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DETERGENT-INDUCED PROTEOLYSIS OF RABBIT INTESTINAL BRUSH BORDER VESICLES

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Proteolysis of brush border vesicle proteins is induced by detergent solubilisation. This proteolysis is selective in that some of the proteins are more susceptible than others. The rate of induced proteolysis is decreased by decreasing the temperature, has a minimum at about pH 6 and is effectively prevented by a combination of the protease inhibitors, EDTA, diisopropylfluorophosphate and iodoacetamide.

Introduction

The disruption of cell membranes frequently results in the release of proteolytic activity. For example, it has been found that in the preparation of ghosts from red blood cells the activity of at least three proteases is stimulated [1,2]. The resistance of the proteins associated with the ghost membrane towards these proteases differs. Whereas some of the glycoproteins are relatively resistant, the majority of the proteins in the ghost membrane and cytoskeleton are not [3]. The rate of this proteolysis is stimulated by detergent [3]. As detergent disruption is an essential step in the isolation of integral membrane proteins, it is necessary, for a given membrane preparation, to determine the factors that tend to minimise proteolysis. To this end the effect of detergent induced proteolysis of brush border vesicles derived from rabbit small intestine has been studied. These vesicles are known to contain, as integral membrane proteins, a variety of proteases [4].

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; Taps, 3-(tris(hydroxymethyl)methyl)aminopropanesulphonic acid.

Materials and Methods

Brush border vesicles were prepared from rabbit small intestine, which had been stored at −50°C, essentially after the methods described by Schmitz et al. [5], Kessler et al. [6] and Hauser et al. [7]. However the isolation medium consisted of 300 mM mannitol and 5 mM Hepes plus potassium hydroxide to pH 7.6, and, unless otherwise stated, 10 mM magnesium chloride was used in the precipitation step. Brush border vesicles were incubated with detergents over a 20-h period. Unless otherwise stated this was at room temperature (20 ± 2°C). Aliquots were taken at timed intervals and were frozen in liquid nitrogen prior to storage at −35°C. In some experiments further aliquots (100 to 200 μl) were taken at the same time intervals and were centrifuged for 30 min at 25 000 × *g*. Supernatants and pellets were separated and treated as above.

Prior to electrophoresis the pelleted material was made up to the volume from which it was precipitated. The protein samples were dissolved, boiled and then electrophoresed on 8% polyacrylamide, sodium dodecyl sulphate gels as described by Laemmli [8]. After electrophoresis the gels were stained with 0.1% Coomassie blue in methanol, acetic acid and water (50 : 7 : 43, v/v) and destained

in methanol, acetic acid and water (20 : 7 : 73, v/v).

Gels were not scanned directly. They were photographed and the negatives were rephotographed to give diapositives. These were scanned using an Integraph CH gel scanner (Bender and Hobein AG).

Results

Effect of various detergents on inducing proteolysis

The efffect of incubating brush border vesicles for different lengths of time in the presence of various detergents is shown in Fig. 1. At the concentration used, which was 24 mM, these detergents solubilised the majority of the Coomassie blue stainable material; brush border vesicles without detergent were precipitable (at 25 000 × *g* for 30 min) leaving no stainable material in the supernatant, whereas, the detergent-treated vesicles were not precipitable, the Coomassie blue stainable material being in the supernatant. The electrophoretic patterns show that detergent disruption of brush border

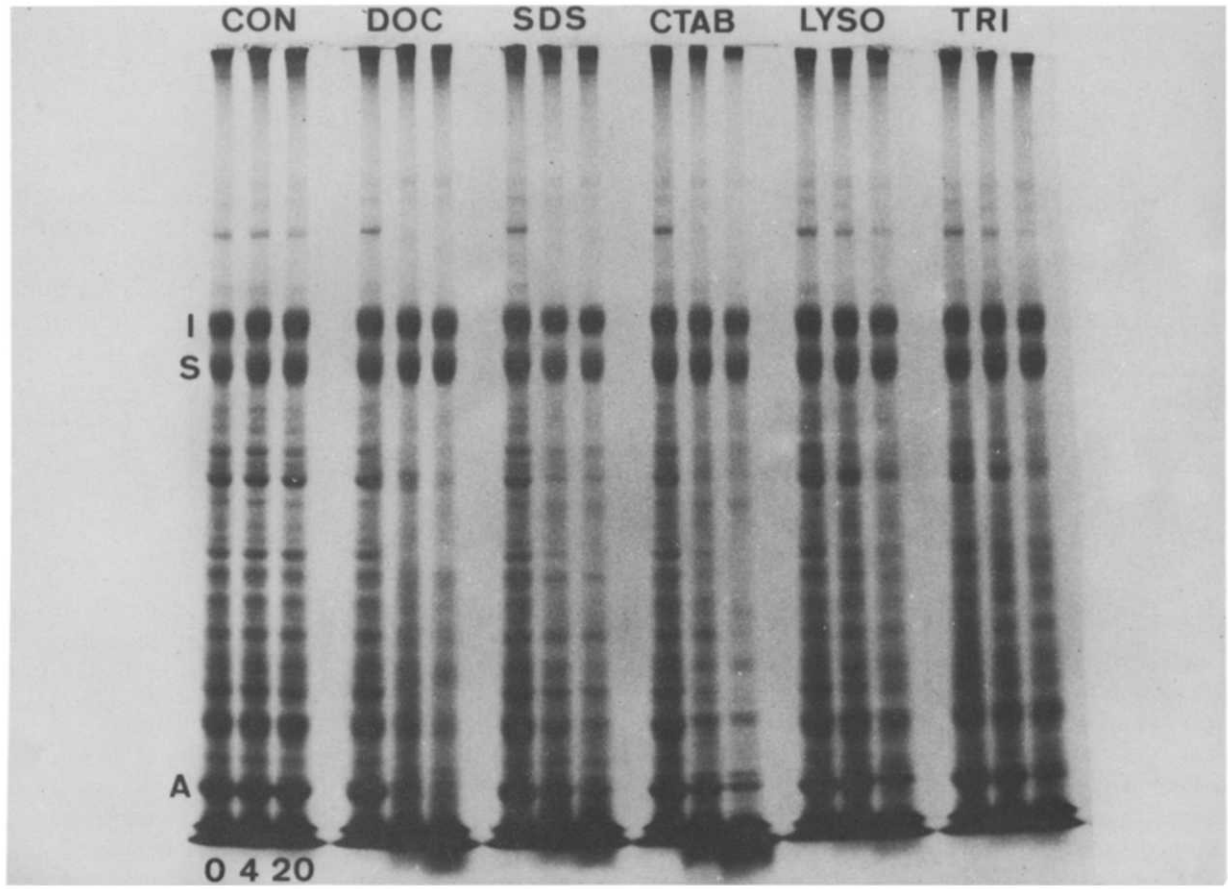

Fig. 1. Effect of detergent induced proteolysis, after 0, 4 and 20 h, on the sodium dodecyl sulphate, polyacrylamide gel electrophoresis pattern of brush border vesicle proteins. The control (CON) was brush border vesicles, at 8 mg of protein per ml, in 300 mM mannitol and 5 mM Hepes plus potassium hydroxide to pH 7.6. To this the following detergents were added at 24 mM: sodium deoxycholate (DOC), 10 mg · ml^{-1}; sodium dodecyl sulphate (SDS), 7 mg · ml^{-1}; cetyltrimethylammonium bromide (CTAB), 9 mg · ml^{-1}; lysophosphatidylcholine (LYSO), 13 mg · ml^{-1}; and Triton X-100 (TRI), 16 mg · ml^{-1}. Sodium chloride was added at 24 mM where Na^+ was not the counterion of the detergent. By comparison to calibrated gels the two major bands migrating with apparent molecular weights between 110 000 and 130 000 have been identified as containing sucrase (S) and isomaltase (I) [9] and the band with an apparent molecular weight of 45 000 as containing actin (A) [10].

vesicles induces proteolysis, that at a given concentration, some detergents are more effective than others and that some of the protein bands are more resistant than others. Of the three bands identified on the gel that labelled actin (A) shows little resistance to proteolysis while those containing the glycoproteins sucrase (S) and isomaltase (I) are far more resistant.

For the experiment shown in Fig. 1 two samples of brush border vesicles were isolated from the same preparation of disrupted enterocytes by using either 10 mM magnesium chloride or 10 mM calcium chloride in the precipitation step. The results shown are for the isolation using the magnesium chloride precipitation. The protein pattern and the extent of the detergent induced loss of Coomassie blue stainable material was independent of whether magnesium chloride or calcium chloride was used in the precipitation step.

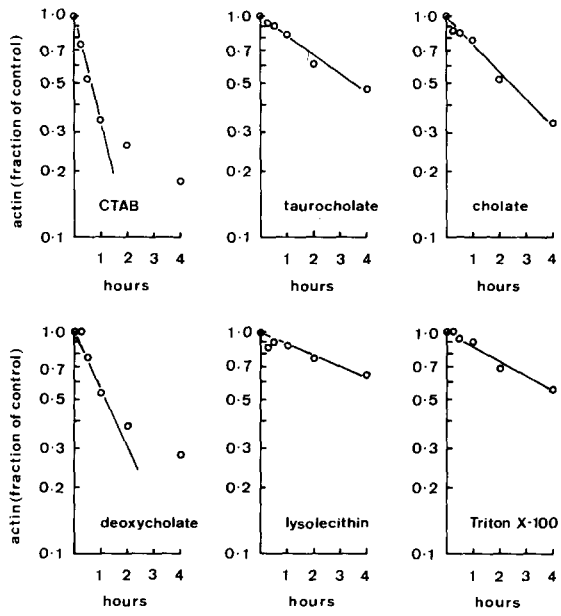

Fig. 2. The rate of proteolysis of actin in brush border vesicles induced by various detergents. Brush border vesicles, at 11 mg of protein per ml, were incubated in 300 mM mannitol and 5 mM Hepes plus potassium hydroxide to pH 7.6 with detergent at 11 mg · ml^{-1}. The detergents with their half-time of induced proteolysis are: 30 mM cetyltrimethylammonium bromide (CTAB), 35 min; 20 mM taurocholate, 210 min; 26 mM cholate, 145 min; 27 mM deoxycholate, 70 min; 20 mM lysophosphatidylcholine, 350 min; and 17 mM Triton X-100, 275 min.

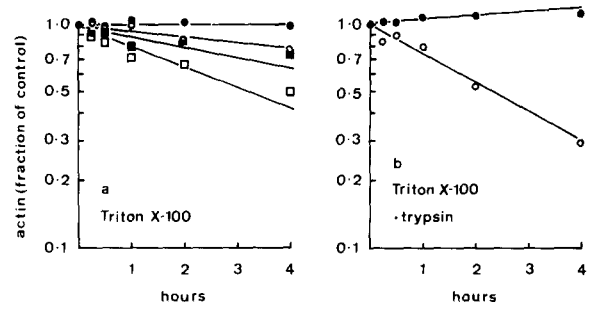

Fig. 3. The effect of Triton X-100 concentration on the inducible proteolysis of actin in brush border vesicles. Brush border vesicles, at 10 mg of protein per ml, were incubated in 300 mM mannitol and 5 mM Hepes plus potassium hydroxide to pH 7.6 with various concentrations of Triton X-100. These were: •, 1.2 mM (0.78 mg · ml^{-1}); ○, 2.4 mM (1.6 mg · ml^{-1}); ▪, 4.8 mM (3.2 mg · ml^{-1}); and □, 9.6 mM (6.5 mg · ml^{-1}). When present trypsin was added at 0.4 mg · ml^{-1}.

Some of the data from a similar experiment to that shown in Fig. 1 were quantified. Diapositives of the gels were scanned and at a given time the ratio of the height of the actin peak to the height of the isomaltase peak was calculated; the relatively stable isomaltase peak being used as an internal standard. The ratio at time zero was given the arbitrary value of unity and the ratios at subsequent time intervals were normalised to this value. The log of this ratio against time is plotted in Fig. 2. For several of the detergents the loss of the actin band follows a single exponential decay for which half-times may be derived (see legend to Fig. 2). For those detergents which are more effective at inducing proteolysis the plot is distinctly curved, in these cases a half time value has been derived from the straight line drawn through the first part of the curve.

Effect of detergent concentration on induced proteolysis

The time course of proteolysis of the actin band was followed at several Triton X-100 concentrations. The data derived from scanning diapositives of the Coomassie blue stained gels are plotted in Fig. 3a in the same form as in Fig. 2. At a concentration of 1.2 mM Triton X-100 or less there was no appreciable proteolysis of the actin band over a 20-h period; the time course in Fig. 3 is only shown to 4 h. Proteo-

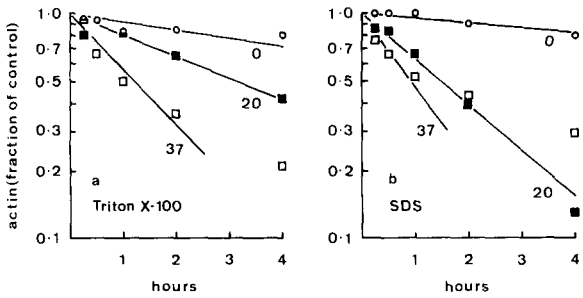

Fig. 4. The effect of temperature on the rate of proteolysis of actin in brush border vesicles induced by Triton X-100 and sodium dodecyl sulphate. Brush border vesicles, at 16 mg of protein per ml in (a) and 11 mg per ml in (b), were incubated in 300 mM mannitol and 5 mM Hepes plus potassium hydroxide to pH 7.6 with in (a) 25 mM Triton X-100 (16 mg · ml^{-1}) and in (b) 39 mM sodium dodecyl sulphate (11 mg · ml^{-1}). The incubations were at 0, 20 and 37°C.

lysis was induced at a Triton X-100 concentration of 2.4 mM and increased with increasing Triton X-100 concentration. Proteolysis is induced on solubilisation of the vesicles in that at or below 1.2 mM Triton X-100 the vesicles were pelletable (25 000 × *g* for 30 min) whereas at or above 2.4 mM they were not. Trypsin was also included in a duplicate experiment,

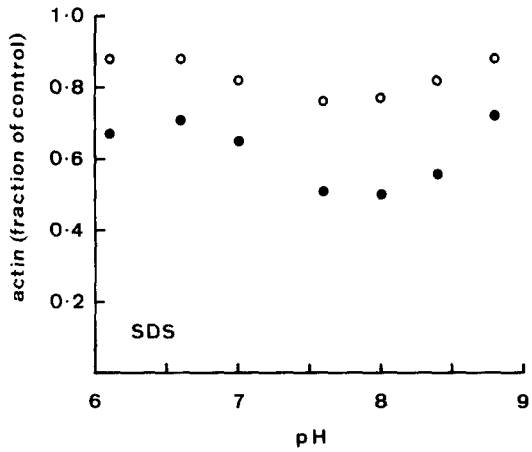

Fig. 5. The effect of pH on the rate of sodium dodecyl sulphate-induced proteolysis of actin in brush border vesicles. The buffer contained 300 mM mannitol, 39 mM sodium dodecyl sulphate (11 mg · ml^{-1}) and the following at 5 mM, sodium acetate, Mes, Hepes, Taps and piperidine plus sodium hydroxide and hydrochloric acid to give a range of pH values. Brush border vesicles were then added at 5 mg of protein per ml and the pH was measured. ○, after 4 h; ●, after 20 h.

Fig. 3b, using brush border vesicles from the same preparation as for Fig. 3a. Similarly, at or below 1.2 mM Triton X-100 proteolysis of the actin band did not occur whereas at or above 2.4 mM Triton X-100 it did. The rate of proteolysis was considerably faster than in the absence of trypsin, and at 4.8 mM Triton X-100 and above, it was too rapid to measure with the time scale used.

Similar experiments were made using sodium cholate, from 2.3 to 74 mM, and sodium dodecyl sulphate, from 0.9 to 28 mM (otherwise the experimental conditions were the same as those described in the legend to Fig. 3a). With sodium dodecyl sulphate there was a transition, in that below 7 mM the majority of the protein was pelletable and at 14 mM and above there was no pelletable material. Proteolysis was only markedly stimulated at 14 mM and above. The results were similar with cholate in that proteolysis was not stimulated if the majority of the protein was pelletable. However, the extent of proteolysis was not as great, and further, as the detergent concentration was increased there was no abrupt transition from the majority of the protein being pelletable to the majority of the protein being unpelletable.

Effects of temperature and pH on the rate of proteolysis

The effect of temperature on Triton X-100 and sodium dodecylsulphate induced proteolysis of actin is shown in Fig. 4. The data are plotted as in Figs. 2 and 3. Reducing the temperature reduces the rate of proteolysis.

The pH dependence of sodium dodecylsulphate induced proteolysis of actin is shown in Fig. 5. This is a plot of the ratio of the height of the actin peak after 4 and 20 h to the height of the actin peak at time zero plotted against pH. As the resistance of the isomaltase peak to proteolysis varied with pH it was not used as an internal standard for the loss of the actin peak. In the pH range from 6 to 9 the induced protease activity is minimal at about 6.5 and maximal in the region of 7.5 to 8.0.

Inhibition of the detergent-induced proteolysis

The effect of iodoacetamide, diisopropylfluorophosphate and EDTA singly and in combination on the sodium dodecyl sulphate induced proteo-

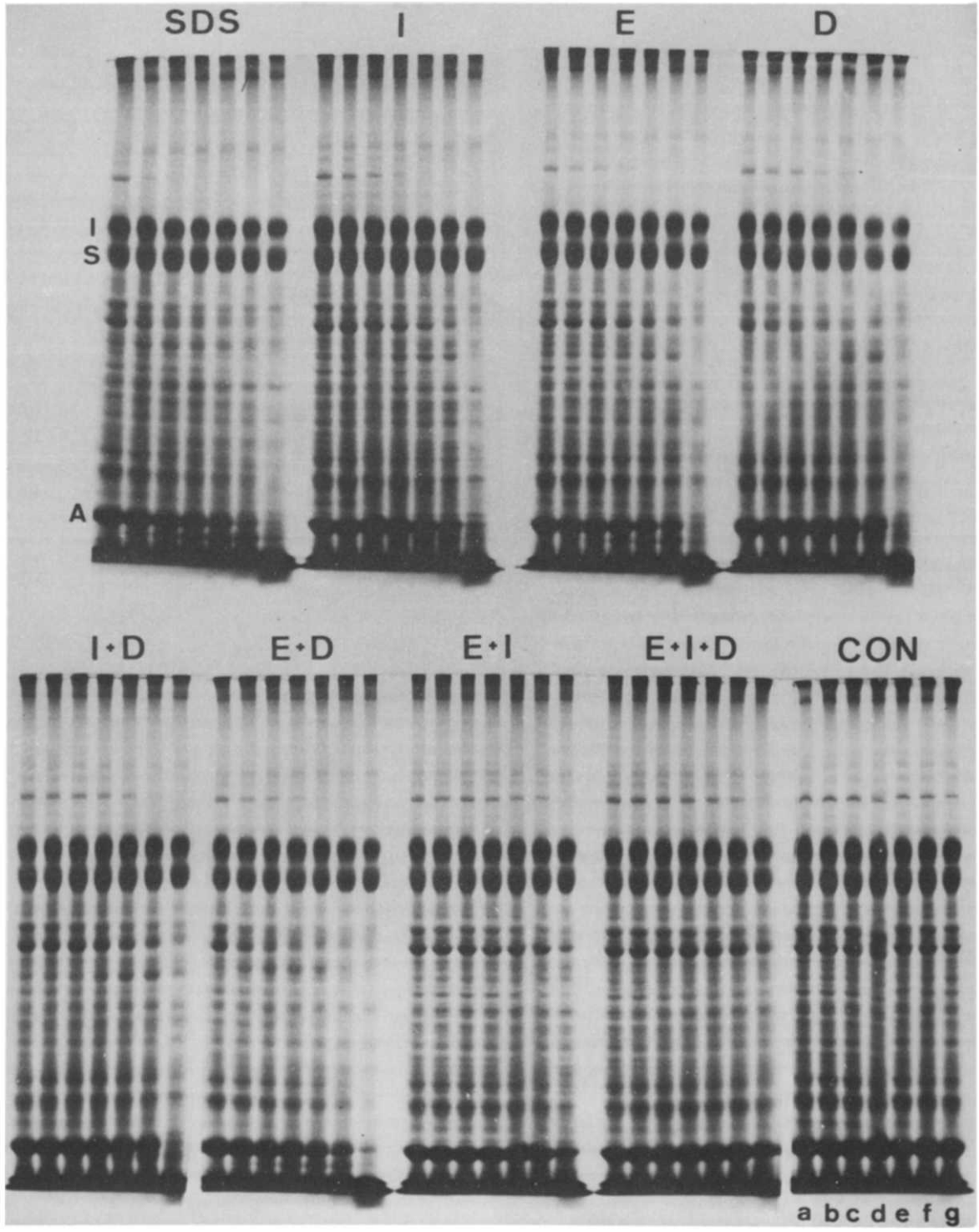

Fig. 6. Inhibition of sodium dodecyl sulphate-induced proteolysis of brush border vesicles. Brush border vesicles, at 11 mg of protein per ml, were incubated in 300 mM mannitol and 5 mM Hepes plus potassium hydroxide to pH 7.6. The following protease inhibitors were added singly or in combination, each at 4 mM, iodoacetamide (I), EDTA (E) and diisopropylfluorophosphate (D). After a 15-min preincubation at 37°C sodium dodecyl sulphate was added, to 9 mM (2.5 mg · ml^{-1}), and the incubation was carried out at room temperature. Samples were taken at the same time intervals for each of the nine gels. These were, as indicated for the control (CON): a, 0 min; b, 15 min; c, 30 min; d, 1 h; e, 2 h; f, 4 h; g, 20 h. The bands indicated on the gel contain isomaltase (I), sucrase (S) and actin (A).

lysis of brush border vesicles is shown in Fig. 6. Each of the nine sets of seven columns is a time course of proteolysis from 0 to 20 h. Top left (SDS) is for brush border vesicles incubated with sodium dodecyl sulphate and bottom right (CON) is for brush border vesicles incubated without sodium dodecylsulphate. The loss of the actin band (A) is the most obvious to follow. Although iodoacetamide (I), EDTA (E), diisopropylfluorophosphate (D), iodoacetamide plus diisopropylfluorophosphate (I + D), and EDTA plus diisopropylfluorophosphate (E + D) slow down the rate of proteolysis, in that the actin band after 4 h is larger than in the control with sodium dodecyl sulphate and without inhibitor (SDS) there is little or no actin present after 20 h. However, EDTA plus iodoacetamide (E + I) substantially reduces proteolysis even after 20 h and this is reduced further by the addition of diisopropylfluorophosphate (E + I + D). Even the combination of all three inhibitors does not completely inhibit proteolysis over a 20-h period, as compared with the control (CON).

Qualitatively similar results were found when Triton X-100 was used instead of sodium dodecyl sulphate (conditions as in the legend to Fig. 6, Triton X-100 replacing sodium dodecyl sulphate at the same concentration, 2.5 mg · ml^{-1}).

Discussion

In general terms the organisation of brush border vesicles is known [4]. They consist of a structural core, a major component of which has been identified as actin [10]. The core is surrounded by a continuous membrane; this is inferred from transport studies that show that under the appropriate conditions substrates can be accumulated [6,11] and also from the observation that the actin is resistant to added proteases unless the membrane is disrupted by detergents [12–14]. Thus the resistance of actin to added proteases can be taken as an indication of membrane integrity. External to the membrane, but anchored to it, are a variety of hydrolases, including proteases, which can only be removed from the undisrupted membrane by the action of proteases such as papain. This releases the majority of the hydrolases from the membrane in a soluble, active form [4].

Under similar conditions the rate of proteolysis induced by a given detergent varied from preparation to preparation. This may in part reflect the extent of contamination by proteases of cytosolic [15,16] or pancreatic [17,18] origin or may be caused entirely by variations in the intrinsic protease activity of the isolated preparation. The latter has been found for red blood cell ghosts [2].

Also as found for red blood cells [3] proteolysis is selective, in that some proteins are more susceptible than others (Figs. 1 and 6). The experiment shown in Fig. 3a indicates that proteolysis occurs when there is sufficient detergent to cause disruption of the vesicle membrane, in that detergent concentrations that are too low to liberate proteins into the supernatant do not induce proteolysis. This is shown more clearly in the parallel experiment in which trypsin was included (Fig. 3b). Under these conditions this enzyme displays no proteolytic activity unless the detergent concentration is sufficient to solubilise the vesicles. As judged by the lack of proteolytic activity towards the internal marker protein actin, trypsin entry into the brush border vesicles does not seem to be induced at sublytic concentrations of Triton X-100. In both instances, with and without trypsin, the only major bands that are resistant to proteolysis are those containing sucrase and isomaltase. As there is no proteolysis without disruption, it would therefore seem probable that the majority of the protein bands that are not resistant to detergent induced proteolysis are in the absence of detergent enclosed by the membrane.

The data in Fig. 3 indicate that provided that there is sufficient detergent to induce lysis then the rate of proteolysis increases with increasing detergent concentration. From Figs. 1 and 2 it would seem that some detergents are more effective at inducing proteolysis than others. This may in fact be so, however as the concentration dependence of each detergent was not investigated this may merely reflect the efficiency of the detergent in solubilising the brush border vesicle membrane or the extent to which a particular detergent can enhance proteolysis by causing protein denaturation.

Proteases can be classified into three main groups [19]: serine proteases, cysteine proteases and divalent metal ion dependent proteases. Each of these three classes has a different type of inhibitor, in this study

they are represented by diisopropylfluorophosphate, iodoacetamide and EDTA, respectively. The presence of all three types of protease activity in brush border vesicles is indicated as over a 20-h period detergent induced proteolysis was only effectively inhibited when all three types of protease inhibitors were present.

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