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Heat Inactivation and Sephadex Chromatography of the Small-Intestine Disaccharidases of the Chick

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1. The maltase, sucrase, isomaltase and palatinase activities of the chick small intestine are localized in particles that sediment when centrifuged at 100000*g* for 90min. 2. Solubilization of the particle-bound disaccharidases without loss of activity was achieved by digestion with papain. Trypsin was less effective and caused a preferential solubilization of the sucrase, isomaltase and palatinase activities. 3. On Sephadex G-200 columns, the solubilized preparations yielded two disaccharidase peaks. The first peak was eluted close to the void volume of the column and contained all the sucrase, isomaltase and palatinase activities and some of the maltase activity. The remainder of the maltase activity was eluted beyond the total volume of the column. 4. Precipitation with ethanol did not affect the behaviour of the disaccharidases of gel filtration. 5. The maltase activity of the second peak on rechromatography in a buffer containing 0.01M-maltose was eluted close to the void volume. 6. Similar pH optima but different K_m values were obtained for the maltase activities of the two peaks. 7. Heat-inactivation studies showed that the first peak contained two disaccharidase enzymes; one hydrolysed sucrose and maltose and the other hydrolysed isomaltose, palatinose and maltose. The second peak contained three disaccharidase enzymes all specific for the hydrolysis of maltose. 8. It is proposed that the intestinal disaccharidases of the chick exist in the form of two complexes: a sucrase–isomaltase complex and a maltase complex.

Siddons (1969) showed that in many ways (K_m values, pH optima, distribution along the small intestine, location within the mucosal cells and development with age) the maltase, sucrase and palatinase activities in the small intestine of the chick are very similar to the corresponding activities in the weaned mammal.

In the mammalian small intestine, studies on the nature of the enzymes responsible for the hydrolysis of maltose, sucrose and palatinose have established the existence of several α-D-glucosidases with different specificities. For example, in the human small intestine there are at least four enzymes that hydrolyse maltose, and of these one hydrolyses sucrose and another hydrolyses isomaltose and palatinose (Auricchio, Semenza & Rubino, 1965; Dahlqvist & Telenius, 1969). In the pig the intestinal hydrolysis of maltose is caused by three different enzymes (Dahlqvist, 1959).

In the present paper, evidence is presented for the existence of a similar multiplicity of enzymes responsible for the hydrolysis of disaccharides in the small intestine of the chick.

MATERIALS AND METHODS

Preparation of homogenates. Rhode Island Red × Light Sussex chicks that had been reared to 4 weeks of age on a practical-type chick mash (Siddons, 1969) were killed by fracture of the neck. The small intestine, i.e. that section of the intestine between the pylorus and the ileo-caecal junction, was removed and the pancreas and any adhering mesentery were cut away. The intestine was slit open, washed with 0.15M-NaCl, gently blotted with a piece of cloth and homogenized with 0.15M-NaCl for 3min in an MSE homogenizer. The homogenate was centrifuged at 1000*g* for 5min at 4°C. The supernatant was decanted, filtered through gauze and stored at −20°C. This supernatant contained approximately 10mg of protein/ml. Homogenates of embryo small intestines were prepared in the same way except that the intestines were not washed out. The embryos were obtained from eggs produced by Light Sussex hens mated with Rhode Island Red cocks by incubating them for 18 days in a standard commercial incubator.

Solubilization of the disaccharidases. The term 'soluble' refers to material that does not sediment on centrifugation at 100000*g* for 90min. The solubilization of the disaccharidases was achieved by digestion with either trypsin or

papain: 10ml of homogenate was mixed with either (i) 1.2ml of 0.5M-sodium–potassium phosphate buffer, pH7.0, and 0.8ml of a solution containing 3mg of trypsin (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.)/ml of water, or (ii) 1.2ml of 0.5M-potassium phosphate buffer, pH7.0, and 0.8ml of a solution containing 3mg of papain and 7.5mg of cysteine hydrochloride/ml of water. The solution was incubated at 37°C for 60min and then cooled in ice before being dialysed against 5l of 0.01M-sodium–potassium phosphate buffer, pH7.0, for 18h at 4°C. The dialysis residue was centrifuged at 100000*g* for 90min at 4°C.

Precipitation of the solubilized disaccharidases. Precipitation of the solubilized disacccharidases prepared as above was carried out with ethanol by the procedure of Dahlqvist & Telenius (1969).

Heat inactivation. Preliminary studies indicated that the inactivation of the small-intestinal disaccharidases occurred between 55 and 70°C (Fig. 1). Inactivation was therefore studied at 55, 60, 65 and 70°C. For this purpose 5.0ml of sample was mixed with 5.0ml of 0.2M-sodium phosphate buffer, pH7.0, in a thin-walled glass tube. The temperature of the solution was raised quickly by immersing the tube in a water bath at a temperature 7–8°C higher than the temperature being studied. The tube was shaken continuously, and the desired temperature was reached without being exceeded within 1min. The tube was then transferred to a bath maintained at the temperature being studied. Samples (1.0ml) were taken at the moment the required temperature was reached (zero-time sample) and at intervals of 10min thereafter, and transferred to tubes cooled in ice.

Sephadex chromatography. Sephadex G-200 (Pharmacia AB, Uppsala, Sweden) was swollen in 0.01M-sodium–potassium phosphate buffer, pH7.0, containing 0.02% of sodium azide, and then packed to a height of 60cm into a glass column (70cm×1.5cm diam.). This buffer was also used for the elution of the column. It was chosen since K^+ stabilizes the disaccharidases (Auricchio, Dahlqvist & Semenza, 1963*a*) and Na^+ activates them (Semenza, Tosi, Vallotton-Delachaux & Mülhaupt, 1964). The sample (1–3ml) prepared in the same buffer was applied and eluted at a flow rate of 5–6ml/h. All operations were performed at 4°C.

Disaccharidase assays. Activities were assayed by the method of Dahlqvist (1964). Maltase activity was assayed at pH5.8, sucrase and palatinase at pH6.2 and isomaltase at pH6.4. A substrate concentration of 28mM in 50mM-sodium maleate buffer was used in all the assays except for most of the isomaltase assays, when for economy 2.8mM-substrate was used. One unit of disaccharidase activity is defined as the amount required to hydrolyse 1μmol of disaccharide in 60min at 37°C.

Determination of protein. Protein content was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine plasma albumin was used to prepare a standard curve. The protein content of the eluate from the Sephadex columns was estimated by measuring E_{280}.

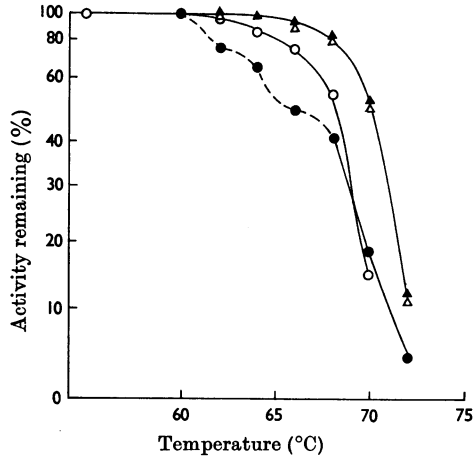

Fig. 1. Effect of continuously increasing temperature on the maltase (●), sucrase (○), isomaltase (△) and palatinase (▲) activities of a homogenate of chick small intestine. The homogenate was prepared in 0.1M-sodium phosphate buffer, pH7.0, and the temperature was increased at 0.5°C/min.

RESULTS

Localization of the disaccharidases. When the disaccharidase activity was measured in the supernatants and sediments obtained by the differential centrifugation of a small-intestinal homogenate, it was found that most of the activity was located in particles that sedimented at 100000*g* (Table 1). After centrifugation of the homogenate for 20min at 7000*g*, about 70% of the activity remained in the supernatant, but of this over 90% sedimented when the 7000*g* supernatant was centrifuged at 100000*g* for 90min.

Solubilization of the disaccharidases and precipitation with ethanol. No solubilization of the disaccharidases occurred when a homogenate prepared in 0.05M-potassium phosphate buffer, pH7.0, was incubated at 37°C for 4h. A partial solubilization was obtained by digestion with trypsin (Table 2). However, there

Table 1. *Differential centrifugation of a homogenate of chick small intestine*

The homogenate was centrifuged at 7000*g* for 20min. The supernatant was decanted and centrifuged at 100000*g* for 90min. The sediments were resuspended in 0.15M-NaCl to the original volume of the homogenate.

	Activity (units/ml)		
	Maltase	Sucrase	Palatinase
Homogenate	256	38.3	8.7
7000*g* supernatant	177	26.7	5.9
7000*g* sediment	51	8.9	2.0
100000*g* supernatant	11	3.5	0.7
100000*g* sediment	160	23.3	5.3

Table 2. *Digestion of a homogenate of chick small intestine with either trypsin or papain and precipitation of the solubilized disaccharidases with ethanol*

The isomaltase activity was assayed with 2.8 mM-substrate, and the activity of the other enzymes with 28 mM-substrate. For conditions of digestion and ethanol precipitation see the Materials and Methods section.

		Activity (units/ml)				Protein
		Maltase	Isomaltase	Sucrase	Palatinase	(mg/ml)
Untreated	Homogenate	194	66	35	7.0	9.55
Trypsin-treated	Homogenate	138	55	26	6.0	
	100000*g* supernatant	26	28	14	2.9	1.80
	Ethanol precipitate	19	20	11	2.1	0.76
Papain-treated	Homogenate	177	57	31	6.9	
	100000*g* supernatant	136	52	26	5.1	3.74
	Ethanol precipitate	128	48	25	4.6	1.12

was some inactivation of the enzymes during the digestion, and there was also a preferential solubilization of the isomaltase, sucrase and palatinase activities. Thus, after digestion with trypsin, 50% of the remaining isomaltase, sucrase and palatinase activities was in soluble forms, but less than 20% of the remaining maltase activity was soluble. A more complete solubilization of the particle-bound enzymes in a homogenate was achieved by digestion with papain (Table 2). During the digestion with papain less than 10% of the activity was lost and of the remaining activity at least 75% was in soluble form.

Precipitation of the trypsin-solubilized disaccharidases with ethanol and redissolving of the precipitated enzymes resulted in a fourfold increase in the specific activity of the isomaltase, sucrase and palatinase activities compared with the original homogenate, whereas there was little change in the specific activity of the maltase. Ethanol precipitation of the papain-solubilized enzymes resulted in a fivefold increase in the specific activities of all the disaccharidases.

Sephadex chromatography. Figs. 2 and 3 show the chromatograms obtained when samples of solubilized disaccharidases prepared by papain or trypsin digestion were subjected to gel filtration on Sephadex G-200. With both samples the disaccharidases were separated into two peaks. All the isomaltase, sucrase and palatinase activities were eluted in a sharp peak close to the void volume of the column, indicating large molecular size. This peak also contained maltase activity which, in the sample solubilized with trypsin, accounted for 70% of the total maltase applied to the column; in the sample prepared by papain digestion less than 25% of the maltase applied to the column was eluted in this peak. This peak was found to have maltase:isomaltase:sucrase:palatinase activities in the proportions 1.2:0.8:1.0:0.2 (the isomaltase activity was assayed with 2.8 mM-substrate). The remainder of the maltase activity, which accounted for most of the maltase activity in the papain-solubilized sample but only a small proportion of the maltase activity in the trypsin-solubilized sample, was eluted more slowly and formed a second peak that extended beyond the total volume of the column, suggesting an interaction with the Sephadex. Ethanol precipitation of the solubilized disaccharidases had no effect on the behaviour of the disaccharidases on gel filtration. The disaccharidases in the intestines of 18-day-old embryos behaved similarly on Sephadex chromatography to the chick disaccharidases. Thus the activity was separated into two peaks. The first peak contained all the isomaltase and sucrase activity (palatinase was not measured) and some of the maltase activity, with maltase:isomaltase:sucrase proportions the same as those found for the chick. The second peak contained only maltase activity, but whereas with the papain-solubilized preparations of the chick small intestine the activity in this peak accounted for 75% of the total maltase activity applied to the column, with similar preparations of the embryo intestine less than 50% of the total maltase activity was in this peak.

Heat inactivation. The use of heat inactivation as a method of identifying individual enzymes is based on the fact that the inactivation of enzymes usually shows a linear relationship when the logarithm of the remaining activity is plotted against time (Moelwyn-Hughes, 1940). Heat-inactivation studies of the particle-bound disaccharidases in a homogenate of chick small intestine (Fig. 4) suggested that the sucrase and the isomaltase and palatinase activities are due to single enzymes and that the maltase activity is due to at least three different enzymes. The sucrase enzyme could be distinguished from the isomaltase and palatinase since it was more heat-labile, but the isomaltase and palatinase activities could not be separated and are probably due to the same enzyme.

The heat inactivation of the disaccharidases in the two peaks obtained on Sephadex chromatography

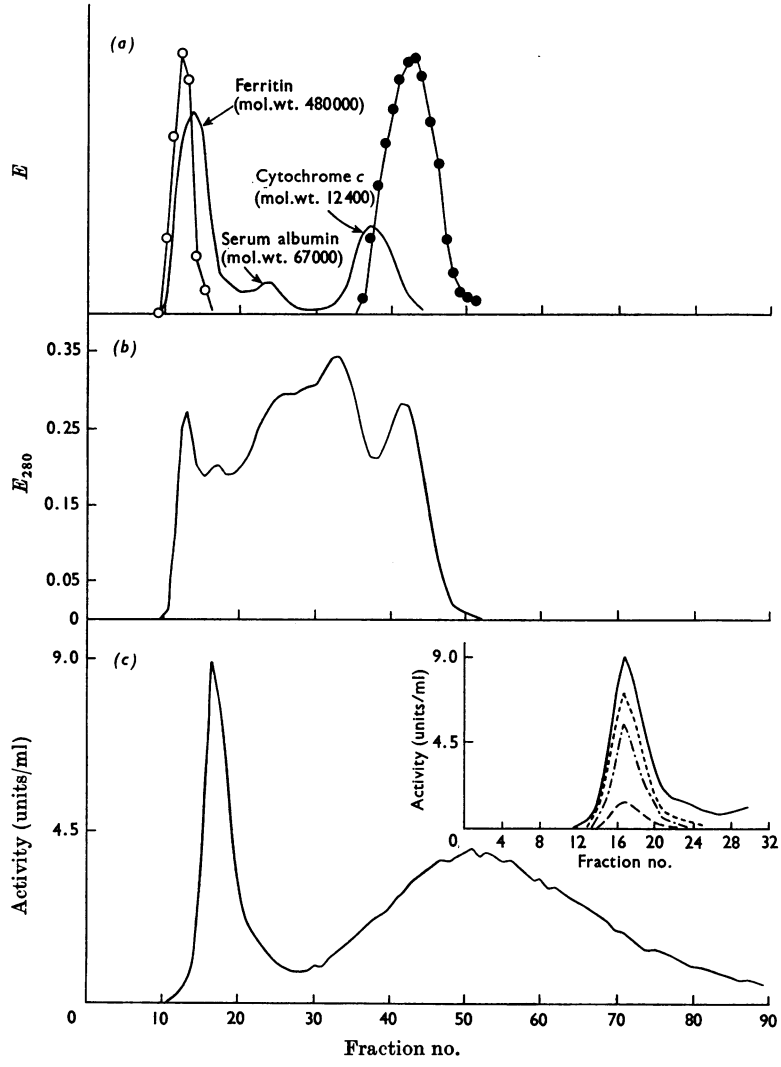

Fig. 2. Sephadex G-200 chromatogram of chick small-intestinal disaccharidases solubilized with papain. Fractions (3 ml) were collected. (*a*) Calibration of the column with Blue Dextran (○) indicating the void volume and potassium dichromate (●) indicating the total volume of the column, and with a mixture of proteins of different molecular weights (——). (*b*) Protein as indicated by E_{280}. (*c*) Maltase activity. (*c*) Inset: ——, maltase activity; – – – –, sucrase activity; –·–·–·, isomaltase activity; — —, palatinase activity.

was also studied (Fig. 5). For this purpose the fractions constituting the two peaks (fractions 14–25 for the first peak and fractions 30–90 for the second peak) after chromatography of the ethanol-precipitated enzymes were pooled separately and concentrated by ultrafiltration. The isomaltase, sucrase and palatinase activities behaved similarly to the particle-bound enzymes of the homogenate, though they were slightly less stable. Again the decrease in both activities followed first-order kinetics, and the velocity of the inactivation of the sucrase was greater than that of the isomaltase. The inactivation of the maltase activity of the first peak did not follow first-order kinetics, and the rate was intermediate between those of sucrase and isomaltase, suggesting that the maltase activity is due partly to the sucrase enzyme and partly to the isomaltase enzyme.

Heat inactivation of the maltase activity of the second peak showed that this activity was due to at least three different enzymes; one constituting 80%

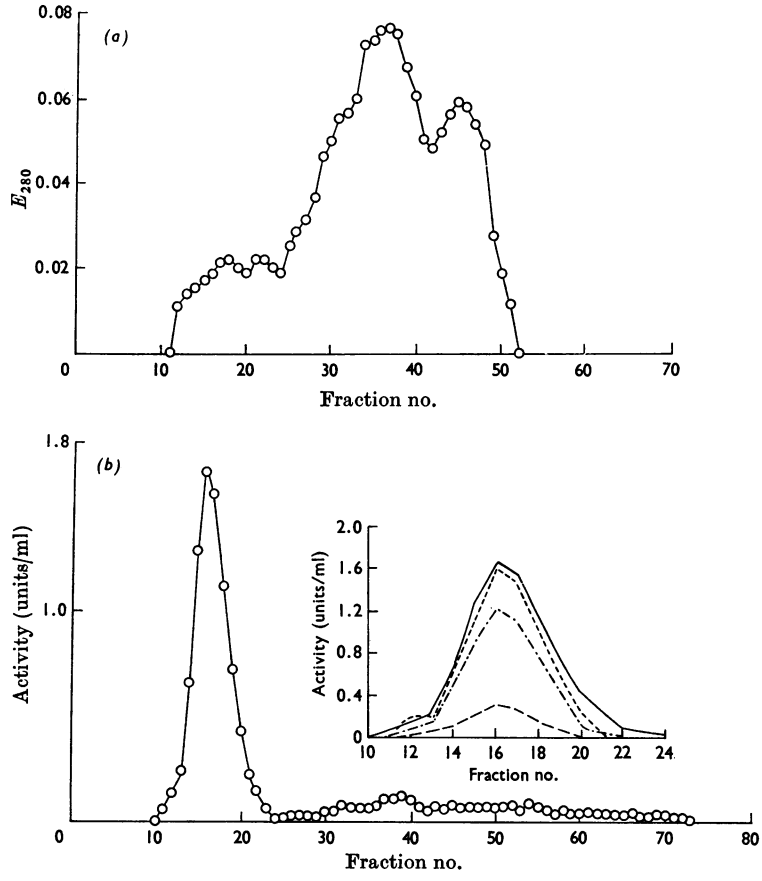

Fig. 3. Sephadex G-200 chromatogram of chick small-intestinal disaccharidases solubilized with trypsin. Fractions (3ml) were collected. (*a*) Protein as indicated by E_{280}. (*b*) Maltase activity. (*b*) Inset: ——, maltase activity; – – – –, sucrase activity; -·-·-·, isomaltase activity; — —, palatinase activity.

of the total was inactivated at 60°C, the second constituting 15% of the total was inactivated at 65°C, and the remaining 5% was more heat-stable.

Effect of pH *and substrate concentration on the maltase activity.* The maltase activities in the two peaks obtained on Sephadex chromatography both exhibited optimum activity at pH 6.2, which was the same as the pH optimum of the maltase activity of the homogenate. The maltase activity in the three preparations, however, showed different responses to varying substrate concentration (Fig. 6). Thus K_m values of 1.18, 5.56 and 3.12mM were found for the maltase activity in the first peak, second peak and homogenate respectively. The K_m value of the soluble maltase activity produced by papain digestion was the same as that of the homogenate. The K_m of the trypsin-solubilized maltase was 1.47mM. This value is similar to that of the maltase activity of the first peak, and is consistent with the behaviour of the trypsin-solubilized disaccharidases on Sephadex chromatography.

DISCUSSION

The work of Miller & Crane (1961) with isolated brush-border membranes and the use of histochemical staining techniques (Jos, Frezal, Rey & Lamy, 1967) have demonstrated that the disaccharidases of the mammalian small intestine are localized in the brush-border region of the intestinal epithelial cells. Further, Johnson (1967) has shown by electron microscopy of isolated brush borders from epithelial cells of hamster intestine before and after papain digestion that the disaccharidases are contained in knobs 60Å in diameter, which are attached to the luminal surface of the plasma membrane but are not an integral part of it. Eicholz (1968) also proposed, from his studies with isolated brush-border membranes, that the

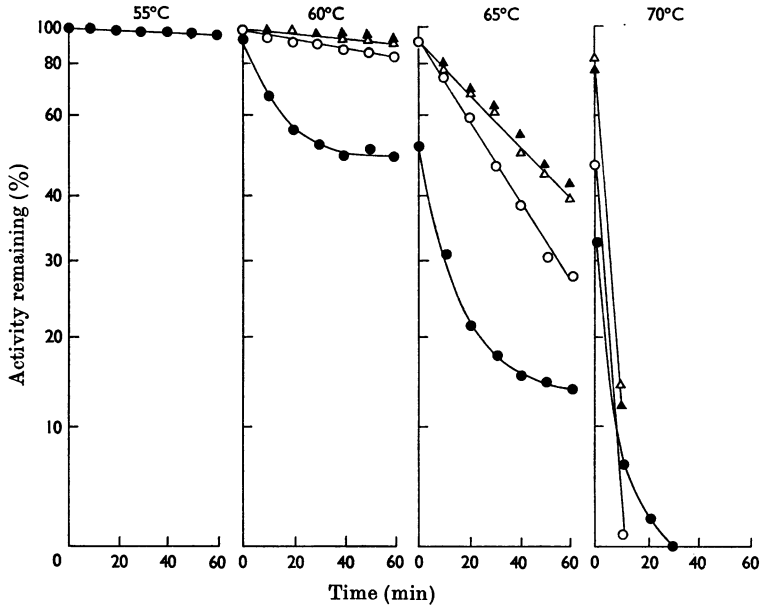

Fig. 4. Inactivation of the maltase (●), sucrase (○), isomaltase (△) and palatinase (▲) activities in a homogenate of chick small intestine at different temperatures. The homogenate was prepared in 0.1 M-sodium phosphate buffer, pH 7.0.

disaccharidases are arranged into discrete morphochemical subunits and are not diffusely distributed throughout the protein portion of the membrane matrix.

The present demonstration by heat inactivation of a number of disaccharidases in the small intestine of the chick and the failure to separate these enzymes chromatographically is consistent with the localization of the small-intestinal disaccharidases of the chick in similar multi-enzyme complexes.

The particulate nature of the disaccharidases in a homogenate was demonstrated by the sedimentation of the activities at 100000*g*. The particles that contain the disaccharidases and sediment at this centrifugal force are probably fragments of the brush borders produced during the homogenization of the mucosa. Miller & Crane (1961) showed that this breakdown of the brush border can be prevented by the inclusion of EDTA in the homogenizing buffer. The release of the particle-bound disaccharidases was achieved by digestion with papain, and subsequent Sephadex chromatography of the solubilized disaccharidases showed that they exist in the form of two complexes. One of the complexes is eluted from Sephadex columns close to the void volume of the column and contains enzymes hydrolysing maltose, sucrose, isomaltose and palatinose. Heat-inactivation studies showed that this complex contains two active sites. The more heat-labile site hydrolyses sucrose and the other site hydrolyses isomaltose and palatinose. The heat-inactivation kinetics of the maltase activity suggests that both sites also hydrolyse maltose. A similar sucrase–isomaltase complex has been isolated from the rabbit (Kolinska & Semenza, 1967), the rat (Dahlqvist, 1963) and the human (Semenza, Auricchio & Rubino, 1965). In the human it has also been shown that the isomaltase and palatinase activities are due to the same enzyme, isomaltose being the preferred substrate (Auricchio *et al.* 1963*b*; Dahlqvist, Auricchio, Semenza & Prader, 1963; Auricchio *et al.* 1965). This is consistent with our observations in the chick.

The second disaccharidase complex present in the small intestine of the chick contains three enzymes all specific for maltose. It can be separated from the sucrase–isomaltase complex since it is retained on Sephadex columns. The maltase activity of this complex has the same pH optimum as the maltase activity of the sucrase–isomaltase complex, but the two activities can be distinguished by a difference in their K_m values.

Dahlqvist & Telenius (1969) and Dahlqvist & Lindberg (1966) have shown that in the intestine of the 20-week-old human foetus the maltase activity associated with the isomaltase and sucrase activities is well developed, whereas other maltases that are present in the adult human are missing. In the intestine of 18-day-old chick embryos both the

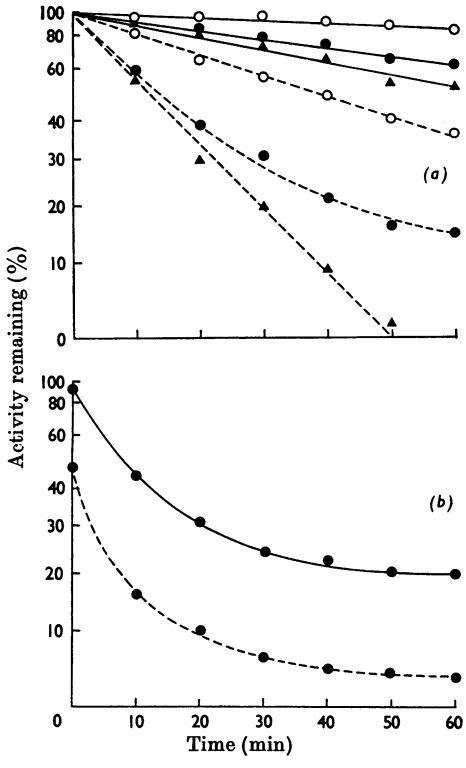

Fig. 5. Inactivation at 60°C (——) and 65°C (----) of the maltase (●), sucrase (▲), and isomaltase (○) activities in (*a*) the first peak and (*b*) the second peak obtained on Sephadex chromatography of papain-solubilized chick small-intestinal disaccharidases.

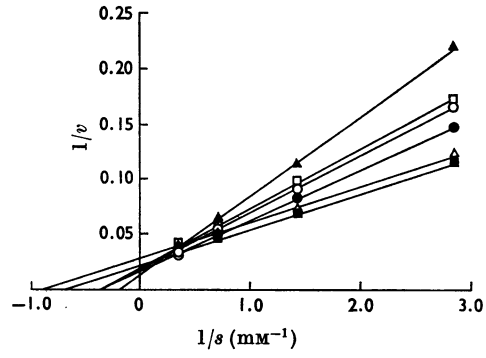

Fig. 6. Effect of substrate concentration on the maltase activity of chick small-intestinal homogenate (●), 100000*g* supernatant of papain-treated homogenate (○), ethanol precipitate of 100000*g* supernatant of papain-treated homogenate (□), 100000*g* supernatant of trypsin-treated homogenate (■), first peak (△) and second peak (▲) obtained on Sephadex chromatography of papain-solubilized disaccharidases.

sucrase–isomaltase complex and the maltase complex were present. However, the proportion of the total maltase activity contributed by the maltase complex was lower than in the chick, suggesting that the development of the maltase complex may occur later in embryonic life than that of the sucrase–isomaltase complex.

Many methods have been employed in attempts to solubilize the intestinal disaccharidases (Borgström & Dahlqvist, 1958; Gitzelmann, Davidson & Osinchak, 1964) but with very little success. However, in all the animals so far studied, digestion with proteolytic enzymes has proved successful, suggesting that the disaccharidases are linked to the particles in which they are localized by covalent peptide bonds. There appears to be a species difference in the nature of this bonding, since trypsin solubilizes the disaccharidases of the rat (Dahlqvist, 1963) and the pig (Borgström & Dahlqvist, 1958) but not those of man (Auricchio *et al.* 1963*b*). In the chick, complete solubilization of the disaccharidases is obtained with papain whereas trypsin preferentially solubilizes the sucrase–isomaltase complex, suggesting that there is a difference in the nature of the bonds joining the two complexes to the particles in which they are located.

It was suggested above that the separation of the disaccharidases on Sephadex was due to an interaction between the maltase complex and the Sephadex since the activity was eluted beyond the total volume of the column. This was substantiated by the finding that on rechromatography of this complex in 0.01 M-sodium–potassium phosphate buffer, pH 7.0, containing 0.01 M-maltose the activity was eluted close to the void volume of the column. This further suggested that the interaction was between the active site of the maltase and the Sephadex. Kolinska & Semenza (1967) showed that the separation of the mammalian intestinal disaccharidases is also based on an enzyme–Sephadex interaction. However, they found that it was the sucrase–isomaltase complex that was retained and, since after inactivation of the isomaltase by aging the remaining sucrase activity was not retained, they suggested that the retention is due to a substrate–enzyme interaction mainly involving the isomaltase site. Dahlqvist & Telenius (1969) reported, however, that the retention of the sucrase–isomaltase complex of the human ileum only occurred if the ethanol-precipitation step was included in the preparation of the enzymes. In the chick the sucrase–isomaltase complex is not retained whether ethanol precipitation has or has not been performed.

Apart from this difference in behaviour on Sephadex the disaccharidases of the chick appear to be very

similar to the mammalian enzymes. Thus, although it has not been possible to separate the individual enzymes, a combination of Sephadex chromatography and heat inactivation has shown that the hydrolysis of disaccharides in the chick small intestine is carried out by five enzymes. All five hydrolyse maltose, one of them also hydrolyses sucrose and another hydrolyses palatinose and isomaltose. Failure to isolate the individual enzymes can be attributed to the molecular arrangement of the activities in multienzyme complexes that are attached to the brush-border membrane (Eicholz, 1966). Obviously the degradation of these complexes is a necessary prerequisite for the isolation of the individual enzymes.

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