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ISOLATION AND CHARACTERIZATION OF THE PROXIMAL AND DISTAL FORMS OF LACTASE-PHLORIZIN HYDROLASE FROM THE SMALL INTESTINE OF THE SUCKLING RAT

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Summary

The complex between lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23) and phlorizin hydrolase (glycosyl-*N*-acylsphingosine glycohydrolase, EC 3.2.1.62) has been purified from the proximal and distal regions of the small intestine of suckling rats. The two enzymes behaved differently on DEAE-cellulose ion-exchange chromatography and during electrophoresis in the presence and absence of sodium dodecyl sulphate (SDS), but they had very similar cyanoge bromide cleavage patterns. Kinetic studies on the proximal and distal enzymes showed the same pH optimum of 6.0 and the same heat stability at 45°C, but a small difference in K_m. Treatment of both enzymes with fucosidase, mannosidase or *N*-acetylhexosaminidase did not affect enzymic activity or electrophoretic mobility. Neuraminidase digestion abolished the electrophoretic differences and gave two active enzymes with similar isoelectric points.

Introduction

The major role of the intestinal disaccharidase enzyme lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23) in the nutrition of neonatal mammals is well established. In man, lactase activity develops progressively in utero, reaching postnatal values late in gestation, and usually remains high throughout life [1,2]. The occurrence of cases in which lactase activity is absent from the human intestine, as in congenital lactose malabsorption or adult hypolactasia, has stimulated much research and debate on the molecular events leading to the decline of lactase activity [3,4]. Studies with the rat have shown that lactase

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activity is well developed in the foetus and reached a maximum between 10 and 20 days after birth but declines rapidly thereafter to a low adult level [5,6].

Lactase is strongly associated with another enzyme, phlorizin hydrolase (glycosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.62), in the form of an enzyme complex with two independent, or partly independent, catalytic sites [7—9]. Whether these two enzymic activities develop simultaneously in the neonatal rat intestine is controversial [7,10,11]. Recently, it has been shown that the lactase enzymes from the proximal and distal small intestine are electrophoretically distinct and exhibit different postnatal development patterns [12]. To characterize further these two isoenzymes, we have purified lactase-phlorizin hydrolase complex by an established method [8] from the proximal and distal regions of the small intestine of suckling rats, and now report the results of an extensive electrophoretic, kinetic and structural analysis of the pure enzymes. Preliminary results have already been published [13].

Material and Methods

Purification of lactase-phlorizin hydrolase. The entire small intestine from the pylorus to the ileocaecal junction was removed from 13-day-old rats under ether anaesthesia. The intestines were cut into four segments of equal length. The first two quarters were combined to give the proximal sample, the third quarter was discarded and the last quarter was taken as the distal sample. After the pieces of intestine had been slit open and their contents washed off with 0.15 M NaCl, the lactase-phlorizin hydrolase complex was solubilized directly by papain digestion [14] and purified by the method of Schlegel-Haueter et al. [8]. For the distal lactase an additional chromatographic step was performed on Ultrogel AcA 34 (LKB, Bromma, Sweden) eluted with 50 mM phosphate buffer, pH 6.0.

Assays. Lactase activity was measured with lactose as substrate in 50 mM sodium maleate buffer, pH 5.8, in the presence of 0.2 mM *p*-chloromercuribenzoate [15]. Phlorizin (Fluka AG, Buchs, Switzerland) was purified before use by chromatography on Sephadex LH-20 with 38% (v/v) propan-2-ol in water [16]. Phlorizin hydrolase activity was measured in McIlvaine's buffer (citric acid/Na_2HPO_4) pH 5.8, with either phlorizin or *N*-palmitoyl dihydrogluco-cerebroside (Miles, Slough, U.K.) as substrate according to the method of Leese and Semenza [17]. Free glucose was determined with a glucose ultraviolet test kit (Boehringer, Mannheim, F.R.G.) by following either the change in fluorescence of NADPH at 470 nm for cerebroside or the change in absorbance of NADPH at 365 nm for phlorizin. Maltase activity was assayed by the method of Dahlqvist [18] with maltose as substrate. Protein was measured by the procedure of Lowry et al. [19] using crystalline bovine serum albumin as standard.

To determine the pH activity curve for lactase, purified enzyme was incubated at 37°C for 30 min with 0.1 M lactose in either 20 mM sodium acetate buffer (pH range 4.2—5.4) or 20 mM sodium maleate buffer (pH range 5.0—7.4), both containing 0.45 M NaCl. The reaction was stopped by dilution (1 : 5) with water at 4°C followed by boiling for 4 min. For the pH activity

curve of phlorizin hydrolase, purified enzyme was incubated in either 40 mM citric acid/KOH buffer (pH range 3.0—6.0) or 40 mM Tris/maleic acid buffer (pH range 5.0—8.0), both containing 25 mM sodium phosphate. The reaction was stopped by the addition of perchloric acid which was then neutralized with K_2CO_3 as described by Leese and Semenza [17].

Electrophoresis. Gel electrophoresis under strongly dissociating conditions was performed in the presence of 0.1% sodium dodecyl sulphate (SDS) on 7% acrylamide gels as described by Weber and Osborn [20]. For the estimation of molecular weights on gels the Pharmacia electrophoresis calibration kit was used. The system of Davis [21] as modified by Cogoli et al. [14] was adopted for electrophoresis under non-dissociating conditions on 5.25% acrylamide gels in 0.1 M sodium phosphate buffer, pH 7.2. A third system used was that of Laemmli [22] as modified by Hauri et al. [23] since is resolves the components of brush-border membranes with the retention of enzymic activity, and was the method originally used to identify the lactase isoenzymes [12]. After electrophoresis the gels were split longitudinally; one half was stained for protein with Coomassie blue and the other half was sliced into 1 mm sections. Each slice was eluted overnight with 200 µl 50 mM sodium maleate buffer, pH 5.8, and then the eluates were assayed for enzymic activity.

Isoelectric focusing was carried out with the LKB Multiphor apparatus on Ampholine polyacrylamide gel plates (pH range 3.5—9.5) for 90 min at 24 W. The focused pH gradient was determined in a 10 mm strip of gel sliced into 5-mm sections and eluted with 1 ml boiled 10 mM NaCl. Lactase activity was measured in a 4 mm strip of gel sliced into 2-mm sections and eluted with 150 µl 0.1 M sodium maleate buffer, pH 5.8.

CNBr cleavage. CNBr cleavage was performed on lyophilized enzyme with a 100-times molar excess of CNBr over methionine residues in 70% formic acid for 24 h at room temperature. The reaction mixture was then diluted with water and lyophilized. The peptides thus obtained were subjected to electrophoresis on 8% acrylamide gels in the presence of 8 M urea and 0.1% SDS [24].

Tryptic digestion. Purified lactase-phlorizin hydrolase complex was incubated at room temperature with trypsin (Worthington, 250 IU/mg) at a ratio of 7.5 : 1 (w/w) in 0.1 M sodium phosphate buffer, pH 7.0. At various time intervals aliquots were removed and a 10-fold excess, by weight over trypsin, of soybean trypsin inhibitor (Worthington) was added. Lactase and phlorizin hydrolase enzymic activities were measured in the digest. Polyacrylamide gel electrophoresis in the presence of SDS was used to analyse the protein components of the digest [20].

Glycosidase treatment. Neuraminidase (*Vibrio cholera*) was purchased from Calbiochemical; L-fucosidase (*Charonia lampus*), α-mannosidase (*Turbo cornutus*) and β-*N*-acetylhexosaminidase (*T. cornutus*) were obtained from Miles. Purified lactase-phlorizin hydrolase (56 µg) in a final volume of 200 µl 50 mM sodium acetate buffer, pH 5.6, containing 1 mM $CaCl_2$, was incubated with 5 units of neuraminidase for 24 h at 37°C. Fucosidase (0.04 units) and mannosidase (0.04 units) were used in 50 mM sodium acetate buffer at pH 5.0, *N*-acetylhexosaminidase (0.2 units) was used in 50 mM sodium citrate buffer at pH 5.4; these three incubations were for 45 h at 37°C. The reactions were stopped by dilution 1 : 2 with water at 4°C, and the remaining lactase and

phlorizin hydrolase activities were measured. Changes in electrophoretic mobility brought about by enzymic digestion were followed by gel electrophoresis under non-dissociating conditions and by isoelectric focusing as detailed above.

Results and Discussion

Purification

The method of Schlegel-Haueter et al. [8] was found to be effective for the purification of the proximal and distal forms of lactase-phlorizin hydrolase complex. By carrying out the initial papain solubilization on intact intestinal segments, as opposed to homogenates, the yield of enzyme was more than doubled without affecting the final specific activity of 22 μmol glucose released per min by 1 mg enzyme protein. Proximal and distal enzyme preparations behaved similarly throughout the purification procedure. However, a small difference in their elution profiles from DEAE-cellulose was observed and investigated further. The proximal lactase eluted at a concentration of 0.08 M potassium phosphate (Fig. 1a) whereas, the distal lactase eluted at 0.11 M (Fig. 1b). A mixture of pure proximal and distal lactase applied to the same column was resolved into two peaks, the first eluting at 0.085 M potassium phosphate corresponded to proximal lactase, and the second at 0.11 M to distal lactase (Fig. 1c). This result was a further indication of a fundamental difference in structure between proximal and distal lactase, and was the first direct evidence for this difference being retained after papain solubilization. Following chromatography on DEAE-cellulose the distal enzyme preparation was still contaminated with maltase activity. These two enzymic activities were well resolved by a final chromatographic step on Ultrogel AcA 34.

Kinetic properties

At constant ionic strength, both the proximal and distal forms of lactase had indistinguishable activity profiles against changing pH, with an optimum at pH 6.0. Similarly, the proximal and distal phlorizin hydrolase enzymes had identical pH optima of 6.0. These results are in agreement with earlier reports in the literature [5,8,17]. In heat-inactivation experiments both proximal and distal phlorizin hydrolase were stable for 60 min at 45°C whereas proximal and distal lactase were rapidly inactivated with a half-life of about 20 min. The relative heat stability of phlorizin hydrolase in contrast to lactase is well known and has been interpreted as evidence for there being two independent catalytic sites [7–9].

K_m values were obtained by the revised version of the direct linear plot method [25] and found to be 14.8 ± 2.6 mM (S.D., $n = 4$) for proximal lactase and 19.1 ± 5.7 mM for the distal lactase. The difference was statistically significant ($P < 0.01$) but is probably not important physiologically. For the proximal and distal phlorizin hydrolase enzymes K_m values of 0.73 ± 0.11 mM (S.D., $n = 4$) and 1.12 ± 0.32 mM, respectively, were obtained using *N*-palmitoyl dihydrocerebroside as substrate at pH 6.0. The difference between these K_m values was not statistically significant in Student's *t*-test. A value of 0.62 mM

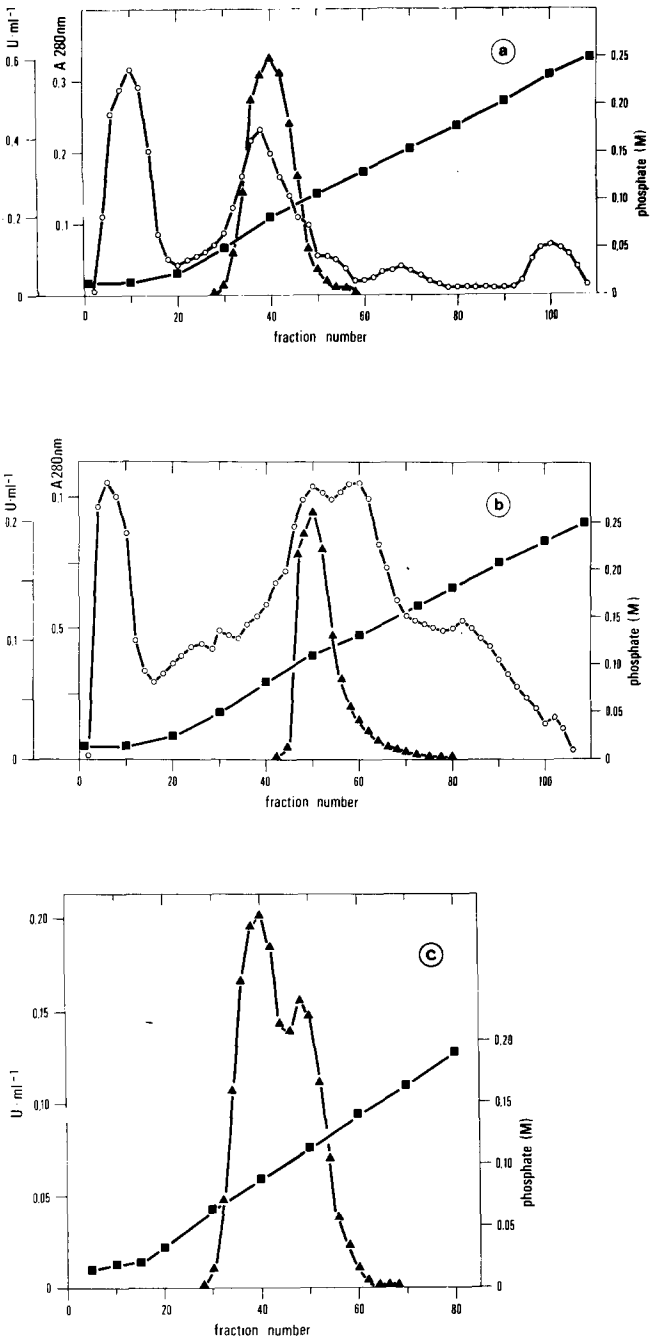

Fig. 1. Ion-exchange chromatography of lactase-phlorizin hydrolase complex on DEAE-cellulose. The column (1 X 21 cm) was equilibrated with 0.02 M potassium phosphate buffer, pH 6.0, and eluted with a 220 ml linear gradient of potassium phosphate 0.02 M—0.25 M, pH 6.0. Fractions of 2 ml were collected. a, proximal lactase; b, distal lactase. In c, a mixture of proximal and distal lactase was applied to the column and eluted with a 120 ml linear gradient of potassium phosphate 0.02 M-0.2 M, pH 6.0. Fractions of 1.5 ml were collected. The absorbance of the eluted fractions was measured at 280 nm (○———○), lactase was assayed as described in Material and Methods (▲———▲) and the phosphate gradient was monitored by conductivity measurements (■———■).

has been reported for phlorizin hydrolase isolated from the entire small intestine [17].

Electrophoretic properties

The electrophoretic pattern of the purified lactase-phlorizin hydrolase isoenzymes on polyacrylamide gels run under non-dissociating conditions is illustrated in Fig. 2. The proximal enzyme gave a single stained protein band which comigrated with lactase activity in eluates of a sliced gel. However, for the distal enzyme two distinct, adjacent bands of similar intensity, both possessing lactase activity, were observed. Incubation of the distal enzyme overnight at 37°C resulted in the disappearance of the slower migrating band with the concomitant intensification of the faster band. This observation suggested dissociation or denaturation but the addition of mercaptoethanol had no effect. In similar experiments with a purified lactase preparation that was homogeneous in the ultracentrifuge, Schlegel-Haueter et al. found two bands by gel electrophoresis [8]. It could not be established whether these bands resulted from denaturation, aggregation or impurity. Birkenmeier and Alpers [7], using a lactase purified from the entire small intestine of the young rat, observed on non-dissociating gels three electrophoretically distinct bands, each of which had lactase activity.

Illustrated in Fig. 3 are the results obtained when the purified lactase-phlorizin hydrolase isoenzymes were subjected to electrophoresis in the presence of SDS. If the enzymes were boiled in SDS prior to electrophoresis, both the proximal and distal forms were resolved into two bands having apparent molecular weights of approx. 200 000 and 160 000. In the distal preparation the bands were of equal intensity (Fig. 3d), whereas for the proximal enzyme the slower moving band was more intense (Fig. 3b). No enzymic activity was detectable in eluates of gels loaded with enzyme that had been boiled in SDS. With the omission of boiling but still in the presence of SDS an additional higher molecular weight band could be detected either by staining with Coomassie blue or in eluates of sliced gels as enzymic activity. For the distal enzyme the additional band exhibited lactase activity and was clearly visible after staining (Fig. 3c). On gels of the proximal enzyme a higher molecular

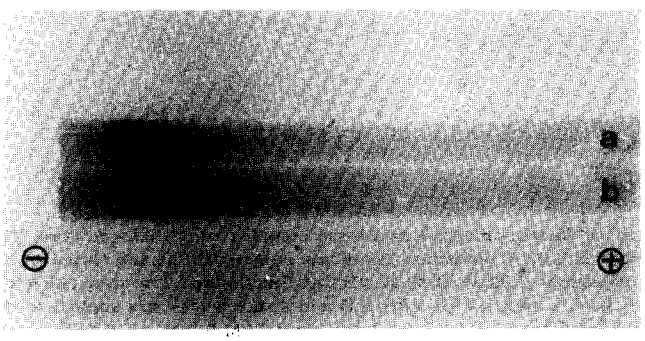

Fig. 2. Polyacrylamide gel electrophoresis of purified lactase-phlorizin hydrolase complex under non-dissociating conditions on the modified Davis system. a, proximal; b, distal. Further details given in Material and Methods.

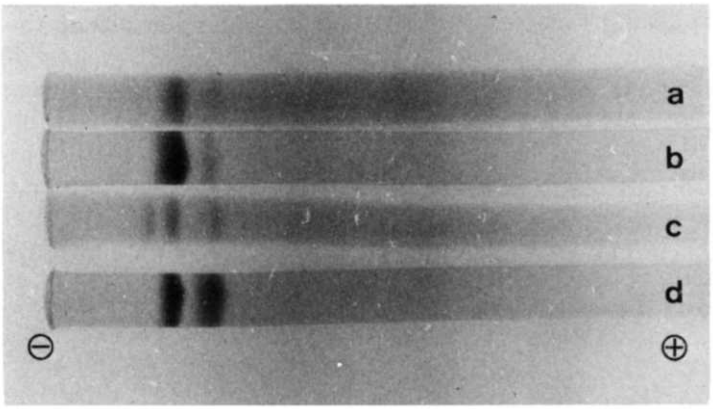

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified lactase-phlorizin hydrolase complex on the Weber and Osborn [20] system. Samples were suspended in 0.1% SDS and 0.1% mercaptoethanol in 10 mM sodium phosphate buffer, pH 7.0 and either applied directly to the gel a and c, or boiled for 2 min and then applied to the gel b and d. Proximal enzyme a and b; distal enzyme c and d. Further details in Material and Methods.

weight region of lactase activity was again detectable but it stained only faintly in comparison with the two major bands and is not visible in Fig. 3a.

On the third electrophoresis system used, that of Laemmli [22] as modified by Hauri et al. [23], it had previously been shown that the proximal and distal enzymes in isolated brush-border membranes ran as single enzymically-active bands, the distal lactase having a slightly higher electrophoretic mobility than the proximal [12].

However, with the purified soluble lactase-phlorizin hydrolase complex two faster bands were observed for both the proximal and distal enzymes (Fig. 4) but no comigrating enzymic activity was detectable in either of these regions. As lactase activity was retained on gels of the Weber and Osborn type (0.1%

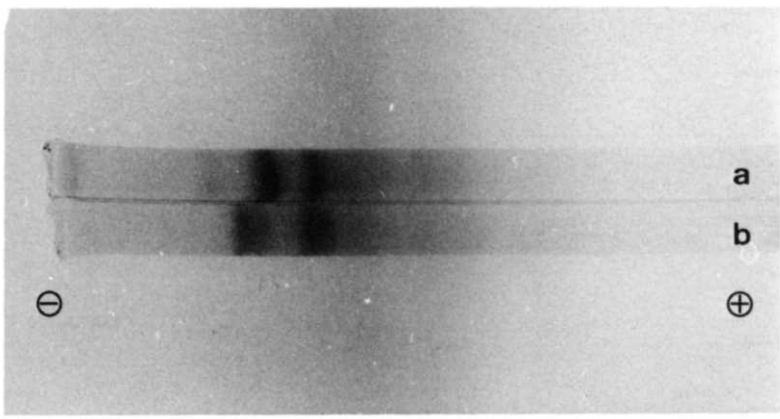

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified lactase-phlorizin hydrolase complex on the modified Laemmli system. Proximal enzyme (a) and distal enzyme (b) were dissolved at room temperature in 0.0625 M Tris-HCl buffer, pH 6.8, containing 2% SDS. Further details in Material and Methods.

SDS) but lost in the Laemmli system (2% SDS) it would appear that the increased SDS concentration inactivated the enzyme. Similarly, the loss of activity from purified enzyme but not from brush-border membranes during electrophoresis in the Laemmli system may be attributed to the higher amount of protein and lipid, thus lowering the SDS/protein ratio in the latter. In an earlier study with lactase purified from the entire small intestine of young rats, five stained protein bands were observed on SDS gels [7]. Another intestinal glycosidase, maltase-glucoamylase, has been dissociated into a complex mixture of partially active fragments in 1% SDS solutions by heating or lowering the pH [26].

Isoelectric focusing

Isoelectric focusing of the proximal enzyme gave a complex pattern of bands with two predominant stained regions, each comprising a doublet of bands, one at pH 4.2—4.3 and the other at pH 4.7—4.8 (Fig. 5). Lactase activity was detected in the region from pH 4.2 to 4.8 with maximum activity at pH 4.4. The pattern of the distal enzyme was less complex. A group of bands with a major stained doublet was again present at pH 4.2—4.3 which possessed lactase activity. A weaker band devoid of enzymic activity was visible at pH 3.5 but at acidic pH both lactase and phlorizin hydrolase are known to be rapidly inactivated [8,27]. The isoelectric point of rat-intestinal lactase has been reported previously to be in the range 4.3—4.5 [8].

CNBr cleavage

To investigate possible differences in the primary amino acid sequence of the proximal and distal forms of the lactase-phlorizin hydrolase complex, CNBr cleavage was employed, followed by electrophoretic analysis of the fragments. The cleavage patterns for both enzymes were very similar, suggesting that the two enzymes have a similar distribution of methionine residues and thus may

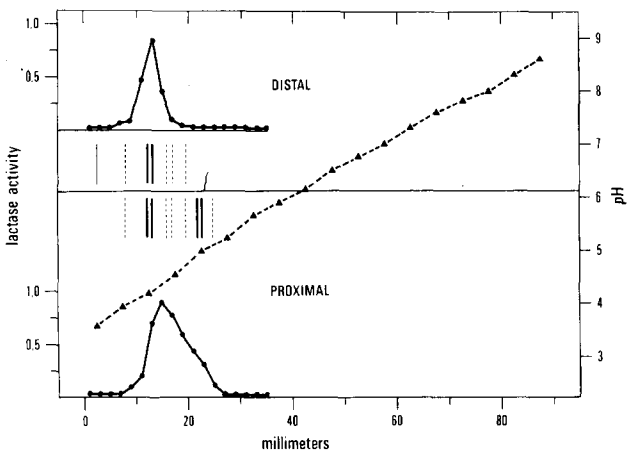

Fig. 5. Isoelectric focusing on a single Ampholine polyacrylamide gel plate of proximal and distal lactase. After 90 min at a constant power of 24 W the pH gradient (▲———▲) and lactase activity (●———●, in arbitrary units) were measured in eluates of gel slices. The centre insert shows the pattern of protein bands after staining with Coomassie blue.

share a common amino acid sequence. The high molecular weight bands from the distal lactase were more intense than those from the proximal enzyme. This was probably due to a difference in cleavage rate with CNBr; variation in carbohydrate content could account for this.

Tryptic digestion

Treatment of the proximal and distal enzymes for 3 h at room temperature with trypsin affected neither lactase-phlorizin hydrolase activity nor electrophoretic mobility of the enzymes on SDS-polyacrylamide gels. For lactase to be effective in vivo it must be resistant to trypsin, a property apparently retained by the purified enzyme after papain solubilisation.

Glycosidase treatment

Lactase has been reported to contain 17% carbohydrate [7]. As ion-exchange chromatography on DEAE-cellulose (Fig. 1) and gel electrophoresis (Fig. 2) demonstrated a charge difference between the proximal and distal enzymes, the possibility of this being due to variations in carbohydrate structure was investigated. Incubation with neuraminidase, fucosidase, mannosidase or *N*-acetylhexosaminidase for 24 h or 45 h at 37°C had no effect, in comparison to controls, on the enzymic activity of lactase or phlorizin hydrolase.

Due to the low amount of purified lactase-phlorizin hydrolase available it was not possible to measure the carbohydrate released during incubation, so the effects of digestion were followed by observing changes in electrophoretic mobility on gels under non-dissociating conditions. After treatment with fucosidase, mannosidase or *N*-acetylhexosaminidase no change in mobility was detected for either the proximal or distal lactase in comparison to their respective controls.

Neuraminidase treatment, however, reduced the different electrophoretic mobilities of the proximal and distal lactase enzymes to a single comigrating band (Fig. 6.). Control experiments showed that it was neither the presence of $CaCl_2$ nor the acetate buffer at pH 5.6, but rather the action of neuraminidase that was responsible for this alteration in mobility of the lactase enzymes. Isoelectric focusing revealed that neuraminidase digestion abolished most of the heterogeneity from the proximal and distal lactases and increased their isoelectric points to give a major comigrating doublet of bands for both enzymes. The position of a minor band in the proximal enzyme preparation remained unchanged after neuraminidase treatment.

Taken as a whole these results obtained with the purified enzymes substantiate the preliminary evidence for there being a proximal and distal form of lactase-phlorizin hydrolase in the intestinal brush-border membrane of the suckling rat [12]. The close similarities in kinetic properties and CNBr cleavage patterns are indicative of a fundamentally common amino acid sequence, whereas a major difference in charge is suggested by the results of ion-exchange chromatography, gel electrophoresis and isoelectric focusing. The abolition of these differences by neuraminidase treatment implies the involvement of sialic acid residues. The possibility cannot be excluded that the effect we observed with neuraminidase digestion was due to some contaminant protease or glycosidase activity, but the fact that no similar effect was observed with the three

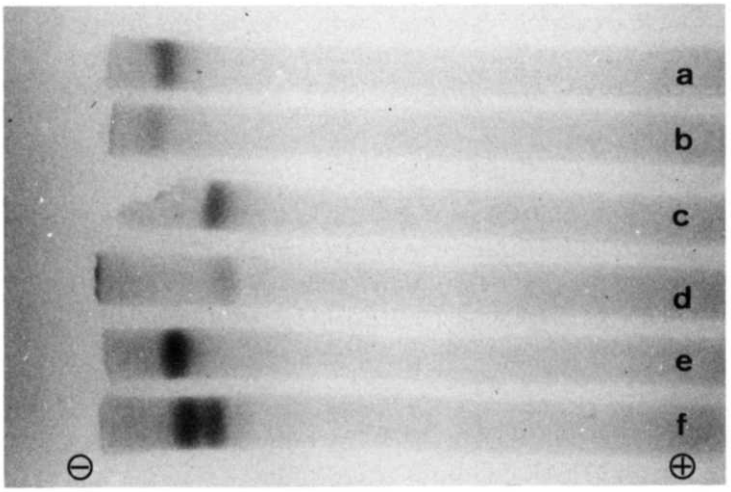

Fig. 6. Effect of neuraminidase treatment on the electrophoretic mobility of purified lactase-phlorizin hydrolase complex. a, neuraminidase-treated proximal enzyme; b, neuraminidase-treated distal enzyme; c, proximal and d, distal enzyme incubated for 24 h at 37°C with 1 mM $CaCl_2$ in 50 mM sodium acetate buffer, pH 5.6; e, proximal and f, distal enzyme incubated for 24 h at 4°C with 1 mM $CaCl_2$ in 50 mM sodium acetate buffer, pH 5.6.

other glycosidase enzymes used weighs against this. Although intestinal lactase has a high carbohydrate content Birkenmeier and Alpers [7] were unable to detect, by gas-liquid chromatography, the presence of sialic acid residues in purified lactase. However, the incorporation of radioactivity from CMP-[^{14}C]-sialic acid into the surface membrane of rat intestinal villus cells has been demonstrated by Weiser [28]. Furthermore, the activity of several glycosyltransferase enzymes varies along the length of the gut [29] and this may directly affect the amount of sialic acid incorporated into membrane components, or modulate the number of available receptor sugars to which sialic acid can be attached, and thus generate isoenzymes.

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