J. Membrane Biol. 15, 227–248 (1974)

Membrane Proteins Related to Anion Permeability of Human Red Blood Cells

II. Effects of Proteolytic Enzymes on Disulfonic Stilbene Sites of Surface Proteins

Z. I. Cabantchik* and A. Rothstein

Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada

Received 8 August 1973

Summary. The proteolytic enzymes, pronase, chymotrypsin and trypsin, release a small fraction of covalently bonded 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonate or (^{3}H)DIDS, a specific inhibitor of anion permeability, from intact human red cells. The rate of release is parallel to the digestion of the sialoglycoprotein, indicating that the released (^{3}H)DIDS was bound to that component. Most of the label is associated with a protein that behaves on SDS acrylamide gel electrophoresis as though its molecular weight was 95,000 Daltons (95K). Trypsin has no effect on this protein, but after pronase or chymotrypsin treatment of the cells, the label is found in three peaks of 95, 65 and 35K in proportions of 5, 85 and 10%. In parallel, the enzyme treatment results in a shift of most of the 95K protein to 65 and 35K. The digestion of the glycoprotein and splitting of the 95K protein can occur without any appreciable effects of the enzymes on anion permeability or on the inhibitory effects of DIDS treatment either before or after proteolytic attack. It is suggested that the sialoglycoprotein and the 35K segment of the 95K protein are not involved directly in anion permeation. The most likely location of the inhibitory site is in a portion of the 65K segment, exposed to the outside surface. Some additional observations are presented concerning the shielding effects of the negatively charged sialoglycoprotein and the arrangement of the 95K protein in the membrane.

In two previous papers, it was demonstrated that the disulfonic stilbene derivative, (^{3}H)DIDS[1] can be used as a covalent label for superficial

* Z. I. Cabantchik was the recipient of an International Atomic Energy Agency Fellowship.

1. Abbreviations used throughout the paper are as follows:

(^{3}H)DIDS	4,4′-Diisothiocyano-2,2′-ditritiostilbene disulfonic acid
SITS	4-Acetamido-4′-isothiocyano-2,2′-stilbene disulfonic acid
TBS	Tris-buffer-saline
PBS	Phosphate buffer saline
PAS	Periodic Acid Schiff staining (for carbohydrates)
CB	Coomassie Blue staining (for proteins)
TNBS	2,4,6-Trinitrobenzene sulfonic acid
FDNB	1-Fluoro-2,4-dinitrobenzene
EDTA	Ethylene diamine tetra acetic acid

proteins of the human red blood cell, and that the degree of binding is linearly related to the inhibition of anion permeability (Cabantchik & Rothstein, 1972, 1974). The only membrane components with significant amounts of label were the major sialoglycoprotein, and the protein that behaves on SDS acrylamide gels as though its molecular weight was 95,000 daltons (95K). The former contains about 5% of the label and the latter 95%. Because (^{3}H)DIDS and other disulfonic stilbenes do not penetrate the membrane (Maddy, 1964; Cabantchik & Rothstein, 1972, 1974) it has been concluded that both proteins are located on the outer surface of the membrane (Cabantchik & Rothstein, 1974), confirming observations based on the use of other, nonpenetrating labels (Bretscher, 1971; Philips & Morrison, 1971; Segrest, Kahane, Jackson & Marchesi, 1973).

Because of their location, both "exposed" proteins are susceptible to proteolytic attack with release of a sialoglycopeptide in one case (Cook & Eylar, 1965; Winzler, 1969), and digestion from 95K to 65K in the other (Bender, Garan & Berg, 1971; Triplett & Carraway, 1972). Furthermore, intensive attack with pronase leads directly to inhibition of anion permeability (Knauf & Rothstein, 1971; Passow, 1971) and to greater susceptibility to the inhibitory effects of chemical agents (Passow, 1971).

Both the proteases and the disulfonic stilbenes have revealed useful information concerning those "exposed" proteins that potentially play a functional role in anion permeability. In the present paper the two kinds of "modifiers" were used in combination to gain insight into the location of the DIDS-binding sites relative to the site of attack of the enzymes, into the arrangements of protein on the surface of the membrane and into the role of proteins or protein components in anion permeability.

Materials and Methods

Red blood cells washed with isotonic saline or phosphate buffer-NaCl (PBS) at 300 milliosmolar and pH 7.4, were reacted with 0.5 to 1.0 μM (^{3}H)DIDS (1.47 millicuries/micromole) for 10 to 20 min at 5 °C in a final suspension at 30% Hct. They were subsequently washed in isotonic Tris-buffer-NaCl, pH 7.4, in Tris-buffer-NaCl at pH 7.4 containing a 0.5 to 1.0% albumin (to remove any noncovalently bound reagent) and finally in isotonic PBS. Labeled cells were exposed to one of the proteases, pronase (Calbiochem), chymotrypsin (Sigma) or trypsin (Sigma) for different lengths of time at 37 °C. Proteolytic attack with pronase can continue even after extensive washing due to residual bound enzyme. This problem was overcome by washing three times with 20 volumes of PBS containing 0.5% albumin, and three times with 20 volumes of PBS. Such digested, washed cells could be stored, if necessary, in 0.5% albumin for at least 30 hr without any evidence of further proteolysis. The course of proteolysis was followed by measuring the appearance of sialic acid and of radioactivity in the supernatants of the cell suspensions. Sialic acid was measured by the method of Warren (1959) following a 1-hr hydrolysis in 0.1 N H_2SO_4 at 80 °C. Tritium radioactivity of samples was measured

in 10 ml toluene/PPO containing 10% Protosol (New England Nuclear) with a Beckman Scintillation Counter. With extensive proteolysis small amounts of hemolysis may occur. Hemoglobin was measured by the method of Dacie and Lewis (1968) and corrections were introduced to compensate for hemolysis where necessary.

In some experiments, the cells were first subjected to proteolysis, then were washed with albumin and PBS as described, and were subsequently reacted with (^{3}H)DIDS to determine whether the proteases exposed new binding sites.

Ghosts were prepared from normal and modified cells by the method of Dodge, Mitchell and Hanahan (1963). The ghosts from albumin-washed cells were stable for at least 48 hr at 5 °C with no evidence of further proteolysis in terms of changes in membrane protein patterns on acrylamide gels. Protein determinations were performed either by a modified biuret method or by fluorescence (Cabantchik & Rothstein, 1974). The isolated ghosts were, in some instances, delipidated by three repetitive extractions with ethanol ether (3:1) (Rosenberg & Guidotti, 1968). Glycoproteins were extracted by the method of Hamaguchi and Cleve (1972) and precipitated with ethanol (60%).

Polyacrylamide-SDS *Gel Electrophoresis and Fractionation*

SDS-acrylamide gels (7.5%) were prepared, run and stained according to a modified Fairbanks, Steck and Wallach (1971) method (Cabantchik & Rothstein, 1974). Gels were stained for protein with Coomassie Blue (CB) and for carbohydrate with periodic-acid Schiff (PAS). The patterns were photographed and also scanned with a Gilford 240 spectrophotometer with a 2410 linear transport accessory, at 530 nm for protein and at 560 nm for carbohydrate.

Gels with radioactively labeled proteins were fractionated on a Maizel Autogel Divider (Savant Instruments Inc.) into 40 to 50 fractions that were collected directly in scintillation vials (Savant Unifrac Collector). The gel fractions were dried at 80 °C, resuspended in 0.1 ml water and digested overnight with 10 ml toluene/PPO, 10% Protosol. The radioactivity was counted in the same vial. The recovery of count was not less than 90% of the quantity initially loaded on the gels.

The gels were calibrated for molecular weights using iodinated (^{131}I) samples of β-galactosidase, α-phosphorylase, bovine serum albumin and ovalbumin (Cabantchik & Rothstein, 1974).

Results

When (^{3}H)DIDS-treated red cells are exposed to proteolytic attack with trypsin, chymotrypsin or pronase a small but finite proportion of the bound label is released into the medium (Figs. 1 and 2), the amount depending on the enzyme used, its concentration and the time of treatment. In parallel, sialic acid is also released. Pronase is more effective in terms of release of both (^{3}H)DIDS and sialic acid than either chymotrypsin or trypsin. The maximum release of (^{3}H)DIDS is about 7% for pronase (Fig. 1), and 3 to 4% for trypsin and chymotrypsin (Fig. 2). The maximum release of sialic acid is over 85% for pronase, 62% for chymotrypsin and 50% for trypsin, based on an analyzed value for total sialic acid content of 16×10^{-15} g per cell (range 15 to 17)[2].

2 The published values for sialic acid content of human red blood are comparable to those reported by Winzler (1969).

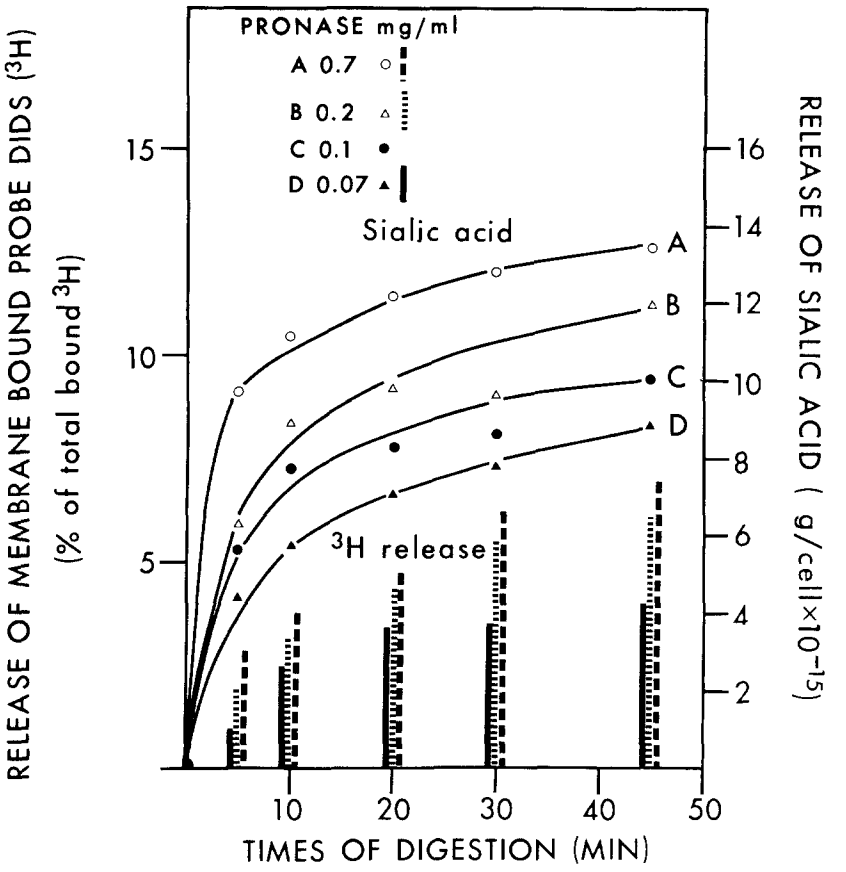

Fig. 1. The time course of sialic acid and (^{3}H)DIDS release from labeled cells exposed to different concentrations of pronase. Cells labeled with 0.25 μM (^{3}H)DIDS were extensively washed, divided into aliquots and exposed to different pronase treatments (pH 7.4, 37 °C). (^{3}H)DIDS and sialic acid were measured in aliquots of the supernatant taken after different times of digestion. Corrections were made for some hemolysis that took place after heavy pronase treatments

From Figs. 1 and 2, it seems evident that a correlation exists between the release of (^{3}H)DIDS and of sialic acid. The correlation is more clearly expressed if, for the pronase data of Fig. 1 (plus an additional experiment at another pronase concentration), one parameter is plotted against the other (Fig. 3). The correlation suggests that the (^{3}H)DIDS released by the proteolytic enzymes is that fraction bound to the sialoglycoproteins, a suggestion reinforced by the fact that the maximal amount released, 6%, is about the same as the amount estimated to be associated with these components by their isolation using the method of Hamaguchi and Cleve (1972) (Fig. 4 in Cabantchik & Rothstein, 1974).

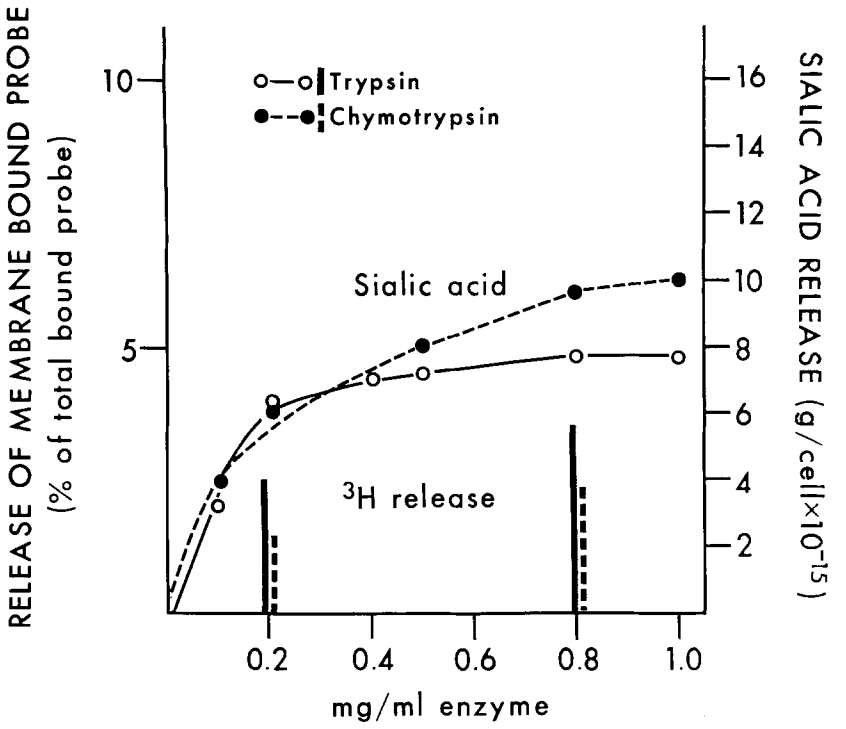

Fig. 2. The effect of trypsin and chymotrypsin at different concentrations on the release of sialic acid and (^{3}H)DIDS from labeled red cells. The experimental conditions are the same as those shown in Fig. 1

The digestion of sialoglycoproteins by the proteases is demonstrated in more detail by SDS acrylamide gel electrophoresis. The glycoproteins are visualized in the gels by PAS staining as in Fig. 4. They are also scanned to provide densitometric tracings (Fig. 5) and referred to by roman numerals (Steck, Fairbanks & Wallach, 1971). Band I contains the sialic acid and is referred to as the sialoglycoprotein. Band II of Steck *et al.* (1971) was found to consist of two distinguishable components IIa and IIb. Because the same electrophoresis procedure was used (Fairbanks *et al.*, 1971), the explanation for the fine splitting observed in the present electrophoretograms can be attributed to the improvement of the carbohydrate staining procedure (*see* Materials and Methods).

The most intensive digestion of glycoproteins in the intact cells is produced by pronase. Even with 0.02 mg/ml of enzyme, Band I is digested (Fig. 4). The other bands are less susceptible but can be degraded with higher concentrations of pronase. Treatment of the cells with trypsin or chymotrypsin results in more subtle effects. Band I, the most intensely stained glycoprotein,

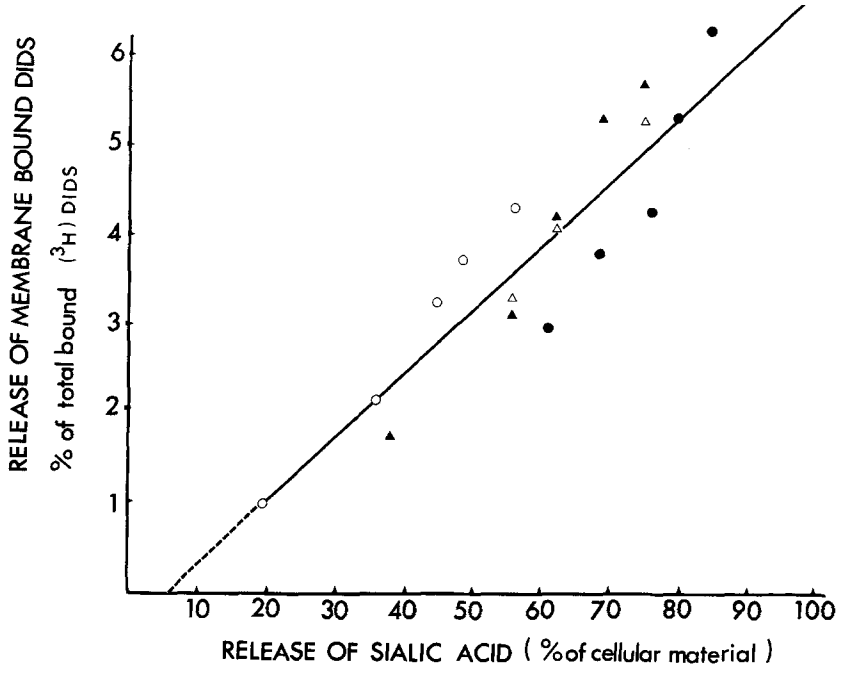

Fig. 3. The release of sialic acid and (^{3}H)DIDS from red blood cells by different pronase treatments. The per cents of bound (^{3}H)DIDS and sialic acid released by different pronase treatments are plotted against each other (data from Fig. 1). Labels represent the values obtained in the course of digestion with four different concentrations of pronase. The line is calculated by the method of least squares

disappears accompanied by the appearance of a new band (I') of lower apparent molecular weight (Figs. 4 and 5). Bands IIa, IIb and III are more resistant but can be digested by more intense treatments. These results are essentially the same as those reported by Steck *et al.* (1971) for proteolysis of glycoproteins in ghosts.

Proteolytic activity of pronase on intact cells is also reflected in the observed changes in the relative amount of bound (^{3}H)DIDS to total ghost protein. Cells were labeled and one portion was treated with 0.1 mg/ml of pronase for 30 min. Ghosts were prepared from the control and pronase-treated cells and analyzed for protein and for (^{3}H)DIDS content. Despite the known loss of up to 6% of the label (Fig. 1), the ratio of label to protein increased by $19 \pm 10\%$ in the pronase-treated cells. These data indicate that unlabeled membrane proteins other than the sialoglycoprotein are lost as a result of pronase treatment either as a result of direct proteolysis, or because the proteolyzed ghosts retain less protein during their preparation. The only information bearing on the latter possibility is the observa-

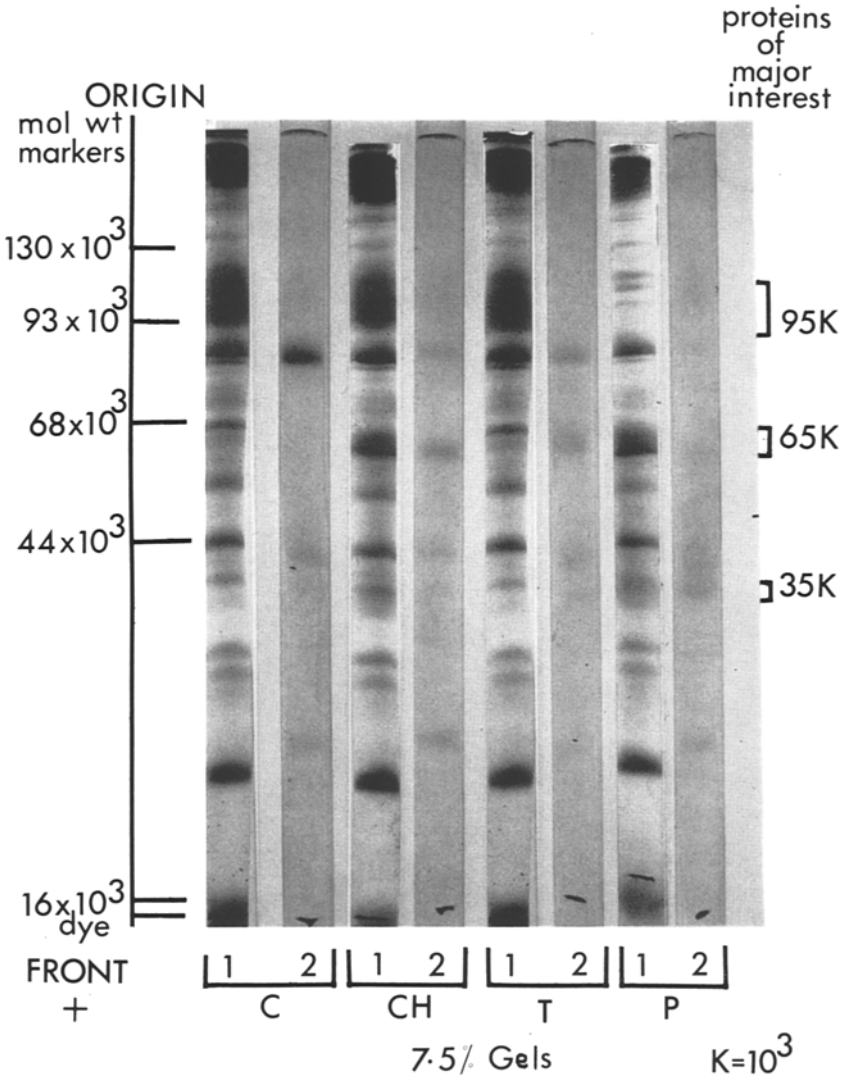

Fig. 4. Effects of proteolytic enzymes on the proteins as displayed by SDS acrylamide gel electrophoresis. Cells (30% suspension) were digested with 0.2 mg/ml enzyme for 20 min at 37 °C in PBS-buffer, pH 7.4, and washed with 1% albumin and in the same buffer. Membranes were prepared as described in Materials and Methods. *C:* Control; *CH:* Chymotrypsin; *T:* Trypsin; *P:* Pronase-treated cells; 1: Coomassie Blue staining (proteins); 2: Periodic acid-Schiff staining (carbohydrates). Arrows on the left show the position and the molecular weight of the protein markers: β-galactosidase, phosphorylase-A, bovine serum albumin, ovalbumin, and hemoglobin (from top to bottom). Proteins of major interest are shown on the right as multiples of 1,000 daltons (= 1K)

tion that hemoglobin retention is increased (not decreased) in ghosts prepared from pronase-treated cells confirming published findings (Triplett & Carraway, 1972).

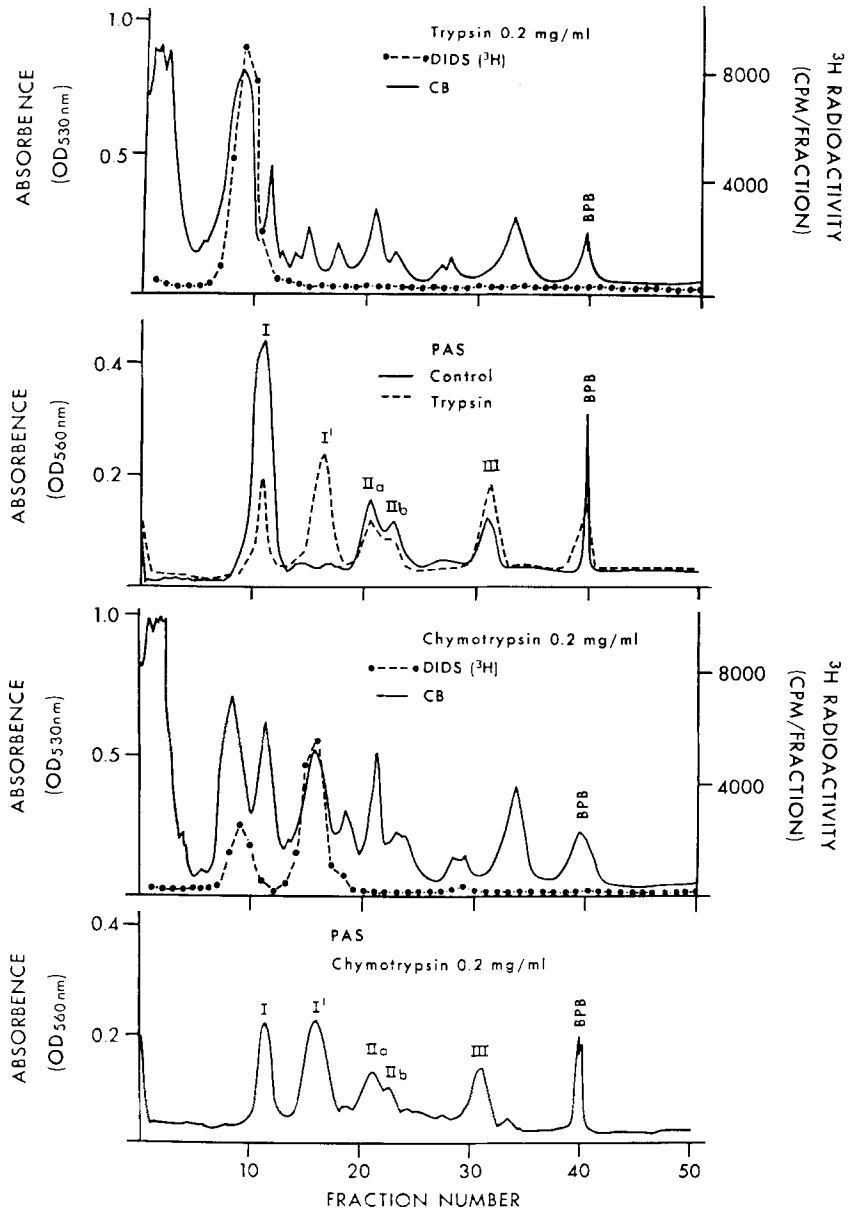

Fig. 5. Densitometry tracings and labeling profiles of (^{3}H)DIDS-labeled cells digested with trypsin and chymotrypsin. The enzyme treatment was with 0.2 mg/ml for 20 min at 37 °C. Details of procedures are the same as in Fig. 4

The only other protein known to be susceptible to proteolytic attack in the intact cell is designated 95K based on its apparent molecular weight of 95,000 daltons as estimated by SDS acrylamide gel electrophoresis. This

protein contains about 95% of the bound (^{3}H)DIDS (Cabantchik & Rothstein, 1972, 1974). Proteolysis seems to result in little release of its label into the medium. For example, after treatment with pronase, the (^{3}H)DIDS is distributed in three peaks of apparent molecular weights 95K, 65K and 35K, rather than in a single band of 95K observed in untreated cells. The shift in the location of the label can be followed by exposure to increasing concentrations of pronase (Fig. 6). As the label decreases in the 95K peak, approximately equivalent amounts appear in the 65K plus 35K components with most in the former. At the highest concentration of pronase used in the experiment (0.7 mg/ml), the distribution of label was approximately 5, 85 and 10% for the 95, 65 and 35K components. Even with 1 mg/ml of pronase (not shown), a residual 5% of label remains at 95K. Evidently some of the protein at 95K is resistant to pronase attack.

The chymotrypsin attack on the 95K protein based on the distribution of (^{3}H)DIDS is similar to that of pronase but the proteolysis is less vigorous and little 35K component was found in this particular experiment (Fig. 5). Higher concentrations of the enzyme or longer times of exposure result in more of the label moving from 95K to 65K and some appearance at 35K. Trypsin, on the other hand, in the concentrations used, had no effect whatsoever on the distribution of the label in the 95K (Fig. 5).

In parallel to the shift of the label after pronase treatment, a shift of protein (visualized by staining with Coomassie Blue) is also observed. The staining of the 95K band diminishes and two new bands appear at 65K and 35K corresponding to the locations of the (^{3}H)DIDS label (Fig. 6 for gels and Fig. 7 for densitometric tracings). The 65K band has been previously reported (Bender *et al.*, 1971; Triplett & Carraway, 1972), but the 35K band is only observed if the pronase-treated cells are washed with albumin as outlined in Materials and Methods. The albumin apparently absorbs residual proteolytic activity and preserves the band because it is not seen if the washing is done only with PBS.

The rèmoval of much of the protein from the 95K location by pronase treatment reveals three individual bands (Fig. 6) confirming the published findings of Bender *et al.* (1971). Presumably the residual (pronase resistant) (^{3}H)DIDS label at 95K is associated with these bands but the resolution of the procedure does not allow any conclusions concerning the particular band that carries the binding site.

The finding of three bands at 95K after pronase treatment and the unusual width of the bands suggest that it may be a heterogeneous collection of proteins that display similar behavior in SDS acrylamide gel electrophoresis. Heterogeneity is also suggested by the relative behavior of the protein as

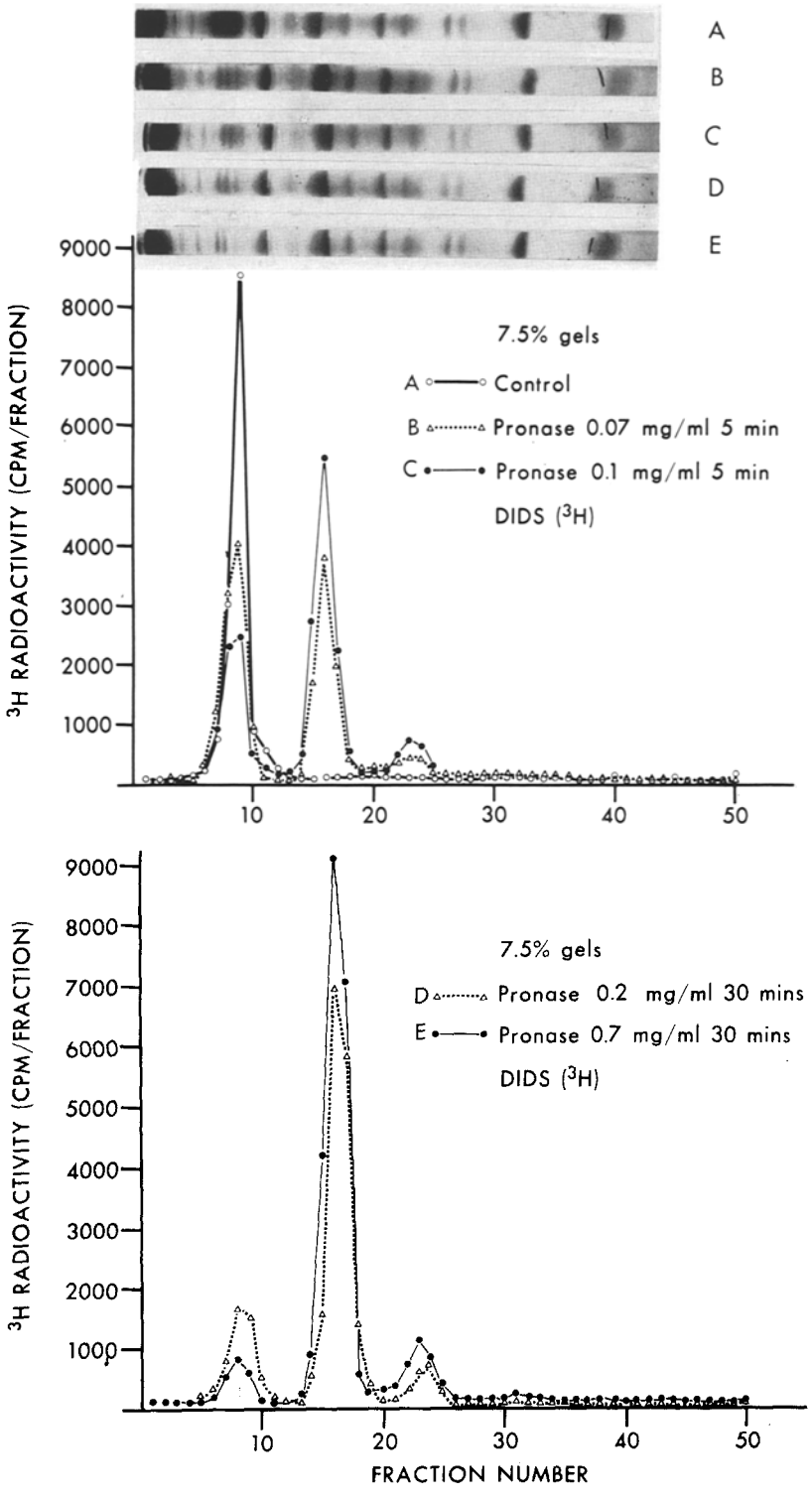
A
B
C
D
E
9000
8000
7000
6000
5000
4000
3000
2000
1000
^{3}H RADIOACTIVITY (CPM/FRACTION)
7.5% gels
A Control
B Pronase 0.07 mg/ml 5 min
C Pronase 0.1 mg/ml 5 min
DIDS (^{3}H)
10
20
30
40
50
7.5% gels
D Pronase 0.2 mg/ml 30 mins
E Pronase 0.7 mg/ml 30 mins
DIDS (^{3}H)
FRACTION NUMBER

Fig. 6

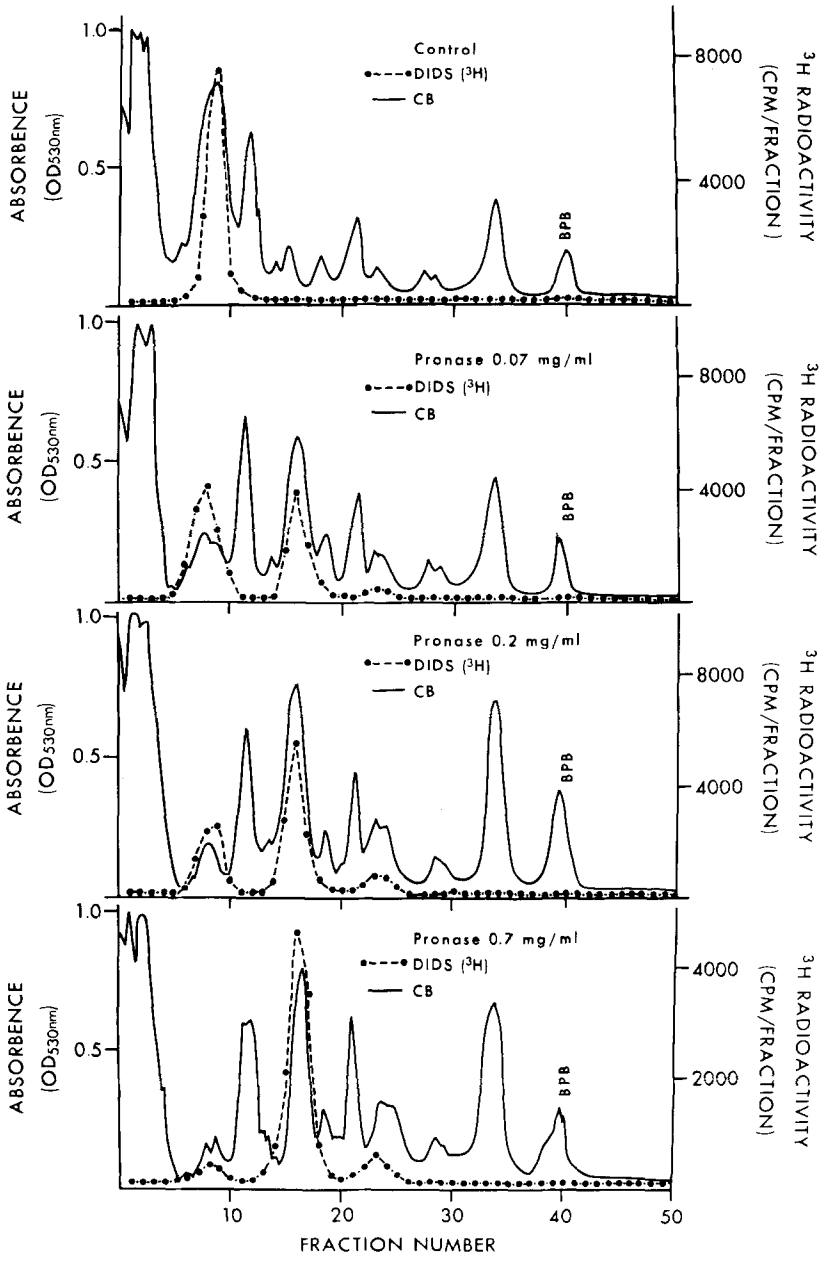

Fig. 7. The effect of pronase treatment on the (^{3}H)DIDS distribution and on protein patterns. Densitometric tracings are of CB-stained gels shown in Fig. 6, and distribution of (^{3}H) label measured in the same gels

Fig. 6. The effect of pronase treatment on the (^{3}H)DIDS distribution and on protein patterns. Red blood cells (30% hematocrit) labeled with 1.0 μM (^{3}H)DIDS, were subjected to different concentrations of pronase at 37 °C for 5 or 30 min (*A* to *E*) washed extensively, and analyzed as described in Materials and Methods. Polyacrylamide gel electrophoresis was performed on 7.5% gels in 1% SDS, Coomassie Blue staining

Table 1. Specific activity of protein bands expressed as the ratio of counts to Coomassie Blue staining

Pronase (mg/ml)	Specific activity	
	95K band	65K band
0	0.7	–
0.07	1.8	0.8
0.20	1.7	0.8
0.70	0.7	1.4

The data are taken from Fig. 7. The specific activities are calculated from the ratio of areas under the peaks of radioactivity and of staining (measured densitometrically) at 95K and 65K.

determined by staining and of the DIDS-binding sites as determined by the tritium label to graded doses of pronase. Inspection of figure 7 and comparison of the densitometric versus radioactivity scans at 95 and 65K suggest that changes in the relative size of the peaks occur at certain pronase concentrations. The attempt to quantitate the differences are presented in Table 1. Tracings of the peaks (staining and radioactivity) were cut out, by eye, and weighed to determine areas under the curves. Specific activities were calculated as the ratio of areas. The staining intensity although of doubtful value in comparing the amount of protein in different bands, is assumed to give relative amounts of protein in a given band prepared at the same time under identical conditions. Granted that the technique is crude, it does suggest that with mild pronase treatment the specific activity of the protein remaining at 95K is increased. Furthermore, with heavier pronase treatment, the specific activity of the 65K band increases. These data suggest that the pronase first attacks 95K protein that has relatively fewer DIDS sites and secondly attacks protein with higher specific activity. They are also compatible with the finding, discussed previously, that the specific activity of the total ghost protein is increased with mild pronase attack.

In all of the experiments so far discussed, the cells were first labeled with (^{3}H)DIDS and then were treated with proteolytic enzymes. If the order is reversed with proteolysis preceding labeling, the binding of the agent is increased by a factor of more than two (Table 2). The increased labeling is observed in the lipid fraction, extracted with solvents, as well as in delipidated proteins.

By gel electrophoresis, it is clear that both pronase (Fig. 8) and chymotrypsin (Fig. 9) treatments result in an increased labeling in each of the

Table 2. The labeling of components of cells predigested with proteolytic enzymes and subsequently exposed to (^{3}H)DIDS

Treatment		Total ghosts	Protein fraction	Lipid fraction
Control		2.20	2.00	0.12
Chymotrypsin	0.2 mg/ml	4.83	5.49	0.23
Trypsin	0.2 mg/ml	4.62	4.10	0.30
Pronase	0.1 mg/ml	5.42	4.85	0.55
Pronase	0.2 mg/ml	5.38	4.45	0.57
Pronase	0.7 mg/ml	5.90	5.05	0.60

Values are expressed in nmoles $\times 10^{-10}$ per ghost.

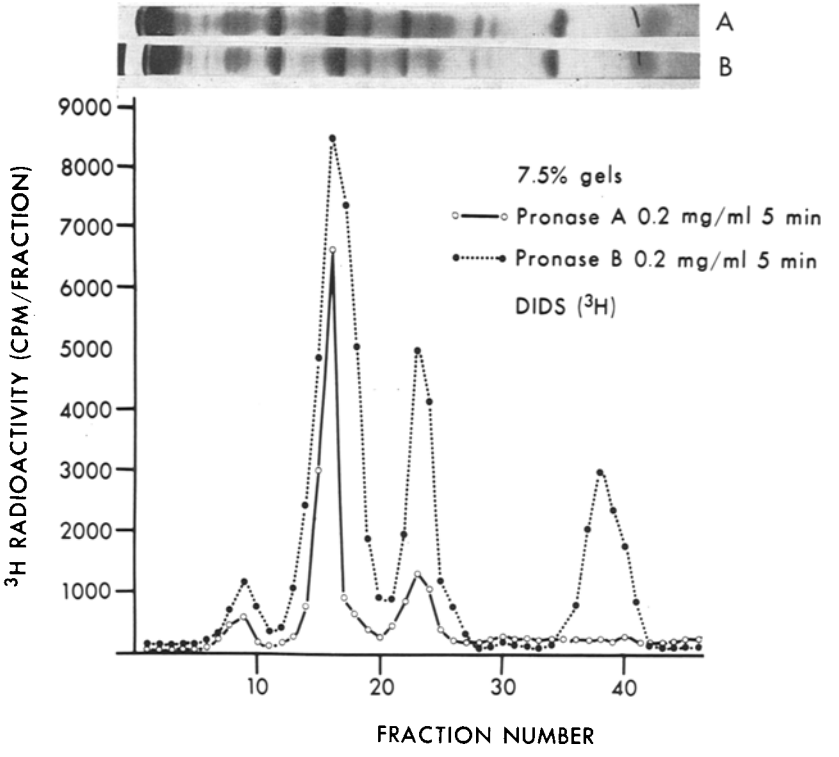

Fig. 8. The (^{3}H)DIDS labeling patterns in cells predigested with pronase. A comparison of labeling patterns in the proteins of ghosts isolated from labeled cells (^{3}H)DIDS treated with pronase (*A*) versus predigested cells that were subsequently reacted with (^{3}H)DIDS under the same conditions (*B*). Polyacrylamide SLS electrophoresis and (^{3}H) distribution were performed as described in Materials and Methods

three protein peaks, 95K, 65K and 35K. With trypsin no 65 or 35K components appear, but an increased labeling of the 95K band is found. With all three proteolytic enzymes, a new band is found at the SDS front. This

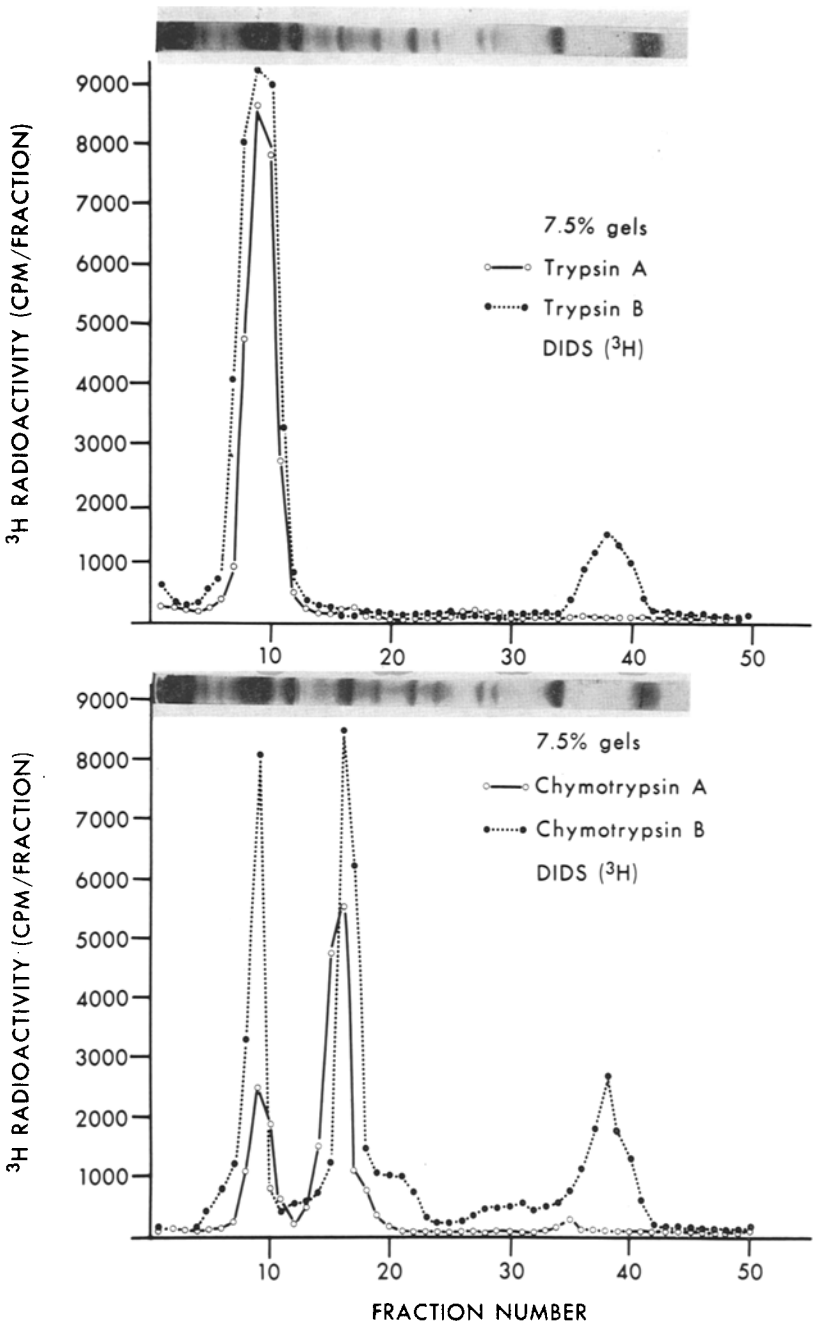

Fig. 9. The (^{3}H)DIDS labeling patterns in cells predigested with chymotrypsin or trypsin. A comparison of labeling patterns in the proteins of ghosts isolated from labeled cells (^{3}H)DIDS treated with proteases (*A*) versus predigested cells subsequently reacted with (^{3}H)DIDS (*B*) in similar conditions as (*A*). Polyacrylamide SLS electrophoresis and (^{3}H) distribution were analyzed as described in Materials and Methods

band is presumed to be lipid on the basis of its location at the SDS front and also because pre-extraction of the membrane material with ethanol-ether before the electrophoresis specifically removes much of it. The count is recovered in the organic phase.

Pronase produces several effects on the intact cell, including the digestion of the sialoglycoproteins, the splitting of the 95K protein as reported here and elsewhere (Bender *et al.*, 1971; Triplett & Carraway, 1972), the inhibition of anion permeability (Knauf & Rothstein, 1971; Passow, 1971) and increase in cation permeability (Passow, 1971). The relative sensitivity of these systems varies considerably. The sialoglycoprotein is particularly sensitive to pronase, chymotrypsin and trypsin with a parallel release of small amounts of bound (^{3}H)DIDS (Fig. 1). The splitting of the 95K band is about equally sensitive to pronase and to chymotrypsin, but is insensitive to trypsin (Figs. 5, 6 and 7). The anion permeability is much less sensitive. In fact, the digestion of the sialoglycoprotein and the splitting of the 95K protein are largely complete at concentrations of pronase (below 0.2 mg/ml) that have little effect on permeability (Figs. 6, 7 and 10). Concentrations that do reduce the permeability (above 0.2 mg/ml) are not associated with any obvious specific action on proteins. A small increase in sialic acid release occurs (Fig. 1), a small additional amount of (^{3}H)DIDS is moved from the 95K position, and the staining at 95K of the three residual bands reduced, particulary the one farthest from the origin (Fig. 6). The lack of relationship of the splitting of the 95K to permeability is also suggested by the fact that chymotrypsin (2 mg/ml) effectively splits over 95% of the 95K band yet has no effect on anion permeability. Finally, treatment with pronase does not alter the inhibitory effects of DIDS reacted with the cells either before or after proteolysis. Inhibition is over 98% in controls and pronase-treated cells.

Discussion

The proteolytic enzymes are useful probes to determine which proteins of the membrane are exposed to the outside and potentially to determine the functional role of those proteins. The present investigation confirms observations in the literature (Triplett & Carraway, 1972) that in the human red blood cell, only two classes of proteins, the sialoglycoprotein and the 95K protein (or proteins) are exposed and susceptible to proteolytic attack. One new detail is the finding that the initial proteolytic attack on the 95K protein involves a splitting into two segments, one at 65K that has been reported previously (Bender *et al.*, 1971; Triplett & Carraway, 1972) and a newly observed one at 35K. The latter is digested by residual enzyme acti-

vities remaining in washed ghosts unless they are removed by albumin solutions.

The 35K band is usually broad judged either by the width of the stained band or by the width of the labeled peak. Its exact molecular weight cannot be assigned by its location in SDS gels since its relative position varies with the porosity of the gel. For example, on 5.6% gels its apparent molecular weight is 45K, compared to 35K on 7.5% gels. This anomaly in mobility results from its relatively high content of carbohydrate residues (T. L. Steck, *personal communication*).

The two proteins that are attacked by proteolytic enzymes are the only two membrane components that covalently bind any significant quantities of the disulfonic stilbene, (^{3}H)DIDS, a highly specific inhibitor of anion permeability (Cabantchik & Rothstein, 1972, 1974). The inhibitory sites are small in number (300,000 per cell, maximum), possess a unique architectural arrangement (based on inhibitory potencies of the various stilbene analogues) and follow a one-to-one relationship of binding to inhibition, a series of properties compatible with the conclusion that the DIDS site is also the substrate binding site of the anion transferring mechanism (Cabantchik & Rothstein, 1974).

The experiments with proteolytic enzymes eliminate the sialoglycoprotein as playing a possible role in anion permeability even though they bind a small proportion of the DIDS (5%). This conclusion is based on several observations. Firstly, treatment with neuraminadase to remove the sialic acid has no effect on anion permeability (Knauf & Rothstein, 1971), demonstrating that this component is not essential. Secondly, trypsin that removes half of the sialic acid and a large proportion of the sialoglycopeptide-bound DIDS (Fig. 2) has no effect on anion permeability (Passow, 1971). Thirdly, treatment with sufficient pronase to remove 90% of the sialic acid and most of the sialoglycopeptide-bound DIDS (Fig. 1) has no effect on anion permeability (Fig. 10). These observations rule out any role of the carbohydrate-peptide segment of the major sialoglycoprotein in anion permeability.

The remaining DIDS-binding component, the 95K protein is not digested by trypsin, but is split by chymotrypsin and pronase into two components, one of 65K and the other 35K. A detailed study with pronase demonstrates that after mild proteolytic digestion, the (^{3}H)DIDS label is found in three locations, 95K, 65K and 35K, but that the splitting of most of the 95K is not associated with any appreciable effect on anion permeability (Fig. 10) or on the inhibitory effects of DIDS. Furthermore, the 35K segment and its label is digested away under certain conditions (failure to

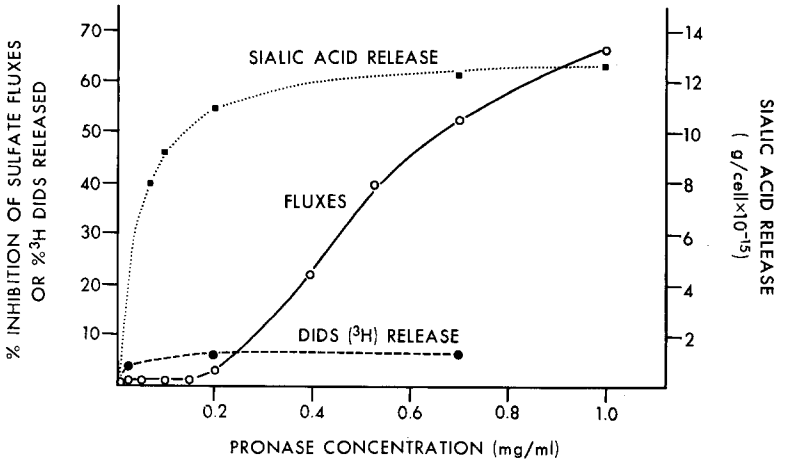

Fig. 10. The effect of pronase on different properties of the red blood cell. The values of sialic acid and (^{3}H)DIDS release are taken from Fig. 2. The effect of pronase on anion fluxes ($^{35}SO_4^2$ exchange) were performed as described in Materials and Methods, and are presented as per cent inhibition of the untreated cells

wash with albumin, as described in Results) that do not in themselves influence anion permeability. It can be concluded, therefore, that the 35K segment of the 95K protein plays no role in anion permeability and that the binding of DIDS to this segment (15% of the total binding) is nonspecific, unrelated to the inhibition. The remaining components are the 65K segment with 80% of the total DIDS-binding sites, and a residual pronase resistant component at 95K with 5% of the sites. At present, there is no basis for selecting between the two as potential components of the anion permeation system.

A serious complication in proposing a role for the 95K protein or the 65K segment in permeability is the apparent heterogeneity of the 95K protein revealed by its behavior to proteolytic attack (Table 1). Another possibility that cannot be ignored is the existence of a small permeability component that contains such a small fraction of the total DIDS-sites that its labeling is below the sensitivity of the procedures. It would have to be a component that binds less than 1 to 2% of the total membrane-bound DIDS (less than 3000 to 6000 sites per cell).

Pronase itself at high concentrations inhibits anion permeability (Knauf & Rothstein, 1971; Passow, 1971). It is not at all clear whether the mechanism is related to the DIDS inhibition. No particular change in labeling with (^{3}H)DIDS can be correlated with the pronase inhibition.

The experiments with proteolytic enzymes reveal some interesting information concerning the arrangement of proteins on the outer surface of the cell. The major sialoglycoprotein is presumably extended out from the membrane because of its high negative charge density and is, therefore, accessible to proteolytic attack. The first attack, however, with trypsin or chymotrypsin only removes a small segment leaving a lower molecular weight glycoprotein visible in the gels (Figs. 3 and 4).

The proteolytic attack exposes a small amount of lipid component to reaction with DIDS (Table 2 and also the peak of label at the SDS front in Figs. 8 and 9). This effect is greatest with pronase, but is also appreciable with trypsin. Because the latter only attacks the sialoglycoproteins, and not the 95K protein, the exposure of lipids must be attributed in part to the digestion of the former. The screening effect of the high density of negative charge of the sialic acid residues on the access of the negatively charged DIDS to the lipid surface might account for the low binding to lipid except in cells treated with proteolytic enzymes. It is also possible as recently observed (Bretscher, 1972; Gordesky & Marinetti, 1973) that lipids are asymmetrically arranged in the red blood cell membrane with the majority of the phosphatidyl-ethanolamine and phosphatidyl-serine facing the inside surface of the membrane scarcely available to compounds reacting at the outer surface. Thus, proteolysis may cause some localized rearrangements of lipids exposing chemical species which otherwise would be inaccessible to the chemical probes. It should be noted, however, that Bretscher (1972) using another surface label, found no increase in its reaction with phosphatidyl-ethanolamine of red blood cell membranes predigested with pronase. The present studies agree, on the other hand, with Gahmberg and Hakomori (1973) who have recently shown an increased surface labeling of glycolipids of red blood cells pretreated with pronase or trypsin and postlabeled by the galactose oxidase-(^{3}H)-sodium borohydride method. Treatment with trypsin, pronase and chymotrypsin also allows a somewhat increased labeling in the 95K protein (Figs. 8 and 9), an effect also explicable by the removal of the screening effect of the sialic acid residues or the exposure of targets by the physical removal of carbohydrate containing peptides (Phillips & Morrison, 1973). Those rather localized effects would suggest a close geographical proximity of the 95K and the sialoglycoprotein in the membrane matrix. The very large increase in labeling of the 65K and 35K segments resulting from pronase or chymotrypsin treatment (Figs. 8 and 9) could be due either to removal of the sialic acid residues or to exposure of new sites because of the splitting of the 95K protein. This suggests that those sites in the 65K segment that are inaccessible to (^{3}H)DIDS either before or after

proteolysis are inaccessible or highly resistant to enzymes like pronase or chymotrypsin in the intact cell. The fact that no additional proteins other than 95K or its products are labeled after proteolytic treatment indicates that no major rearrangements of membrane proteins has occurred at the outer surface.

After proteolytic treatment less than 5% of the bound (H^3)DIDS is released into the medium, accounted for by hydrolysis of the sialoglycoprotein. This data suggests that the sialoglycoprotein and the 95K protein are the only proteins on the outer surface. If, for example, another major protein component were bound to the surface it would be expected to bind (^{3}H)DIDS but might not be seen in the ghosts because it dissolved during hemolysis and washing. It should, however, after attack by pronase in the intact cell, be hydrolyzed with release of its (^{3}H)DIDS into the medium. No such release was observed.

A more interesting speculation relates to the possible arrangement of the 95K protein in the membrane and its relationship to anion permeability. The 95K protein seems to be at least in part hydrophobically bonded, a conclusion based on its difficulty of solubilization (Tanner & Boxer, 1972; Yu, Fishman & Steck, 1973). It is not extracted by high or low ionic strength, high pH, EDTA or 8 M urea whereas almost all other proteins except about 25% of the sialoglycoproteins are extracted (Juliano & Rothstein, 1971). Furthermore, several kinds of evidence suggest that it is a major component of the intralipid particles seen by the freeze-fracture technique of electron-microscopy (Rothstein & Cabantchik, 1973). On the other hand, it must also be at least partly exposed to the outside insofar as it is labeled by nonpenetrating probes such as (^{3}H)DIDS (Cabantchik & Rothstein, 1972) and others (Bretscher, 1971), it is iodinated by peroxidase (Phillips & Morrison, 1971) and it is split by pronase and chymotrypsin as demonstrated in this paper. Because in the intact cell the 35K segment can be digested and the 65K segment cannot [except in leaky ghosts where all proteins are digested (Triplett & Carraway, 1972)], it is proposed that the 65K segment is hydrophobically bonded, associated with the intralipid particles, whereas the 35K segment is associated with the outer face of the lipid layer. Part of the 65K segment must, however, also be partly exposed because it contains most of the bound (^{3}H)DIDS. Furthermore, after proteolytic attack it is found to bind considerably more of the label. It has also been reported that the inhibitory potency of the disulfonic stilbene, SITS (Takeshita & Rothstein, *unpublished observations*) and of FDNB (Passow, 1971) on anion permeability are increased after pronase treatment, reinforcing the concept that the inhibitory site is located on a portion of the 65K segment that is at

the outer surface of the membrane. Studies by Lepke and Passow (1973) support the view that phloridzin inhibition of anion permeability is exerted on the outer surface of the red blood cell membrane.

We have recently observed (Cabantchik, Balshin & Rothstein, *submitted for publication*) that pyridoxal-phosphate, a slowly penetrating anion, will form Schiff bases with amino groups on either side of the membrane of intact red blood cells and can be irreversibly fixed with sodium borohydride. The reaction with sites in the 95K protein is equally effective at the inner and outer surface of the intact cell, and the anion permeability is substantially inhibited from both sides, unlike Lepke and Passow's (1973) recent findings with phloridzin.

The 95K protein band, however, may not only be associated with anion permeability, but with other functions as well. The phosphorylated intermediate of the (Na^+–K^+)-ATPase activity is found in the same location by acrylamide gels (Avruch & Fairbanks, 1972; Knauf, Proverbio & Hoffman, 1973) as is TNBS (Arroti & Garvin, 1972), an inhibitor of sugar transport and anion permeability (Rothstein, Knauf, Cabantchik & Balshin, 1973).

These findings, and other evidence of heterogeneity discussed earlier, would suggest that the 95K band is a collection of proteins of similar molecular weights and of similar location and orientation in the membrane, some of which are apparently involved in specific transport and permeability functions.

This work was supported in part by the Medical Research Council of Canada, Grant No. MA-4665.

References

Arroti, J. J., Garvin, J. E. 1972. Selective labelling of human erythrocyte components with tritiated trinitrobenzenesulfonic acid and picryl chloride. *Biochem. Biophys. Res. Commun.* **49**:205

Avruch, J., Fairbanks, C. 1972. Demonstration of a phosphopeptide intermediate in the Mg^{++}-dependent Na^+- and the K^+-stimulated adenosine triphosphatase reaction of the erythrocyte membrane. *Proc. Nat. Acad. Sci.* **69**:1216

Bender, W. W., Garan, W., Berg, H. C. 1971. Proteins of the human erythrocyte membrane as modified by pronase. *J. Mol. Biol.* **58**:783

Bretscher, M. S. 1971. Human erythrocyte membranes: Specific labelling of surface proteins. *J. Mol. Biol.* **58**:775

Bretscher, M. S. 1972. Phosphatidylethanolamine: Differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. *J. Mol. Biol.* **71**:523

Cabantchik, Z. I., Rothstein, A. 1972. The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Membrane Biol.* **10**:311

Cabantchik, Z. I., Rothstein, A. 1974. Membrane proteins related to anion permeability of human red blood cells. I. Localization of disulfonic stilbene binding sites in proteins involved in permeation. *J. Membrane Biol.* **15**:207

Cook, G. M., Eylar, E. H. 1965. Separation of the M and N blood group antigens of the human erythrocyte. *Biochim. Biophys. Acta* **101**:57

Dacie, J. V., Lewis, S. M. 1968. Practical Hematology. p. 481. Grune and Stratton Inc., New York

Dodge, J. T., Mitchell, C., Hanahan, D. 1963. The preparation and chemical characteristics of hemglobin free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **110**:119

Fairbanks, G., Steck, T. L., Wallach, D. F. H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2806

Gahmberg, C. G., Hakomori, S. J. 1973. External labelling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. *J. Biol. Chem.* **248**:4311

Gordesky, S. E., Marinetti, G. V. 1973. The asymmetric arrangement of phospholipids in the human erythrocyte membrane. *Fred. Proc.* **32**:674

Hamaguchi, H., Cleve, H. 1972. Solubilization of human erythrocyte membrane glycoproteins and separation of the MN glycoprotein from a glycoprotein with I, S, and A activity. *Biochim. Biophys. Acta* **278**:271

Juliano, R. L., Rothstein, A. 1971. Properties of an erythrocyte membrane lipoprotein fraction. *Biochim. Biophys. Acta* **240**:227

Knauf, P. A., Proverbio, F., Hoffman, J. F. 1974. Chemical characterization and pronase susceptability of the Na:K pump associated phosphoprotein of human red blood cells. *J. Gen. Physiol.* (*In press*)

Knauf, P. A., Rothstein, A. 1971. Chemical modification of membranes. I. Effect of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell. *J. Gen. Physiol.* **58**:190

Lepke, S., Passow, H. 1973. Asymmetric inhibition by phorizin of sulfate movements across the red blood cell membrane. *Biochim. Biophys. Acta* **298**:529

Maddy, H. 1964. A fluorescent label for the outer components of the erythrocyte membrane. *Biochim. Biophys. Acta* **88**:390

Passow, H. 1971. Effects of pronase on passive ion permeability of the human red blood cell. *J. Membrane Biol.* **6**:233

Phillips, D. R., Morrison, M. 1971. Exposed protein on the intact human erythrocyte. *Biochemistry* **10**:1766

Phillips, D. R., Morrison, M. 1973. Changes in accessibility of plasma membrane protein as the result of trypsin hydrolysis. *Nature, New Biol.* **242**:213

Rosenberg, S. A., Guidotti, G. 1968. The protein of human erythrocyte membranes. I. Preparation, solubilization and partial characterization. *J. Biol. Chem.* **243**:1985

Rothstein, A., Cabantchik, Z. I. 1973. Protein structures involved in the anion permeability of the red blood cell membrane. *In:* Comparative Biochemistry and Physiology of Transport. K. Block, L. Bolis and S. E. Luria, editors. North Holland Publishing Co. (*In press*)

Rothstein, A., Knauf, P. A., Cabantchik, Z. I., Balshin, M. 1973. The location and chemical nature of drug "targets" within the human erythrocyte membrane. *In:* Proceedings of the Biological Council Symposium on Drugs and Transport Processes. B. A. Callingham, editor. The Macmillan Press, London (*In press*)

Segrest, J., Kahane, I., Jackson, R. L., Marchesi, V. T. 1973. Major glycoprotein of the human erythrocyte membrane: Evidence for an amphipathic molecular structure. *Arch. Biochem. Biophys.* **155**:1973

Steck, T. L., Fairbanks, G., Wallach, D. F. H. 1971. Disposition of the major proteins in the isolated erythrocyte membrane. Proteolytic dissection. *Biochemistry* **10**:2617

Tanner, M. J. A., Boxer, O. J. 1972. Separation and some properties of the major proteins of human erythrocyte membranes. *Biochem. J.* **129**:333

Triplett, R. B., Carraway, K. L. 1972. Proteolytic digestion of erythrocytes, resealed ghosts and isolated membranes. *Biochemistry* **11**:2897

Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**:1971

Winzler, R. J. 1969. A glycoprotein in human erythrocyte membranes I. *In:* Red Cell Membrane: Structure and Function. G. A. Jamieson and T. J. Greenwalt, editors. p. 157. J. B. Lippincott, Philadelphia

Yu, J., Fishman, D. A., Steck, T. L. 1973. Selective solubilization of proteins and phospholipids from red blood cell membranes by anionic detergents. *J. Supramol. Struct.* **1**:233