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The Solubilization, Thermolability, Chromatographic Purification and Intracellular Distribution of some Glycosidases of Rabbit Small Intestine

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Carnie & Porteous (1962) showed that treatment of a 0·25 M-sucrose suspension of the epithelial cells of rabbit small intestine in a Waring Blendor followed by centrifuging for 15 min. at 10 000*g* gave a supernatant suspension from which part of the invertase and maltase activities of the homogenate could be obtained in particulate form (the 10^5g fraction) by centrifuging at 100 000*g* for 45 min. Transglycosylation by the maltase and invertase activities was demonstrated and the probable nature of one of three sugars formed by trans-α-glucosylation during incubation with sucrose was also determined. The kinetic characteristics of the invertase activity and optimum conditions for assay of its hydrolytic activity were elucidated.

Borgström & Dahlqvist (1958) concluded that the invertase, maltase and trehalase activities of hog intestine were confined to the microsomal fraction of the epithelial cells. We have now attempted to determine the intracellular distribution of the invertase activity of the epithelial cells of rabbit small intestine. The solubilization of the invertase, maltase and trehalase activities of the 10^5g fraction (Carnie & Porteous, 1962), the thermolability of these enzyme activities and their partial chromatographic resolution are described.

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EXPERIMENTAL

Cell preparations. Mucosal cells of rabbit small intestine were isolated as described by Carnie & Porteous (1962). All subsequent operations were carried out in vessels immersed in chipped ice or contained in centrifuges operated at 2–5°.

Homogenizers. The Potter & Elvehjem (1936) type was used. The pestle was fashioned from a cylindrical block of Teflon (20 mm. diam. × 40 mm.), one end of which was turned down to a cone of cross-sectional apex angle 45°, the other end of which was attached to a stainless-steel shaft. The 'mortar' was made from Veridia precision-bore glass tubing (overall length 17 cm.), the closed end of the mortar being formed to the same cone dimensions as the pestle. The radial tolerance between mortar and pestle was 0·125 mm. and the operational area of the pestle was approximately 30 cm.^{2} The pestle was driven at 2000 rev./min. while the mortar was reciprocated (about 25–30 complete strokes/min.) for the time stated for each experiment. In some experiments a homogenizer with a greater radial tolerance (0·2 mm.) between pestle and mortar was used and a loose-fitting hand-operated homogenizer of this kind was always used for resuspending centrifugal sediments.

Cell homogenization. A weighed portion (usually 5–10 g.) of freshly isolated mucosal cells was, unless otherwise stated, suspended in 9 vol. of ice-cold 0·25 M-sucrose. Approximately 10 ml. portions of the suspension were treated in turn in the homogenizer and the homogenates transferred to a measuring cylinder. In some experiments larger volumes (100–200 ml.) of a 20% (v/v) suspension of mucosal cells in 0·25 M-sucrose were homogenized in a Waring Blendor or top-drive macerator as described by

Carnie & Porteous (1962) and similarly transferred to a measuring cylinder. Measured portions of the homogenate were preserved for analysis and enzyme assay.

Differential centrifuging. The centrifuges and rotors used were those described by Carnie & Porteous (1962) with the addition of a MSE Magnum centrifuge and rotor no. 6875 for low-speed centrifuging. Centrifugal forces were calculated for the mean radius of rotation of the volume of material present in a centrifuge tube. Homogenates of mucosal cells were prepared (*a*) with the loose-fitting Teflon homogenizer, (*b*) with the close-fitting Teflon homogenizer and (*c*) with a Waring Blendor. Sediments obtained from homogenates (*a*) and (*c*) by 10000*g*-min. were resuspended with homogenization in 0·25 M-sucrose (one-fifth of the volume of the original homogenate) and a 'nuclear' fraction sedimented by recentrifuging at 10 000*g*-min. The combined supernatant suspensions were subjected to 32000*g*-min., the sediment was resuspended with homogenization in 0·25 M-sucrose (one-third of the volume of the original homogenate) and the 'heavy mitochondrial' fraction sedimented by recentrifuging at 32 000*g*-min. 'Light mitochondria' were isolated by a similar procedure involving a single resuspension and recentrifuging of material sedimented from the previous supernatant suspensions by 150 000*g*-min. A 'microsomal' fraction was sedimented from the combined supernatant suspensions by 4 500 000*g*-min., the supernatant solution constituting the cell-sap fraction. In the fractionation of homogenate (*c*) a single 'mitochondrial' fraction was prepared by omitting the step leading to the isolation of the 'heavy mitochondria'. Subcellular fractions were isolated from homogenate (*b*) in a similar manner except that the 'nuclear' fraction was sedimented by 6000*g*-min.

Microscopy. Cell suspensions, cell homogenates and centrifugal sediments (after resuspension in cold 0·25 M-sucrose) were examined by bright-field microscopy immediately they became available. In each case two individual drops of suspension were placed on a microscope slide; to one was added 1 drop of water, to the other 1 drop of aq. 0·05% methylene blue. After the individual preparations had been mixed, they were viewed under a coverslip with a dry objective (N.A. 0·70) and with an oil-immersed objective (N.A. 1·37).

Reagents. Sucrose, glucose, NaCl, $NaHCO_3$, $CaCl_2,6H_2O$, $Na_2H_2P_2O_7$, $NaH_2PO_4,2H_2O$ and KH_2PO_4 were AnalaR chemicals (British Drug Houses Ltd.). Diethylaminoethylcellulose (DEAE-cellulose) was obtained from H. Reeve Angel and Co. Ltd., London, as Whatman D.E. 50 powder. Other chemicals were obtained as follows: sodium deoxycholate from Hopkin and Williams, Chadwell Heath, Essex; tris from Boehringer und Soehne, through Courtin and Warner, Lewes, Sussex; pancreatic lipase (crude) from L. Light and Co. Ltd., Colnbrook, Bucks.; dried snake venom (*Agkistrodon piscovorus piscovorus*) from Ross Allen's Reptile Institute, Fla., U.S.A.; salt-free crystalline trypsin from Armour Pharmaceutical Co., Eastbourne, Sussex; trypsin inhibitor (ex-soya bean, 5 times crystallized) from Sigma Chemical Co., through G. T. Gurr and Son, London; trehalose dihydrate (which was chromatographically pure) from British Drug Houses Ltd. Acetone was refluxed over excess of $KMnO_4$, and distilled on to and redistilled from anhydrous K_2CO_3; butan-1-ol and ethanol were purified as described by Carnie & Porteous (1962).

Analytical methods. The measurement of pH values and determination of total nitrogen were carried out as described by Carnie & Porteous (1962).

Dialysis. All dialyses were carried out at 2° in the apparatus described by Porteous (1962). Cell homogenates and subcellular fractions were dialysed against 0·04 M-sodium phosphate buffer, pH 6·7; other dialyses were carried out against solutions specified in the text.

Enzyme assay procedures. Invertase activity was determined under the optimum conditions described by Carnie & Porteous (1962); the enzyme at 2° was added to 0·1 M-sucrose in 0·04 M-Na_2HPO_4–NaH_2PO_4, pH 6·7, preincubated at 37° for 10 min. Trehalase and maltase activities were determined by replacing the sucrose with 0·1 M-trehalose or -maltose respectively. Disaccharase activity was measured as the difference between the free hexose in the assay system at zero time and after incubation for 60 min. at 37°.

Unless stated to the contrary, deproteinization of samples withdrawn from the assay system for the determination of free hexoses was shown to be unnecessary for the enzyme preparations used, and was omitted. When deproteinization could be omitted invertase and trehalase activities were terminated as described by Carnie & Porteous (1962) and reducing sugar was determined in samples from the assay system by the method of Nelson (1944). Substrate hydrolysis did not exceed 5% in these assays.

Since maltose is a strongly reducing sugar, the glucose released in the maltase assay was determined after separating the hexose by paper chromatography (Carnie & Porteous, 1962). The appropriate area of the unsprayed chromatogram, or a blank area, was transferred to a centrifuge tube and extracted with a known volume of water for 30 min. at room temperature, then compressed with a glass rod. After being centrifuged (500*g*, 10 min.) to remove cellulose fibres, a sample of the supernatant solution was analysed for glucose (Carnie & Porteous, 1962). Because there is an upper limit to the total quantity of sugar (maltose plus glucose) which can be applied to a chromatogram and because there is a lower limit to the amount of glucose which can be quantitatively recovered from the chromatogram, it was necessary to allow up to 20% hydrolysis of the maltose in the assay system in order to determine with any accuracy the glucose released by maltase activity. Despite this disadvantage and the tedious nature of the technique adopted, it was preferred to the method of Tauber & Kleiner (1932), which is reputed to determine reducing monosaccharides in the presence of reducing disaccharides but proved unreliable in our hands. Attempts to use glucose oxidase to determine glucose released in the three disaccharase assay systems were frustrated by the presence of powerful disaccharase activities in all commercial preparations of glucose oxidase tested.

Enzyme units. One unit of disaccharase (invertase, maltase or trehalase) activity is defined as the amount of enzyme required to release 1 μmole of free hexose from 0·1 M-disaccharide (sucrose, maltose or trehalose respectively) in 0·04 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 6·7, in 60 min. at 37°.

Specific enzyme activities. These are expressed as the number of enzyme units/mg. of total nitrogen or as the number of enzyme units/$E^{1\,cm.}_{280\,m\mu}$.

Relative specific activity. This is used in the sense defined by de Duve, Pressman, Gianetto, Wattiaux & Applemans (1955).

Ultrasonic treatment. Ultrasonic treatment of particulate enzyme preparations was carried out with a 60w (18–20 kcyc./sec.) apparatus (Measuring and Scientific Instruments Ltd., London) with a stainless-steel probe of end-ratio 9:1. A measured volume of material was placed in a hard-glass centrifuge tube immersed in ice; ultrasonic treatment was applied for a measured period (3–5 min.), during which time the temperature of the treated material usually rose to 10° but never exceeded 14°. The centrifuge tube was transferred to fresh ice, the contents were stirred gently but continuously and the temperature was decreased to 2° in approximately 3 min. The cycle of ultrasonic treatment and cooling was repeated to give a total time of treatment detailed in the text.

Preparation of chromatographic columns. DEAE-cellulose (8 g.) was washed twice by alternate suspension and centrifuging (1000*g*, 10 min.) in 0·5 N-NaOH (250 ml., prepared in de-ionized water), the supernatant suspension being discarded on each occasion. The DEAE-cellulose was then similarly washed four times with 250 ml. portions of de-ionized water and allowed to sediment in 300 ml. of de-ionized water for 12 hr. before removal of the fine supernatant suspension. Coarse material was removed by decanting the supernatant suspension after another sedimentation (2 min.) from 300 ml. of de-ionized water. The graded material was recovered by centrifuging (500*g*, 20 min.) and washed six times by alternate suspension in 250 ml. of 0·005 M-sodium phosphate buffer, pH 7·5 (prepared in de-ionized water), and centrifuging as before, the supernatant being discarded on each occasion. A column (1 cm. × 13 cm.) of DEAE-cellulose (approx. 4 g. dry wt.) in 0·005 M-sodium phosphate buffer, pH 7·5 (prepared in de-ionized water), was packed under pressure (20 cm. Hg) to constant column height. The column was then washed with 250 ml. of the same buffer at 2° overnight.

Table 1. *Invertase activities of homogenates*

Rabbit small-intestine mucosal cells were homogenized in 0·25 M-sucrose at 2°. Further treatments (where indicated) were: D, the homogenized sample was treated with sodium deoxycholate (final concn. 0·5%, w/v) at pH 7·6 for 15 min. at room temperature; F, the homogenized sample was frozen, kept at −20° for 60 min. and thawed. The relative invertase activities were determined as described in the text after dialysis of the homogenates against water at 2° for 24 hr. The results shown refer to a single experiment.

Homogenizer	Total homogenization time (min.)	Further treatment	Relative invertase activity
Teflon homogenizer (annular clearance 0·2 mm.)	1	—	100
Teflon homogenizer (annular clearance 0·125 mm.)	1	—	140
	1	D	130
Waring Blendor	0·5	—	103
	1	—	123
	5	—	140
	5	F	123
	5	D	100
Top-drive macerator	0·5, 1, 2, 5, 10	—	100
	5	D	111

Table 2. *Apparent intracellular distribution of invertase activity of rabbit small-intestine epithelial cells*

Cell disruption was carried out by three methods (*a*, *b* or *c*) and centrifugal fractionation as described in the text. Briefly, the methods were: (*a*) cell suspension in 0·25 M-sucrose homogenized with loose-fitting Teflon homogenizer for 1 min. at 2°; (*b*) cell suspension in 0·25 M-sucrose homogenized with close-fitting Teflon homogenizer for 1 min. at 2°; (*c*) 160 ml. of cell suspension in 0·25 M-sucrose homogenized with a Waring Blendor (Carnie & Porteous, 1962) for 5 min. at 2°. Invertase activity and total nitrogen were determined as indicated in the text. The relative specific activity is defined as percentage of total invertase activity/percentage of total nitrogen.

	Percentage of total invertase activity			Percentage of total N			Relative specific activity		
Fraction	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
Homogenate	100	100	100	100	100	100	1·0	1·0	1·0
'Nuclei'	60	36	10	36	21	17	1·7	1·7	0·6
'Heavy mitochondria'	6	9	36	5	11	13	1·2	0·8	2·8
'Light mitochondria'	3	14		5	6		0·6	2·3	
'Microsomes'	17	30	55	13	15	28	1·3	2·0	2·0
Cell sap	5	4	2	32	43	38	0·2	0·1	0·05
Recoveries	91	93	103	91	96	96	—	—	—

RESULTS

Effect of various homogenization treatments

The results of initial experiments are recorded in Table 1. Microscopic observations showed that either of the two Teflon homogenizers appeared to disrupt almost all cells in the original suspension; the closer-fitting of these two homogenizers elicited 40% more enzyme activity from the original cell suspension than did the looser-fitting Teflon homogenizer. A top-drive macerator released no more activity in ½–10 min. than did the loose-fitting Teflon homogenizer in 1 min. Treatment in the close-fitting Teflon homogenizer for 1 min. was as effective in eliciting enzyme activity as was treatment in the Waring Blendor for a total of 5 min.; but microscopic observation showed that no nuclei had survived homogenization in the blender or macerator, whereas the nuclei survived in the Teflon homogenizer.

Failure to dialyse the various homogenates (Table 1) before enzyme assay did not affect the results obtained but gave inconveniently high reducing-sugar blanks (arising from hydrolysis of the sucrose of the homogenization medium) in the enzyme assays.

Subcellular distribution of enzyme activity after homogenization of the cells

The apparent subcellular distribution of invertase activity after homogenization of mucosal cells (*a*) with the loose-fitting Teflon homogenizer, (*b*) with the close-fitting Teflon homogenizer and (*c*) with the Waring Blendor is shown in Table 2. Light-microscopic observations were of most value in examining the homogenate and the 'nuclear' sediments but did not permit complete characterization of 'mitochondrial' and 'microsomal' sediments. The latter terms were arbitrarily assigned to the sediments obtained under the conditions stated above. Homogenates and the 'nuclear' fractions were examined with and without staining with methylene blue; the remaining fractions were not stained before examination. Homogenates (*a*) and (*b*) contained free nuclei and granules together with some intact or partially disrupted cells, which were particularly evident in homogenate (*a*). Homogenate (*c*) contained no intact or partially disrupted cells and very few nuclei. The 'nuclear' fraction from homogenate (*a*) was contaminated with some tissue, intact individual cells and with cell debris; that from homogenate (*b*) contained only a few intact cells and little cell debris; and that from homogenate (*c*) contained very few intact nuclei and no intact cells. All nuclear fractions contained some mucin. All 'mitochondrial' fractions contained granules under Brownian movement and were free of any other microscopically identifiable structures; the 'light mitochondria' were smaller than the 'heavy mitochondria'. The 'microsomal' and cell-sap fractions were optically clear.

In the above experiments assays for invertase activity were terminated and protein was removed as follows. Samples from the assay system were measured into known volumes of barium hydroxide and mixed; after 10 min. an equal volume of 5% (w/v) zinc sulphate was added, and the solutions were mixed and centrifuged (500*g*, 20 min.). The concentration of the barium hydroxide solution was previously adjusted so that Ba^{2+} ions not precipitated as barium phosphate were precipitated as barium sulphate. The protein-free supernatant solutions were analysed for reducing sugars as described above.

Solubilization of invertase activity

Mucosal cells from the small intestine of a rabbit were suspended in 5 vol. of 0·25M-sucrose and homogenized in a Waring Blendor (Carnie & Porteous, 1962); the sediment obtained at 10 000*g* for 15 min. was discarded and the supernatant suspension centrifuged at 100 000*g* for 45 min. The 10^5g sediment in various experiments contained 55–60% of the invertase activity of the original homogenate. Attempts to solubilize the invertase activity of the particulate material (10^5g fraction) from 6 g. of mucosal cells were carried out as follows.

Deoxycholate treatment. The 10^5g fraction was treated at 2° for 15 min. in water (pH 6·6), in 0·5% (w/v) sodium deoxycholate solution (pH 7·6), or in sodium deoxycholate [0·5% (w/v), in 0·1M-sodium phosphate buffer, pH 7·6]. The suspensions were centrifuged (100 000*g* for 60 min. at 2°), the supernatant extracts were dialysed at 2° for 24 hr. against water and the non-diffusible material was centrifuged as described above. On the assumption that these final supernatants contained only soluble enzyme, extraction of the particulate enzyme preparation with water solubilized 5% of the invertase activity. The dialysed deoxycholate extracts contained 89–100% of the original particulate invertase activity; 56–79% appeared to be in true solution in four experiments but only 9% in a fifth experiment, the remainder in each case being found in the final high-speed sediment. Maltase, invertase and trehalase activities were present in these solubilized preparations in essentially the same proportions as they were in the 10^5g fraction from which they were derived. The wide variations in the proportion of particulate invertase activity brought into solution in these experiments may be explained by variations in the amount of deoxycholate removed during dialysis; the enzyme activities were possibly held in solution as a deoxycholate–protein complex from which deoxycholate was not readily removed by dialysis. In the fifth experiment mentioned above an unusually thin-walled variety of dialysis tubing was employed.

Samples of the dialysed deoxycholate extracts retained all their invertase activity for 6 weeks at −20° but centrifuging of the thawed samples at 100 000*g* for 60 min. sedimented 75% of the activity, leaving 25% in the supernatant fractions. Samples of the original 10^5g fraction or of dialysed aqueous suspensions of this fraction lost about 50% of their residual activity per week on storage at −20°. Extraction with deoxycholate therefore stabilized the invertase activity but did not give a preparation which was particularly suitable for purification of the enzyme.

Acetone-drying and extraction with bicarbonate. Acetone-drying at −10° or acetone-drying followed by extraction with butan-1-ol at −10° destroyed about 20% of the invertase activity, and very little activity could be extracted with 0·01 M-sodium phosphate buffer, pH 6·9, from these solvent-dried preparations. Extraction with $NaHCO_3$ (Hultin, 1957) of a sample of the 10^5g fraction followed by centrifuging (100 000*g* for 60 min. at 2°) solubilized 9% of the invertase activity; 85% was recovered from the sediment.

Snake venom, pancreatic lipase and trypsin. Snake venom (phospholipase A) did not destroy any invertase activity but failed to solubilize more than 5% of the invertase activity of the 10^5g fraction or of an acetone-dried preparation of this fraction under the conditions used by Rendina & Singer (1959). Incubation of the 10^5g fraction with pancreatic lipase [20 mg. in 20 ml. of 0·5 M-NH_4Cl–aq. NH_3 buffer, pH 8·0, containing 0·4% (w/v) of $CaCl_2,6H_2O$] at 37° for 3 hr., termination of the reaction by the addition of 5 vol. of ethanol at −20° and centrifuging (6000*g* for 20 min. at 2°) sedimented all the invertase activity; extraction of the sediment with water followed by centrifuging (100 000*g* for 60 min. at 2°) left 17% of the activity in the supernatant solution. Digestion of the 10^5g fraction with trypsin (10 mg. or 40 mg. in 20 ml. of 0·1 M-sodium phosphate buffer, pH 6·7 or 7·6) at 37° for 3 hr., termination of the reaction with trypsin inhibitor followed by dialysis (against 0·01 M-sodium phosphate buffer, pH 6·9, at 2° for 24 hr.) and centrifuging of the non-diffusible material (100 000*g* for 60 min. at 2°) left 3% of the invertase activity in the supernatant solution. Termination of a similar digestion with trypsin by the addition of ethanol at −20° followed by centrifuging (as in the lipase-digestion experiment) sedimented all the invertase activity. Extraction of the sediment with water and centrifuging (as in the lipase-digestion experiment) left 15–21% of the invertase activity in the supernatant solution.

The trypsin, trypsin inhibitor, lipase and snake venom were each shown to be free of maltase, invertase and trehalase activity.

None of the soluble enzyme preparations obtained by the above methods had a specific invertase activity greater than that of a dialysed aqueous suspension of the 10^5g fraction of the mucosal cells.

Ultrasonic treatment. The results of attempts to solubilize the invertase activity of the 10^5g fraction are shown in Table 3. These results showed that suspension of the 10^5g fraction in water or in pyrophosphate buffer gave the same invertase activity (subfractions A of treatments 1a and 2a) from a single batch of epithelial cells. Ultrasonic treatment for 3, 6 or 9 min. of a suspension of cells in pyrophosphate did not alter significantly the total invertase activity of the suspensions (subfractions A of treatments 3a, 3b and 3c). Extraction with pyrophosphate without ultrasonic treatment yielded 3% of the original activity as an apparently soluble enzyme (treatment 2a, subfraction B); ultrasonic treatment of a suspension of cells in pyrophosphate yielded a supernatant fraction B (treatments 3a, 3b and 3c) containing an additional amount of enzyme which was roughly proportional to the period of the ultrasonic treatment. The specific activities of these supernatant fractions also increased with the duration of the treatment, but the highest specific activity obtained was less than half that of the original 10^5g fraction, whereas each of the residual sediments (subfractions C) had a specific activity approximately twice that of the original material.

Ultrasonic treatment of larger amounts of a 10^5g fraction for longer periods gave supernatant fractions B (treatments 3d and 3e) containing 33 and 42% respectively of the original enzyme activity; dialysis of these supernatant fractions increased the specific activity of the preparations to 60 and 80% respectively of that of the original 10^5g fraction. High-speed centrifuging of these dialysed supernatant fractions gave very slight sediments leaving optically clear and colourless supernatants with little change in absolute or specific activity. Similar results were obtained in a third experiment with a 10^5g fraction isolated from rabbit XII.

Supplementary experiments showed that subfraction A of treatment 2b (Table 3; rabbit XII) contained maltase, invertase and trehalase activities in the proportions 1·50:1·0:0·49, whereas in the corresponding subfraction D of treatment 3e the activities were present in the proportions 1·44:1·0:0·25. Subfraction A (i) of treatment 1b and subfractions D of treatments 3d and 3e (Table 3, rabbit XI) had similar ratios of maltase to invertase (1·52, 1·45 and 1·48 respectively). These results suggest that the trehalase was less readily extracted or more readily destroyed by ultrasonic treatment of the 10^5g fraction of mucosal cells than were the maltase and invertase activities. There was no loss of invertase activity during dialysis (Table 3; rabbit XI); the maltase and trehalase activities were also stable under the same conditions of dialysis.

Thermolability of invertase, maltase and trehalase

The 10^5g fraction from 6 g. of intestinal mucosal cells was treated with deoxycholate in phosphate buffer, pH 7·6, centrifuged, the supernatant solution dialysed and the non-diffusible material again centrifuged exactly as described above. The final supernatant solution contained 76% of the

Table 3. *Effects of ultrasonic treatment of the 10^5g fraction*

The 10^5g fraction of rabbit small-intestine mucosal cells was obtained as described by Carnie & Porteous (1962). Samples of this fraction were then subjected to the following treatments. Treatment 1a: 10^5g fraction from 6 g. of mucosa was suspended in 20 ml. of water at 2° for 30 min. to give subfraction A. Treatment 1b: 10^5g fraction from 8 g. of mucosa was suspended in 25 ml. of water at 2° for 75 min. to give subfraction A. Treatment 2a: 10^5g fraction from 3 g. of mucosa was suspended in 10 ml. of 0·02M-pyrophosphate buffer, pH 8·4, at 2° for 30 min. to give subfraction A. Treatment 2b: 10^5g fraction from 8 g. of mucosa was suspended in 25 ml. of 0·02M-pyrophosphate buffer, pH 8·4, at 2° for 75 min. to give subfraction A. Treatments 3a, 3b and 3c: as described for treatment 2a, but the suspension was immediately subjected to ultrasonic treatment for 3, 6 or 9 min. respectively to give subfractions A. Treatments 3d and 3e: as described for treatment 2b, but the suspension was immediately subjected to ultrasonic treatment for 20 and for 40 min. respectively to give subfractions A. Centrifuging of subfractions A at 100 000g for 60 min. at 2° gave supernatant subfractions B, and sediments which on resuspension and dilution to a known volume in water were designated subfractions C. Some fractions, before assay and analysis, were dialysed at 2° for 24 hr. against large volumes of: (i) water, (ii) 0·01M-sodium phosphate buffer, pH 6·9, or (iii) 0·005M-sodium phosphate buffer, pH 7·5, in de-ionized water. Further centrifuging (at 100 000g for 60 min. at 2°) of optically-clear dialysed fractions B was carried out in three instances (treatments 3d and 3e) to yield supernatant subfractions D and slight sediments which on resuspension and dilution to a known volume in water were designated subfractions E. Invertase activity and nitrogen were determined as indicated in the text; total invertase activity and total nitrogen were calculated for subfractions derived from 24 g. of mucosal cells. N.D., Not determined.

Rabbit	Treatment of 10^5g fraction	Subfraction	Treatment of subfraction before assay and analysis	10^{-3} × Total invertase activity (units)	Relative invertase activity	Total N (mg.)	Specific invertase activity (units/mg. of N)
X	1a	A	—	29·2	100	124	236
	2a	A	—	29·9	102	121	247
		B	—	0·85	3	44	19
		C	—	28·7	98	77	373
	3a	A	—	29·2	100	119	246
		B	—	3·05	10	64	48
		C	—	25·6	87	58	443
	3b	A	—	29·0	99	119	243
		B	—	4·50	15	64	70
		C	—	24·8	85	56	445
	3c	A	—	29·2	100	119	246
		B	—	6·85	23	70	98
		C	—	22·2	76	50	445
XI	1b	A	—	27·3	100	108	252
		A	(i)	27·3	100	77	355
	3d	A	—	23·6	87	108	218
		B	—	8·85	32	71	125
		B	(ii)	8·95	33	57	157
		C	—	15·6	57	40	391
		D	—	7·00	26	47	149
		E	—	1·45	5	9	161
	3e	A	—	21·9	81	109	201
		B	—	11·4	42	68	169
		B	(ii)	11·4	42	55	208
		C	—	11·6	42	41	283
		D	—	10·1	37	47	216
		E	—	1·66	6	10	166
XII	2b	A	(iii)	16·4	100	65	253
	3e	A	—	N.D.	N.D.	N.D.	N.D.
		B	(iii)	N.D.	N.D.	N.D.	N.D.
		D	—	7·7	47	38	203

total invertase activity of the original 10^5g fraction. The extract also contained maltase and trehalase activities and was subjected to elevated temperatures for 10 min., cooled to 14° and immediately assayed, together with untreated samples of the same extract, for enzyme activities. The results (Table 4) showed that the trehalase activity was considerably more stable at elevated tempera-

tures than was either the maltase or the invertase activity; and that the maltase activity was slightly more stable, particularly in unbuffered solution at lower pH values, than was the invertase activity under similar conditions.

Chromatographic behaviour of the invertase, maltase and trehalase activities

A portion (10 ml.) of subfraction D from rabbit XII (Table 3) containing 990 invertase units, 1430 maltase units and 250 trehalase units was applied to a DEAE-cellulose column (1 cm. × 13 cm.). The enzyme solution was followed by 5 ml. of the dialysis buffer (Table 3), the flow rate adjusted to 0·5 ml./min. and the column developed at 2° with the series of buffers detailed in Fig. 1. Fractions (10 ml.) were collected as soon as buffer I was applied to the column. All fractions were dialysed for 12 hr. at 2° against 0·01M-sodium phosphate buffer, pH 6·7, and diluted to a known volume with the same buffer before determining the enzyme content and the extinction at 280 mμ.

No trehalase activity was detected in the eluted fractions from the column. Invertase activity and maltase activity were each found in two main overlapping regions of the elution pattern (fractions 28–34 and 36–40, Fig. 1). Each region contained a peak of invertase activity which was incompletely resolved from a peak of maltase activity. The ratio of maltase to invertase activity in the solution originally applied to the column was 1·44. The ratio (in parentheses) of these two enzyme activities for each of the relevant fractions eluted from the column showed that fractions 28(∞), 29(∞), 32(3·75), 33(3·5), 34(2·9), 35(2·78), 36(2·76) and 38(2·15) were significantly enriched with maltase activity, and that fractions 37(0·87), 39(0·88), 40(0·77) were significantly enriched with invertase activity. Of these enriched fractions, fractions 32 and 37 contained the highest maltase and invertase activity respectively. On the basis of extinction measurements at 280 mμ the specific maltase

Table 4. *Thermolability of invertase, maltase and trehalase activities*

The residual enzyme activities of deoxycholate extracts of the 10^5g fraction of rabbit intestine (Carnie & Porteous, 1962) were determined, as described in the text, after treatment for 10 min. under the conditions indicated. Enzyme preparations were buffered (where indicated) in 0·06M-sodium phosphate during heat treatment. pH values were unchanged at the end of the incubations. N.D., Not determined.

Conditions of treatment		Percentage of activity remaining		
pH	Temp.	Invertase	Maltase	Trehalase
Unbuffered, pH 6·1	45°	95	100	100
	50	91	96	100
	55	17	29	100
	60	0	7	56
Buffered, pH 6·7	45	100	95	100
	50	100	94	100
	55	73	84	99
	60	2	10	54
Unbuffered, pH 5·6	51	50	60	N.D.
	53	12	23	N.D.
	55	0	17	N.D.
	60	0	2	N.D.
Buffered, pH 6·7	50	100	91	N.D.
	55	84	85	N.D.
	57	54	71	N.D.
	60	3	20	N.D.

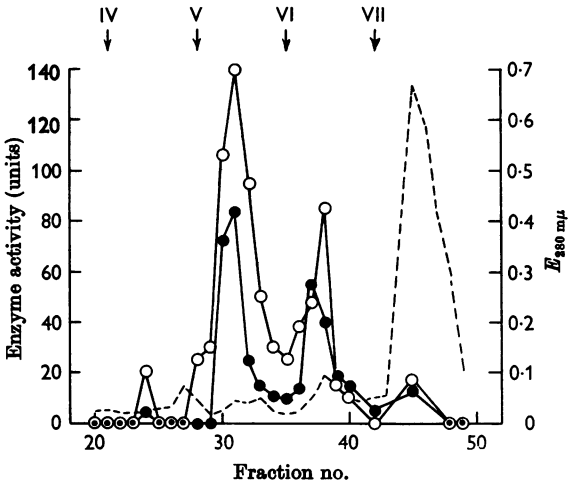

Fig. 1. Chromatographic elution of maltase (○) and invertase (●) activities from a DEAE-cellulose column with a discontinuous buffer gradient. The broken line indicates the extinction values at 280 mμ. The overall recoveries of the maltase and of the invertase activities applied to the column were 45 and 36% respectively. All fractions were dialysed, as indicated in the text, before assay for enzyme activity and determination of the extinction values at 280 mμ. The phosphate buffers were prepared from $Na_2HPO_4,12H_2O$ and $NaH_2PO_4,2H_2O$ (Carnie & Porteous, 1962). Fractions 1–7 were eluted with (I) 0·005M-phosphate buffer, pH 7·5; fractions 8–14 were eluted with (II) 0·02M-phosphate buffer, pH 7·0; fractions 15–21 were eluted with (III) 0·05M-phosphate buffer, pH 7·0. None of these fractions contained maltase, invertase or trehalase activities and the extinction values did not exceed 0·05. Immediately after the collection of the fraction indicated in the Figure, the following buffers were applied to the column: (IV) 0·075M-phosphate, pH 6·5; (V) 0·075M-phosphate–0·1M-NaCl, pH 6·0; (VI) 0·1M-phosphate–0·2M-NaCl, pH 5·5; (VII) 0·2M-phosphate–0·5M-NaCl, pH 5·2. The results shown are from a single experiment; essentially the same elution pattern was obtained in a subsequent experiment.

activity of fraction 32 was 60 times that of the solution applied to the column; the specific invertase activity of the same fraction was 25 times that of the original solution. The specific maltase and specific invertase activities in fraction 37 were 30 and 47 times greater than the respective specific activities of the original solution. Fraction 31 represented an approximately 80-fold purification of the two glycosidases. Fractions 28 and 29 contained maltase activity only but these fractions together contained only about 11% of the maltase activity recovered from the column.

DISCUSSION

Rupture of mucosal cells with a loose-fitting Teflon homogenizer left some cells unbroken and others partially disintegrated. Such cells, and cell debris, sedimented with the free nuclei and may have contributed to the high invertase activity of that fraction which nevertheless had the highest relative specific activity of all fractions isolated in this experiment (Table 2). In contrast, rupture of the mucosal cells with a close-fitting Teflon homogenizer left very few cells unbroken or partially broken. The invertase activity of the nuclear sediment was markedly decreased though its relative specific activity remained unchanged; concomitantly, the activities and the relative specific activities of the 'light mitochondria' and of the 'microsomes' increased markedly. The shift in invertase activity from the nuclear sediment to the 'light mitochondrial' and 'microsomal' fractions (Table 2) may be attributable partly to the more complete disruption of cells which was achieved with the close-fitting homogenizer and partly to the fact that the nuclei were isolated at lower values of the time–force integral. These three subcellular fractions between them contained 80% of the invertase activity, and only these three fractions, of the five isolated, exhibited relative specific activities significantly greater than that of the original homogenate. These last results [experiment (*b*), Table 2] would seem superficially to give the most accurate picture of the intracellular distribution of the mucosal-cell invertase. Our results (Table 2) differ from those of Borgström & Dahlqvist (1958) on hog intestine but our findings, like those of Borgström & Dahlqvist, are open to the criticism that the techniques used failed to differentiate between the brush-border region and the rest of the mucosal-cell structure. Since this work was completed Miller & Crane (1961) have isolated intact brush-border elements from the epithelial cells of golden-hamster intestine and have shown that the brush border contains most if not all the invertase and maltase activities of the small intestine. These findings, if applicable to rabbit intestine, suggest that the least vigorous of the present homogenization techniques (Table 2) disrupted the brush border in such a way that 60% of its invertase activity sedimented with the nuclei and 17% with the microsomes; that more vigorous homogenization produced from the brush border a larger proportion of finer particles with the sedimentation characteristics of mitochondria and microsomes; and that after treatment in the Waring Blendor 90% of the particles produced from the brush border had the sedimentation characteristics of mitochondria and microsomes. Further work will be necessary to decide whether these interpretations are valid and whether the findings of Miller & Crane (1961) are applicable to rabbit intestine. Work now in progress in this Laboratory has confirmed that little of the brush border of rabbit small intestine survives as a microscopically identifiable structure after homogenization in 0·25 M-sucrose with a Teflon homogenizer.

As a source of invertase, maltase and trehalase for further work, it was desirable to use a fraction of the disrupted mucosal cells which was reasonably homogeneous and readily isolated, and which contained a high proportion of the enzymes in a partially purified form. The 'nuclear' fractions of experiments (*a*) and (*b*) and the 'microsomal' fraction of experiment (*c*) (Table 2) were considered. The first of these fractions was rejected since it contained significant amounts of intact tissue, whole cells and mucin; the second of these fractions likewise contained some intact cells and mucin. The third of the above fractions (in effect the $10^5 g$ fraction described by Carnie & Porteous, 1962) appeared to be the best available source of enzymes for solubilization studies.

Extraction with bicarbonate, acetone-drying or incubation with lecithinase brought less than 10% of the invertase of the $10^5 g$ fraction of the mucosal cells into solution. Digestion with lipase brought 17% of the enzyme into solution; digestion with trypsin under various conditions brought 3–21% of the enzyme into solution. Borgström & Dahlqvist (1958) were able to solubilize more than 90% of the activity of an analogous particulate invertase preparation from hog intestine by digestion with trypsin. The same authors reported that deoxycholate-solubilized hog-intestine invertase is slowly sedimented on high-speed centrifuging after exhaustive (72 hr.) dialysis of the deoxycholate extract. The present results, with one exception, suggested that deoxycholate treatment of a particulate invertase preparation followed in turn by centrifuging at 100 000g for 60 min., dialysis of the supernatant extract for 24 hr. in a rapid-dialysis apparatus, and centrifuging of the non-diffusible material as before, left 56–79% of the invertase in true solution. In one instance,

however, only 9% of the enzyme activity was solubilized.

In the present work ultrasonic treatment of the 10^5g fraction of mucosal cells was the only reliable method of solubilizing the enzymes; 37–47% of the invertase activity could be brought into apparently true solution with a specific activity which was 80% of that of the original particulate material.

Though the intracellular location of the invertase of rabbit small intestine remains in doubt, it seems clear on present evidence that it is firmly attached to or forms part of a water-insoluble structure of the cells. Further, the invertase activity of this structure is resistant to at least three digestive enzymes and is relatively stable to treatment with deoxycholate at pH 7·6 and 2°, to incubation at pH 8·4 and 37°, and to treatment with acetone, butan-1-ol or ethanol at low temperatures.

Carnie & Porteous (1962) showed that the mucosal cells of rabbit small intestine contained invertase and maltase activity, and that the invertase activity is that of an α-glucosidase. The presence of a third α-glucosidase (trehalase) has now been demonstrated in the same preparation. That the trehalase is distinct from the maltase and invertase activities was shown by the results of heat-inactivation studies (Table 4), by differences in chromatographic behaviour (Fig. 1) and was suggested by the differential loss of trehalase activity during ultrasonic extraction of the 10^5g fraction of mucosal cells (Table 3 and text).

Carnie (1961) showed that the ratio of maltase to invertase activity of the 10^5g fraction of rabbit small intestine remained essentially constant within the range 1·45–1·55 from one rabbit to another. The ratio was unaltered by ultrasonic treatment (Table 1) or by deoxycholate-extraction of the 10^5g fraction. The difference in the thermolability of the maltase and invertase activities (Table 2) suggested but did not prove that these activities were due to separate proteins. The partial chromatographic separation of maltase and invertase activities which has been achieved (Fig. 1) suggests that these two activities were due to at least two distinct proteins.

If the chromatographic elution pattern depicted in Fig. 1 was the result solely of ideal ion-exchange of proteins with a single species of ion-exchanger in the substituted cellulose it would seem that two invertases and two maltases were present in the solution applied to the ion-exchange column. It is also possible that the elution of two peaks of activity for each of two enzymes was an artifact (Ringertz & Reichard, 1960) arising from the elution of only two catalytic proteins with two species of ion-exchange group in the substituted cellulose (Peterson & Sober, 1961) may account for the results obtained.

SUMMARY

1. Attempts have been made to determine the intracellular location of the invertase activity of the mucosal cells of rabbit small intestine.

2. The results obtained suggest that the invertase is distributed between the 'nuclear', 'light mitochondrial' and 'microsomal' fractions of the cells but other interpretations are possible since the technique used did not differentiate between the brush-border region and the residual parts of the structure of the mucosal cells of the small intestine.

3. At least half of the invertase activity of the small intestine can be isolated in particulate form by differential centrifuging after complete disintegration of the mucosal cells and the nuclei.

4. Attempts have been made to solubilize this particulate enzyme. Extraction with bicarbonate, acetone-drying and aqueous extraction, digestion with lecithinase, digestion with lipase and digestion with trypsin failed to solubilize more than one-fifth of the enzyme and in most instances the yield was much lower. Deoxycholate appeared to solubilize 9–79% of the enzyme. Extraction with pyrophosphate solubilized very little enzyme but ultrasonic treatment of a suspension of the particulate enzyme in pyrophosphate brought about 50% of the activity into apparently true solution.

5. The solutions resulting from these deoxycholate and ultrasonic treatments contained invertase, maltase and trehalase activities.

6. The trehalase activity was shown to be distinct from the maltase and invertase activities since the trehalase was more thermostable than the other two α-glucosidases. Further, part at least of the maltase and invertase activities was recovered from an ion-exchange column but none of the trehalase activity was recovered.

7. Partial resolution of the maltase and invertase activities by ion-exchange chromatography suggested that these activities were due to at least two distinct proteins.

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Copper Concentration and Cytochrome-Oxidase and Ribonuclease Activities in the Brains of Copper-Deficient Lambs

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The association of degenerative changes in the central nervous system of lambs in enzootic ataxia or 'swayback' with low concentrations of copper in blood and liver has been clearly established (Innes & Shearer, 1940; Bennetts & Beck, 1942). Gallagher, Judah & Rees (1956*a*, *b*), working with rats, and Gubler, Cartwright & Wintrobe (1957), using pigs, demonstrated that copper deficiency resulted in a marked fall of cytochrome-oxidase activity in several tissues. These later findings, coupled with the work of Wainio, Wende & Shimp (1959), which provided clear evidence that copper is an integral part of the cytochrome-oxidase molecule, prompted Howell & Davison (1959) to compare the cytochrome-oxidase activity of brain tissue from normal and swayback lambs. They demonstrated a significant reduction in cytochrome-oxidase activity in homogenates of frontal cortex, cerebellar peduncle and caudate nucleus prepared from brain tissue of lambs with ataxia. The copper content of these brain fractions from ataxic lambs was lower than in corresponding tissues from the normal lambs used in their study. The mean copper concentration of the liver of their clinically normal lambs (109 ± 13 p.p.m.) falls within the range (74–430 p.p.m.) quoted by Cunningham (1946) as being that found in young lambs reared outside copper-deficient areas. However, it has been our experience and that of other workers that many lamb flocks fail to show cases of ataxia even though the animals would be regarded as severely copper-deficient on the basis of the low copper contents of liver and blood.

This point led us to examine possible relationships between liver and brain copper and brain cytochrome-oxidase activity in swayback lambs and in lambs from flocks of low liver copper content in which clinical signs of swayback had not appeared. This study was also extended to a number of lambs reared by ewes that had received dietary supplements of sodium sulphate and ammonium molybdate during pregnancy (a procedure that results in copper deficiency and development of typical histological lesions of swayback in the lamb; Mills & Fell, 1960; Fell, Williams & Mills, 1961). In these earlier studies the first detectable sign of degenerative change in the central nervous system was the absence of cytoplasmic RNA in the large motor neurones of the red nucleus. This absence of RNA could arise presumably from either a failure of RNA synthesis or an increase in ribonuclease activity. This latter possibility was examined in the work reported.

MATERIALS AND METHODS

Lambs suffering from 'delayed' swayback were drawn from a number of farms in north-east Scotland. Clinically normal lambs with tissues of low copper content were taken from the Duthie Stock Farm of the Rowett Research Institute; previous work had shown that many lambs from this farm have a low copper content of blood and liver. Animals were killed by bleeding after intraperitoneal administration of sodium pentobarbitone and their brains rapidly dissected out on to ice prepared from glass-distilled water. Lateral slices of the medulla between the trapezoid body and the point of entry of the hypoglossal nerve, taken to include the area of the red nucleus, were dissected from both sides of the anterior median fissure. Approximately 0·6 g. of fresh tissue was homogenized in 10 ml. of ice-cold water for cytochrome-oxidase and ribonuclease assays.

Cytochrome oxidase. Cytochrome-oxidase activity was determined manometrically by the method of Schneider &