the medium containing it had relative viscosity 3·9 and specific gravity 1·01 g./ml. The initial homogenate contained no recognizable structures as seen by bright-field microscopy with unstained preparations, but after centrifuging at 1700*g* for 5 min. and washing the sediment by resuspending and recentrifuging three times in Krebs–Ringer phosphate, pH 7·4, the sediment was seen to consist almost entirely of whole cells very similar in appearance to those prepared in dextran. The initial supernatant was almost perfectly clear and the sediment was well packed. There was very little succinate-dehydrogenase activity in the combined supernatants, but 36% of the total protein was extracted into these combined supernatants. A homogenate in 6% Glaxo dextran (mol.wt. 15×10^4) prepared at the same time gave 35% of the total protein in the combined supernatants.

Subcellular fractionation of rat intestinal mucosa. Method 1. Fractions 1.2–1.10 were examined by bright-field microscopy with and without methylene blue staining. Fraction 1.2 consisted of some intact tissue, many free nuclei that appeared to be a little distorted, free microvillus sheets (Porteous & Clark, 1965), microvillus sheets joined together end to end to form strips, and many free granules both large and small. In addition there were large amounts of unrecognizable material. The appearance of fraction 1.3 was almost identical with that of fraction 1.2. Fractions 1.4 and 1.6 appeared to be similar to fraction 1.3, except that they contained fewer strips of microvillus sheets and more single microvillus sheets. There was no apparent difference between fractions 1.4 and 1.6. Fractions 1.7 and 1.8 contained mainly small granules, and fractions 1.9 and 1.10 were optically clear.

Each fraction was analysed for protein and succinate-dehydrogenase activity. The results are shown in Table 3.

The nuclear and microvillus-sheet fractions 1.3–1.6 contained 41·3% of the total protein and 68·3%

Table 3. *Distribution of protein and succinate-dehydrogenase activity in subcellular fractions of rat intestinal mucosa isolated by two different methods*

The methods of isolating the subcellular fractions and of determining protein and succinate-dehydrogenase activity are described in the text. Only one subcellular fractionation was carried out by method 1. The results are given as percentages of the content or activity of the filtrate (fraction 1.2) of the cell homogenate and the recoveries are the sum of the protein contents or succinate-dehydrogenase activities of subcellular fractions 1.3–1.10 described in Scheme 1. Four fractionations were carried out by method 2: succinate-dehydrogenase activity was estimated in three, and protein in all four of these. The mean results (±S.E.M.) are given as percentages of the content or activity of preparation A (the homogenate of the mucosal scrapings in 6% dextran in Krebs–Ringer phosphate). The recoveries are the sum of the protein contents (or succinate-dehydrogenase activities) of the combined supernatants (b_1–b_4) obtained after preparing the cell 'ghosts' together with the contents (or succinate-dehydrogenase activities) of the isolated subcellular fractions 2.3–2.10 obtained from the cell 'ghosts' and described in the text. Attention is drawn in the text to the fact that part of the protein of the combined supernatants (b_1–b_4) could be sedimented at higher *g*-min. values.

Method 1				Method 2			
Preparation or subcellular fraction	Sedimented at *g*-min.	Protein	Succinate dehydrogenase	Preparation or subcellular fraction	Sedimented at *g*-min.	Protein	Succinate dehydrogenase
				Preparation A	—	100	100
				Combined 8500*g*-min. supernatants (b_1–b_4)	—	49±6	12±3
				Preparation B	8500	56±6	89±3
1.2	—	100	100				
1.3	6000	33·0	54·0	2.3	500	19±3	17±7
1.4	4000	5·0	8·5	2.4	4000	3·6±0·9	11±2
				2.5	6000	1·3±0·3	7±2
1.6	1×10^4	3·3	5·8	2.6	1×10^4	1·3±0·2	7±2
1.7	$3{\cdot}2\times10^4$	3·4	15·4	2.7	$3{\cdot}2\times10^4$	5·6±0·7	38±9
1.8	15×10^4	3·6	11·5	2.8	15×10^4	5·2±0·2	16±4
1.9	$4{\cdot}5\times10^6$	7·3	4·3	2.9	$4{\cdot}5\times10^6$	9·0±1·2	4·0±0·6
1.10	—	39·0	1·4	2.10	—	9·0±1·5	3·0±1·7
	Recoveries ...	94·6	100·9		Recoveries ...	102±3	114±9

of the total succinate-dehydrogenase activity. The two mitochondrial fractions, 1.7 and 1.8, together contained 7% of the protein and 26·9% of the dehydrogenase activity. The microsomal fraction, 1.9, contained 7·3% of the protein and 4·3% of the dehydrogenase activity. The cell-sap fraction, 1.10, contained 39% of the total protein but only a small proportion of the total succinate-dehydrogenase activity (1·4%).

Method 2. The microscopic observations on preparations A and B and on the combined supernatants from preparation B have been described above. Fractions 2.3–2.10 were also examined.

Fractions 2.3 and 2.4 consisted predominantly of intact microvillus sheets and well-shaped nuclei together with a few granules, and a few whole cells. Microvillus sheets appeared to be relatively more numerous in fraction 2.4 (cf. Porteous & Clark, 1965). Fraction 2.5 contained a few nuclei, microvillus sheets and large clear granules and small granules. Large clear granules predominated. Fraction 2.6 was similar to fraction 2.5, except that there were very few nuclei and microvillus sheets. Fractions 2.7 and 2.8 contained mainly small granules under Brownian movement. Fractions 2.9 and 2.10 were optically clear.

Analyses on preparation A, preparation B, the supernatants b_1–b_4 and the isolated subcellular fractions are shown in Table 3. The combined 8500*g*-min. supernatants obtained from homogenates of mucosa in 6% dextran in Krebs–Ringer phosphate medium contained 49% of the protein and 12% of the succinate-dehydrogenase activity of preparation A. The combined nuclear and microvillus-sheet fractions (2.3 and 2.4) contained 23% and 38% of the protein and succinate-dehydrogenase activity respectively of preparation A. The fractions containing predominantly large granules (2.5 and 2.6) together contained 3% and 14% of the protein and succinate-dehydrogenase activity respectively. The mitochondrial fractions (2.7 and 2.8) together contained 11% of the protein

and 54% of the succinate-dehydrogenase activity of preparation A. The microsomal fraction (2.9) contained 9% of the protein and only 4% of the succinate-dehydrogenase activity. The final clear supernatant (2.10) contained only 9% of the protein and 3% of the succinate-dehydrogenase activity of preparation A.

DISCUSSION

It is generally assumed that the properties of a tissue are a reflection of the properties of its individual cells. Ideally, segregated intact cells should therefore have properties identical with those of the cells in the intact tissue. They should respire in the presence of suitable substrates without the addition of other cofactors, and should contain the same amount and kind of protein, DNA, RNA, enzymes, low-molecular-weight substrates, cofactors and salts as cells in whole tissue. The metabolism of a given compound should be the same in isolated cells as in whole tissue.

Numerous attempts have been made to isolate viable cells from various tissues by several methods. Liver cells have been prepared by such methods as forcing the tissue through sieves of various mesh sizes (Kaltenbach, 1954; Lata & Reinertson, 1957; Henley, Sørenson & Pollard, 1959), by incubation in pH 5 solutions (Longmuir & ap Rees, 1956), by shaking with glass beads (St Aubin & Bucher, 1952), by digestion with trypsin (Dulbecco & Vogt, 1954), and by dispersion of the tissue with loosely fitting homogenizers (Anderson, 1953; Branster & Morton, 1957; Kalant & Young, 1957; Zimmerman, Devlin & Pruss, 1960; Berry, 1962; Exton, 1964). Some of these methods have also been applied to kidney and tumour tissues (Branster & Morton, 1957; Kalant & Young, 1957; Zimmerman *et al.* 1960). Isolated parietal cells from rabbit gastric mucosa (Walker & Lunseth, 1963) and fat cells from rat epididymal fat pads (Rodbell, 1964) have been prepared by incubating the tissue with collagenase at 37°. Certain cells such as ascites-tumour cells (Wu & Racker, 1958) and lymph-node cells (Helmreich & Eisen, 1959) have been prepared without mechanical or chemical dispersion of the tissues. With but few exceptions (Helmreich & Eisen, 1959; Zimmerman *et al.* 1960; Rodbell, 1964) the isolated cells were deficient in one or more metabolic activities associated with the intact tissue. One particular feature observed was the loss of one or more of the cell-sap enzymes from the isolated cells into the suspending fluid (Henley *et al.* 1959; Wu & Racker, 1958; Zimmerman *et al.* 1960; Exton, 1954). Several of the methods for the isolation of cells from liver tissue were tested by Laws & Stickland (1956), who found in each case a lack of endogenous respiration and a failure of glucose, pyruvate, citrate or serum to stimulate respiration.

It is possible that the integrity of the outermost membrane of cells isolated from any animal tissue mass is dependent on the cell type as well as on the method of isolation used. The role of high-molecular-weight compounds in the dispersion media used to isolate partially intact cells in the present and in other similar work is not clear. Some results suggest an improved membrane integrity, others a loss of membrane integrity or an increase in membrane permeability in the presence of dextran and similar compounds. Uvnäs & Thon (1959) found that histamine was more completely retained in isolated mast cells that had been separated by density-gradient centrifugation in solutions of Ficoll (a polymer of sucrose with mol.wt. approx. 400000) than in those cells that had been prepared in sucrose solutions. Birbeck & Reid (1956) showed that the integrity of mitochondria isolated from rat liver improved with the molecular weight of the dextran used in the homogenization medium, and Dalton & Felix (1953) found better preservation of mitochondria in the intact cells when the tissue was dispersed in 6% dextran rather than in 0·85% sodium chloride. On the other hand, Halpern (1956) has shown that there is an increased capillary permeability after injection of dextran into albino rats, and Åberg, Bloom & Hansson (1961) found that cats given [^{14}C]dextran intravenously excreted intact dextran (mol.wt. 66000) via the small intestine. Birbeck & Reid (1956) found that 59% of the total nitrogen of rat-liver homogenates appeared in the final cell sap when the medium containing dextran was used. A similar homogenate prepared in 0·25M-sucrose gave only 49% of the total nitrogen in the final cell sap.

Although dextran solutions of widely different viscosity served equally well for the preparation of epithelial-cell 'ghosts' (Table 2), it is possible that a certain minimum viscosity or colloid osmotic pressure is required to preserve at least part of the cellular integrity during dispersion of the tissue. Thus omitting the dextran altogether from the dispersion medium resulted in almost complete cell rupture as judged by light-microscopy of preparation B, and turbidity and succinate-dehydrogenase activity measurements on the 8500*g*-min. supernatant (Table 2). An 8% solution of polyethylene glycol that had a relative viscosity of 3·9 (cf. Table 2) served equally well in the preparation of epithelial-cell 'ghosts'. The complete cell breakage observed when the tissue was homogenized in 6% dextran (mol.wt. 15×10^4) dissolved in water instead of Krebs–Ringer phosphate was probably due to osmotic rupture of the cells in the hypo-osmotic solution.

The isolated intestinal epithelial cells described

in the present paper did not respire in the absence of added cofactors and could not metabolize glucose or glucose 6-phosphate.

The isolated cell 'ghosts' contained about half of the protein of the original tissue homogenate, the remainder appearing in the combined supernatants b_1–b_4 (Tables 1 and 3). Since the material that was extracted by dextran included most of the two glycolytic-pathway enzyme activities studied (phosphoglucose isomerase and lactate dehydrogenase; Table 1), it is possible that it was mainly cell-sap protein that leaked out. This suggestion is supported by the fact that subsequent thorough homogenization of the cell 'ghosts' and fractionation of the homogenate by differential centrifugation yielded only 9% of the total protein in the cell-sap fraction 2.10 as compared with 39% in the cell-sap fraction 1.10 isolated by the more conventional method (Table 3; see also Porteous & Clark, 1965). The total protein of the initial 8500*g*-min. dextran supernatant and the final cell-sap fraction 2.10 together accounted for 58% of the protein in the original cell homogenate. Taking into account the fact that a small proportion (18%) of the combined supernatant protein (of method 2) was particulate, the amount of protein that can be considered to represent the cell-sap fraction is decreased to 49% of the total protein. Further evidence that the protein extracted into the 8500*g*-min. dextran supernatants consisted mainly of the soluble material of the cytoplasm is given by the distribution of a number of particulate components. Thus the greater part of the activity of the microvillus-membrane enzymes that were measured (71% of the aminopeptidase, 82% of the alkaline-β-glycerophosphatase and 92% of the invertase activities) remained in the cell 'ghosts'.

Most of the activity (96%) of the mitochondrial enzyme, succinate dehydrogenase, also remained in the cell 'ghosts' (Table 1). Similarly, acid-phosphatase activity, which may be representative of lysosome particles (Appelmans, Wattiaux & de Duve, 1955) or may be distributed more generally through the intestinal epithelial cell (Porteous & Clark, 1965), and non-specific esterase activity, which is possibly associated with the endoplasmic reticulum (Underhay, Holt, Beaufay & de Duve, 1956; Schotz, Rice & Alfin-Slater, 1954), were both largely retained in the cell-'ghost' preparation (83% and 76% respectively). The small amount of particulate enzyme activity that appeared in the 8500*g*-min. supernatants (b_1–b_4) probably represented a release of intact particles from disrupted cells. Thus 60% of the succinate-dehydrogenase activity of the combined 8500*g*-min. supernatants, b_1–b_4 (Tables 1 and 3), was sedimented after centrifuging at 10000*g* for 15min. and a further 6% after centrifuging at 100000*g* for 45min., and only 24% (i.e. 2–3% of the total present in preparation A) remained in the final supernatant. Despite the loss of soluble protein and other cell components (43–47% of the total RNA), the isolated cell 'ghosts' appeared to be morphologically intact (Figs. 1 and 2) as seen by light-microscopy. Varying the concentration or the molecular weight of the dextran in the solution used for homogenization of mucosal scrapings had little effect on the appearance of the resulting isolated cell 'ghosts', even though there was a large variation in the viscosity of the medium. The high succinate-dehydrogenase activity and turbidity of the supernatants (Table 2) obtained from homogenates prepared in either 12% dextran (mol.wt. 15×10^4) or 6% dextran (mol.wt. 200×10^4) was probably due to the incomplete sedimentation of the epithelial-cell 'ghosts'. The slightly increased specific gravity and markedly increased viscosities of these solutions (Table 2) would diminish the rate of sedimentation of particles of a given size and density.

Difficulties in fractionating homogenates of intestinal mucosa by differential centrifugation have been reported by some authors (Busch, Davis & Anderson, 1958; DiNella, Meng & Park, 1960; Robinson, 1963; Porteous & Clark, 1965) and were usually caused by the formation of a gel from which the nuclei would not sediment cleanly at low centrifugal forces. Porteous & Clark (1965) overcame these difficulties by filtering the homogenate of rabbit intestinal mucosa through nylon cloth. However, rabbit mucosa does not seem to contain as much mucin as tissue from rat. Some authors have discarded the nuclear fraction and taken the nuclei-free supernatant as a starting material (DiNella *et al.* 1960; Borgström & Dahlqvist, 1958; Hübscher, Clark, Webb & Sherratt, 1963; Ailhaud, Samuel & Desnuelle, 1963). Other authors do not record any particular difficulties in fractionating mucosa of the small intestine (Doell & Kretchmer, 1962, 1963; Allard, de Lamirande & Cantero, 1957; Gallo & Treadwell, 1963).

Attempted fractionation of homogenates of rat intestinal mucosa in 0·3M-sucrose–5mM-EDTA was always frustrated by the formation of a gel that failed to sediment at the low centrifugal forces necessary for the preparation of clean nuclear fractions (see the Experimental section), and therefore the use of the isolated cell-'ghost' preparation as a starting material for further fractionation by differential centrifugation was investigated. The fractionation was accomplished without the difficulties encountered in the more conventional method (see the Experimental section), and cleaner fractions were obtained as judged by light-microscopy. It is possible that most of the mucin was extracted by the initial dextran treatment, thus preventing any major interference

by this material in the later fractionation of the cell 'ghosts'. The use of the cell-'ghost' preparation as a starting material for subcellular fractionation allowed the separation of two fractions (2.5 and 2.6; Table 3) containing large granules that were similar in appearance to the granule fractions isolated from rabbit small intestine (Porteous & Clark, 1965). The intracellular function of the large granules isolated in fractions 2.5 and 2.6 is not known. The nuclear fractions 2.3 and 2.4 isolated from the cell-'ghost' preparation contained less protein (24%) than did corresponding fractions (Table 3) isolated by the conventional method (38%). The nuclear fractions isolated by the method in which original mucosa was used as the starting material (method 1) were grossly contaminated with mitochondria (Table 3). Thus fractions 1.3 and 1.4 together contained 63% of the total succinate-dehydrogenase activity with correspondingly less in the mitochondrial fractions (27% in fractions 1.7 and 1.8). The trapping of mitochondria by the mucin of the nuclear fractions has been observed by Robinson (1963). The nuclear fractions isolated by the method in which the cell-'ghost' preparation was used as the starting material (method 2) contained only 28% of the total succinate-dehydrogenase activity (Table 3). A very much higher proportion (54%) of the total succinate-dehydrogenase activity was recovered in the mitochondrial fractions isolated by the second method (Table 3).

Although the preparation of epithelial-cell 'ghosts' described in the present paper is not suitable as a model system for the study of the metabolism of whole cells, it may be of use in other investigations. The value of the preparation as a starting material for subcellular fractionation has been discussed above, and it may prove to be a useful material from which to separate microvillus sheets and nuclei. The separation of these two cell components has so far not been possible (Porteous & Clark, 1965), except by techniques that destroy the nuclei (Miller & Crane, 1961; Gallo & Treadwell, 1963). Preparations of epithelial-cell 'ghosts' could also be of use in the study of membrane phenomena and the active transport of ions, sugars and amino acids across the microvillus membrane.

During the preparation of this paper for publication two relevant reports have appeared. The first (Harrer, Stern & Reilly, 1964) describes a method, involving treatment with trypsin, for the preparation of intact viable cells from rat gastrointestinal epithelium. The second (Stern & Reilly, 1965) describes respiration and glycolysis in cells isolated by this method.

We thank Mrs L. Stevens of the Department of Pathology, University of Aberdeen, for taking the photomicrographs shown in Figs. 1 and 2, Dr G. Hübscher for the gift of materials for the aminopeptidase assay, Dr S. Ball of Glaxo Research Laboratories (Barnard Castle, Co. Durham) for the gift of dextran, the Medical Research Council for generous financial aid, and Miss Helen Brough for capable technical assistance.

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