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TOPOLOGICAL STUDIES ON THE HYDROLASES BOUND TO THE INTESTINAL BRUSH BORDER MEMBRANE*

I. SOLUBILIZATION BY PAPAIN AND TRITON X-100

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SUMMARY

Papain digestion of closed, right side out vesicles from pig, rat and rabbit jejunum brush border induces the release of the hydrolases bound to the membrane without grossly affecting the lipid bilayer limiting the vesicles. This observation definitely proves that intestinal hydrolases are surface components attached to the external side of the membrane. All proteins released by papain could be identified by electrophoresis and immunoelectrophoresis to already known intestinal hydrolases, with the exception of an unidentified substance strongly stained by the Schiff's reagent.

The early observation that the aminopeptidase form released from pig brush border by Triton X-100 is different from that released by papain was extended to other hydrolases from pig, rat and rabbit. In some cases, the Triton-released form could be converted by further proteolytic digestion into a new form similar to that liberated by papain. These facts may be related to the existence of hydrophobic anchors retaining the intestinal hydrolases to the membrane surface.

INTRODUCTION

The brush border membrane of mature enterocytes is known to contain a variety of hydrolases or transferases (disaccharidases, aminopeptidase, alkaline phosphatase, γ-glutamyl transferase) involved in the last steps of intraluminar digestion [1] and perhaps also in the transport of digested products across the membrane [2]. The determination of the position occupied by the above enzymes with respect to each other and to the lipid bilayer is of fundamental importance for a better understanding of their function. An apparently promising experimental approach to this problem is to measure the rate at which the enzymes are solubilized under the influence of proteases or detergents. The potentialities of the method are

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well illustrated by a recent report on the proteolytic digestion of right side out and inside out vesicles from erythrocyte membranes [3].

Intestinal hydrolases have already been shown some years ago to be released by papain from the so called "brush borders" [4, 5] prepared according to Crane[6] or from isolated brush border membranes [7]. The first part of the present work deals with the papain digestion of closed brush border vesicles obtained from pig, rat and rabbit jejunal mucosa by a technique recently worked out in our group [8]. It will be shown that the hydrolases are also released by papain from the vesicles, although at markedly different rates.

Moreover, the known solubilizing properties of the non ionic detergent Triton X-100 towards a variety of membrane proteins have been utilized last year in the course of the purification of pig intestinal aminopeptidase for the initial release of the enzyme from the brush border [9]. It was of considerable interest to find on this occasion that the enzyme form solubilized by Triton had not the same behavior as that released by papain.

These early observations were confirmed by further assays reported in the second part of the present work and they were extended to other brush border hydrolases from pig, rat and rabbit intestine. They were finally discussed in relation with the existence of "anchors" which are assumed to bind externally located proteins to the membrane surface.

MATERIALS AND METHODS

Materials

Enzymes and special reagents. Glucose oxidase and papain were purchased from Merck and NBC, respectively, agarose and Triton X-100 from Touzart et Matignon. Dithiothreitol was a Calbiochem product. Fast TR Salt, diazoblue B, phenazine methosulfate and 3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyl tetrazolium bromide were purchased from Sigma. Baryum chloranilate was obtained from Fluka.

Substrates. The aminopeptidase substrates l-alanine-β-naphthylamide and all the l-aminoacid *p*-nitroanilides were Cyclo Chemical products. Sucrose, maltose and trehalose were purchased from Merck. All other reagents were of the best available grade.

Methods

Preparation of brush border membrane vesicles. The method worked out in this laboratory for pig duodenal and jejunal mucosa [8] could be applied without modification to rabbit and rat jejunum. The closed vesicles obtained from the three species were used immediately. Storage in liquid N_2, as practised earlier, was not observed to affect enzyme activities, but to accelerate markedly solubilization by papain.

Analytical techniques. Total proteins in the membrane were determined as previously reported after dissolution in 1 % sodium dodecylsulphate [8]. Neutral and amino sugars were evaluated [8] by a modification of the phenol–H_2SO_4 and Elson–Morgan techniques, respectively. Methods already described in a previous paper [8] were employed for phospholipids and total cholesterol. After separation by silicic acid chromatography, the glycolipids were measured by the technique of Forstner. Sulfuric ester bonds were hydrolyzed according to Muir [11] and the resulting sulfate

was determined by the baryum chloranilate method with anhydrous potassium sulfate as the standard [12].

Enzymatic activities. Aminopeptidase and alkaline phosphatase activities were measured by spectrophotometry with the aid of the substrates, l-alanine *p*-nitroanilide and *p*-nitrophenyl phosphate, respectively [8]. γ-Glutamyl transpeptidase was also determined spectrophotometrically, using γ-glutamyl *p*-nitroanilide as the substrate, Mg^{2+} as the activator and the dipeptide glycylglycine as the receptor for glutamyl radicals [13]. The glucose generated by disaccharidases from their respective substrates was determined by the Tris–glucose oxidase reagent [14].

Digestion of vesicles by papain. A 39 mg/ml commercial papain suspension in a 50-mM potassium phosphate buffer at pH 6.2 was incubated for 15 min. at 0 °C in the presence of 5 mM cysteine and 0.03 mM dithiothreitol. When phospholipids had to be determined in the same preparations, the phosphate was replaced by 0.1 M citrate at the same pH.

Vesicle suspensions (5 mg of proteins per ml) in the phosphate buffer (pH 6.2) were incubated at 37 °C with 0.5 mg/ml activated papain. Aliquots (100 μl) were removed, diluted 10-fold at 4 °C with a 10-mM phosphate buffer, pH 7.2, 150 mM in NaCl (phosphate-buffered saline) and spun for 15 min at 18000 × *g* in the angular rotor of a Haereus-Chris centrifuge. The solubilized enzyme activities in the supernatant were expressed as per cent of the initial values. No inactivation of enzymes by papain was observed in the course of these assays.

For preparative purposes, larger quantities of vesicles were employed but the general conditions of the papain digestion were the same as for the above reported kinetic assays. The digestion mixtures were chilled to 4 °C and spun for 45 min at 105 000 × *g* in the 50 Ti rotor of an L2 65B Spinco centrifuge. The pellet was resuspended in the same volume of the phosphate-buffered saline and again centrifuged. The 2 supernatants were pooled and the final pellet was suspended in phosphate-buffered saline (1/5 of the initial volume).

Treatment of the vesicles with Triton X-100. The vesicles (5–10 mg of proteins per ml) were incubated overnight with 1 % Triton X-100 at 4 °C in the presence of 15 % (w/w) sucrose. The mixture was centrifuged at 18 000 × *g* for 15 min.

Preparation of the antipapain supernatant sera. The papain supernatants prepared as indicated above were dialyzed for 2 days against water, lyophilized and kept frozen at −20 °C until use. Samples of the resulting powder (2 mg) were dissolved in 0.5 ml of physiological saline, the solutions were emulsified in the same volume of complete Freund's adjuvant and the emulsions thus obtained were injected subcutaneously (4–6 shots) into the back of a rabbit. After 6 weeks, 2 mg of the same material were injected intramuscularly. The next day and a day later, 4 mg were injected into an ear vein of the animal. The rabbit was bled one week after the second intraveneous injection. The serum was filtered through 0.45-μm Millipore plates and kept at 4 °C in the presence of NaN_3 (0.01 %).

Gel electrophoresis and subsequent stainings. An imidazole rather than a Tris buffer was used for the preparation of the 7.5 % polyacrylamide gels [15]. The advantage of the imidazole buffer was to give better resolutions, especially for the enzyme forms liberated by Triton X-100 and to exert no adverse effects on disaccharidase activities. Gels were charged with 50–150 μl of the papain supernatants or Triton X–100 extracts (100–300 μg of proteins in both cases). After electrophoresis, the

protein bands were stained by analine blue black. The gels were destained by transversal electrophoresis as described by Canalco [15]. Glycoproteins were specifically stained by the Schiff's reagent [16]. Individual enzyme activities were revealed in the gel with the aid of specific chromogenic substrates and of techniques similar to those employed by Uriel [17]. Aminopeptidase: The gels were plunged in the dark for 30 min into a freshly prepared and filtered mixture of the substrate l-leucyl-β-naphthylamide (200 mg in 2 ml of dimethylformamide and 1 ml of water) and diazoblue B (10 mg in 7 ml of 50 mM diethylmalonyl urea at pH 8.0) The slabs were rinsed with water and fixed in 7 % acetic acid (orange-red band). γ-Glutamyl transpeptidase: The same general procedure was used for this enzyme, but with the specific substrate γ-l-glutamyl-β-naphthylamide (200 mg) dissolved in 2 ml of dimethylformamide and 1 ml of water. As already mentioned earlier, an activator (20 mM $MgCl_2$) and a receptor (4 mM glycylglycine) were added to the assay mixtures (orange-red band). Alkaline phosphatase: An aqueous solution of Naphtol AS-MX Phosphoric Acid disodium salt (2 mg in 5 ml) was mixed just before use with a solution of fast red TR salt (30 mg) in 0.2 M Tris at pH 8.0. The gels were immersed in the dark for 30–60 min into the above mixture, rinsed and fixed with 7 % acetic acid (red band). Disaccharidases: To a solution of 3-(4, 5-dimethyl-thiazolyl-2)-2, 5-diphenyltetrazolium bromide (12 mg) in a 10-mM potassium phosphate buffer at pH 6.0 (2.5 ml) were added just before use, in the dark and at 4 °C, 3 mg of phenazine methosulfate and 2 mg of glucose oxidase. This reagent was mixed with 0.5 ml of a 0.15-M solution of the suitable substrate in the phosphate-buffered saline and 2.5 ml of a 1.6 % agarose solution in a 10 mM phosphate buffer (pH 6.0) at 45 °C. The gel was immersed into this mixture and the agarose was quickly solidified by cooling down the tube in crushed ice. After 30–60 min in the dark, the disaccharidases materialized as black bands. All R_F values were measured by reference to the migration of bromophenol blue under identical conditions.

Immunoelectrophoresis. They were performed in 7 % polyacrylamide gels which were observed to give better resolutions than agarose. The gel slabs were subsequently inserted into agarose plates [18]. After a 48-h diffusion against an antipapain supernatant serum, the plates were rinsed at room temperature with physiological saline for 48 h and with water for 1 h. They were dried overnight at 37 °C and the proteins in the precipitin lines were stained with amido black. Individual enzyme activities were revealed with the chromogenic substrates mentioned above. The plates were not dried in this case and the incubations with the suitable substrates were performed immediately after rinsing.

Preparative and analytical centrifugations. Preparative high speed centrifugations were performed in the 50 Ti angular rotor of a Spinco-Beckman centrifuge Model L2-65B. The reported g values were for the upper part of the tubes. Sedimentation coefficients were measured at 20 °C in an analytical ultracentrifuge Spinco–Beckman Model E equipped with Schlieren optics. For these assays, the brush border vesicles were suspended (5 mg of proteins per ml) in phosphate-buffered saline.

Centrifugations in sucrose or Ficoll gradients. Linear 30–50 % (g per 100 g of solution) sucrose gradients were prepared in an Isco Former gradient Model 570. The concentrations of the 2 sucrose solutions were checked to ± 0.5 % with the aid of an Abbe refractometer. Native or papain-digested vesicle suspensions (5 mg of

protein per ml) were charged at the top of the gradient and the tubes were centrifuged in a SW 36 rotor at 35 000 rev./min for 16 h at 4 C Amino peptidase, alkaline phosphatase and total proteins were determined in 0.6-ml samples collected in an Isco density gradient Fractionator Model 540. The linearity of the gradient was also checked by refractometry after centrifugation in the eluted fractions.

The concentrations of Ficoll solutions (weight of Ficoll per 100 g of solution) were derived from density values measured with a digital microdensimeter (Parr, Austria). All Ficoll solutions were buffered with 1 mM Tris, pH 8.0, and contained 1 mM $MgCl_2$. The vesicles (5 mg of proteins per ml) were charged at the top of linear 1–45 % gradients prepared as indicated above for sucrose. Fractions (0.6 ml each) were collected after a 20-h centrifugation in a SW 36 rotor at 35000 rev. min and 4 C. The density of the vesicles before and after papain digestion was identified to that of the fractions containing the highest enzyme concentration.

Electron microscopy. The vesicles were negatively stained by 1 % uranyl acetate and immediately observed in a Siemens Elmiscop microscope 1-80 kV equipped with a double condensor and an objective aperture of 50 μm.

RESULTS

Papain digestion

Time course of enzyme solubilization. Preliminary assays performed 2 years ago in this laboratory had already indicated the optimal conditions for enzyme solubilization from pig jejunal vesicles [19]. These assays have now been extended to rat and rabbit with the results reported in Fig. 1. For the 3 investigated species,

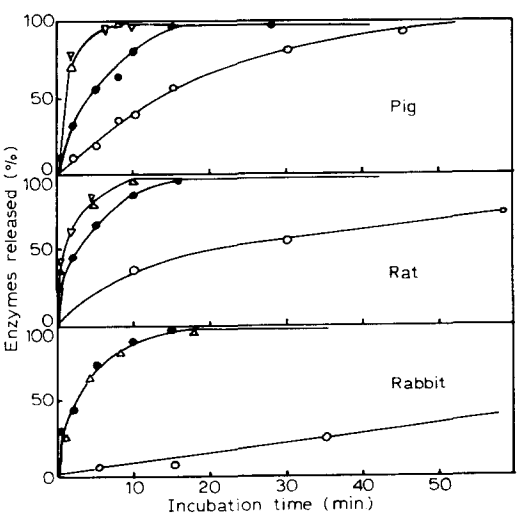

Fig 1. Time course of the papain-induced solubilization of maltase (△ – △), sucrase (▽ – ▽), aminopeptidase (● – ●) and alkaline phosphatase (○ – ○) from the jejunal brush border membrane of pig, rat and rabbit. All enzymes were stable towards papain under the conditions of the assays. Assays were performed three times in the case of pig, once in the case of rat and twice in the case of rabbit with identical results. Each assay is made with different membrane preparations from 2 pigs, 10 rats and 2 rabbits.

disaccharidases were found to be solubilized very fast followed by aminopeptidase and alkaline phosphatase. The rate of alkaline phosphatase solubilization was always observed to be the lowest, especially with rabbit vesicles. The fact that most hydrolases are completely solubilized at the end of papain treatment is consistent with the view that all or almost all vesicles are closed in the same manner, which should be right side out since the knobs have been observed to be external in both intact brush border membrane and vesicles [4, 8, 20]. This apparently important finding confirms earlier histological observations showing that intestinal hydrolases are bound to the outer side of the membrane. Right side out vesicles are likely to be stabilized in the present case by the filamentous material originating from the microvilli and seen by electron microscopy to partly fill the vesicles [8].

Nature and proportions of the liberated products. The nature and proportions of the enzymes and several other compounds released by papain from pig jejunal vesicles are given in Tables I and II. In each assay, the vesicles were incubated with papain for 10 min at 37 °C and the resulting mixture was centrifuged to give a supernatant and a pellet which were analyzed separately.

TABLE I

ENZYMES LIBERATED BY PAPAIN FROM PIG JEJUNAL VESICLES

See text for the conditions used for the papain digestion of vesicles. Three independent assays were performed and averaged in each case.

Fraction	Percent recovered					
	Disaccharidases			γ-Glutamyl transferase	Amino peptidase	Alkaline phosphatase
	Maltase	Sucrase	Trehalase			
Supernatant	90	87	45	88	67	33
Pellet	10	13	55	12	33	67

TABLE II

SOLUBILIZATION BY PAPAIN OF SURFACE COMPONENTS OF PIG BRUSH BORDER MEMBRANE

Assays were run in duplicate.

Fraction	Percent recovered						
	Proteins	Sugars		H_2SO_4 esters	Glyco-lipids	Phospho-lipids	Choles-terol
		Neutral	Amino				
Supernatant	50	42	61	32	44	0	0
Pellet	50	58	39	68	56	100	100

The results listed in Table I again show that maltase, sucrase and aminopeptidase are liberated faster than alkaline phosphatase. They also indicate, however, that trehalase is released much more slowly than the other disaccharidases and that, on the contrary, γ-glutamyl transferase appears early in the supernatants.

Moreover, papain is seen in Table II to solubilize approximately half of the proteins, neutral sugars, amino sugars and glycolipids of the vesicles, and about one third of the H_2SO_4 esters. By contrast, no traces of phospholipids and cholesterol can be detected in the supernatants. Papain digestion, therefore, does not appear to affect the lipid bilayer of the membrane but rather to remove a variety of surface components.

Centrifugation of negative and papain-digested vesicles. Intact vesicles from pig jejunal mucosa were observed by analytical centrifugation to give a single, slightly asymmetrical peak. The migration of the top of the peak was consistent with a sedimentation coefficient of 450 S. The shape of the peak was not essentially modified after papain digestion of the vesicles. But, the sedimention coefficient measured under the same conditions was found in this case not to exceed 350 S.

Moreover, brush border vesicles were centrifuged through sucrose and Ficoll concentration gradients. Small sucrose molecules could be assumed to equilibrate relatively fast outside and inside the vesicles so that sedimentation was a function of the density of the membrane [21, 22]. Observed values were 1.20 and 1.17 before and after papain digestion. The slight decrease of the sedimentation coefficient of the vesicles and of the density of the membrane is consistent with the above reported selective removal of heavier, non-lipid components from this membrane. In the same connection, the fact that the heterogeneity of the vesicle population is not appreciably increased by papain digestion confirms that papain attack proceeds at about the same rate on all vesicles.

Moreover, it was of interest to find that centrifugation of native or papain-digested vesicles through Ficoll gradients led to the formation of a single, very thin band with a density of 1.10. This value, which is lower than those obtained with sucrose gradients (see above), probably corresponds to the density of the vesicles [21, 22]. It appears, therefore, that the large Ficoll molecules do not penetrate inside the vesicles and, consequently, that the lipid bilayer is not grossly affected by papain treatment.

Electron microscopy. A confirmation of the chemical observation reported above was obtained by electron-microscopy observations on negatively stained vesicles before and after papain digestion. Fig. 2 shows that digested vesicles have lost their granular appearance, indicating that the "knobs" have been removed. But, the size and shape of the vesicles remain the same.

Electrophoresis and Immunoelectrophoresis of the solubilized material.

In order to obtain a more general view of all solubilized proteins, papain supernatants prepared from pig, rat and rabbit jejunal vesicles were submitted to electrophoresis in anionic and cationic gels under non-dissociating conditions. Bands were stained as usual by aniline blue black and the chromogenic substrates listed in the preceding section were employed for ascertaining the position of the bands corresponding to known enzymatic activities. Separation was only observed with anionic gels, indicating that the bulk of the solubilized material was anionic. R_F values are reported in Table III under the heading "Papain".

The aniline blue-black staining was found to reveal in the 3 investigated species 2 major protein bands related, respectively, to aminopeptidase and to one of the maltases (designated maltase M_2 in Fig. 3). The more sensitive staining by

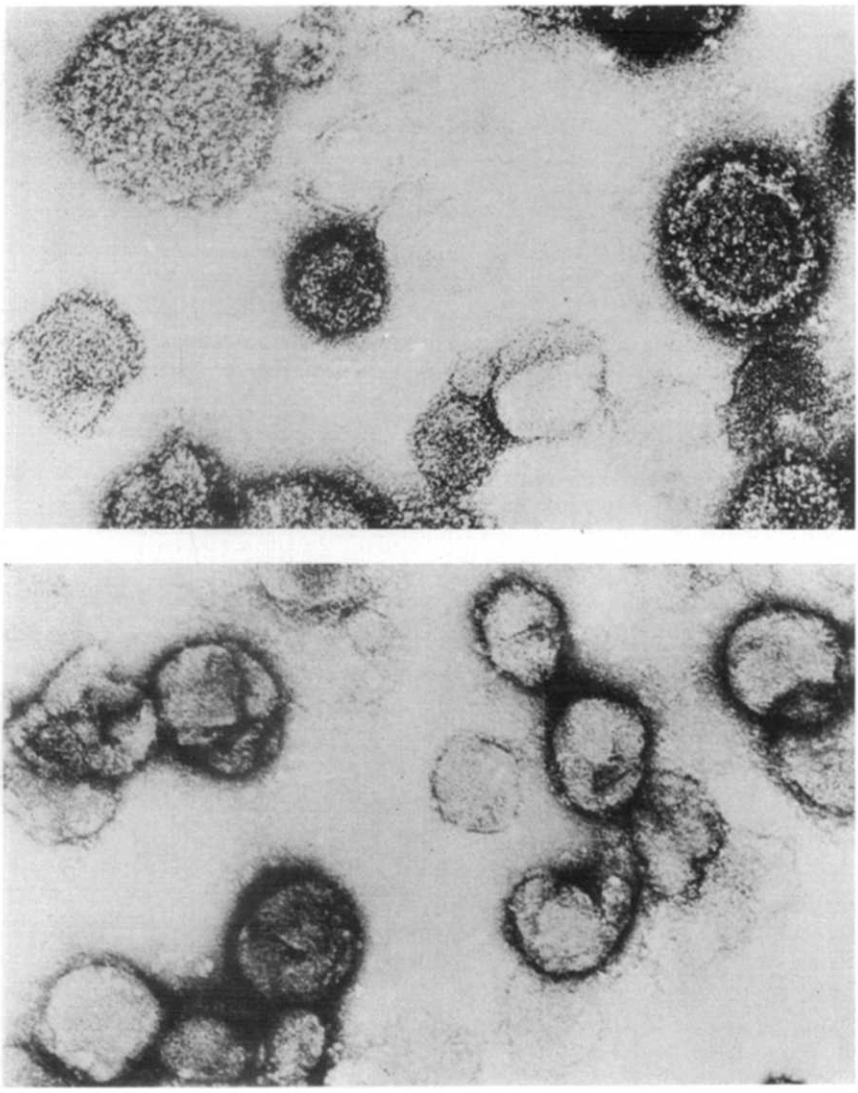

Fig. 2. Electron micrograph of negatively stained vesicles before (upper picture) and after (lower picture) papain digestion. Magnification × 180 000.

chromogenic substrates yielded some additional bands corresponding to alkaline phosphatase, sucrase and other maltase forms. It was of interest that the disaccharidase pattern varied from one species to the others. For rabbit, the band designated sucrase was given by the well documented sucrase–isomaltase complex with maltase activity [23]. The same complex did not appear to exist in pig jejunal mucosa, unless its maltase activity is very low. Moreover, a single maltase devoid of sucrase activity was identified in rat whereas 2 were present in pig and rabbit intestine.

In assays performed with pig jejunal vesicles, 2 strong glycoprotein bands again related to aminopeptidase and maltase M_2 were revealed by Schiff's reagent. A 3rd

TABLE III

R_f VALUES ON 7 % POLYACRYLAMIDE GELS AT pH 7.4 OF 4 INTESTINAL HYDROLASE ACTIVITIES SOLUBILIZED BY PAPAIN OR TRITON X-100 FROM JEJUNAL VESICLES

See text for the symbols M_2 and M_3. The R_f values of the fainter bands are indicated in parenthesis. Same number of assays as in Fig. 1.

Material	Pig		Rat		Rabbit	
	Papain	Triton	Papain	Triton	Papain	Triton
Aminopeptidase	0.300	0.100	0.270	0.195	0.630	0.284
Sucrase	0.350	0.040	0.250	0.023	0.440	0.233
Maltases	0.410 (M_2)	0.130	0.090	(0.023)	0.065	0.070
	(0.730) (M_3)	(0.410)	(0.270)	0.150	(0.250) 0.440	0.235
Phosphatase	0.470	0.210	0.380	0.190 0.230	0.607	
Proteins	0.300	0.000	0.085	0.000	0.070	0.000
	0.410	0.100	(0.200)	0.170	(0.160)	0.070
	(0.730)	0.140	0.260	0.310	(0.180)	0.230
					0.430	0.280
					0.630	

positive band designated X in the diagram was also observed. The corresponding material was not likely to be a glycolipid, since it was not stained by Sudan III and also not extracted by a chloroform–methanol (1:1, v/v) mixture.

Additional and probably more precise information about the number and nature of the surface components released by papain from pig vesicles was obtained by immunoelectrophoresis using anti-papain supernatant sera. A photographic reproduction of the precipitin lines after staining with aniline blue-black is given in the upper part (Part A) of Fig. 3. A composite drawing of the lines revealed by successive incubations of the gels with the specific chromogenic substrates already used before is presented in the lower part (Part B) of the same figure. It is noteworthy that relatively few lines are visible in the upper diagram, demonstrating that the total number of surface components solubilized by papain is not high. Moreover, each protein line can be identified to an enzyme activity line, except for the already mentioned component X. This latter remark is consistent with the view that the majority of the surface components detached by papain are known intestinal enzymes.

Treatment of the vesicles by Triton X-100.

When pig jejunum vesicles were incubated overnight with 1 % Triton X-100 at 4 °C, 80–100 % of the aminopeptidase and maltase activities were readily solubilized whereas alkaline phosphatase released under the same conditions did not exceed 30–50 % of the total. Thus, solubilization of alkaline phosphatase by both papain and Triton appears to be especially difficult. Moreover, the material solubilized by Triton was obviously different from that released by papain as shown by a slower migration during gel electrophoresis (Table III).

Another observation made earlier with Triton-released pig aminopeptidase was that the electrophoretic migration of this material was restored after trypsin incubation to the values normally observed with papain [9]. Table IV shows that a

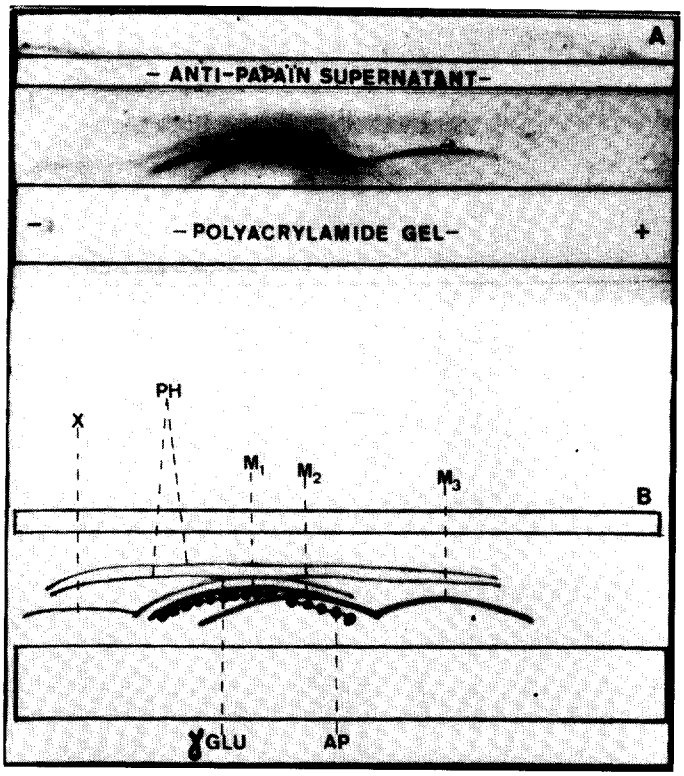

Fig. 3. Identification by immunoelectrophoresis of the proteins and enzymes present in the papain supernatants from pig vesicles. Above (A), the protein lines were stained with aniline blue black (photographic reproduction). Below (B), composite drawing of the lines revealed by chromogenic substrates: Aminopeptidase (AP), γ-glutamyl transferase (γ-Glu), alkaline phosphatase (PH) and the maltases (M_1, M_2 and M_3).

similar conversion also occurs with maltase, but not with sucrase and alkaline phosphatase. Furthermore, papain is seen to achieve a complete conversion of the Triton-released sucrase. But, alkaline phosphatase is again observed in this case to be more resistant.

TABLE IV

R_F VALUES AFTER TRYPSIN OR PAPAIN TREATMENT OF A TRITON X-100 SUPERNATANT FROM PIG JEJUNAL VESICLES

The R_F value of the material present in the Triton X-100 or papain supernatant of the vesicles are indicated here for the 2nd time for purpose of comparison. Each experiment was carried out in duplicate with identical results.

Material	Triton X-100 supernatant			Papain
	Trypsin	Papain	None	Supernatant
Aminopeptidase	0.300	0.300	0.100	0.300
Sucrase	0.037	0.350	0.040	0.350
Maltases	0.420	0.410	0.130	0.410
	(0.710)	(0.700)	(0.410)	(0.730)
Phosphatase	0.200	0.200	0.210	0.470
		(0.470)		

DISCUSSION

Previous investigations on the solubilization of intestinal hydrolases by papain had so far been performed with the aid of open "brush borders" [4, 5] isolated membrane structures [7]. In this latter case, the tightness of the vesicles towards macromolecules was not checked and it could be suspected that papain was able to penetrate inside and consequently to solubilize internally as well as externally located molecules. By contrast, the migration of the vesicles used in the present work through Ficoll gradients indicated that they were impervious to high molecular weight compounds. Considering that they were also found to be impervious to inulin (unpublished experiments) with a molecular weight not exceeding 5000, it could be assumed with a considerable degree of certainty that papain did not enter the vesicles. Since all the vesicles in question appear by electron microscopy to be right side out, the obvious conclusion is that the molecules found in the papain supernatants are external components of the membrane. It is of great interest to find that the proteins solubilized in this manner are relatively few in number and that all, with the single exception of component "X", can be identified to already known intestinal hydrolases. These proteins amount to as much as 50 % of the total proteins of the vesicles, including the filamentous material located inside.

Papain digestion also releases 50 % of the total sugars and glycolipids of the vesicles, but no trace of phospholipids and choresterol. In addition, the digested vesicles have nearly the same hydrodynamic properties as native ones on sucrose or Ficoll gradients. This fact strongly suggests that complete removal of the surface hydrolases is possible with no appreciable dislocation of the bilayer matrix. Electron microscopy after negative staining shows that enzyme removal runs parallel with the disappearance of the "knobs" which give a characteristic granular appearance to the external side of the membrane [4, 8, 20]. This observation is consistent with the idea of Johnson [4] that hydrolases form the "knobs". However Benson et al. [20] have shown that it is not possible to correlate the release of disaccharidases with the removal of "knobs".

The mechanism of the solubilization of intestinal hydrolases by papain is still unknown, although the enzyme may be expected to split a limited number of peptide bonds, either in the hydrolases themselves, or in other proteins perhaps involved in their binding to the lipid bilayer (see below). The only well established fact in this respect is that the hydrolases are fully active after solubilization and that, with the exception of enterokinase, they are quite stable against further degradation by papain [19].

It may be relevant at this point of the discussion to evoke again the problem already briefly mentioned above of how surface membrane components, as most brush hydrolases appear to be, are firmly bound to the lipid bilayer. A plausible assumption is that the components in question are anchored, either by an hydrophobic region of their own molecule which would be "buried" in the bilayer, or by a separate molecule with which they would interact non covalently. Experimental support for these views has been recently provided by the observation that Triton X-100 extracts of mitochondrial inner membranes contain the total ATPase complex [24]. Triton X-100 has also been reported to extract from the electric organ of the electric eel an "elongated form" of acetylcholine esterase containing several enzyme molecules

and a stem supposed to be the anchor [25]. Moreover, cytochrome b_5 solubilized by action of the detergent on liver microsomes also appears to be bound to an anchor fragment [26]. In all cases, these primary forms were found to be converted into a smaller and more globular form by violent mechanical stirring (ATPase) or limited proteolysis (acetylcholine esterase and cytochrome b_5).

Although direct experimental support is still lacking in the specific case of intestinal hydrolases, these enzymes may, as a working hypothesis, also be assumed to be attached to the brush border lipid bilayer by suitable anchors. According to this assumption, Triton would extract from the membrane the covalent or non-covalent hydrolase–anchor associations whereas the hydrolases proper would be obtained, either directly from the native membrane by papain action, or by extracting first the association and then splitting it with papain or trypsin. In this perspective, the apparent high molecular weight values indicated for theTriton-extracted aminopeptidase by its partial exclusion from Sepharose 4B in the absence of the detergent and the slow electrophoretic migration of hydrolases can be assumed to result from protein–protein aggregation processes induced by the presence of presumably hydrophobic anchors. Furthermore, the aminopeptidase solubilized by papain action has no affinity for the lipids obtained from brush border membrane vesicles whereas the Triton X-100-extracted enzyme readily binds to these lipids (Verger et al., unpublished).

The return now to the papain-solubilized hydrolases, it was noteworthy that the same enzymes derived from pig, rat and rabbit vesicles were often observed to migrate differently during gel electrophoresis. Very large variations were noted for instance between the pig and rabbit hydrolases. Moreover, no cross reaction could be discerned between a papain supernatant from rat vesicles and an antiserum prepared with a supernatant from pig vesicles. It seems, therefore that relatively little structural homology should exist between given hydrolases from different species. By contrast, a certain level of homology between the maltases in the same species is suggested by the fusion of the corresponding precipitin lines (Fig. 3).

A last remark is related to the fact that, in a given species, the hydrolases are not released at the same rate by papain, alkaline phosphatase being the slowest. This fact, already observed by several authors, has so far been interpreted in terms of peptide bond accessibility, alkaline phosphatase, for instance, being considered more deeply buried in the lipid bilayer than the other enzymes. This interpretation is not fully supported by the finding made in the course of the present work that papain action on alkaline phosphatase is not facilitated by previous Triton extraction. An alternative hypothesis is that the rate at which any hydrolase is released depends on several parameters including a positive fit between the geometry of papain active site and the structure of the hydrolase–anchor association in the vicinity of the strategic peptide bond(s). Papain is known to display a surprisingly high specificity in certain circumstances. It solubilizes for instance the leucyl-β-naphthylamidase activity of the plasma membrane of liver cells [27] and the surface glycoproteins of 3T3 mouse fibroblasts [28]. In this latter case, it induces the loss of contact inhibitors for concentrations well below those observed with trypsin and chymotrypsin. In the same connection, it is of interest that other proteolytic enzymes such as trypsin, chymotrypsin and subtilisin are not able to solubilize the hydrolases from pig brush border membrane vesicles [19] and rat intestinal mucosa [29].

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