Biochem. J. (1974) **144**, 281–292
Printed in Great Britain

Soluble Neutral and Acid Maltases in the Suckling-Rat Intestine

THE EFFECT OF CORTISOL AND DEVELOPMENT

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(*Received* 20 *May* 1974)

The 100000*g* supernatants from 13-day-old suckling-rat intestinal homogenates contained 43.5% of the total intestinal maltase activity, compared with 7.1% in weaned adult rats aged 40 days. The soluble maltase activity was separated on Sepharose 4B into two quantitatively equal fractions at pH6.0, one containing a maltase with a neutral pH optimum and the other a maltase with an acid pH optimum. The neutral maltase was shown to be a maltase–glucoamylase identical with membrane-bound maltase–glucoamylase in molecular weight, heat-sensitivity, substrate specificity, K_m for maltose and K_i for Tris. The soluble enzyme was induced by cortisol, but the ratio of the soluble to bound enzyme fell during induction. Solubility of the neutral maltase was not accounted for by the action of endogenous proteinases under the preparative conditions used. It is postulated that the soluble neutral maltase is a membrane-dissociated form of the bound enzyme and that the relationship between these two forms is modulated by cortisol. The acid maltase generally resembled acid maltase of liver, muscle and kidney. It was shown to be a maltase–glucoamylase with optimal activity at pH3.0, and molecular weight of 136000 by density-gradient centrifugation. At pH3.0 its K_m for maltose was 1.5mM. It was inhibited by turanose ($K_i = 7.5$mM) and Tris ($K_i = 5.5$mM) but not by *p*-chloromercuribenzoate or EDTA. Some 55% of its activity was destroyed by heating at 50°C for 10min. The acid maltase closely resembled β-glucuronidase and acid β-galactosidase in its distribution in the intestine, response to tissue homogenization in various media, and decrease in activity with cortisol treatment and weaning, indicating that it was a typical lysosomal enzyme concentrated in the ileum.

The hydrolysis of maltose is a recognized function of several different enzymes (Kolinska & Semenza, 1967; Kolinska & Kraml, 1972; Kelly & Alpers, 1973), attached to the apical brush-border membrane of the small intestine (Miller & Crane, 1961; Forstner *et al.*, 1968), and this membrane is generally accepted to be the only site of maltase activity in this tissue (Miller & Crane, 1961; Donaldson *et al.*, 1973).

The high-molecular-weight, or heavy, maltase (Auricchio *et al.*, 1965; Eggermont, 1964) is readily released from membranes by papain (Eichholz, 1968; Forstner, 1971), and has been extensively purified in both the suckling (Schlegel-Haueter *et al.*, 1972) and the adult rat (Kolinska & Kraml, 1972) and in man (Kelly & Alpers, 1973). It is a maltase–glucoamylase, optimally active at neutral pH and quite heat-stable in comparison with other brush-border maltases (Auricchio *et al.*, 1965; Kolinska & Kraml, 1972). It has relatively weak isomaltase activity (Dahlqvist & Telenius, 1969; Kolinska & Kraml, 1972). In addition maltose is hydrolysed, by two other brush-border disaccharidases, sucrase and isomaltase (Kolinska & Kraml, 1972). Neither of these maltases exhibits glucoamylase activity (Dahlqvist & Thomson, 1963). Total maltase activity is relatively low in the suckling rat intestine but increases strikingly at weaning (Rubino *et al.*, 1963). During the suckling period there is virtually no sucrase–isomaltase activity in the intestine (Rubino *et al.*, 1963) and the heavy maltase–glucoamylase is the only membrane maltase present (Schlegel-Haueter *et al.*, 1972).

In extra-intestinal tissues, acid maltases (Lejeune *et al.*, 1963; Auricchio *et al.*, 1968; Palmer, 1971; De Barsy *et al.*, 1972), as well as neutral maltases (Lejeune *et al.*, 1963; Torres & Olivarria, 1964; Angelini & Engel, 1973) are well known. Acid maltases appear to be lysosomal enzymes (Lejeune *et al.*, 1963; Jeffrey *et al.*, 1970*a*,*b*), whereas the neutral maltases are derived from plasma membrane (Berger & Sacktor, 1970) and microsomal fractions

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(Lejeune *et al.*, 1963; Angelini & Engel, 1973), and may also be soluble (Lejeune *et al.*, 1963; Torres & Olivarria, 1964; Angelini & Engel, 1973). Acid maltases have been characterized in detail by a number of investigators, the interest stemming from the fact that acid maltase deficiency is the cause of type II glycogen storage disease (Hers, 1963; Van Hoof *et al.*, 1972).

While studying the development of disaccharidases in the suckling rat intestine it became obvious that the soluble fraction of maltase activity was very high in suckling as compared with adult rat intestine. In the present paper we describe the separation of soluble maltase activity into two components, one of which is a heavy maltase–glucoamylase which appears to be identical with the membrane-bound enzyme except for its dissociation from the membrane. The second maltase is a lysosomal acid maltase not previously described in intestinal homogenates, with properties similar to those found for lysosomal enzymes present in liver and other tissues. The activity of the acid maltase is greatly decreased at the time of weaning.

Materials and Methods

Chemicals and reagents

Sepharose 4B and Dextran T250 were purchased from Pharmacia (Uppsala, Sweden). EDTA, maltose and sucrose were purchased from Fisher Scientific Co. (Montreal, P.Q., Canada). Shellfish glycogen, Tris, glucose oxidase, peroxidase, *o*-dianisidine, mercuripapain, and phenolphthalein glucuronic acid (sodium salt) were obtained from Sigma (St. Louis, Mo., U.S.A.). *p*-Chloromercuribenzoate was purchased from Calbiochem (La Jolla, Calif., U.S.A.). *Escherichia coli* β-galactosidase (57 units/mg) was obtained from Worthington Biochemical Co. (Freehold, N.J., U.S.A.). Cortisol acetate was obtained from Merck, Sharp and Dohme Ltd. (Kirkland, P.Q., Canada).

Animals

White rats of the Wistar strain of either sex were used. Litters were decreased to eight animals 2 days after birth. Food and water were given *ad libitum*. Suckling rats were killed at 13 days, adult rats at 40 days.

Preparative techniques

Rats were killed by a blow on the head, the small intestine was removed from the ligament of Treitz to the terminal ileum and rinsed through with ice-cold 0.154 M-NaCl. The intestine was homogenized without inversion in ice-cold 5.0 mM-disodium EDTA buffer, adjusted to pH 7.4 with NaOH (10 ml/g of intestine), in a Waring blender for 15 s. To separate soluble from insoluble membrane-bound enzymes the homogenate was centrifuged at 100000*g* for 1 h in an International preparative ultracentrifuge model B60 (rotor type no. A 237). The supernatant was dialysed against 30 vol. of water for 2 days, freeze-dried and resuspended in water (50 mg of protein/ml). All procedures were carried out at 4°C.

Membrane-bound enzymes were solubilized by suspending the 100000*g* precipitate from 30 suckling rats in 10 mM-potassium phosphate buffer, pH 6.0 (0.1 ml/mg of protein), and incubating with mercuripapain (150 μg/mg of protein) and cysteine (15 μg/mg of protein) in a Dubnoff shaking incubator at 30°C for 40 min. The mixture was centrifuged at 100000*g* for 1 h at 4°C. The supernatant was dialysed against water for 2 days at 4°C, freeze-dried and resuspended in 5.0 ml of water (25.0 mg of protein/ml).

Brush-border membranes were prepared as described by Welsh *et al.* (1972) except that intestinal slices from three suckling rats were homogenized in 160 ml of 5 mM-EDTA, pH 7.4, and the pellet, obtained by centrifugation at 40000*g* for 30 min, was disrupted in 15.0 ml of 1.0 M-Tris–HCl buffer, pH 7.4, for 5 min at 4°C. Membrane fractions were harvested from a discontinuous gradient of 10 to 50% sucrose containing 0.5 M-$MgCl_2$.

Sepharose 4B chromatography

Samples (10.0 ml) containing concentrated 100000*g* supernatants or 5.0 ml samples of papain-solubilized material were placed on the top of a Sepharose 4B column (2.4 cm × 90 cm) previously equilibrated with 10 mM-potassium phosphate buffer, pH 6.0, and eluted with the same buffer at a constant flow rate of 10 ml/h. Fractions (5.0 ml) were collected.

Enzyme assays

Maltase, sucrase and lactase activities were determined by incubation with the corresponding 0.028 M-substrate in 0.05 M-sodium maleate buffer, pH 6.0, at 37°C (Dahlqvist, 1964). Glucoamylase activity was measured in the presence of glycogen (20 mg/ml) in 0.05 M-sodium maleate buffer, pH 6.0 (Eggermont, 1969). Acid maltase and acid glucoamylase were incubated in the presence of 0.028 M-maltose or 20 mg of glycogen/ml in 0.05 M-glycine–HCl buffer, pH 3.0. Released glucose was measured with a Tris–glucose oxidase reagent, pH 7.0, at 37°C as described by Dahlqvist (1964). Alkaline phosphatase was determined with *p*-nitrophenyl phosphate substrate under conditions described by Forstner *et al.* (1968). *E. coli* β-galactosidase was measured with lactose as substrate as for the lactase assay. Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Acid β-galactosidase was measured with 0.028 M-lactose as substrate in 0.05 M-glycine–HCl buffer,

Table 1. *Total maltase activity in intestinal homogenates and cell sap of adult and suckling rats*

Suckling rats were 13 days old and adult rats 40 days old. Intestinal homogenates were centrifuged at 100000*g* for suckling rats and 40000*g* for adult rats for 1h. Maltase activity was determined at pH6.0 in adult rats and at pH6.0 and 3.0 in suckling rats as described in the Materials and Methods section. Activities are expressed as μmol of substrate hydrolysed/min per rat, ±S.E.M. for four separate experiments. In the experiments with suckling rats three to four intestines were homogenized simultaneously, whereas in adult rats the homogenate was obtained from a single intestine.

	Assay condition (pH)	Homogenate maltase activity (total units)	Supernatant maltase activity (Total units)	Supernatant maltase activity (% of homogenate)
Adult rats	6.0	141.5±26.9	10.9±3.2	7.1±1.0
Suckling rats	6.0	4.05±0.62	1.73±0.25	43.5±2.9
	3.0	2.34±0.19	1.67±0.16	71.3±2.9

pH3.0. β-Glucuronidase was measured with 0.01M-phenolphthalein glucuronic acid in 0.1M-sodium acetate buffer, pH4.5 (Fishman, 1965).

K_m and K_i were determined graphically by the methods of Lineweaver & Burk (1934) and Dixon (1953).

Density-gradient ultracentrifugation of maltases

Continuous linear mannitol gradients (4.6ml) were prepared, ranging from 5% (w/v) mannitol at the top to 20% (w/v) mannitol at the bottom. The gradient solution contained 0.2% (w/v) Triton X-100, 0.01M-KCl and 0.02M-potassium phosphate buffer, pH 6.0. The maltase solution (100μl; 0.05–0.2mg of protein) was placed on the top of the gradient with 0.6mg of *E. coli* β-galactosidase (57units/mg). The gradient was centrifuged for 5h at 40000rev./min in a model L Spinco preparative ultracentrifuge with a swinging-bucket rotor (SW 50L). After centrifugation the contents of the tubes were collected in 27 fractions of 10 drops each. To each fraction 0.5ml of water was added and the fractions were assayed for maltase and β-galactosidase activities. The sedimentation coefficients and molecular weights of the different maltases were calculated as described by Martin & Ames (1961) by comparison with *E. coli* β-galactosidase marker (mol.wt. 540000; $s_{20,w} = 16.0$S, as the tetramer; Craven *et al.*, 1965).

Cortisol acetate injection protocol

Each litter of suckling rats was divided into two groups of four. One group was not given cortisol by injection and served as control. The other group was given subcutaneous injections of cortisol acetate on postnatal days 10 (5.0mg/100g body wt.), 11 (2.5mg/100g) and 12 (2.5mg/100g). Both control and injected rats were killed at 13 days.

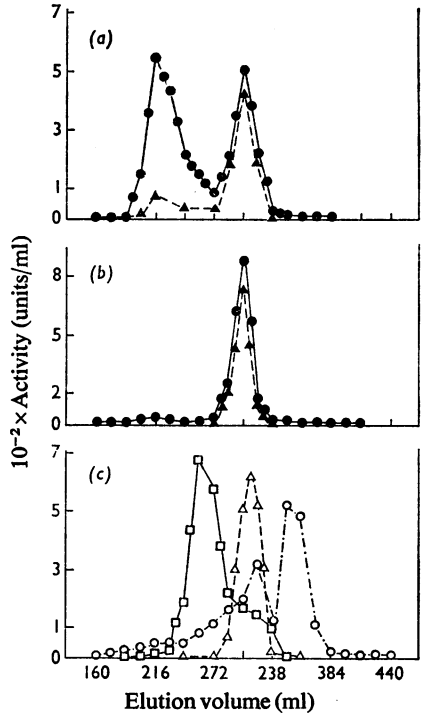

Fig. 1. *Chromatography of a concentrated* 100000*g* *supernatant from intestinal homogenates of* 13*-day-old rats on a Sepharose* 4*B column*

For details see the Materials and Methods section. The void volume was 120ml and the total volume 370ml. Enzymic activity and protein were determined as described in the Materials and Methods section. ●, Maltase; ▲, glucoamylase; (*a*) assays performed at pH6.0; (*b*) assays performed at pH3.0; (*c*) □, lactase (pH6.0); △, alkaline phosphatase (pH9.2); ○, protein.

Results

Initial results (Table 1) indicated that high-speed supernatants from rat intestinal homogenates contained 43.5% of the total mucosal maltase activity in the suckling rat as compared with 7.1% in adult rats. Since we were aware of lysosomal maltases in

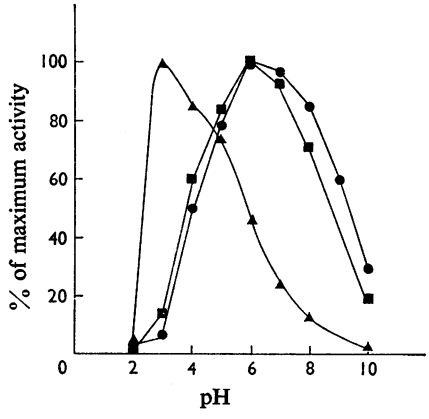

Fig. 2. *pH-dependence of 100000g supernatant maltases and maltase solubilized from membranes by papain, obtained from Sepharose 4B column eluates*

For details see the text. ▲, Low-molecular-weight supernatant maltase; ●, high-molecular-weight supernatant maltase; ■, papain solubilized membrane.

other tissues with low pH optima (Lejeune *et al.*, 1963) as well as the high lysosomal activity in suckling rat intestine (Koldovsky & Palmieri, 1971), maltase activity was measured at pH 3.0, which is the optimum pH for intestinal acid β-galactosidase in rats (Alpers, 1969). Under these conditions 71.3% of the maltase activity of the intestine was present in the supernatant, indicating that this fraction of the homogenate contained significant acid maltase activity.

The 100000*g* supernatant from 13-day-old suckling rats was dialysed and concentrated as described in the Materials and Methods section, and chromatographed on Sepharose 4B (Fig. 1). At pH 6.0 (Fig. 1*a*) two maltase peaks were obtained, the first being eluted before lactase (Fig. 1*c*), and the second appearing well beyond the lactase peak but slightly before alkaline phosphatase, which was estimated previously to have a molecular weight of 70000 (Forstner, 1971). Fig. 1(*b*) shows that the high-molecular-weight maltase was inactive at pH 3.0, whereas the activity of the low-molecular-weight maltase was enhanced at this pH. At pH 6.0 each maltase contributed approximately equally to total soluble activity. Figs. 1(*a*) and 1(*b*) also indicate that both maltases had glucoamylase activity and that the low-molecular-weight glucoamylase was considerably more active per unit of maltase activity than the larger.

The behaviour of the soluble neutral maltase–glucoamylase on Sepharose 4B corresponded closely in our experience with the papain-solubilized maltase–glucoamylase derived from the microvillus plasma membrane (Forstner, 1971). In order to compare these two enzymes in greater detail membrane-bound maltase from suckling rats was solubilized by incubation with papain and chromatographed on Sepharose 4B. As expected, since suckling rat intestine contains no sucrase or isomaltase, a single maltase peak was obtained in approximately the same elution volume as the first peak in Fig. 1 (results not shown). Material from all three maltase peaks was combined independently, dialysed overnight against water and concentrated by freeze-drying. When reconstituted at a concentration of 1.0 unit of maltase/ml in water and frozen at −10°C activity remained constant over several months.

The influence of pH on the activity of the three maltases is illustrated in Fig. 2. The neutral, high-molecular-weight maltases displayed virtually identical pH dependency, with maximum activity at pH 6.0 and little activity at pH 3.0. The low-molecular-weight maltase was optimally active at pH 3.0, but possessed approximately 45% of its maximum activity at pH 6.0.

The apparently similar size of the membrane-bound maltase, solubilized by papain, and the naturally soluble neutral maltase was examined more

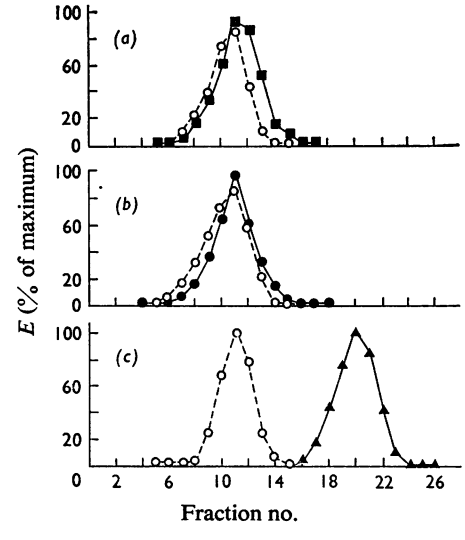

Fig. 3. *Sedimentation patterns of membrane maltase solubilized by papain* (■), *neutral* 100000*g supernatant maltase* (●), *acid* 100000*g supernatant maltase* (▲) *and E. coli β-galactosidase* (○) *on linear mannitol gradients*

Conditions were as described in the Materials and Methods section for the neutral maltases and β-galactosidase; each point represents the mean of three experiments. The sedimentation behaviour of acid maltase was determined once.

critically by density-gradient ultracentrifugation (Fig. 3). Both enzymes travelled almost identically with the β-galactosidase marker (mol.wt. 540000; $s_{20,w}$ 16.0S) and appeared to have the same molecular weights within an experimental range of error of 50000 (equal to one ten-drop fraction). The acid maltase also shown in Fig. 3 had a sedimentation constant of 6.4S, corresponding to a molecular weight of 136000.

Comparison of neutral maltases

The similarity in pH optima and molecular weight exhibited by the spontaneously soluble maltase from the 100000*g* supernatant and maltase derived from the plasma membrane seemed sufficiently striking to suggest that the one might be a membrane-dissociated but otherwise identical form of the other. Both enzymes were found to have similar affinity for maltose at pH6.0, with $K_m = 1.7$mM for the spontaneously soluble maltase, and $K_m = 1.25$mM for the papain-solubilized enzyme (results not shown). Neither enzyme was inhibited by turanose, in agreement with previous results obtained for the papain-solubilized enzyme (Schlegel-Haueter *et al.*, 1972).

When the susceptibility of both enzymes to inhibition by Tris at pH6.0 was investigated a major difference became apparent, however. As shown by Dixon plots in Figs. 4(*a*) and 4(*b*) the papain-solubilized enzyme was much more sensitive to Tris ($K_i = 2.5$mM) than the spontaneously soluble enzyme ($K_i = 9.5$mM). Fortunately, because the neutral maltase–glucoamylase is the only maltase bound to the brush-border membrane in the suckling rat, it was possible to examine the maltase in its membrane-bound state. When this was done, the K_i for Tris (Fig. 4*c*) was identical with that of the spontaneously soluble enzyme. The implications of this experiment are threefold: (1) papain altered the functional activity of the membrane-bound enzyme while solubilizing it; (2) the spontaneously soluble enzyme resembled the membrane-bound enzyme closely; and (3) the spontaneously soluble enzyme was probably not released from the membrane by proteolysis.

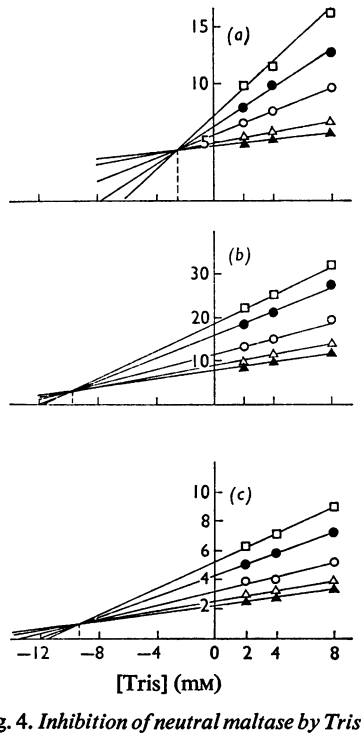

Fig. 4. *Inhibition of neutral maltase by Tris*

(*a*) Membrane-bound maltase after solubilization by papain; (*b*) 100000*g* supernatant maltase; (*c*) membrane-bound maltase. Maltose concentrations were 1.25mM (□), 1.5mM (●), 2.5mM (○), 5.0mM (△), 10.0mM (▲). Activity is plotted according to Dixon (1953). The K_i for Tris is indicated on the abscissa by the interrupted line. Assays were performed at pH6.0. *v* is expressed as μmol of maltose hydrolysed/min.

Comparison of the three enzymes with regard to heat-stability also disclosed a rather unexpected behavioural relationship. Neither soluble maltase was affected by heating at 50°C for 40min (Fig. 5), conditions which inactivate maltases associated with sucrase–isomaltase, but do not affect the maltase–glucoamylase solubilized by papain (Auricchio *et al.*, 1965). In contrast with the soluble enzymes, however, the membrane-associated maltase was partially inactivated after 20min. At 60°C all three enzymes were affected, but again the membrane-associated enzyme was much less stable than either of the soluble enzymes. These results are similar to those obtained with Tris in that the behaviour of only two of the three neutral maltase forms was comparable, but in this case the soluble form of the membrane maltase rather than the associated form most closely resembled the spontaneously soluble supernatant maltase.

The 100000*g*-supernatant neutral maltase hydrolysed maltose, glycogen, turanose, trehalose, palatinose and sucrose in descending rates corresponding to 100, 6.2, 1.2, 0.4, 0.3 and 0.3. The activity of the papain-solubilized enzymes for these substrates was of a similar order except that no activity was found with palatinose or sucrose. Although otherwise similar the membrane-bound enzyme differed from both soluble enzymes in possessing twice as much activity with glycogen. However, particularly with regard to the membrane-bound maltase, slight differences in substrate specificity have to be interpreted with caution, owing to the possibility of interaction with other enzymes such as amylase.

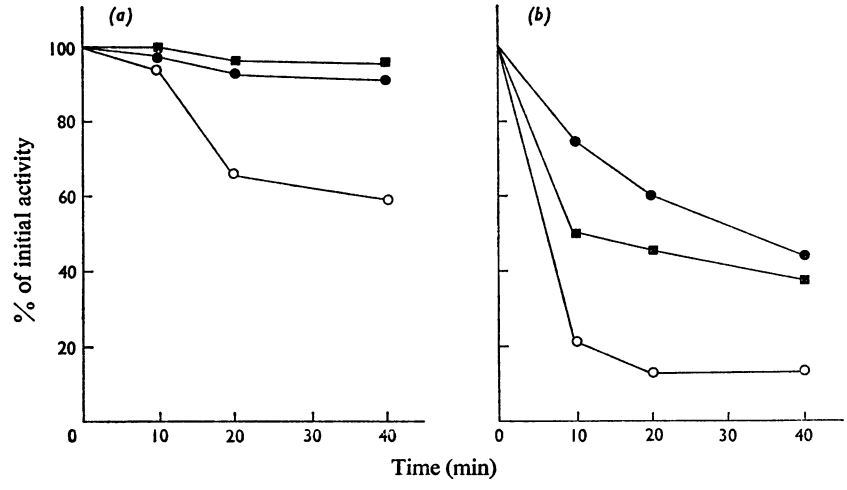

Fig. 5. *Heat-inactivation of neutral maltases*

Tubes containing maltase in a total volume of 1.0ml were incubated at either (*a*) 50°C or (*b*) 60°C for 10–40min. Incubations were terminated by transfer to iced water, and residual maltase activity was determined. ■, Membrane maltase solubilized by papain; ●, neutral 100000*g* supernatant maltase; ○, membrane-bound maltase.

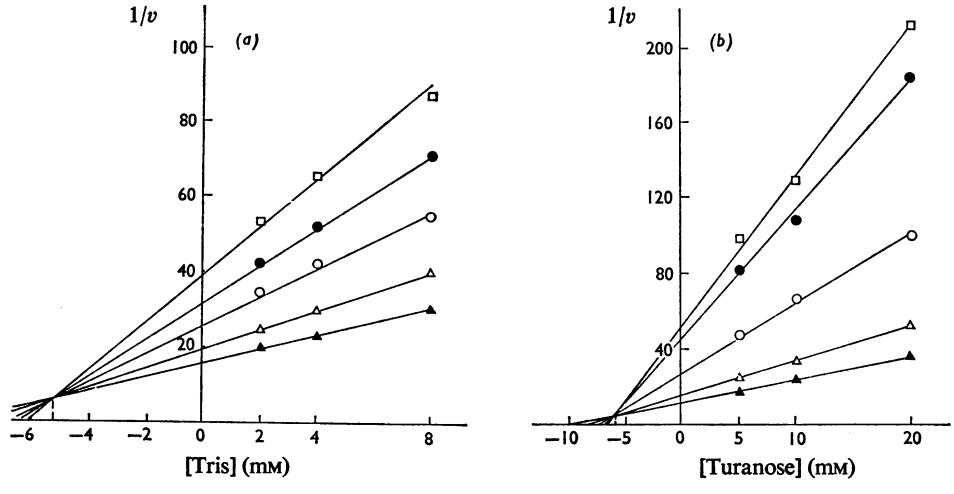

Fig. 6. *Inhibition of acid maltase by (a) Tris and (b) turanose*

Maltose concentration was 1.25mM (□), 1.5mM (●), 2.5mM (○), 5mM (△), 10mM (▲). Activity is plotted according to Dixon (1953). The respective K_i values are indicated on the abscissa by interrupted lines, as in Fig. 4. Assays were performed at pH3.0 with turanose and at pH6.0 with Tris. Units of v are μmol of maltose hydrolysed/min.

The substrate specificities do suggest, however, that all three enzymes are maltase–glucoamylases with little specificity for other substrates.

Characterization of acid maltase

The relative activity of the acid maltase against various substrates, determined as mol of glucose released/min, was for maltose 100, glycogen 44, palatinose 1.4, turanose 1.4, trehalose 0.4. No activity was observed with dextran, lactose, cellobiose or melobiose. At pH3.0 the K_m for maltose was 1.5mM. Both turanose ($K_i = 7.5$mM) at pH3.0 and Tris ($K_i = 5.5$mM) at pH6.0 inhibited acid maltase activity competitively (Fig. 6). Inhibition by Tris was

Table 2. *Effect of homogenization in iso-osmotic and hypo-osmotic media, plus incubation at 37°C for 30min, on the solubility of acid maltase and two lysosomal enzymes*

The scraped mucosa from the small intestine of a 13-day-old suckling rat was homogenized with four strokes in 11.0ml of 0.25M-mannitol or water in a Potter–Elvehjem homogenizer in ice. A 0.2ml sample was removed for determination of total enzyme activity. A 5.0ml portion of the 0.25M-mannitol homogenate was incubated for 30min at 37°C. Immediately after homogenization, and at the end of the incubation, the homogenates were centrifuged at 40000*g* for 10min. The supernatant was removed by decanting and soluble enzyme activity determined. Activity is expressed as total μmol of substrate hydrolysed/min per fraction.

	Enzyme activity of homogenate in:		
		Mannitol (0.25M)	
Enzyme	Mannitol (0.25M)	and incubation at 37°C for 30min	Water
Acid maltase			
Total	0.283	0.306	0.453
Soluble	0.122	0.287	0.354
Soluble (% of total)	43.2	93.8	78.3
Acid β-galactosidase			
Total	0.340	0.308	0.626
Soluble	0.088	0.248	0.325
Soluble (% of total)	25.8	80.5	52.0
β-Glucuronidase			
Total	0.216	0.235	0.312
Soluble	0.082	0.208	0.196
Soluble (% of total)	38.0	88.5	63.0

strongly pH dependent, as has been noted by Semenza & von Balthazar (1974) for sucrase, and could not be demonstrated at pH3.0. No inhibition was noted with *p*-chloromercuribenzoate or EDTA, and heating at 50°C for 40min. destroyed 65% of the activity, 55% within the first 10min The acid maltase was greatly retarded, well beyond the total volume, on a Sephadex G-200 column (results not shown).

Source of acid maltase

Under the high shear forces and hypo-osmotic conditions used for homogenization (5.0mM-EDTA in a Waring blender) pronounced lysosomal disruption with release of lysosomal enzymes into the supernatant could have taken place. This suspicion was confirmed by the experiment shown in Table 2 in which the solubility of acid maltase was compared in suckling rat intestine with two other intestinal lysosomal enzymes (Alpers, 1969; Koldovsky & Palmieri, 1971) under conditions chosen to preserve, or maximally disrupt, lysosomal integrity. In 0.25M-mannitol only 43% of the acid maltase was solubilized in comparison with 71% in 5.0mM-EDTA (Table 1). Acid β-galactosidase and β-glucuronidase were solubilized to a similar extent. In water, or after incubation of the 0.25M-mannitol homogenates for 30min at 37°C (Lejeune *et al.*, 1963), the solubility of all three enzymes increased two- to three-fold. Acid maltase was therefore released by the same conditions and to a similar degree as two other lysosomal enzymes. In a second experiment the small intestine of suckling rats was scraped lightly to remove superficial mucosa, and to leave behind the underlying muscle (Forstner, 1969). The activity of acid maltase was then determined in the superficial and deep layers, and compared with acid β-galactosidase and β-glucuronidase. Acid maltase was 2.4-fold, acid β-galactosidase 2.4-fold and β-glucuronidase 2.6-fold more active in the superficial layer. Acid maltase is therefore predominantly a superficial mucosal enzyme with a vertical distribution in the intestinal mucosa similar to that of other lysosomal enzymes.

In the suckling rat lysosomal activity is found predominantly in the ileum, and after the 9th postnatal day activity is decreased in response to cortisone (Koldovsky & Palmieri, 1971). Fig. 7 shows that soluble acid maltase is distributed predominantly in the distal small intestine along with other lysosomal enzymes. The administration of cortisol acetate to

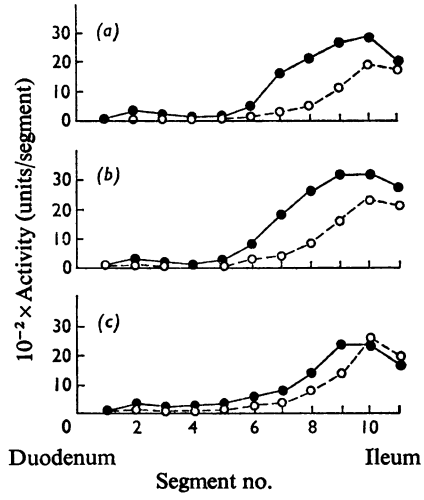

Fig. 7. *Longitudinal distribution of (a) acid maltase, (b) acid β-galactosidase and (c) β-glucuronidase in small-intestinal segments of control 13-day-old rats* (●) *and of 13-day-old rats injected with cortisol acetate* (○)

The intestine was cut into 11 pieces. Each segment was homogenized in 5.0mM-EDTA buffer, pH7.4, and centrifuged for 60min at 100000*g*. The 100000*g* supernatant was analysed at pH3.0 for β-galactosidase and maltase activity, and at pH4.5 for β-glucuronidase activity. Activities are expressed as μmol of substrate hydrolysed/min per intestinal segment.

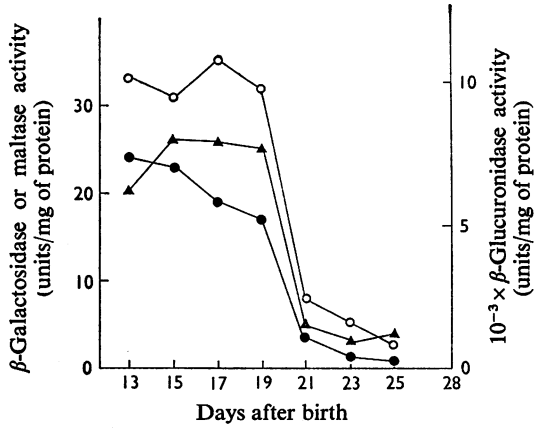

Fig. 8. *Change in the soluble activity of lysosomal enzymes and acid maltase in the small intestine with development*

Rats were killed at 2-day intervals between 13 and 25 days of age. The small intestine was homogenized in 5.0mM-EDTA as described in the Materials and Methods section, and centrifuged for 60min at 100000*g*. The 100000*g* supernatant was analysed for acid β-galactosidase (▲), β-glucuronidase (○), and acid maltase (●), as described in Fig. 7. Activity is expressed as μmol of substrate hydrolysed/min per mg of protein.

littermates in doses known to induce precocious development (Doell & Kretchmer, 1964) diminished the soluble activity of all three enzymes. This change was most marked in proximal segments, in this respect resembling the sequential cessation of macromolecular uptake in the intestine with age (Clarke & Hardy, 1969), which is probably correlated with loss of lysosomes (Cornell & Padykula, 1969). Again with weaning (Fig. 8) soluble acid maltase activity diminished sharply and in parallel with similar decreases in acid β-galactosidase and β-glucuronidase activity. These results in total leave no doubt that acid maltase is a lysosomal enzyme distributed similarly to, and sharing an identical developmental pattern with, other intestinal lysosomal enzymes.

Effect of hydrocortisone on soluble maltase activity

The results in Fig. 7 indicate that treatment with cortisol should decrease soluble acid maltase activity in the whole intestine. It was of interest, however, to determine whether cortisol affected the soluble maltase–glucoamylase, since if related to the membrane-associated enzyme its activity might be enhanced. Fig. 9 compares Sepharose 4B chromatograms of the 100000*g* supernatant material from control rats, aged 13 days, given no cortisol (Fig. 9*a*), 13-day-old rats which had received cortisol injections for 3 days (Fig. 9*b*), and adult rats aged 40 days (Fig. 9*c*). In the cortisol-treated rats acid maltase activity fell in both absolute and relative terms in comparison with neutral maltase activity. In the supernatant from adult rats acid maltase activity was not detectable. Neutral maltase activity increased greatly after cortisol treatment (Fig. 9). The apparent augmentation of activity at pH3.0 in the neutral maltase–glucoamylase area is largely explained on the basis of a marked increase in maltase activity since pH3.0 activity still accounted for less than 10% of the maximal activity at pH6.0. The changes induced by cortisol are placed in perspective by Table 3, which compares the relative activities of the various maltases in the entire intestine in control and cortisol-injected 13-day-old suckling rats. Values for total and total soluble maltase and sucrase activities were derived from multiple experiments, and that for bound maltase–glucoamylase activity was

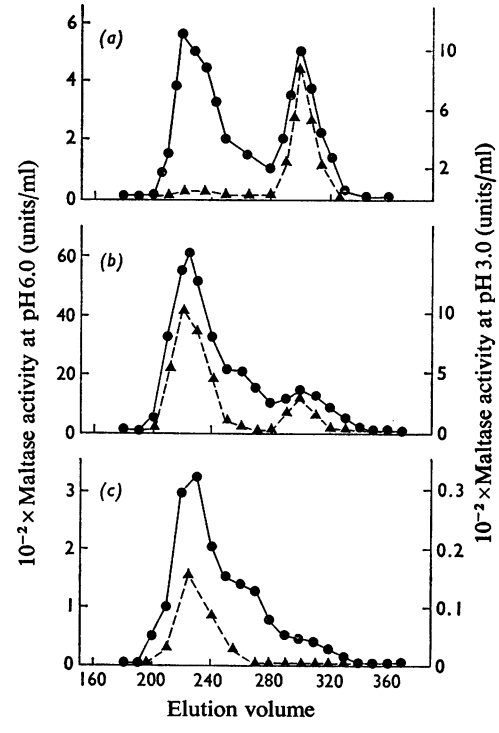

Fig. 9. *Maltase elution profiles from concentrated* 100000*g* *supernatants of intestinal homogenates from* (*a*) 13*-day-old control rats,* (*b*) 13*-day-old rats given cortisol acetate by injection for* 3 *days as described in the Materials and Methods section and* (*c*) 40*-day-old rats*

Conditions were the same as in Fig. 1. ●, Maltase activity at pH6.0; ▲, maltase activity at pH3.0. The column contained Sepharose 4B and recovery of maltase applied to the columns was always in excess of 80%.

Table 3. *Estimated activity of various maltases in the intestine in control 13-day-old rats and 13-day-old rats injected with cortisol acetate for 3 days*

The activity of the soluble maltases was measured by triangulation of the elution profiles obtained from Sepharose 4B chromatography, and suitably corrected for the small amounts of maltase of intermediate molecular weight assumed to be associated with sucrase and isomaltase in injected rats. Total maltase associated with sucrase–isomaltase was calculated from total sucrase activity, assuming that the maltase activity of this fraction was 1.9 times the sucrase activity (Kolinska & Kraml, 1972). Total bound maltase–glucoamylase activity was calculated by subtraction of bound maltase associated with sucrase from the total bound maltase activity in cortisol-injected rats. When more than one experiment was performed total activity (μmol of substrate hydrolysed/min) was calculated per rat to facilitate comparison.

	Control		Cortisol-treated		
Enzyme	Total units (A)	% of total	Total units (B)	% of total	B/A
Maltase					
Total	4.05±0.62 (4)		29.68±1.43 (8)		7.3
Soluble	1.73±0.25 (4)	43.5±2.9 (4)	6.54±1.76 (8)	18.0±2.5 (8)	3.8
Neutral maltase–glucoamylase					
Bound	2.27	56.0	17.13	57.7	7.5
Soluble	1.01	25.0	3.71	12.5	3.7
Acid maltase					
Soluble	0.77	19.0	0.59	2.0	0.8
Sucrase					
Total			3.44±0.21 (7)		
Soluble			0.24±0.08 (7)	4.9±0.7 (7)	
Maltase associated with sucrase			6.55	22.0	

arrived at by difference as described in Table 3. Soluble acid maltase activity fell fractionally in response to cortisol, but in terms of total mucosal maltase activity was decreased from 19% to a negligible 2%. Cortisol increased bound maltase–glucoamylase 7.5-fold, to the same extent as the increase in total maltase activity. There was little change therefore in the proportion of total maltase activity accounted for by the bound enzyme. The soluble neutral maltase–glucoamylase also increased in total activity, indicating that like the bound enzyme, its activity was enhanced by cortisol. Significantly, however, the percentage of total activity accounted for by this enzyme fell from 25% to 12.5%. Since bound maltase–glucoamylase did not change as a percentage of total activity, the ratio of soluble to bound neutral maltase–glucoamylase also fell proportionately. These results clearly indicate that the soluble neutral maltase–glucoamylase is, like the membrane-associated form, responsive to cortisol, and therefore closely related to that form. At the same time, if the soluble enzyme is a naturally dissociated form of the membrane-bound enzyme it is obvious that cortisol altered the ratio of associated to dissociated species in favour of the former.

Source of soluble neutral maltase

Table 4 indicates that membrane-bound maltase is not released by enzymes in lysosome-rich homogenates from suckling rat ileum under conditions pertaining to the preparative procedures used in our experiments. Freshly prepared homogenates from adult rat jejunum and suckling rat ileum were adjusted to similar total maltase activity and mixed in varying proportions. After 5 min at 4°C, mixed and unmixed homogenates were centrifuged to separate bound from soluble maltase activity. Supernatant maltase activity was predicted for each mixture, from the values found for the starting homogenates, assuming 0, 2 and 5% release of bound maltase from adult rat membranes. In all but one supernatant the determined results indicated that no more than 2% of the bound maltase could have been released. If maltase attached to suckling rat membranes is also included, all the results are compatible with less than 2% solubilization. Similar results were obtained in experiments with ileum from suckling rats given cortisol (not shown).

In other experiments suckling rat intestine was homogenized in iso-osmotic buffer (0.278 M-mannitol–0.01 M-sodium phosphate, pH 7.4) at varying time-intervals from 5 to 30 s in a Waring blender, and with a Potter–Elvehjem homogenizer with as few as four vertical strokes of the pestle. Under all of these conditions approximately 25% of the neutral maltase activity was found in the high-speed supernatant (100000*g* × 60 min).

It was concluded therefore that the soluble neutral maltase either exists free of membrane within the cell, or represents a bound maltase which is attached to membranes by unusually weak forces.

Table 4. *Mixing of homogenates from 13-day-old suckling and adult rats*

Homogenates were prepared in 5.0mM-EDTA, pH7.4, as described in the Materials and Methods section, and maltase activity was determined immediately. Concentrations were adjusted with 5.0mM-EDTA buffer, pH7.4, to contain similar maltase activity and the homogenates were mixed in the proportions indicated in a final volume of 2.0ml. A, Adult rat homogenate; B, suckling rat homogenate. After 5min at 4°C the tubes were centrifuged for 30min at 40000*g*. The resulting supernatant was analysed for maltase activity at pH6.0. Expected results are expressed for 0, 2 and 5% solubilization of maltase attached to the membranes in the adult rat homogenate.

Mixing conditions for homogenates		Supernatant maltase activity (units/2.0ml)			
		Predicted for % released from membrane			
A (%)	B (%)	0%	2%	5%	Determined
100	0				0.048
75	25	0.096	0.100	0.108	0.100
					0.098
50	50	0.150	0.152	0.156	0.152
					0.152
25	75	0.202	0.204	0.206	0.210
					0.194
0	100				0.260

Discussion

Soluble intestinal maltase activity has been found to represent a very small fraction of total intestinal maltase activity whenever measured previously, ranging from 1.2 to 11.0% in a variety of species, including rabbit (Gitzelmann *et al.*, 1963), chicken (Siddons, 1970), human (Auricchio *et al.*, 1963) and monkey (Swaminathan & Radhakrishnan, 1970). Foetal or neonatal mammals have not been studied, however, so that this experience must be regarded only as typical of the weaned animal. The very large soluble maltase fraction described in the present paper appears to be a phenomenon limited to the neonatal period, in that it was decreased by cortisol which induced precocious intestinal development in suckling rats (Clark, 1959; Doell & Kretchmer, 1964) and was very low in the adult. As illustrated in Table 3, total soluble maltase activity actually rose with development, so that this fraction, if truly representative of a distinctive maltase pool, may continue to have an importance throughout adult life.

Our results show that the principal soluble maltase, after treatment with cortisol and in the adult rat, is a high-molecular-weight neutral maltase–glucoamylase strikingly similar to the maltase–glucoamylase bound to the microvillus membrane. The soluble enzyme could not be distinguished from membrane-bound maltase–glucoamylase by its affinity for maltose, pH-dependency, molecular size, inhibition by Tris or heat-stability. Two features, the response to Tris and to heating, merit additional comment. Papain has been used exclusively to obtain a soluble intestinal maltase–glucoamylase for subsequent purification (Kolinska & Kraml, 1972; Schlegel-Haueter *et al.*, 1972; Kelly & Alpers, 1973). Our experiments with Tris provide the first indication that papain may affect the function of maltase (by increasing sensitivity to the inhibitor) and suggest the advisability of comparing the purified, proteolytically derived maltase, with the spontaneously soluble enzyme in future studies. In contrast with the response to Tris, which distinguished bound and proteolytically solubilized forms of the membrane-associated enzyme, heat-stability was shared by both soluble maltases and appeared to lessen with membrane association. Superficially these results are at variance with those of Dahlqvist (1959), who found identical heat-inactivation curves for particulate maltases and trypsin-solubilized maltase in the pig. However, the particulate maltase preparation used in his experiments was a deoxycholate extract of washed membranes, which in retrospect might be considered soluble. On the other hand the significance of our findings with intact membranes is obscured by the fact that aggregation or other responses of membrane constituents may have effects such as limiting accessibility to substrate which have little relevance to the function of maltase in its normal membrane environment. It is thus premature to conclude that the local environment within the microvillus membrane may influence some properties of the enzyme.

The soluble neutral high-molecular-weight maltase–glucoamylase does not appear to be artificially derived from microvillus plasma membrane. Pancreatic enzymes are known to release disaccharidases (Dahlqvist, 1963), but pancreatic protein ases are less active in the suckling intestine when the soluble maltase fraction is highest (Jones, 1972). Also the comparable inhibition by Tris observed in the associated and spontaneously soluble maltases argues that the latter was not attacked by proteinases. Our mixing experiments (Table 4) also indicate that intracellular proteinases do not affect the ratio of bound to unbound enzyme under our conditions of homogenization. The most reasonable conclusion to be drawn from our findings is that the soluble enzyme in the suckling rat is either free of membranes within the cell, or bound so loosely to the plasma membrane that it is physically dissociated by mild homogenization. There is suggestive evidence that proteins destined for incorporation within chloroplasts (Hoober *et al.*, 1969) and mitochondrial membranes (Beattie, 1968) must exist in the cytoplasm. Dehlinger & Schimke (1971) have suggested

that some membrane proteins are actually in equilibrium between associated and dissociated forms, and that the rate of dissociation could control degradation of membrane proteins if solubility were a prerequisite for lysosomal attack (Dehlinger & Schimke, 1971). They have also suggested that association of soluble enzymes with membranes might permit continuous membrane self assembly without requirement for direct ribosomal interaction. In the present studies the change in the ratio of free to bound enzyme in suckling rats under the influence of cortisol suggests that membrane binding affinity for the neutral maltase, and possibly the equilibrium between soluble and bound forms of the enzyme, may be subject to hormonal control and change with development. It is evident that further exploration of the relationship in terms of establishing the characteristics of enzyme–membrane interaction, and the rates of synthesis and degradation of the two forms, may be extremely productive in probing the importance and significance of soluble forms of membrane enzymes.

In addition to the neutral maltase we have shown that suckling rat intestine also contains a lysosomal acid maltase–glucoamylase accounting for 19% of the total intestinal maltase activity when assayed at pH 6.0. After cortisol injection or weaning activity is decreased to negligible values. Acid maltase has not been described in intestinal homogenates previously.

Schlegel-Haueter *et al.* (1972) reported that 'much' α-amylase activity in the suckling rat was eliminated when the homogenate was centrifuged at 105000*g* for 60 min and the supernatant discarded. The nature of the α-amylase activity was not further explored, but others have found that 78% (Dahlqvist & Thomson, 1963) to 90% (Alpers & Solin, 1970) of the total amylase activity of the intestine is soluble. The soluble activity closely resembles pancreatic amylase in heat-sensitivity, absence of maltase activity, neutral pH optimum (Alpers & Solin, 1970), inhibition by EDTA (Rutloff *et al.*, 1966), and is decreased in the intestinal mucosa after pancreatic duct ligation (Alpers & Solin, 1970). The acid maltase–glucoamylase activity cannot have arisen from chance association of maltase and α-amylase in column eluates, since it was more active at pH 3.0 when α-amylase is inactive (Alpers & Solin, 1970) than at pH 6.0 and its activity was not affected by EDTA. Maltase activity has not been fractionated frequently in foetal or developing intestine. However, neither Dahlqvist & Telenius (1969) nor Siddons (1970) found evidence of a maltase distinctly different from brush-border enzymes in human foetus or chick embryo. In both situations, however, the dominant maltase activity appeared to be associated with sucrase–isomaltase, suggesting that early development in these species differs fundamentally from that of the rat. Like acid maltases of liver (Lejeune *et al.*, 1963; Auricchio *et al.*, 1968; Jeffrey *et al.*, 1970*b*), and muscle (Palmer, 1971) the intestinal acid maltase is inhibited by turanose, while showing little affinity for the sugar as substrate. Inhibition was competitive, as reported by others (Lejeune *et al.*, 1963; Bruni *et al.*, 1969) but the K_i for turanose was slightly higher than values reported for rat liver (2.8 mM) (Jeffrey *et al.*, 1970*b*), ox liver (1.4 mM). (Bruni *et al.*, 1969) or rabbit muscle (2.0 mM) (Palmer, 1971). The rather low pH optimum is an interesting feature, since acid maltases in other tissues generally show maximum activity between pH 3.5 and 4.5 (Lejeune *et al.*, 1963; Bruni *et al.*, 1969; Auricchio *et al.*, 1968; Palmer, 1971). It is of interest, however, that lysosomal acid β-galactosidase in rat intestine has a pH optimum of 3.0, whereas in the human the pH optimum lies between 3.5 and 4.0 (Alpers, 1969). The marked retardation noted in Sephadex column chromatography has also been reported for human liver and kidney acid maltase (De Barsy *et al.*, 1972) and ox liver acid maltase (Bruni *et al.*, 1969). Molecular weights of 114000 (Jeffrey *et al.*, 1970*a*) and 107000 (Bruni *et al.*, 1969) have been reported for rat and ox liver respectively, in the same general range as the molecular weight of 136000 estimated for intestinal acid maltase. In other respects as well, such as its K_m for maltose, glucoamylase activity, sensitivity to polyols, insensitivity to *p*-chloromercuribenzoate, and slight isomaltase activity, reflected in the low affinity for palatinose, the enzyme appears to share the general characteristics of acid maltases in other tissues. Whether it has an important intracellular digestive function in the newborn is at present unclear.

This work was supported by a grant from the Medical Research Council of Canada and was completed while G. G. was on leave of absence from the Laboratory of Physiology, Faculty of Sciences, Reims, France. We express our gratitude to Mrs. A. Madapallimattam for her careful technical assistance.

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