The Isolation and Characterization of Subcellular Components of the Epithelial Cells of Rabbit Small Intestine

By J. W. PORTEOUS and B. CLARK
Department of Biological Chemistry, Marischal College, University of Aberdeen

(*Received* 3 *September* 1964)

1. Homogenization of the epithelial cells of rabbit small intestine in 0·3 M-sucrose–5 mM-EDTA, pH 7·4, maintains intact the microvillus sheets that form the lumenal surface of the cells, the nuclei, the mitochondria and the vesicles (microsomes) formed from the endoplasmic reticulum. 2. These particulate components of the cell, and the cell-sap fraction, have been isolated by differential centrifuging of cell homogenates. 3. The nuclei and microvillus sheets sediment together and it has been impossible to separate these subcellular components by centrifugal methods. 4. The isolated subcellular fractions have been identified by a combination of light-microscopic examination, electron-microscopic examination, chemical analysis and assay for selected enzyme activities.

The intact small intestine consists of four principal layers of tissue: the innermost mucosa, the submucosa, the circular muscle coat and the longitudinal muscle coat. A fifth structure, the peritoneum, covers most of the intestine and all of its supporting mesenteries, which in turn carry the blood vessels, lymph ducts and nerve fibres supplying the intestine. The innermost mucosal tissue is itself complex and consists of three distinct parts: (i) a continuous unicellular layer of simple columnar epithelial cells, interspersed with a variable number of mucin-secreting goblet cells, covers the entire surface of (ii) approximately cylindrical villi that arise as perpendicular extrusions of a layer of subepithelial tissue; the latter extends over (iii) a thin muscle layer, the lamina muscularis mucosae. The nerves, blood vessels and lymph vessels traverse all four main layers of intestinal tissue as far as the interior of the villi but do not enter the epithelial cells of the mucosa. The progression of these epithelial cells up the sides of the villi has been described by Leblond & Stevens (1948), by Leblond, Stevens & Bogoroch (1948) and by Leblond & Walker (1956).

Recent publications (Crane & Neuberger, 1960; Dahlqvist & Borgström, 1961; Wolstenholme & Cameron, 1962) have given more precise information than has hitherto been available on the intralumenal appearance and the biochemical and physiological activities of the normal and pathological small intestine *in vivo*. A variety of preparations of intact intestine or of disrupted intestinal mucosa have been employed *in vitro* in recent years (Fisher & Parsons, 1950*a*,*b*; Wilson & Wiseman, 1954*a*,*b*; Agar, Hird & Sidhu, 1954; Crane & Wilson, 1958; McDougal, Little & Crane, 1960; Crane & Mandelstam, 1960; Hakim, Lester & Lifson, 1963), primarily to study transport of simple molecules into or across intestinal tissue (see also Wiggans & Johnston, 1959; Smyth, 1961; Taylor, 1963), but occasionally to study metabolic processes in association with or independently of transport phenomena (Ginsburg & Hers, 1960; Salomon & Johnson, 1959; Hübscher, Clark, Webb & Sherratt, 1963). Although an integrated picture of lipid digestion, absorption, resynthesis and transport in intestine is now emerging (Palay & Karlin, 1959*b*; Millington, Forbes, Finean & Frazer, 1962; Hübscher *et al.* 1963), relatively little is yet known about other metabolic activities of the small intestine. No satisfactory mechanism has yet been elucidated to account for the active transport of various simple compounds across the intact intestine (Crane, 1960; Wilson, 1962; Parsons, 1963; Nissim, 1964), but it seems probable that the major part if not all of the mechanism is associated with the epithelial cells. These cells must in any case be traversed by substances that suffer passive, 'facilitated' or active transport across the intestine. It is becoming apparent that some at least of the digestive enzymes of the small intestine are integral parts of the structure of the epithelial cells and not secretions from them (Miller & Crane, 1961*a*,*b*; Dahlqvist & Borgström, 1961; Holt & Miller, 1961*a*,*b*; Carnie & Porteous, 1962*b*; Ugolev, Iesuitova, Timofeeva & Fediushina, 1964).

It seemed appropriate therefore to attempt to isolate and characterize all the main recognizable

components of the intestinal epithelial cells as a preliminary to further studies of the metabolism of intestinal epithelium. The complex structure of the intestine imposes certain technical difficulties on such a project. Notable among these difficulties are the separation of epithelial cells (or their components) from the subepithelial mucosa and its components, the preservation of the delicate microvilli of the epithelial cells and the elimination of mucin from cell homogenates. Some previous attempts to fractionate intestinal epithelial cells have taken account of these peculiar difficulties (Hübscher, Clark & Webb, 1962), whereas others have been adopted without modification from procedures designed for a different tissue (Hers, Berthet, Berthet & de Duve, 1951; Morton, 1954; Allard, de Lamirande & Cantero, 1957; Borgström & Dahlqvist, 1958; Triantaphyllopoulos & Tuba, 1959; Ailhaud, Samuel & Desnuelle, 1963). Some procedures have not explicitly included microscopic checks on the isolated fractions, and none has succeeded in preserving intact all the major components of the epithelial cell. The ultrastructure of the intestinal epithelial cells and their supporting villi described by Palay & Karlin (1959*a*) for the rat and the mouse and by Trier (1963) for the human has been confirmed, and extended in some details, for the rabbit small intestine by J. W. Porteous, A. E. Dunn & B. Clark (unpublished work). These electron-microscopic studies have formed a valuable background to the work now described, a preliminary account of which has been presented (Porteous & Clark, 1963).

EXPERIMENTAL

Animals. Rabbits were maintained as described by Carnie & Porteous (1962*a*). They were killed by the injection of saturated $MgSO_4$ (0·5ml./kg. body wt.) into a marginal ear vein after previous light sedation of the animal with Nembutal (0·25ml./kg. body wt.) administered in the same way.

Isolation of epithelial-cell suspensions. Immediately after the death of the animal the abdominal wall was opened, and the small intestine clamped immediately below the stomach and immediately above the junction with the caecum. The small intestine was severed from the stomach and caecum, then immediately emptied, together with attached viscera, from the abdominal cavity into a dish of ice-cold 0·9% NaCl–5mM-EDTA solution, pH7·4. All subsequent manipulations were carried out in vessels immersed in chipped ice or contained in cold rooms or centrifuges maintained at 2–5°; all solutions were cooled before use. In contrast with rabbits killed by neck fracture or decapitation, those killed by the present technique retained a free flow of blood from the viscera for several minutes after death. The excised small intestine was rapidly trimmed free of mesenteries and at the same time transferred to a dish of fresh NaCl–EDTA. The complete length of intestine was then flushed out with NaCl–EDTA (500ml.) while floating in NaCl–EDTA solution and under a hydrostatic head so that the intestine was slightly distended. The washed intestine was transferred to a dish of 0·3M-sucrose–5mM-EDTA, pH7·4, and flushed out with this solution (500ml.) in the same way as before. The washed intestine was drained briefly, laid on a clean glass plate and held down at one end with the edge of a microscope slide. Most of the free intralumenal mucin was removed by gently compressing and stroking the intestine with another glass slide. The mucosa was then expressed by vigorously compressing and stroking the intestine with the edge of the second slide. Alternatively, the mucosa was removed as described by Carnie & Porteous (1962*a*) except that care was taken first to clean away any superficial layer of mucin-like material and the remains of any partially digested food by lightly stroking the surface of the exposed villi with a microscope slide. The former method was more rapid and prevented contamination of the mucosa with fat from the outer surface of the intestine. It was preferred to the latter method, which was only used when, despite starvation of the animal, the gut was not completely free of food and washing failed to remove the last traces of such contamination. Contamination of this kind was invariably associated with a gut that contained more than the usual amount of mucin.

Homogenization of cells and filtration of the homogenate. The weighed cell preparation (usually 15g.) was suspended with a loose-fitting hand-operated Teflon-and-glass homogenizer in 9vol. of 0·3M-sucrose–5mM-EDTA, pH7·4. This suspension was then homogenized in a motor-driven Teflon-and-glass homogenizer (Carnie & Porteous, 1962*b*). The total volume of the final homogenate (fraction I) was noted and a sample put aside for analysis and microscopic examination. The remainder of the homogenate was immediately filtered through nylon cloth (St Martins 9N, 124μ square mesh; supplied by Henry Simon Ltd., Stockport, Cheshire). The cloth was stretched tightly across the bottom perimeter of a cylinder of polythene ($1\frac{1}{2}$in. × 3in. diam.). Several such filters were placed in individual filter funnels inserted into 25ml. measuring cylinders. To each filter was applied a measured volume (not more than 15ml.) of the homogenate, which was rapidly spread over the nylon. Filtration was facilitated by pre-wetting the nylon with sucrose–EDTA solution and by stroking the underside of the nylon with a clean glass rod immediately after applying the homogenate. The total volume of the filtrate (fraction II) was noted and a portion put aside for analysis and for microscopic examination. In some experiments the residue on the filters was removed by agitating the nylon cloth in sucrose–EDTA medium; the resulting tissue suspension was analysed and examined microscopically.

Differential centrifuging. Centrifuges, rotors and the calculation of centrifugal forces were as described by Carnie & Porteous (1962*a*,*b*). All sediments were resuspended in 0·3M-sucrose–5mM-EDTA, pH7·4, with a hand-operated homogenizer (Carnie & Porteous, 1962*b*).

Measured volumes (usually multiples of 20ml.) of the filtrate (fraction II) were centrifuged at 500*g*-min. The supernatant suspension was removed as completely as possible from the loosely packed slightly-pink flocculent sediment and the sediment resuspended to the same volume as the supernatant. Supernatant suspension and resuspended sediment were again centrifuged at 500*g*-min., and the resulting supernatant suspensions removed and com-

bined. The two sediments were combined and resuspended to 20ml. and centrifuged at 1000*g*-min. The supernatant suspension was removed and combined with that already obtained. The loosely-packed white sediment was resuspended and called fraction III. The combined supernatant suspension was centrifuged at 4000*g*-min., the supernatant suspension removed from a small well-packed sediment, and this sediment resuspended in 10ml. of medium and again centrifuged at 4000*g*-min. to give a white sediment surmounting a minute red pellet. The whole sediment on resuspension was called fraction IV. The combined supernatant suspensions were centrifuged at 6000*g*-min. to give a very small well-packed white sediment that on resuspension was called fraction V. The resulting supernatant suspension was centrifuged at 10000*g*-min. to yield another very small white sediment that on resuspension was called fraction VI. Centrifuging the supernatant from the previous sediment at 32000*g*-min. yielded a well-packed buff-coloured sediment called fraction VII after resuspension. The remaining supernatant suspension yielded another well-packed off-white sediment after centrifuging at 150000*g*-min. This sediment was resuspended and called fraction VIII, the remaining supernatant suspension being subjected to 6000000*g*-min. to yield a water-clear supernatant (fraction X) and an almost clear pale-brown jelly-like sediment that on resuspension was called fraction IX. Each of the numbered fractions III–IX was always made to a known volume (usually 10ml.) during the final resuspension; the volume of each supernatant fraction removed from a sediment was noted as a check on the consistency of the centrifuging procedure, and the volume of fraction X was also recorded. The complete isolation procedure occupied approx. 6hr. after injecting the rabbit; the centrifugal fractionation took about 5½hr.

Light-microscopy. Fractions I–X, the residues on the nylon filters and any other tissue preparations were examined, immediately they became available, as described by Carnie & Porteous (1962*b*).

Electron microscopy. (*a*) Intact intestine. The animal was killed as described above, the abdominal cavity opened and narrow rings of intestine were excised into 1% (w/v) OsO_4 in veronal–acetate buffer, pH7·4 (Palade, 1952). Each ring was cut and the resulting strip of intestine quickly trimmed to a series of 1–2mm. cubes under the OsO_4 solution. Fixation was continued at room temperature (18°) for 1hr. Dehydration was carried out, after rinsing the tissue in water, by passage through 50% (v/v), 70% (v/v) and two changes of 100% ethanol. The dehydrated tissue was soaked in two changes of butyl methacrylate–methyl methacrylate mixture (93:7, v/v) during 3hr., then embedded in fresh methacrylate and kept at 55° for 18–24hr. Alternatively, the dehydrated tissue was washed twice with propylene oxide and then embedded in Epon. Blocks were cut with glass knives on a Cambridge–Huxley ultramicrotome to give sections approx. 50mμ thick. Sections were mounted on collodion- and carbon-coated grids, viewed and photographed in a Metropolitan–Vickers EM6 electron microscope. Sections were stained as noted in the legends to the Plates.

(*b*) Cell homogenates and isolated subcellular fractions. Fractions I–IX (1ml. portions) were each added, immediately they became available, to separate portions (4ml.) of cold 0·5% OsO_4 in veronal–acetate buffer (Palade, 1952) made up in 0·3M-sucrose–5mM-EDTA, final pH7·0. After 60min. in chipped ice the flocculent fixed sediments were centrifuged, and the very slightly turbid supernatants removed and discarded. The fixed material was washed once with water and the water discarded after centrifuging. Dehydration was carried out with 70% and 100% ethanol as before; the remaining procedures were those described above.

Preservation of cell homogenates and isolated fractions. Protein determinations and invertase and alkaline-β-glycerophosphatase assays were carried out on freshly isolated dialysed preparations, as described below. All other determinations and assays were carried out on preparations that had been frozen, immediately after isolation, in an ethanol–solid CO_2 mixture, stored at −20°, thawed when required and, if necessary, rehomogenized to procure an even suspension.

Buffers. Phosphate buffers were prepared by adjusting 0·1M-KH_2PO_4 to the desired pH value with 0·1M-Na_2HPO_4. Tris buffers were prepared by adjusting 0·2M-tris (50ml.) to the desired pH value with HCl and then diluting to 100ml. Acetate buffers were prepared by adjusting 0·2M-sodium acetate to the desired pH value with 0·2M-acetic acid. Maleate buffers were prepared by adjusting 0·2M-maleic acid (10ml.) to the desired pH value with NaOH and then diluting to 20ml. with water. pH values were measured at 18–20° with a model 23A pH-meter (Electronic Instruments Ltd., Richmond, Surrey) standardized at the same temperature.

Dialysis. Dialysis was carried out as described by Carnie & Porteous (1962*b*), but for the times and against the solutions specified in the text.

Reagents. Sodium β-glycerophosphate, glucose 6-phosphate (barium salt), sodium succinate, sodium malonate, maleic acid, cholesterol, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, *p*-bromophenylhydrazine hydrochloride, diphenylamine, KF, $Na_2HPO_4,12H_2O$, chloroform, acetone, methanol and ether were obtained as reagent-grade chemicals from British Drug Houses Ltd., Poole, Dorset. β-Naphthylamine (British Drug Houses Ltd.) was recrystallized (m.p. 109–110°) three times from 10% (v/v) ethanol. Bovine serum albumin (fraction V) was obtained from the Armour Pharmaceutical Co., Eastbourne, Sussex; tris and RNA were from C. F. Boehringer und Soehne G.m.b.H., through Courtin and Warner Ltd., Lewes, Sussex; highly polymerized DNA was from the California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A.; *N*-(1-naphthyl)ethylenediamine dihydrochloride was from Eastman–Kodak, Kirby, Lancashire; L-leucyl-β-naphthylamide hydrochloride was from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.; glucose oxidase (crude) and peroxidase (type 1) were from Sigma (London) Chemical Co. Ltd.; *o*-dianisidine was recrystallized three times from pure dry benzene before use. The other reagents were AnalaR chemicals.

Analytical methods. (*a*) Protein. The method of Lowry, Rosebrough, Farr & Randall, as modified by Miller (1959), was applied directly to measured samples of freshly isolated tissue and cell preparations that had been dialysed against water at 2–5° for 12–16hr. Bovine serum albumin was used as a standard over the range 0–100μg. of protein.

(*b*) Nucleic acids. To measured samples of tissue preparations was added an equal volume of ice-cold 10% (w/v) trichloroacetic acid. After standing in ice for 10min. the suspension was centrifuged, the supernatant discarded and

the sediment washed twice by alternate suspension and centrifugation in 5% (w/v) trichloroacetic acid. The final sediment was extracted at room temperature with acetone, then chloroform–methanol–ether (2:1:1, by vol.) and three times with ether. The combined solvent extracts were preserved in some experiments. The dried sediment was extracted at 100° for 10min. with 5% trichloroacetic acid, cooled, centrifuged and the supernatant extract retained. Extraction of the residue was repeated twice more for 5min. at 100° with 5% trichloroacetic acid. The combined trichloroacetic acid extracts were analysed for DNA and RNA.

DNA was determined by the method of Burton (1956); highly purified DNA was used as a standard. Spectrophotometric readings were made at 600 and 650mμ and the difference in extinction values (E_{600}–E_{650}) was taken as a measure of the DNA (Zamenhof, 1957).

RNA was determined by the method of Webb (1956) with the following precautions. The *p*-bromophenylhydrazine hydrochloride was purified by exhaustive ether extraction at room temperature. The full volume (3ml.) of xylene was added before starting the acid hydrolysis. Only xylene that did not give a brown colour in the acid layer after shaking with conc. H_2SO_4 was used. Acid hydrolysis of the tissue extract was carried out in hard-glass centrifuge tubes with standard 'female' ground-glass joints. During the subsequent 3hr. extraction with xylene at 100° a plain air-condenser (approx. 10cm. long × 1cm. diam.) was fitted to the centrifuge tube. These precautions were found to be essential for the production of linear calibration curves (0–100μg. of RNA), the attainment of good recoveries of RNA added to tissues and acceptable matching of duplicate analyses.

(*c*) Inorganic orthophosphate. The method of King (1932) was used.

(*d*) Cholesterol. The combined solvent extract obtained in the course of analysis for nucleic acids was dried with anhydrous Na_2SO_4 for 18hr. The Na_2SO_4 was removed by filtration, the filtrate evaporated to dryness and the residue dissolved in chloroform (5ml.). Acetic anhydride (2ml.) and conc. H_2SO_4 (0·1ml.) were added in turn. After the mixture had been kept in the dark for 10min., spectrophotometric readings were taken at 630mμ (Sackett, 1925).

All these methods of analysis were shown to be unaffected by the presence of sucrose–EDTA in the medium used to suspend tissue preparations.

Enzyme assay procedures. The optimum pH and optimum substrate concentration were determined for each assay procedure. The enzyme-catalysed reactions were shown to follow zero-order kinetics. Tissue preparations were dialysed against glass-distilled water for 18hr. at 2–5° before assay for invertase activity and alkaline-β-glycerophosphatase activity (Clark & Porteous, 1965). Both enzymes were shown to be stable under these conditions of dialysis. Other enzymes were assayed without dialysis of tissue fractions, since the presence of 0·3M-sucrose–5mM-EDTA in the tissue sample did not affect these assay procedures.

(*a*) Invertase activity was determined by incubating dialysed tissue preparations in the assay system described by Carnie & Porteous (1962*b*). Samples (2ml.) of the incubation system were withdrawn into 0·5ml. of $Ba(OH)_2$ to which was then added 0·5ml. of $ZnSO_4$ (Nelson, 1944). A sample (1ml.) of the protein-free supernatant obtained after centrifuging was then added to 4ml. of 0·5M-tris buffer, pH7, containing glucose oxidase (0·25%), peroxidase (0·004%) and *o*-dianisidine (0·01%; added as a 1% solution in ethanol). After incubation at 37° for 75min., reaction was terminated by the addition of 1ml. of 0·5N-H_2SO_4. Spectrophotometric readings were taken at 395mμ. Linear calibration curves were obtained for the range 0–0·5μmole of glucose and were set up for each series of invertase assays. Preliminary experiments established these optimum conditions for the determination of glucose by glucose oxidase. The same experiments showed that the tris buffer [prepared with the commercial material or with tris recrystallized three times from 70% (v/v) ethanol] completely inhibited the invertase and maltase activities of several commercial samples of glucose oxidase and decreased the trehalase activity by about 85% (cf. Dahlqvist, 1961). It was also established that the corresponding intestinal disaccharases were immediately inhibited in the same way by the tris buffer. At low protein concentrations the $Ba(OH)_2$–$ZnSO_4$ deproteinization step could be omitted. The termination of the oxidase reaction by the addition of H_2SO_4 also served to clarify the slight turbidity that developed during the reaction. Acidification shifted the $\lambda_{max.}$ of the solution from 420 to 395mμ.

(*b*) Aminopeptidase activity was assayed in an incubation system similar to that described by Goldbarg & Rutenberg (1958). Enzyme (at 0°) and water were added to 1·37mM-L-leucyl-β-naphthylamide (0·75ml.) and 0·2M-phosphate buffer, pH7 (0·5ml.), preincubated at 37°; the final volume was 3ml. and incubation was continued for 60min. Two control incubation vessels lacked substrate and enzyme respectively. Reaction was terminated by adding 20% (w/v) trichloroacetic acid (1ml.), cooling in ice and centrifuging. Part of the supernatant solution (1ml.) was added to 0·1% sodium nitrite (1ml.), mixed and stood for exactly 3min. at room temperature (18–20°) before adding 0·5% ammonium sulphamate (1ml.). The solutions were mixed and allowed to stand 2min. before adding 2ml. of 0·05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 95% (v/v) ethanol. Extinction values were measured at 580mμ within the next 30min. A linear calibration curve was obtained when β-naphthylamine (0–0·5μmole) was incubated and treated in the same way.

(*c*) Alkaline-β-glycerophosphatase activity was determined by the method of Clark & Porteous (1965), with Co^{2+} as the activating metal ion.

(*d*) Acid-β-glycerophosphatase activity was assayed by adding enzyme (at 0°) to 0·2M-sodium acetate buffer, pH5·6 (1·0ml.), 0·05M-sodium β-glycerophosphate (0·2ml.) and water to bring the volume to 3ml. After incubation at 37° for 15min. 50% (w/v) trichloroacetic acid (0·5ml.) was added, the reaction tubes were transferred to ice and the tube contents were filtered 10min. later through Whatman no. 5 paper. Portions (2ml.) of the filtrate were analysed for inorganic orthophosphate. Preliminary work showed that acid-phosphatase activity increased by approx. 10% of its initial value after freezing and thawing isolated subcellular fractions. Ten repetitions of the freezing-and-thawing procedure brought about no further increase in activity (see the section on Preservation of cell homogenates and isolated fractions).

(*e*) Glucose 6-phosphatase activity was determined by a method adapted from that described by Swanson (1955).

Sufficient water and enzyme (at 0°) to bring the final volume to 1·0ml. were added to 0·1M-maleate buffer, pH6·5 (0·3ml.), and 0·1M-glucose 6-phosphate (0·1ml.) preincubated at 37°. Reaction was terminated after a further 15min. at 37° by the addition of cold 5% trichloroacetic acid (2ml.). After 10min. in ice the tube contents were filtered through Whatman no. 5 paper and 2·0ml. portions of the filtrate analysed for inorganic orthophosphate.

(*f*) Succinate-dehydrogenase activity, as measured by reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium hydrochloride, was determined in the absence of phenazine methosulphate as described by Pennington (1961), except for two modifications: EDTA (2μmoles) was incorporated into the reaction system, and the complete system (and the two controls) were kept at 0° for 30min. before incubating at 37° for 15min. In the absence of added EDTA, reaction velocities were proportional to the amount of enzyme only above a certain minimum amount of enzyme. Below this minimum quantity of enzyme no activity could be detected; with EDTA present a linear plot of activity against protein content of the reaction system was obtained and this plot extrapolated to zero activity at zero enzyme concentration. Enzyme activity was inhibited competitively by malonate (K_i approx. 10^{-5}M).

Enzyme units and presentation of results. One invertase unit is redefined as the amount of enzyme required to release 1μmole of glucose under the conditions given by Carnie & Porteous (1962*b*). This definition is consistent with that adopted for the other activities measured (acid and alkaline β-glycerophosphatase, glucose 6-phosphatase, aminopeptidase, succinate dehydrogenase), namely: one unit of activity is that amount of enzyme which will convert 1μmole of substrate into the product(s) of reaction in the time and under the conditions of assay stipulated. One-fifth of a unit of succinate-dehydrogenase activity gives sufficient formazan, dissolved in 4ml. of ethyl acetate, to give an extinction of 1·0 when measured at 490mμ in cuvettes with a 1cm. light-path. This relationship is deduced from the molar extinction coefficient ($20{\cdot}1 \times 10^3$cm.^{2}/mole) measured on the formazan dissolved in ethyl acetate (Pennington, 1961).

All enzyme activities have been calculated as the number of enzyme units contained by the whole of any one of the fractions I–X, where fraction I represents the original cell homogenate and fraction II the filtrate from this homogenate. The results of chemical analyses have been computed in a similar way. All analyses and enzyme assay results computed for fraction II are presented as percentages of those computed for fraction I, so as to indicate any losses incurred by filtration after correcting for fluid losses, as detailed below. Since fraction II represented the starting material for differential centrifugation, the measured content of each of the isolated subcellular fractions III–X is expressed as a percentage of the content of fraction II. In the histograms the term relative specific activity (or content) of a fraction is defined as the activity (or content) of that fraction expressed as a percentage of the activity (or content) of fraction II divided by the protein content of that fraction expressed as a percentage of the protein content of fraction II. All materials and activities in fraction II have a relative specific activity (or content) of unity; the relative specific activity (or content) is thus an indication of the specific location of a cell component in a particular isolated subcellular fraction (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The specific activities of the individual enzymes (expressed as absolute enzyme units per unit weight of protein) are of course directly proportional to the relative specific activities.

RESULTS

Validity of the cell isolation, homogenization and filtration techniques. The prime aim of the work was to devise a technique for the isolation of the recognizable subcellular components of intestinal epithelial cells. Accordingly, light-microscopic observations served as the main guide during the development of the procedure, and confirmation of these observations was sought by electron microscopy during the final stages of the investigations. Table 1 summarizes the light-microscopic observations on the fractions prepared by the techniques detailed above, and provides cross-references to the electron micrographs.

Histological examination of adjacent lengths of intestine, only one of which had been treated to remove the mucosa, showed that the techniques adopted removed the epithelial cells, the mucosal tissue and parts of the lamina muscularis mucosae, but little more..

The homogenization technique was adopted after a series of trials in which the composition of the medium, the concentration of tissue in the medium, the annular clearance of the homogenizer, the rate and time of homogenization were varied. The effects of these variations were tested by microscopic observations on the homogenates and by preliminary attempts to fractionate the homogenates. Homogenization in 0·25M-, 0·3M- or 0·44M-sucrose under the conditions finally adopted disrupted most but not all of the epithelial cells and the subepithelial tissue. The microscopic picture of the homogenate was similar to that described in Table 1 except that few microvillus sheets survived the treatment. The use of 0·3M-sucrose–5mM-EDTA, pH7·4, under the same conditions again achieved rupture of most of the cells but preserved intact most of the visible subcellular structures, including the microvillus sheets (Table 1).

The concentration of tissue suspension taken for homogenization was adopted from previous experiments with 0·25M-sucrose in which it was shown that homogenization of 1 part of tissue plus 2 or 4 parts of medium resulted in adequate cell breakage, but that the components of the resulting homogenate sometimes agglutinated and would not filter. Attempts to centrifuge these homogenates without filtration frequently resulted in a failure of large particles to sediment. Dilution of the homogenates did not eliminate these difficulties.

In preliminary experiments with 1 part of tissue

Table 1. *Light-microscopic observations on cell homogenates and isolated subcellular fractions*

Fraction	Light-microscopy
I (homogenate of mucosa)	Nuclei, microvillus sheets, free mitochondria, a few erythrocytes mainly in capillaries, mucin, connective tissue, muscle fibres
II (filtrate from I)	Nuclei, microvillus sheets, mitochondria
III ($10^3 g$-min. sediment)	Nuclei and microvillus sheets, the former predominating; a few intact cells (see Plate 2*a*)
IV ($4 \times 10^3 g$-min. sediment)	Nuclei and microvillus sheets, the latter predominating (see Plate 2*a*)
V ($6 \times 10^3 g$-min. sediment)	A few nuclei and many large granules
VI ($10 \times 10^3 g$-min. sediment)	Large granules
VII ($32 \times 10^3 g$-min. sediment)	Heavy mitochondria, i.e. granules under Brownian movement (see Plate 2*b*)
VIII ($15 \times 10^4 g$-min. sediment)	Light mitochondria, i.e. granules smaller than those in VII
IX ($6 \times 10^6 g$-min. sediment)*	Optically clear (see Plate 2*f*)
X (final supernatant)*	Optically clear

* Unstained preparations only examined. All other fractions were examined with and without staining (Carnie & Porteous, 1962*b*).

plus 9 parts of 0·25M-sucrose for homogenization it was nevertheless found that low-speed centrifuging (100–500g-min.) resulted in the appearance of intact tissue, intact cells, nuclei, mitochondria, muscle fibres, connective tissue, capillaries and mucin-like material in a single sediment. The filtration technique adopted removed most of the mucin, intact single cells, intact epithelial tissue and the structures originating from subepithelial tissue (Table 1). Previous work (Summers, 1961) had suggested that serial filtration through two nylon filters of decreasing mesh size was necessary for the preparation of a filtrate free of all contaminants arising from subepithelial tissue, but re-examination of this aspect of the technique showed that serial filtration led to excessive losses of many subcellular components. Blockage of the second filter also frequently occurred.

Success in the filtration step is largely dependent on careful attention to detail and on speed in carrying out the technique. Homogenates must be filtered immediately after they have been prepared and the filtration itself must be rapid. To this end the filter cloths were pre-wetted and drained, a limited volume of homogenate was applied to a large area of filter and the underside of the filter was stroked as described in the Experimental section. Filtration was usually complete within 1–2min. Successful isolation of fractions III and IV was in turn dependent on treatment of the filtrate (fraction II) immediately it became available.

Table 2 contains the results of the quantitative analyses and assays performed on fractions I–X. The mean recovery of the volume of fraction I filtered was 90%. The recovery in fraction II of individual components of fraction I was corrected for this loss of fluid on the filters and supporting glassware. The mean recovery of all the components measured in fraction II was 82%. With but two exceptions the mean recoveries of individual cell components in the filtrate fraction lay between 81 and 97%; the exceptions were DNA (64% mean recovery) and cholesterol (70% mean recovery; recoveries in two experiments were 60 and 79%). Microscopic observations (Table 1) showed that fraction II had been successfully deprived of most of the recognizable subepithelial tissue components present in fraction I; examination of the residue on the filters confirmed this observation but also showed that a few mitochondria and microvillus sheets and very many free nuclei had become trapped by the mucin and connective tissue filtered out by the nylon cloth. The observed differential loss of nuclei during filtration was consistent with the lower recovery of DNA in the filtrate. Whether the differential loss of cholesterol can be accounted for in the same way is not known; it may be that the subepithelial tissue components removed by filtration contain abnormally high concentrations of cholesterol. It was shown in two experiments that all the material lost from fraction I by filtration could be accounted for by analysis or assay of the residues recovered from the filters.

Validity of the differential centrifuging technique. The distribution patterns for DNA, RNA, cholesterol and six enzymes are shown in Fig. 1.

The location of 99% of the DNA of fraction II in fractions III and IV (Table 2), and the high relative specific content of DNA in these fractions (Fig. 1*a*), were consistent with the microscopic identification (Table 1) of these fractions of the epithelial cells as nuclear fractions contaminated with large numbers of microvillus sheets. The microvillus sheets were also confined to these two fractions. Fractions III and IV were retained as separate fractions for several reasons. First, it was found that attempts to isolate the two fractions as one brought about an increased contamination of the nuclear fraction with free mitochondria; secondly, fraction III occasionally contained a few intact epithelial cells or pieces of epithelial tissue, whereas fraction IV was invariably devoid of any such contamination; thirdly, microscopic observation suggested that the nuclei of fraction IV were

Table 2. *Subcellular distribution of components of intestinal epithelial cells*

Absolute values are expressed: in mg./100 ml. of fraction II for protein, DNA, RNA and cholesterol; in units/100 ml. of fraction II for enzyme activities, except aminopeptidase activity, which is stated in hundreds of units/100 ml. of fraction II. Statistics refer to the mean values for the number of experiments shown ± the standard deviation (S.D.) calculated as

$$\text{S.D.} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{N-1}}$$

where x is an individual observed value, $\bar{x}$ the mean of a series of observed values and N the number of observations. Individual deviations from the mean of a series of observations $(x-\bar{x})$ rarely exceeded the mean deviation $(x-\bar{x})/N$ by a factor 2 and none exceeded the mean deviation by a factor 3. Fraction I was the cell homogenate, fraction II the filtrate from fraction I; subcellular fractions III–X are identified in Table 1. Fractions III–IX were isolated by applying 10^3g-min., 4×10^3g-min., 6×10^3g-min., 10×10^3g-min., 32×10^3g-min., 15×10^4g-min. and 6×10^6g-min. to fraction II and to successive supernatant suspensions; fraction X was the final supernatant solution.

Component	No. of expts.	Absolute values for fraction II	Mean percentage values in the filtrate (II) and in each isolated subcellular fraction (III–X)										Recovery (%)
			I	II	III	IV	V	VI	VII	VIII	IX	X	
Protein	6	1280±160	100	85±8·9	26·6± 3·4	6·6± 2·4	2·4±0·4	2·0±0·6	6·6±1·3	4·7±0·8	7·5±1·2	39·4± 3·1	96± 7·1
				100									
DNA	5	63±10·8	100	64±10·5	83·0±18·7	15·9± 2·4	2·5±0·3	0·6±0·2	0·4±0·7	0·1±0·1	0·2±0·1	0·2± 0·1	103±21·8
				100									
RNA	5	40± 6·1	100	81± 6·7	16·4± 2·8	4·8± 1·8	1·8±0·5	1·3±0·7	4·8±0·7	3·1±0·4	26·2±4·5	36·6±10·6	95± 9·0
				100									
Cholesterol	2	20± 2·2	100	70±14·0	34·1± 3·6	12·3± 4·4	3·1±0·2	2·9±0·5	10·7±1·3	9·1±1·3	18·9±5·2	2·2± 1·4	93±0
				100									
Succinate dehydrogenase	5	300±100	100	97±13·0	22·0± 8·0	8·9± 6·0	6·2±2·6	7·2±3·8	34·4±7·0	15·8±4·1	2·5±1·5	1·5± 1·8	99±12·5
				100									
Acid β-glycerophosphatase	6	157±49	100	86±17·4	21·1± 7·0	5·8± 2·9	2·8±1·0	4·0±1·7	18·7±3·3	14·4±6·3	7·5±2·6	4·4± 4·9	79±10·4
				100									
Glucose 6-phosphatase	5	560±153	100	94±13·5	30·0±10·2	11·0± 4·5	4·1±1·6	3·8±1·1	15·5±5·0	13·6±2·4	25·4±2·6	0·3± 0·6	104±10·6
				100									
Alkaline β-glycerophosphatase	4	1800±214	100	83± 7·6	45·2±10·7	12·6± 5·3	3·9±1·1	2·6±1·6	7·8±1·5	8·3±3·7	8·4±3·7	4·9± 9·8	94±22·0
				100									
Invertase	3	2640±128	100	81±23·4	54·5± 2·3	16·8±10·0	2·2±1·0	1·1±0·4	3·1±1·2	6·9±3·6	9·9±4·8	0	93± 5·2
				100									
Aminopeptidase	2	300±14	100	87± 9·0	63·0± 8·0	20·0± 4·5	0·7±2·6	0·6±2·5	1·7±1·3	2·2±1·5	4·4±2·1	3·4± 1·8	96± 9·0
				100									

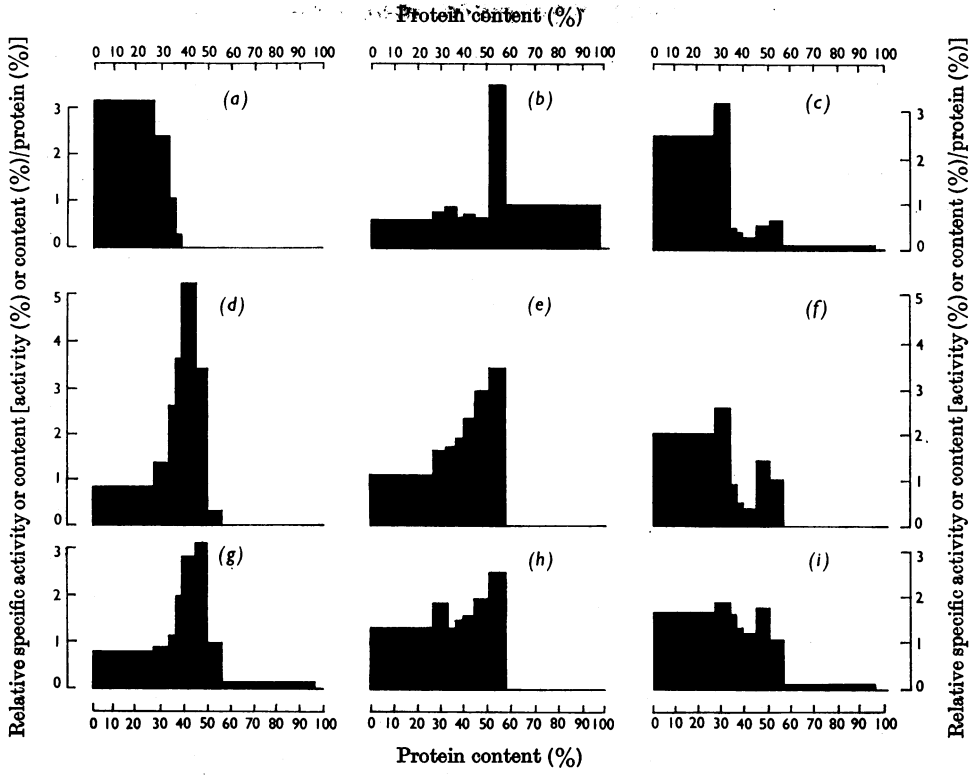
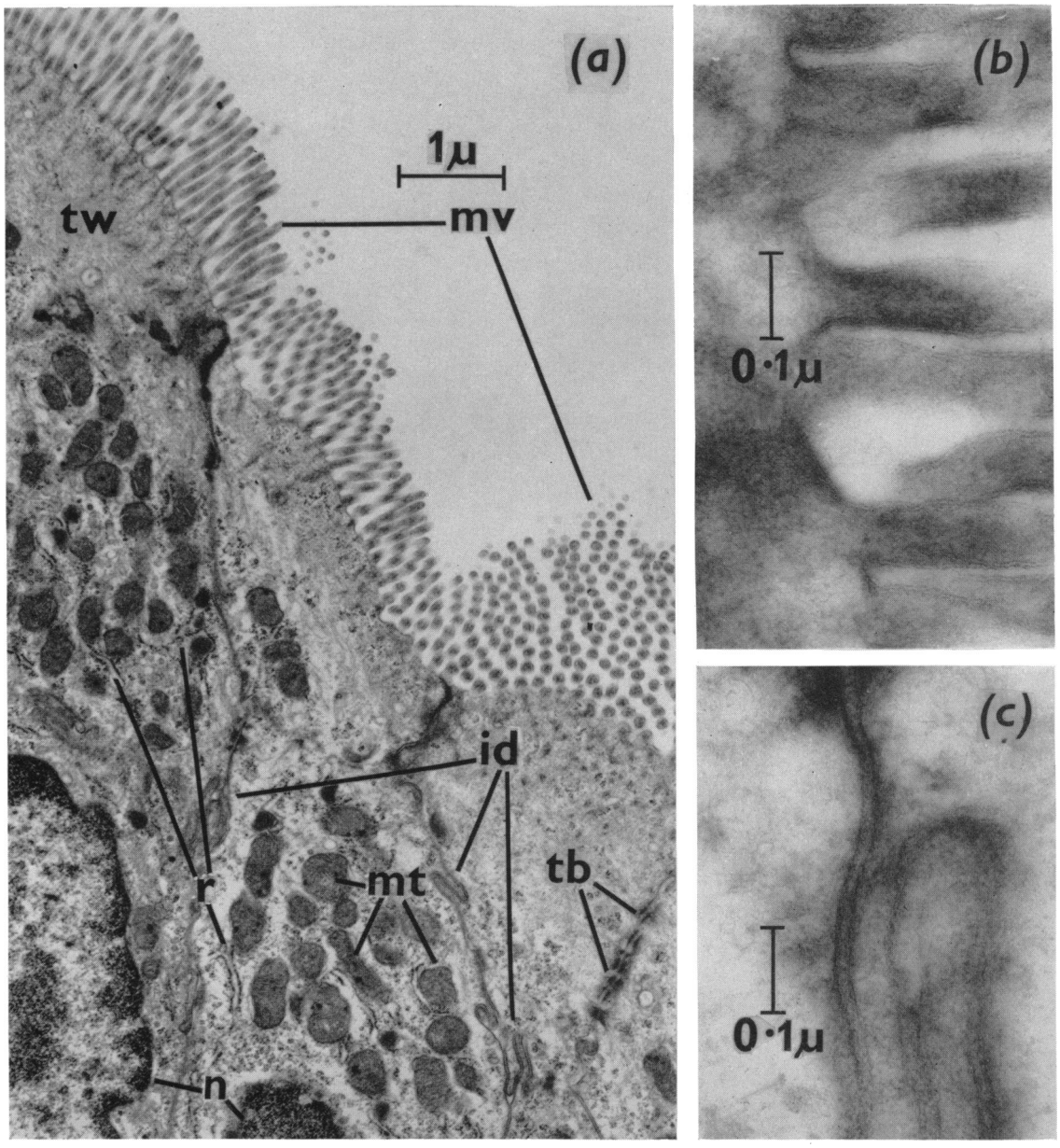


Fig. 1. Subcellular distribution of DNA, RNA, cholesterol and of six selected enzymes. The ordinate indicates mean relative specific activity (or content) of the isolated subcellular fractions; the abscissa indicates the protein content of fractions III–X (Tables 1 and 2). The individual sections of each histogram represent (from left to right) fractions III–X inclusive. (*a*) DNA (5); (*b*) RNA (5); (*c*) aminopeptidase (2); (*d*) succinate dehydrogenase (5); (*e*) glucose 6-phosphatase (5); (*f*) invertase (3); (*g*) acid β-glycerophosphatase (6); (*h*) cholesterol (2); (*i*) alkaline β-glycerophosphatase (4). Numbers in parentheses refer to the numbers of experiments performed.

somewhat smaller than those in fraction III and could possibly have arisen from the immature crypt cells (Leblond & Walker, 1956); fourthly, the microvillus sheets seemed relatively more abundant in fraction IV than they did in fraction III.

All attempts to segregate the nuclei from the microvillus sheets have so far failed. These attempts have included resuspension followed by centrifuging of fraction III or IV in various media including: 0·3M-sucrose–5mM-EDTA–0·01M-tris, pH 7·4; sucrose (0·25–0·5M) with and without the addition of potassium chloride (0·075%), calcium chloride (0·01%) or citric acid (0·1%) (Dounce, 1955); 95% (v/v) glycerol (Woernley & Carruthers, 1957; Woernley, Carruthers, Lilga & Baumler, 1959); the glycerol medium of Philpot & Stanier (1956); the raffinose–dextran medium of Birbeck & Reid (1956). Further attempts to separate the nuclei and microvillus sheets by centrifuging suspensions of fractions III or IV layered on to continuous or discontinuous gradients of glycerol (18–40%, v/v) or of sucrose (0·4–2·0M in water or in 5mM-EDTA, pH 7·4) also failed. The failure to separate the nuclei and the microvillus sheets was unexpected, but subsequent examination showed that they were not markedly different in volume though very different in shape (Plate 2*a*). There was no obvious sign of agglutination of the two subcellular components and a search for any residual physical connexion between the two produced no evidence that they were attached. Such physical attachments might nevertheless be difficult to preserve and detect microscopically.

The identification of fractions VII and VIII as heavy-mitochondrial and light-mitochondrial fractions respectively rested on light-microscopic observations (Table 1), the concentration of a large part of the total succinate-dehydrogenase activity in these fractions (Table 2), the high relative specific activity of this enzyme system in



EXPLANATION OF PLATE 1

Electron micrographs of intact epithelial cells of rabbit small intestine. (*a*) Oblique longitudinal section of the supranuclear region of three adjacent epithelial cells. The tissue was embedded in Epon, and the sections were stained with uranyl acetate: mv, microvilli in longitudinal and cross-section; tw, the terminal web, showing the roots of the microvilli; mt, mitochondria; tb, terminal bars; id, interdigitations of opposed double membranes of adjacent cells; r, rough endoplasmic reticulum; n, nuclei. The supranuclear region of the cell occupies approximately half of the total length of the intestinal epithelial cell; the nucleus occupies most of that half of the cell that is attached to the villi of the subepithelial tissue of the intestine. (*b*) Longitudinal section through parts of several microvilli showing the continuous limiting double membrane. The tissue was embedded in methacrylate, and the sections were stained with permanganate. (*c*) Interdigitation of opposed double membranes of adjacent epithelial cells. The tissue was embedded in methacrylate and the sections were stained with permanganate.

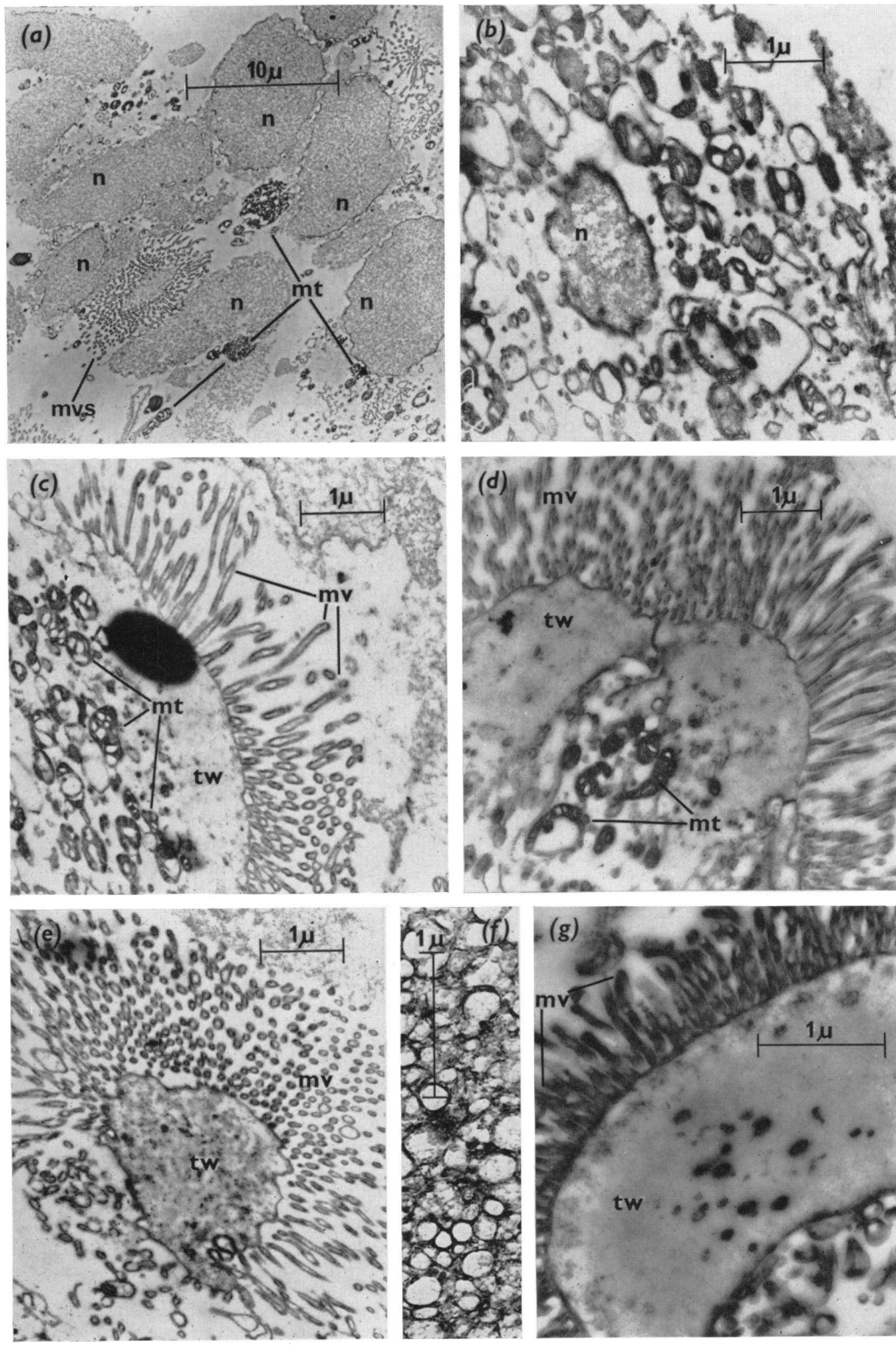


PLATE 2

J. W. PORTEOUS AND B. CLARK

these two fractions (Fig. 1*d*) and the electron-microscopic identification (Plate 2*b*) of mitochondria as the major component of these fractions. Some mitochondrial contamination of fractions III and IV was indicated by the appearance of about 30% of the succinate-dehydrogenase activity in these two fractions (Table 2). This contamination was not apparent under the light-microscope, but electron microscopy (Plate 2*c*, *d*, *e* and *g*) revealed that a proportion of the microvillus sheets still had attached to them some endoplasmic reticulum and many mitochondria. It has been pointed out that fraction IV seemed to contain relatively more microvillus sheets than did fraction III. It may be significant that fraction IV showed a lower specific content of DNA (Fig. 1*a*) and a higher specific activity for succinate dehydrogenase than did fraction III (Fig. 1*d*).

The identification of fraction IX as the microsome fraction of the epithelial cells rested in the first instance on light- and electron-microscopic observations (Table 1 and Plate 2*f*). The high relative specific content of RNA in this single fraction (Fig. 1*b*) is consistent with observations on the microsome fraction of other cells (Palade & Siekevitz, 1956*a*,*b*). The optically clear electron-transparent fraction X contained approx. 40% of the total protein of the cell and 40% of the total RNA of the cell (Tables 1 and 2), thus giving a relative specific content of RNA close to unity (Fig. 1*b*). Fraction X was identified as the cell-sap fraction of the epithelial cells.

de Duve *et al.* (1955) showed that the heavy and light mitochondria and the microsome fraction of rat liver cells between them contained 80% of the total acid-phosphatase activity, although only the light-mitochondrial (or lysosome) fraction showed a high relative specific activity. In intestinal epithelial cells (Table 2 and Fig. 1*g*) the enzyme seemed to be more generally distributed, although the two mitochondrial fractions were the only two that showed a high relative specific activity. Electron-microscopic studies have not yet revealed any lysosomes in the intestinal epithelial cells. It may be significant that repeated freezing and thawing of isolated subcellular preparations of these cells did not give a large increase in acid-phosphatase activity. de Duve *et al.* (1955) also found that approx. 70% of the glucose 6-phosphatase activity of rat liver cells was located in the microsome fraction, which was the only fraction to show a high concentration of the enzyme. In rabbit intestinal epithelial cells this enzyme seems to be much more generally distributed (Table 2 and Fig. 1*e*), although the microsome fraction contained the enzyme at a higher concentration than did any other fraction. If glucose 6-phosphatase is in reality a microsomal enzyme these results would be consistent with some contamination of fractions III and IV with endoplasmic reticulum and marked contamination of the mitochondrial fractions with endoplasmic reticulum. Similarly, the apparent distribution of acid-β-glycerophosphatase activity might suggest the need to introduce a resuspension and recentrifuging step after the initial isolation of the heavy-mitochondrial fraction. These points are discussed below.

Invertase and aminopeptidase activities appeared to be concentrated in fractions III and IV and to a smaller degree in fractions VIII and IX. Fraction IV, which has been shown above to have a greater abundance of microvillus sheets than fraction III, contained the greater concentration of invertase and of aminopeptidase. The proportions of these enzymes in fractions VIII and IX was relatively small and none of the invertase appeared to be soluble (Carnie & Porteous, 1962*b*). Except for the slightly higher concentration of alkaline β-glycerophosphatase in fractions V–VII and the small amount of soluble enzyme (Fig. 1*i*), the distribution of alkaline-β-glycerophosphatase activity among the isolated subcellular fractions of the epithelial cells was very similar to that of the invertase and aminopeptidase activities.

The distribution pattern for cholesterol (Fig. 1*h*) coincided almost exactly with the mean of the distribution patterns for glucose 6-phosphatase and invertase (or alkaline-β-glycerophosphatase) activities.

DISCUSSION

Electron micrographs of longitudinal sections of intestinal epithelial cells show (Plate 1*a*, *b* and *c*) that the outer border of the cell is a double membrane

EXPLANATION OF PLATE 2

Electron micrographs of isolated subcellular components (Table 1) of rabbit intestinal epithelial cells. (*a*) Fraction III: a microvillus sheet (mvs) collapsed into a ring of similar volume to several adjacent nuclei (n); a few contaminating mitochondria (mt) are present. (*b*) Fraction VII: mitochondria showing distortion of the cristae usually found after isolation of the organelles in 0·25–0·3 M-sucrose. (*c*) and (*d*) Fractions III and IV: many isolated microvillus sheets still had mitochondria (mt) attached to them (cf. Plate 1*a*), but the majority, (*e*) and (*g*), were free of mitochondria; the microvilli (mv) and the terminal web (tw) are well preserved. (*f*) Fraction IX: vesicles of the microsome fraction; few ribosomes are apparent. All isolated subcellular components were embedded in methacrylate; sections were stained with permanganate.

that continues across the surface of many hundreds of microvilli and then envelops the rest of the cell (cf. Palay & Karlin, 1959*a*). Attachment of adjacent cells appears to be achieved by interdigitation of the opposed membranes and possibly also by 'terminal bars', one of which invariably girdles each adjacent cell at the level of the 'terminal web' (Plate 1*a*) just below the microvilli (Farquhar & Palade, 1963). Terminal bars also girdle the cells at greater depths from the microvilli. Below the apparently fine fibrous mass of the terminal web lies a large number of mitochondria and rather deep within the cell lies the nucleus. Mitochondria are less densely packed below the nucleus. The endoplasmic reticulum is predominantly 'smooth', but occasionally areas of 'rough' reticulum are seen (Plate 1*a*). High-resolution microscopy suggests that each microvillus contains several threads arranged along its length and that these threads continue some distance into the terminal web (Plate 1*a*). The intestinal epithelial cell thus seems to be capped by a special structure, the terminal web, from which arise the fibrous cores of the microvilli, which in turn are covered by a continuous double membrane. This part of the cell may be expected to have special functions, since it is the first to come into contact with material within the lumen of the intestine, and it is noteworthy that this entire structure is preserved as a separate entity (Table 1 and Plate 2*a*, *c*, *d*, *e* and *g*) during rupture of the cell under specified conditions. This entity we have called the microvillus sheet. Miller & Crane (1961*b*) appear to be the first to have isolated intact microvillus sheets by disrupting golden-hamster intestinal epithelial cells in aqueous EDTA solution with a Waring Blendor. Similar preparations (Eicholz & Crane, 1963) do, however, appear to contain relatively high concentrations of DNA; since intact nuclei were not apparent in these preparations it would seem that DNA had beome rather firmly attached to the isolated microvilli. Attempts to apply the technique of Miller & Crane (1961*b*) to rabbit and golden-hamster intestine have failed to yield intact microvilli free of nuclei in this Laboratory; it may be that the precise speed of the Blendor is critical. Other workers (Gallo & Treadwell, 1963; Bailey & Pentchev, 1964) have modified the method of Miller & Crane (1961*b*). Despite the concomitant presence of microvilli with nuclei in fractions III and IV (Table 1) the relative specific content of DNA was well above unity in these fractions (Fig. 1*a*).

Miller & Crane (1961*b*) obtained a tenfold increase in the specific activity of the invertase of homogenates of golden-hamster intestinal mucosa, merely by isolating the microvillus fraction. In the present work a two- to three-fold increase in specific activity was obtained in fractions III and IV (Fig. 1*f*). The relatively greater abundance of microvillus structures in fraction IV was observed microscopically and was consistent with the DNA and succinate-dehydrogenase distribution in these two fractions. The concentration of invertase activity in fraction IV was also somewhat greater than that in fraction III. This observation would be consistent with the specific location of this digestive enzyme in the microvillus sheet but unequivocal proof of the location of the invertase of rabbit intestine must await the separation of the microvillus sheets from the nuclei of fractions III and IV.

If the invertase is part of the microvillus structure the appearance of a small but significant part of the total activity in the light-mitochondrial and microsomal fractions (Fig. 1*f*) is readily explained. The microvilli are known to be rather delicate structures that tend under adverse conditions to degenerate into much smaller vesicular structures (Millington & Finean, 1963). It seems reasonable to suppose that the invertase activity of fractions VIII and IX is in fact due to the sedimentation in these fractions of small numbers of vesicles derived from the microvilli (Carnie & Porteous, 1962*b*).

Further support for the location of the invertase activity within the microvillus sheets comes from the striking similarity between the intracellular distribution patterns for invertase and alkaline-phosphatase activities (Fig. 1*f* and *i*). There is ample histochemical evidence (Deane & Dempsey, 1945; Emmel, 1945; Johnson & Kugler, 1953; Hancox & Hyslop, 1953; Burgos, Deane & Karnovsky, 1955; Fredricsson, 1956; Brandes, Zetterqvist & Sheldon, 1956; Feigin & Wolf, 1957) for the specific or predominant location of intestinal alkaline phosphatase in the brush border or microvillus region of the epithelial cells.

Reports by previous workers (Hers *et al.* 1951; Morton, 1954; Allard *et al.* 1957; Triantaphyllopoulos & Tuba, 1959; Ailhaud *et al.* 1963; Robinson, 1963) that intestinal-epithelial-cell alkaline phosphatase was located in the microsome fraction probably arose from failure to maintain the integrity of the microvillus sheets during homogenization of the cells. Clark & Porteous (1965) deliberately disintegrated crude preparations of microvilli in the initial purification of intestinal alkaline phosphatase and sedimented the particulate enzyme under conditions similar to those normally used to sediment the microsome fraction of cell homogenates.

The subcellular distribution of aminopeptidase was essentially similar to that of invertase (Fig. 1*c*). A smaller proportion of the total activity appeared in fractions VIII and IX than would have been expected from the observed subcellular distribution of invertase. It is likely that the slight difference in the apparent distribution of the two enzymes is

to be accounted for by chance variations in the fragility of the microvilli. The aminopeptidase distribution was determined in two experiments that were separate from the six experiments that yielded all the other results shown in Table 1 and Fig. 1.

The distribution of RNA in intestinal epithelial cells calls for some comment. The high concentration of RNA in the microsome fraction of liver and pancreas cells is generally attributed to the presence of the RNA-containing ribosomes attached to the endoplasmic reticulum (Palade & Siekevitz, 1956*a*,*b*; Siekevitz, 1963). The paucity of ribosomes in intestinal epithelial cells (Plates 1*a* and 2*f*) contrasts with the high concentration of RNA in the microsome fraction of these cells (Fig. 1*b*). Three conclusions are possible: (*a*) ribosomes in intestinal epithelial cells are rare and the membranes of the endoplasmic reticulum contain extraordinarily high concentrations of RNA; (*b*) ribosomes are present in these cells but are unstable under the conditions used to isolate subcellular fractions; (*c*) ribosomes are present in the intact cells and in the isolated microsome fraction but are labile under the conditions used to prepare, fix and embed tissue sections and subcellular fractions for electron microscopy. The present results do not distinguish between these possibilities.

The RNA distribution pattern (Fig. 1*b*) suggests that the microsome fraction was cleanly separated from the preceding particulate fractions and the following cell sap. The distribution of glucose 6-phosphatase activity (Fig. 1*e*) is open to two interpretations. First, assuming this enzyme to be a microsomal enzyme, as in rat liver cells (de Duve, *et al.* 1955), its broad distribution in the isolated subcellular fractions of rabbit intestinal epithelial cells would indicate some contamination of the heavier particulate fractions with endoplasnic reticulum. Inclusion of EDTA in the cell homogenization medium has been shown to preserve the integrity of the membranous microvillus sheets; it is possible that the endoplasmic reticulum is also stabilized in the presence of EDTA and its rupture from the other structural components and organelles of the cell rendered more difficult. A second interpretation would be that the glucose 6-phosphatase activity is distributed throughout the membrane structures of the intact epithelial cells of the small intestine. de Duve *et al.* (1955) pointed out that EDTA had a protective effect on glucose 6-phosphatase activity and included EDTA in their assay system; it is not clear whether EDTA was included in the homogenization medium in all those experiments in which they assayed the isolated subcellular fractions for glucose 6-phosphatase activity. It is conceivable that some glucose 6-phosphatase activity was lost preferentially from the heavier particulate fractions of rat liver cells when EDTA was not included in the isolation medium.

Since the intracellular distribution of cholesterol is represented by the mean of the distributions of alkaline-phosphatase (or invertase) and glucose 6-phosphatase activities, it would seem that cholesterol is distributed between the microvillus structures and the endoplasmic reticulum or has a more general distribution throughout all membranous components of the cell, including the microvillus structures. The choice between these possibilities depends on the choice between the two interpretations of the glucose 6-phosphatase distribution pattern.

It is clear that any fractionation of intestinal epithelial cells that fails to preserve intact the microvillus sheets as well as the other particulate subcellular fractions will give an erroneous picture of the intracellular distribution of chemical components and enzyme activities. On the other hand, the failure to separate the nuclei and microvillus sheets has presented an obstacle to the unequivocal description of the intracellular location of the cholesterol, invertase, aminopeptidase and alkaline phosphatase of the epithelial cell. Resolution of these two subcellular structures is being attempted by other techniques and if successful should also eliminate one of the alternative interpretations of the apparent intracellular distribution of glucose 6-phosphatase activity.

So far as we are aware there has been no previous description of a technique leading to the isolation and characterization of all the major recognizable subcellular components of intestinal epithelial cells. Contamination of the isolated fractions with those structures that obviously belonged to the subepithelial tissue has been avoided by including a filtration step in the procedure. This step led to a differential loss of DNA and cholesterol, but the magnitude of these differential losses has been recorded.

Some further points call for comment. The large-granule fractions V and VI (Table 1) formed a small part of the homogenate (Table 2), and none of the homogenate components so far examined appeared to be concentrated in these fractions (Fig. 1). Electron-microscopic examination of the contents of these fractions has not led to their unequivocal identification with any component of the intact epithelial cells or subepithelial tissue. Although much if not all of the mucin was removed by filtration of the cell homogenate, no distinction has been made between subcellular structures arising from the epithelial cells proper and from the randomly distributed mucin-producing goblet cells. Similarly, no distinction has been made between subcellular components arising from epithelial cells located at different points along the villi.

Finally, no attempt has been made to distinguish between the functionally different segments of the intestine such as the duodenum, jejunum and ileum. It seems likely that the technique devised for the whole length of the small intestine will be readily applicable to any chosen segment; functional differences between different parts of the intestine will doubtless be reflected in qualitative and quantitative differences in the enzyme activities of the corresponding epithelial-cell homogenates and possibly also in different intracellular distributions of at least some enzymes.

We thank the Medical Research Council for generous financial assistance. The Department of Scientific and Industrial Research donated the electron microscope to the University and a high-speed centrifuge to the Department. Miss Helen Brough assisted in the final stages of the work. Mr A. E. Dunn provided training in electron-microscopic techniques and helped to prepare the sections illustrated. Dr J. E. Fothergill gave valuable advice on photographic methods. We thank Professor Kermack for his continued support and interest.

REFERENCES

Agar, W. T., Hird, F. J. R. & Sidhu, G. S. (1954). *Biochim. biophys. Acta*, **14**, 80.
Ailhaud, G., Samuel, D. & Desnuelle, P. (1963). *Biochim. biophys. Acta*, **67**, 150.
Allard, C., de Lamirande, G. & Cantero, A. (1957). *Cancer Res.* **17**, 862.
Bailey, J. M. & Pentchev, P. (1964). *Proc. Soc. exp. Biol., N.Y.*, **115**, 796.
Birbeck, M. S. C. & Reid, E. (1956). *J. biophys. biochem. Cytol.* **2**, 609.
Borgström, B. & Dahlqvist, A. (1958). *Acta chem. scand.* **12**, 1997.
Brandes, D., Zetterqvist, H. & Sheldon, H. (1956). *Nature, Lond.*, **177**, 382.
Burgos, M. H., Deane, H. W. & Karnovsky, M. L. (1955). *J. Histochem. Cytochem.* **3**, 103.
Burton, K. (1956). *Biochem. J.* **62**, 315.
Carnie, J. A. & Porteous, J. W. (1962*a*). *Biochem. J.* **85**, 450.
Carnie, J. A. & Porteous, J. W. (1962*b*). *Biochem. J.* **85**, 620.
Clark, B. & Porteous, J. W. (1965). *Biochem. J.* **95**, 475.
Crane, C. E. & Neuberger, A. (1960). *Biochem. J.* **74**, 313.
Crane, R. K. (1960). *Physiol. Rev.* **40**, 789.
Crane, R. K. & Mandelstam, P. (1960). *Biochim. biophys. Acta*, **45**, 460.
Crane, R. K. & Wilson, T. H. (1958). *J. appl. Physiol.* **12**, 145.
Dahlqvist, A. (1961). *Biochem. J.* **80**, 547.
Dahlqvist, A. & Borgström, B. (1961). *Biochem. J.* **81**, 411.
Deane, H. W. & Dempsey, E. W. (1945). *Anat. Rec.* **93**, 401.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 105. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Eicholz, A. & Crane, R. K. (1963). *Fed. Proc.* **22**, 416.
Emmel, V. M. (1945). *Anat. Rec.* **91**, 39.
Farquhar, M. G. & Palade, G. E. (1963). *J. Cell Biol.* **17**, 375.
Feigin, I. & Wolf, A. (1957). *J. Histochem. Cytochem.* **5**, 53.
Fisher, R. B. & Parsons, D. S. (1950*a*). *J. Physiol.* **110**, 36.
Fisher, R. B. & Parsons, D. S. (1950*b*). *J. Physiol.* **110**, 281.
Fredericsson, B. (1956). *Exp. Cell Res.* **10**, 63.
Gallo, L. L. & Treadwell, C. R. (1963). *Proc. Soc. exp. Biol., N.Y.*, **114**, 69.
Ginsburg, V. & Hers, H. G. (1960). *Biochim. biophys. Acta*, **38**, 427.
Goldbarg, J. A. & Rutenberg, A. M. (1958). *Cancer*, **11**, 283.
Hakim, A., Lester, R. G. & Lifson, N. (1963). *J. appl. Physiol.* **18**, 409.
Hancox, N. M. & Hyslop, D. B. (1953). *J. Anat., Lond.*, **87**, 237.
Hers, H. G., Berthet, J., Berthet, L. & de Duve, C. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 21.
Holt, J. H. & Miller, D. (1961*a*). *J. Lab. clin. Med.* **58**, 5.
Holt, J. H. & Miller, D. (1961*b*). *J. Lab. clin. Med.* **58**, 327.
Hübscher, G., Clark, B. & Webb, M. E. (1962). *Biochem. J.* **84**, 23P.
Hübscher, G., Clark, B., Webb, M. E. & Sherratt, H. S. A. (1963). *Biochim. biophys. Acta*, Library Vol. 1, p. 201.
Johnson, F. R. & Kugler, J. H. (1953). *J. Anat., Lond.*, **87**, 247.
King, E. J. (1932). *Biochem. J.* **26**, 292.
Leblond, C. P. & Stevens, C. E. (1948). *Anat. Rec.* **100**, 357.
Leblond, C. P., Stevens, C. E. & Bogoroch, R. (1948). *Science*, **108**, 531.
Leblond, C. P. & Walker, B. E. (1956). *Physiol. Rev.* **36**, 255.
McDougall, D. B., Little, K. D. & Crane, R. K. (1960). *Biochim. biophys. Acta*, **45**, 483.
Miller, D. & Crane, R. K. (1961*a*). *Biochim. biophys. Acta*, **52**, 281.
Miller, D. & Crane, R. K. (1961*b*). *Biochim. biophys. Acta*, **52**, 293.
Miller, G. L. (1959). *Analyt. Chem.* **31**, 964.
Millington, P. F. & Finean, J. B. (1963). *Biochim. biophys. Acta*, Library Vol. 1, p. 116.
Millington, P. F., Forbes, O. C., Finean, J. B. & Frazer, A. C. (1962). *Exp. Cell Res.* **28**, 179.
Morton, R. K. (1954). *Biochem. J.* **57**, 595.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Nissim, J. A. (1964). *Nature, Lond.*, **204**, 148.
Palade, G. E. (1952). *J. exp. Med.* **95**, 285.
Palade, G. E. & Siekevitz, P. (1956*a*). *J. biophys. biochem. Cytol.* **2**, 171.
Palade, G. E. & Siekevitz, P. (1956*b*). *J. biophys. biochem. Cytol.* **2**, 671.
Palay, S. L. & Karlin, L. J. (1959*a*). *J. biophys. biochem. Cytol.* **5**, 363.
Palay, S. L. & Karlin, L. J. (1959*b*). *J. biophys. biochem. Cytol.* **5**, 373.
Parsons, D. S. (1963). *Nature, Lond.*, **197**, 1303.
Pennington, R. J. (1961). *Biochem. J.* **80**, 649.
Philpot, J. St L. & Stanier, J. E. (1956). *Biochem. J.* **63**, 214.
Porteous, J. W. & Clark, B. (1963). *Biochem. J.* **88**, 20P.
Robinson, G. B. (1963). *Biochem. J.* **88**, 162.

Sackett, G. E. (1925). *J. biol. Chem.* **64**, 203.
Salomon, L. L. & Johnson, J. E. (1959). *Arch. Biochem. Biophys.* **82**, 179.
Siekevitz, P. (1963). *Annu. Rev. Physiol.* **25**, 15.
Smyth, D. H. (1961). *Proc. R. Soc. Med.* **54**, 769.
Summers, P. (1961). M.Sc. Thesis: University of Aberdeen.
Swanson, M. A. (1955). In *Methods in Enzymology*, vol. 2, p. 541. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Taylor, C. B. (1963). *J. Physiol.* **165**, 199.
Triantaphyllopoulos, E. & Tuba, J. (1959). *Canad. J. Biochem. Biophys.* **37**, 699.
Trier, J. S. (1963). *J. Cell Biol.* **18**, 599.
Ugolev, A. M., Iesuitova, N. N., Timofeeva, N. M. & Fediushina, I. N. (1964). *Nature, Lond.*, **202**, 807.
Webb, J. M. (1956). *J. biol. Chem.* **221**, 635.
Wiggans, D. S. & Johnston, J. M. (1959). *Biochim. biophys. Acta*, **32**, 69.
Wilson, T. H. (1962). *Intestinal Absorption*. Philadelphia: W. B. Saunders Co.
Wilson, T. H. & Wiseman, G. (1954*a*). *J. Physiol.* **123**, 116.
Wilson, T. H. & Wiseman, G. (1954*b*). *J. Physiol.* **123**, 126.
Woernley, D. L. & Carruthers, C. (1957). *Arch. Biochem. Biophys.* **67**, 493.
Woernley, D. L., Carruthers, C., Lilga, K. T. & Baumler, A. (1959). *Arch. Biochem. Biophys.* **84**, 159.
Wolstenholme, G. E. W. & Cameron, M. P. (Eds.) (1962). *Ciba Found. Study Group no.* 14*: Intestinal Biopsy*. London: J. and A. Churchill Ltd.
Zamenhof, S. (1957). In *Methods in Enzymology*, vol. 3, p. 696. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.