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EFFECTS OF INCORPORATED TRYPSIN ON ANION EXCHANGE AND MEMBRANE PROTEINS IN HUMAN RED BLOOD CELL GHOSTS

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SUMMARY

Varying concentrations of trypsin were sealed into human red cell ghosts and the effects on membrane proteins and sulfate equilibrium exchange were studied. After incubation for 45 min at 37 °C, pH 7.2, the following observations were made: above 10 ng/ml the ghosts undergo fragmentation without lysis. Dodecyl sulfate gel electrophoresis shows that the digestion of spectrin and of the protein in band 2.1 (nomenclature of Steck (1974) J. Cell. Biol. 62, 1–19) is nearly complete at 50 ng/ml, that of the protein in band 3 at 25 μg/ml. After digestion at 25 μg/ml, about 60 % of the total protein of the membrane is released and the original bands of conventional dodecyl sulfate gel electropherograms of the remaining protein are nearly completely abolished. In their place three new bands appear representing peptides with molecular weights of 58 000, 48 000 and 34 000, respectively. Sometimes a fourth peptide with a molecular weight of approx. 13 000 is also observed. Using a radioactive labeling technique it is shown that the two peptides with the highest molecular weights are derived from the protein in band 3. Labeling with diazo[^{35}S]sulfanilic acid indicates that in addition to the peptides in the described four Coomassie blue-stainable bands, other peptides with molecular weights up to 100 000 are still present in the exhaustively trypsinized ghosts.

External trypsin has no effect on the sulfate equilibrium exchange in ghosts while internal trypsin causes inhibition. Inhibition becomes apparent at trypsin concentrations exceeding those required to produce a complete digestion of spectrin. It remains incomplete, even at the highest intracellular concentrations which cause maximal effects on all membrane proteins, including the protein in band 3. Under these conditions strong further inhibition can be produced by agents which are known to inhibit anion transport in untreated red cells and ghosts. These agents include the penetrating 1-fluoro-2,4-dinitrobenzene and the nonpenetrating phlorizin, 4-acetamido-4′-isothiocyanato stilbene-2,2′-disulfonic acid, 4,4′-diacetamido stilbene-2,2′-disulfonic acid, and 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′,7-disulfonic

Abbreviations: APMB, 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′,7-disulfonic acid; DAS, 4,4′-diacetamidostilbene-2,2′-disulfonic acid; N_2ph-F, 1-fluoro-2,4-dinitrobenzene; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; EDTA, ethylene diamine tetra acetic acid.

acid (APMB). Unlike the other nonpenetrating inhibitors APMB is not only capable of inhibiting at the outer but also at the inner membrane surface. Treatment with internal trypsin does not significantly reduce the inhibition by incorporated APMB. The described observations suggest that after exhaustive tryptic digestion of the major membrane proteins, the receptor sites for typical inhibitors of anion transport continue to exert their function.

INTRODUCTION

Proteolytic enzymes have been extensively used as tools for the elucidation of various aspects of membrane structure and function (for review, see Steck, 1974, ref. 1). Most protein species in the red blood cell membrane are inaccessible to proteolytic attack at the outer membrane surface while few are resistant to such attack at the inner membrane surface. Thus, external trypsin degrades glycophorin but not the other major protein species which appear as Coomassie blue-stainable bands on dodecyl sulfate gel electropherograms [2, 3, 5]. External pronase [4–6], papain [2], and chymotrypsin [2, 5, 6] cleave among the proteins in the Coomassie blue-stainable bands those in band 3, while all other protein species remain undigested. In contrast, in membrane preparations in which both surfaces are accessible to the enzymes [3–5], or in inside-out vesicles, where the internal membrane surface is exposed to the outside [2], virtually all of the membrane proteins are affected. This applies not only to experiments with the rather non-selective enzymes papain, chymotrypsin and pronase, but also to experiments with the highly selective trypsin. In these experiments, trypsin degrades all protein species with the exception of the protein in band 6 (glyceraldehyde-phosphate dehydrogenase) which is released from the membrane but not hydrolyzed [5, 7, 23].

Exposure of intact red cells to external pronase or papain leads to an inhibition of anion exchange [8, 9]. Although it has been suggested that the protein in band 3 may be involved in the control of anion permeability [17, 19], the cleavage of the protein in this band by external pronase could not be related to the inhibition of anion exchange [6]. External trypsin even at very high concentration has no effect [8].

The present paper is concerned with the action of internally applied trypsin on membrane proteins and anion exchange. After incorporation of trypsin into resealable red cell ghosts [13] the ghosts fragment into small vesicles but do not lyse. Spectrin is digested first, followed by the protein in band 3 and in the other bands of dodecyl sulfate gel electropherograms. After removal of about 60% of the total membrane protein and splitting of the protein in band 3 into two trypsin-resistant moieties, anion movements are partially inhibited. They can be further inhibited by phlorizin and a variety of other agents which are supposed to inhibit anion exchange in the intact cells by combination with the protein in band 3. These experiments demonstrate an asymmetry of the anion transfer system with respect to its sensitivity to trypsin modification and are compatible with the assumption that one or both moieties of the protein in band 3 are capable of performing at least part of the same functions as the intact protein.

Essential results of the present work have been published in a review paper

which appeared in the proceedings of the Ninth FEBS meeting in Budapest 1974 (Passow, H., Fasold, H., Zaki, L., Schuhmann, B. and Lepke, S. (1974) in Biomembranes, Structure and Function, Federation of the European Biochemical Society's Proceedings of the Ninth Meeting, Budapest, 1974 (Gárdos, G. and L. Szász, L., eds.), Vol. 35, pp. 197–214, Publishing House of the Hungarian Academy of Sciences, Budapest).

MATERIALS AND METHODS

The experiments were performed with ORh^+ blood from healthy donors which was used between 2 and 5 days after collection. The blood was preserved in acid citrate dextrose buffer and washed thrice in isotonic saline prior to use.

Prepartion of resealable ghosts

Ghosts were prepared by osmotic hemolysis at 0 °C of one part of a 50 % cell suspension in isotonic NaCl solution in 10 volumes of a medium containing 4 mM $MgSO_4$, 1.3 mM acetic acid, and the desired trypsin concentration. 5 min after hemolysis a concentrated salt solution containing sufficient sodium phosphate, Na_2SO_4 and KCl or NaCl to restore isotonicity was added. If not stated otherwise, the final concentrations of the added electrolytes in the hemolysate were 20.0, 5.0, and 122.5 mM, respectively. The pH was adjusted to 7.2. Subsequently, the ghosts were washed once at 0 °C in a medium containing the listed electrolytes at the indicated concentrations. The wash was performed in the SS 34 angle rotor of a Sorvall centrifuge at 12 000 rev./min (5 min). The sedimented ghosts were resuspended in the described medium (hematocrit 5 %) and incubated at 37 °C for 45 min. At the end of this time period the ghosts were washed another two times at 12 000 rev./min in ice-cold medium. The described procedure leads to an uptake of trypsin during hemolysis. Since resealing for proteins is largely completed immediately after hemolysis, there is little loss of the incorporated trypsin during the wash at 0 °C prior to the incubation at 37 °C. Resealing for small electrolytes is subsequently achieved by incubation at the elevated temperature (see refs. 13, 14). The final preparation of resealed ghosts which contained trypsin and $^{35}SO_4$ was divided into two portions. One served for the preparation of white ghosts for dodecyl sulfate gel electrophoresis. The other was used for measuring SO_4 equilibrium exchange.

Chemical modification

In a number of experiments, the resealed ghosts were prepared from chemically modified red cells; in others, the resealed ghosts were dinitrophenylated prior to the flux measurements. Exposure to diazotized [^{35}S] sulfanilic acid was performed as described in ref. 4 (2.0 mM dissolved in 25 mM sodium phosphate pH 7.4, 135 mM NaCl, 20 mM sucrose, hematocrit 50 %, time of incubation 20 min, temperature 25 °C). After three washes in the absence of diazosulfanilic acid, the modified cells were ghosted, treated with trypsin and resealed as described above. Dinitrophenylation of resealed ghosts was performed prior to the flux measurements and the preparation of white ghosts at 37 °C in the dark under the conditions described in the results section.

Measurement of sulfate equilibrium exchange

The sedimented, $^{35}SO_4$-loaded ghosts were resuspended at 30 °C, pH 7.2 and the appearance of radioactivity in the supernatant was followed. In addition, the total radioactivity in ghosts plus medium was determined. Since the extracellular radioactivity was removed by washing at 0 °C prior to the initiation of the flux measurements at 30 °C, this latter determination represents an approximate measure of the total amount of $^{35}SO_4$ trapped inside the ghosts after resealing. The amount of $^{35}SO_4$ retained by experimentally modified ghosts is expressed as a percent of the total radioactivity found in simultaneously prepared untreated ghosts. This percentage is designated "sulfate retention".

The rate of $^{35}SO_4$ movements is expressed in terms of either rate constants k or half-times $t_{\frac{1}{2}}$. The constants were derived from plots of the time course of the appearance of the radioactivity in the supernatant, k was calculated from initial slopes and equilibrium values. $t_{\frac{1}{2}}$ was read off directly. In populations of untreated ghosts or ghosts containing low concentrations of trypsin, the theoretically expected relationship $k = 0.693/t_{\frac{1}{2}}$ was actually observed. In ghost populations containing sufficient trypsin to cause excessive fragmentation and formation of sealed vesicles this was no longer true. Under these conditions rate constants derived from initial slopes tend to overemphasize the contribution of the loss from small or leaky vesicles to the total loss. For this reason we express our results obtained with high concentrations of trypsin in terms of $t_{\frac{1}{2}}$, with low concentrations in terms of k.

It should be noted that under our experimental conditions, trypsin is still present inside the ghosts when the flux measurements are made. Dodecyl sulfate gel electropherograms of the ghost membranes show that after the resealing periods at 37 °C which preceed the flux measurements, at trypsin concentrations > 10 μg/ml the digestion of the major membrane proteins is virtually complete (see page 358). For this reason, the flux measurements were made at trypsin concentrations exceeding this value. The only exception is the experiment in Fig. 2 where the concentration of internal trypsin was too low to produce much change of sulfate transport.

Preparation of white ghosts and dodecyl sulfate polyacrylamide gel electrophoresis

Resealed ghosts were sedimented and rehemolyzed at 0 °C in 20 times their volume in a solution containing 0.1 % saponin, 10 mM KCl, 5 mM EDTA, 1.0 mg/ml soy bean trypsin inhibitor at pH 7.0; they were subsequently washed in this medium once with trypsin inhibitor present and thereafter without the inhibitor until white. Aliquots of the final sediment were used for dodecyl sulfate gel electrophoresis and protein determinations by the method of Lowry et al. [16]. Washing was performed by centrifugation in an SS 34 Sorvall angular rotor at 20 000 rev./min for 10 min. At 20 000 rev./min the membranes prepared from untreated ghosts sediment completely in this time period. The ratio between proteins and phospholipids in the final sediment is independent of the time of centrifugation at each wash (1.09 (10 min), 1.01 (20 min), 1.07 (30 min)). However, in the trypsin-treated ghosts, the ratio decreases with time of centrifugation (0.63 (10 min), 0.56 (20 min), 0.51 (30 min)), indicating that the distribution of proteins and lipids is not uniform. The preparation of white ghosts was carried out parallel to the measurement of the kinetics of anion exchange. This made it impossible to centrifuge for more than 10 min at each wash and led to a systematic underestimate of the extent of enzymatic degradation of the membrane proteins.

After dissolving the white ghosts in 5 % sodium dodecyl sulfate solution, heating at 100 °C for 3 min, and dilution to 0.5 % sodium dodecyl sulfate, 15–50 μg membrane protein (as determined by the method of Lowry et al. [16]) were layered on top of the gels composed of 5 % acrylamide and 0.25 % bis acrylamide. The gels contained 6 M urea, 0.1 M sodium phosphate, and 0.5 % sodium dodecyl sulfate; the pH was 7.1, the electrode buffer was 0.05 M sodium phosphate, pH 7.1, containing sodium dodecyl sulfate but no urea. After 10 min at 1 mA/tube, the current density was adjusted to 5 mA/tube. Bromophenolblue was used as a tracking dye.

All gels were run in duplicate; one gel was stained with Coomassie blue and the other was cut into about 40 slices for determination of ^{3}H and ^{14}C. Prior to measuring the radioactivity, the slices were digested at 50 °C in Soluene 350 (Packard) and then dissolved in acidified Insta Gel (Packard) for counting at 4 °C in the dark. The recovery of the radioactivity on the gels was of the order of 90 % for ^{14}C and of 80–85 % for ^{3}H.

Molecular weights were determined by using a range of proteins with known molecular weights to establish the semilogarithmic standard curve. The determinations are based on five independent experiments which were evaluated by separate standard curves. The standard error of the mean values was 2.5–5 %.

Materials

Trypsin inhibitor and crystalline trypsin were obtained from Serva. The latter was designated free of Chymotrypsin. [^{14}C]N_2ph-F and [^{3}H]N_2ph-F were purchased from Amersham/Buchler and SITS from British Drug House. AMPB and DAS were gifts from Professor Beyer (Leverkusen) and Fasold (Frankfurt). SITS and APMB were purified as described previously [12].

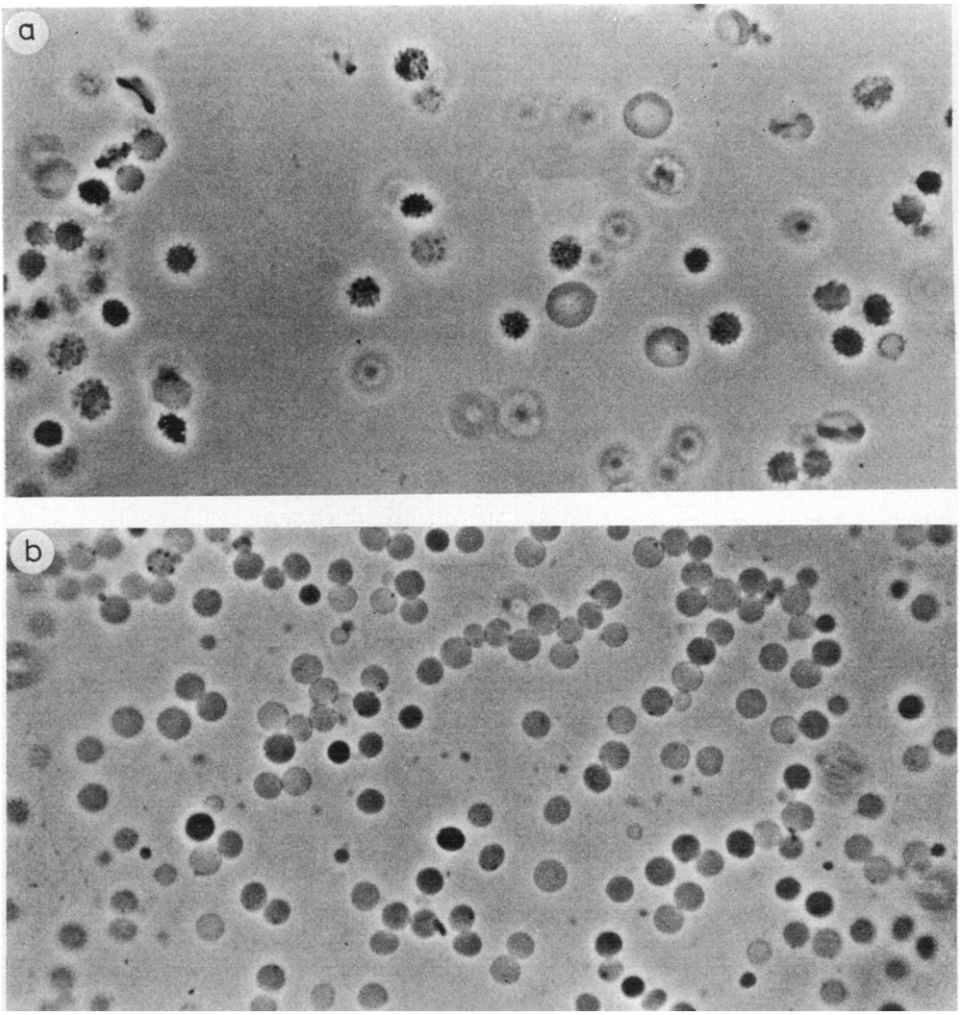
RESULTS

(1) *Fragmentation*

After incorporation of trypsin during hemolysis at 0 °C at concentrations ranging from 50 ng/ml to 50 μg/ml and subsequent incubation at 37 °C, the originally crenated or disk-shaped ghosts (Fig. 1a) are transformed into spherical vesicles of varying sizes with diameters which are smaller than those of the ghosts from which they are derived. At the same time very small globular vesicles are released (Fig. 1b). With increasing time of incubation, the initially shrunken vesicles swell considerably. Centrifugation on a sucrose cushion leads to the retention on top of the cushion of the larger vesicles and of a considerable number of small globules while the remaining globules and a few of the larger vesicles sediment to the bottom of the centrifuge tube (Fig. 1d). Dodecyl sulfate polyacrylamide gel electropherograms of the proteins in the membrane fractions at the top and the bottom of the sucrose cushion show essentially the same pattern. External trypsin (25 μg/ml) also affects the shape of the ghosts and converts a certain fraction of the ghosts into vesicles and globules of an appearance which is similar to that in preparations of the internally trypsinized ghosts (Fig. 1c). Again, the effect varies with time.

(2) *Effects on membrane proteins*

(*a*) *Distribution of Coomassie blue-stained proteins.* After incorporation of



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trypsin at concentrations of 10 ng/ml or more and incubation for 45 min at 37 °C, changes of the membrane proteins become apparent (Fig. 2). Around 50–100 ng/ml the total amount of membrane protein is reduced by about 50 %. Most of the spectrin and virtually all of the protein in band 2,2 has disappeared and the amount of protein in band 3 is significantly reduced. At trypsin concentrations exceeding 10 μg/ml only a trace of band 3 is left at a location on the gel which corresponds to a molecular weight of 106 000 (Fig. 3). In place of the original band pattern, a new pattern is established with three components only, two major components with molecular weights of 58 000 and 48 000, respectively, and one minor component with a molecular weight of about 34 000. Near the bromophenol blue front there is usually but not always an additional component with a molecular weight of about 13 000. The degradation of the membrane proteins ceases when the described band pattern has

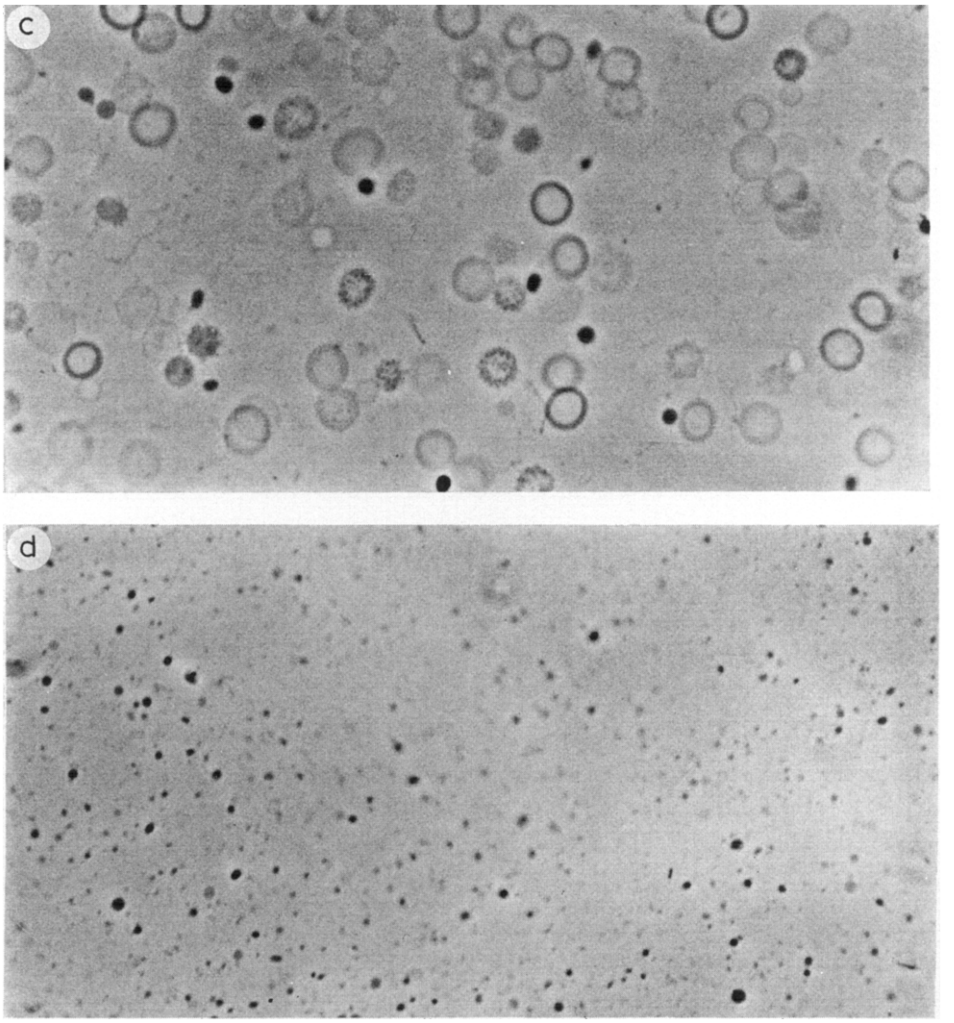


Fig. 1. Effect of trypsin on human red cell ghosts. (a) control, no trypsin; (b) internal trypsin, 25 μg/ml; (c) external trypsin, 25 μg/ml; (d) internal trypsin. "Globules" were collected at the bottom of sucrose cushion after centrifugation for 90 min at 20 000 rev./min in a Sorvall SS34 angular rotor. For experimental conditions see Materials and Methods. Incubation was at 37 °C without or with trypsin for 45 min. Microscopical magnification: × 790.

been established. Since all proteins with molecular weights exceeding those of the proteins in band 3 are hydrolyzed and removed from the membrane before the proteins in band 3 are significantly degraded (cf. Fig. 2), and since the sum of the molecular weights of the proteins in the new bands of 58 000 and 48 000 is virtually identical with our estimate of the molecular weights of the protein in band 3, it is likely that these two major new bands constitute the products of hydrolysis of the protein in band 3. More conclusive evidence for this inference can be derived from the experiment with radioactively labeled membranes presented in section (b).

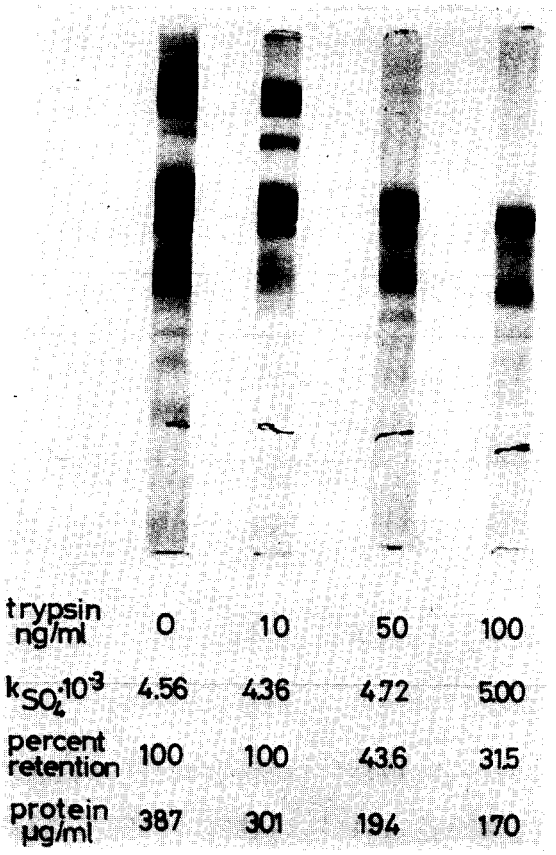
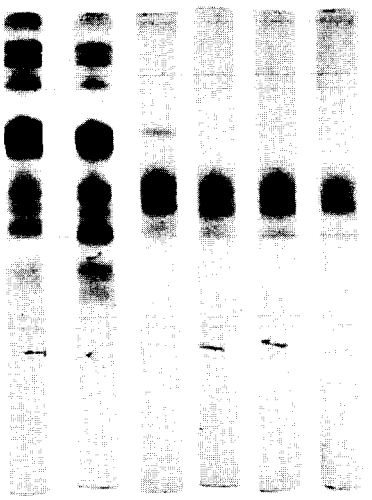


Fig. 2. Effect of internal trypsin on the major membrane proteins, rate of sulfate equilibrium exchange, and sulfate retention (definition see p. 356) in red cell ghosts. Incubation for 45 min at 37 °C. Subsequent measurement of sulfate equilibrium exchange at 30 °C, pH 7.4. k_{SO_4} represents the difference of the rate constants with and without added SITS (0.5 mM). The characteristic doublet close to the origin (better seen on the photographs of the gels in Figs. 3 and 5) is spectrin. The major band below spectrin is band 3. The marks at the lower end of the gels indicate the positions of the tracking dye. Protein was determined in the ghosts derived from a fixed number of red cells.

Control experiments with external trypsin show no significant modification of the distribution pattern of the Coomassie blue stained bands.

(*b*) *Common binding sites for SITS and N_2ph-F.* It has been shown previously that two inhibitors of anion exchange, the nonspecific and penetrating N_2ph-F and the more specific and nonpenetrating SITS combine with the same sites on the protein in band 3 of the dodecyl sulfate polyacrylamide electropherograms [11, 12]. The electropherogram represented in Fig. 4a confirms the previous results. On this electropherogram the membrane proteins of two different batches of ghosts were simultaneously separated. One batch was made from resealed ghosts which had been dinitrophenylated with [^{3}H]N_2ph-F after pretreatment with a maximally inhibitory concentration of SITS, the other batch was made from ghosts which had been dinitrophenylated with [^{14}C]N_2ph-F without pretreatment with SITS. The isolated mem-



trypsin µg/ml	in 0	out 500	in 10	in 50	in 100	in 500
$^{35}SO_4$ retention percent	100	82.4	37.8	34.6	37.4	35.7
protein µg/ml	330	335	128	130	130	98
[^{35}S] sulfanilicacid 10^6molecules/ghost	9.67	8.09	5.56	6.00	6.27	5.35

Fig. 3. Effect of internal trypsin on ghosts derived from red cells labeled with diazo[^{35}S]sulfanilic acid as described in ref. 4. Incubation with trypsin for 45 min, at 37 °C. "in" and "out" refer to internal and external trypsin, respectively. Protein was determined in the ghosts derived from a fixed number of red cells.

branes from both batches were combined, dissolved in sodium dodecyl sulfate solution and subjected to electrophoresis on the same gel. Obviously, pretreatment with SITS considerably reduces the dinitrophenylation of the protein in band 3*. If the ghosts are first incubated with internal trypsin and then treated as described above first with SITS and subsequently with radioactively labeled N_2ph-F, the reduction of dinitrophenylation can still be observed. However, the common binding sites are now located in the bands with molecular weights of 58 000 and 48 000. The protein in band 3 and its binding sites for SITS and N_2ph-F nearly completely disappeared (Fig. 4b). In the electropherogram represented in Fig. 4c, membranes derived from untreated ghosts which had been dinitrophenylated with $^3HN_2$ph-F were mixed with membranes derived from trypsinized ghosts which had been dinitrophenylated with [^{14}C]N_2ph-F. Since there was no spectrin left after trypsination all the radioactivity in the trypsinated ghosts is derived from the protein in band 3. The electro-

* In a previous publication (ref. 12, Fig. 7) we have shown that the number of common binding sites for SITS and N_2ph-F on glycophorin is negligible as compared to the number on the protein in band 3. For this reason in the present experiments, glycophorin was not separated from the protein in band 3.

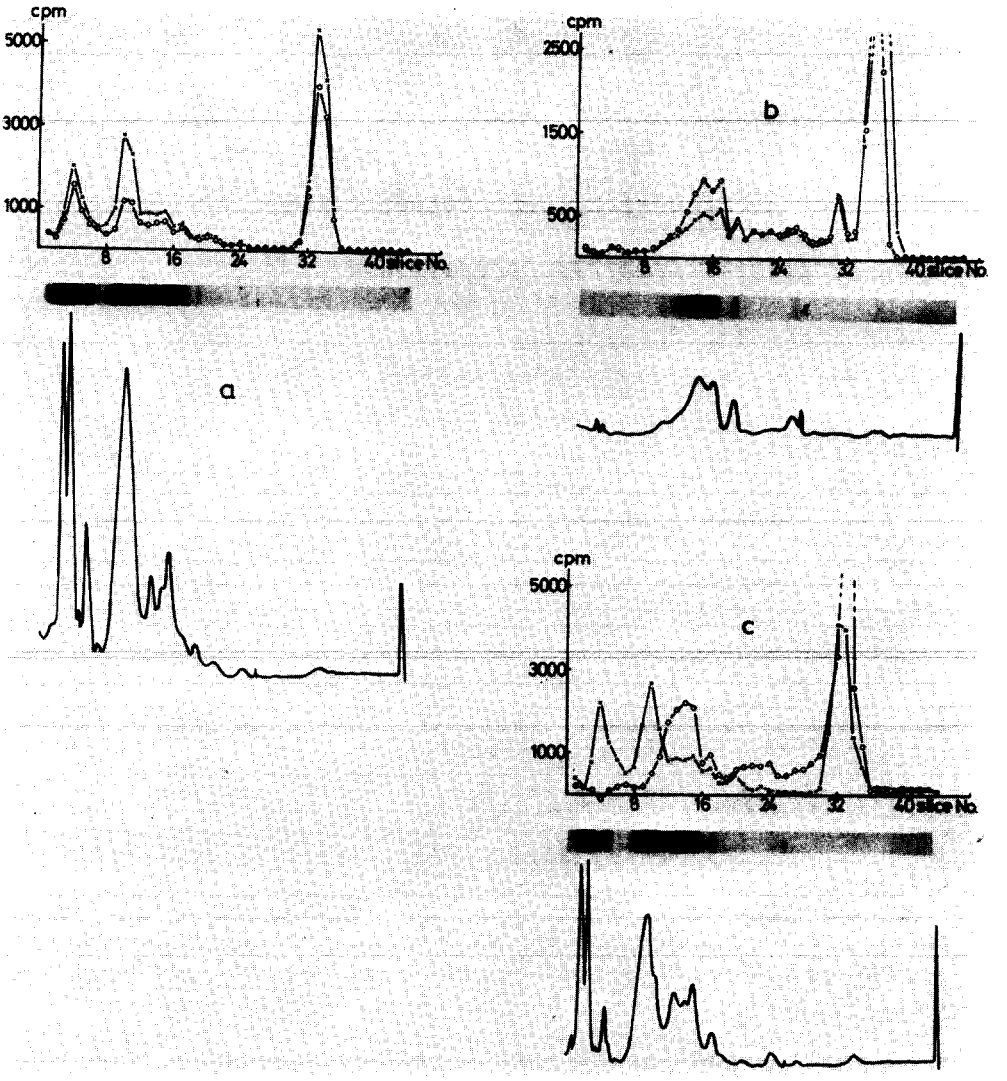


Fig. 4. Effect of treatment with SITS (0.25 mM, 37 °C, 5 min) on subsequent dinitrophenylation (0.28 mM N_2ph-F, pH 7.2, 37 °C, 30 min, hematocrit 10 %) of untreated resealed ghosts and resealed ghosts which had been exposed to internal trypsin (25 μg/ml) for 45 min. at 37 °C prior to the chemical modification. The curves above the photographs of the gels represent the determinations of radioactivity, below the densitogram tracings of the Coomassie blue staining. (a) Untreated ghosts dinitrophenylated in the absence (+) or presence (○) of SITS. (b) Internally trypsinated ghosts dinitrophenylated in the absence (○) or presence (+) of SITS. (c) Mixture of membranes from untreated ghosts (+) and internally trypsinized ghosts (○); both were dinitrophenylated in the absence of SITS. Note that in the different figures the same symbols (○ or +) refer to different experimental conditions.

pherogram shows convincingly that the molecular weight of the split products is lower than that of the undigested protein.

Although the resolution on the gels is not perfect, it is obvious that the common binding sites for SITS and N_2ph-F which were originally associated with band 3 are about equally distributed between the two bands at 58 000 and 48 000 daltons. This would suggest that some protein molecules in band 3 carry more than one stilbene disulfonic acid binding site.

(*c*) *Labeling with diazo*[^{35}*S*]*sulfanilic acid.* The previous section deals exclusively with the chemical and enzymatic modification of the so-called major membrane proteins, i.e. the more abundant membrane proteins which can be identified by Coomassie blue staining of dodecyl sulfate gel electropherograms of the unfractionated membrane. There exist, however, large numbers of "minor" membrane proteins which include, among others, the transport ATPases for Ca^{2+} and alkali ions, acetylcholinesterase and many other enzymes. Since it cannot be excluded that small quantities of such minor membrane proteins may carry some of the common binding sites for N_2ph-F and SITS, but cannot be detected by our technique, it seemed interesting to study whether or not these proteins are also affected by trypsin treatment. There is no simple method of identifying the various members of this group of proteins. However, labeling with [^{35}S] sulfanilic acid should provide a fairly sensitive measure of the presence or absence of membrane constituents which cannot be identified by Coomassie blue or labeling with N_2ph-F [4]. Fig. 5 confirms that the distribution of the radioactive label does not follow the Coomassie blue band pattern. It shows further that although external trypsin has little if any effect on the distribution pattern of the Coomassie stainable proteins, it releases about 15 % of the label and leads to a redistribution of the remaining radioactivity. Internal trypsin releases about 40 % of the label (Fig. 3). The ^{35}S peak in the region of band 3 disappears almost completely. However, even if the protein in band 3 is degraded to the point where it cannot be detected any longer by Coomassie blue staining, a considerable amount of the label still remains attached to membrane constituents in the region of the electropherogram where band 3 was originally located*. The major portion of the ^{35}S, however, is shifted to regions of lower molecular weights which include the locations of the newly formed bands at 58 000 and 48 000 daltons but which cannot simply be attributed to labeling of these two bands (Fig. 5).

(3) *Sulfate retention*

After resealing in $^{35}SO_4$-containing media and subsequent washes at 0 °C, the internally trypsinized ghosts still retain $^{35}SO_4$. However, SO_4 retention decreases with increasing concentration of internal trypsin until, at a trypsin concentration of 100 ng/ml or more, the SO_4 retention reaches about 30–40 % of the value found in untreated ghosts (Fig. 2). Above 100 ng/ml SO_4 retention becomes independent of

* The remaining label does not seem to be associated with surviving band 3 protein. This may be inferred from the sequence of gels represented in Fig. 5. In the untreated ghosts, or in ghosts containing low concentrations of trypsin, the diazo[^{35}S]sulfanilic acid labeling pattern clearly indicates the location of band 3. With increasing trypsin concentration, the label residing on band 3 is gradually removed until, at the highest concentration, band 3 is no longer discernible. The diffuse labeling in the region where band 3 was originally located is, therefore, unlikely to refer to a membrane constituent which could be called band 3 protein.

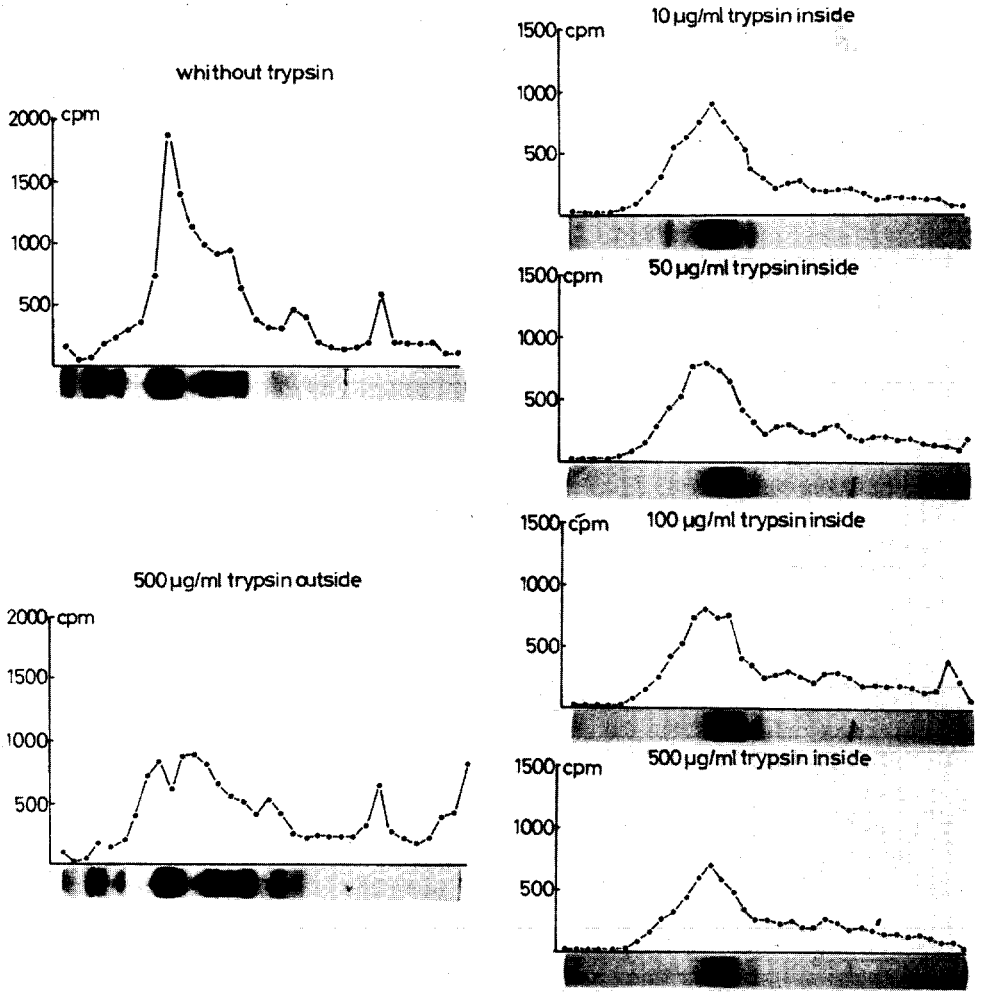


Fig. 5. Effects of external and internal trypsin on the distribution of diazo[^{35}S]sulfanilic acid among the various membrane proteins of resealed red ghosts. Time of exposure to trypsin 45 min, temperature 37 °C.

the trypsin concentration at which the ghosts were loaded prior to resealing. This is illustrated by the data in Fig. 3 where ghosts made from diazotized red cells retain about equal percentages of $^{35}SO_4$ at concentrations of internal trypsin ranging from 10 to 500 µg/ml. Similar observations have also been made with ghosts prepared from untreated red cells (not shown; however, data for a trypsin concentration of 25 µg/ml are represented in Table II). This finding indicates that during the washes at 0 °C the trypsin-treated membrane still constitutes a considerable barrier to the penetration of sulfate.

Sulfate retention as measured under our experimental conditions represents the average of the retention of many ghosts. It remains open whether this average reflects the behavior of most of the ghosts in the population or if some of the ghosts

lost much more $^{35}SO_4$ than the rest. However, the decreased retention must, at least in part, be related to the fragmentation of the ghosts during trypsination. Since the surface area is unlikely to increase, fragmentation should be associated with a decrease of the total volume surrounded by the given amount of membrane material. If one accepts this reasoning, sulfate retention should be a measure of fragmentation. This would imply that under our experimental conditions, fragmentation increases up to about 100 ng/ml internal trypsin, but does not proceed further even if the concentration of internal trypsin is raised up to 500 μg/ml (Figs. 2, 3).

(4) *Sulfate penetration*

Exposure to external trypsin up to 100 μg/ml for 45 min at 37 °C, pH 7.1 had no significant effect on the subsequently measured sulfate equilibrium exchange. The measured rates agreed to within 6 % with those measured in untreated ghosts. After trypsin treatment, anion exchange could still be reduced by the non-penetrating inhibitor DAS (Table I).

Exposure to internal trypsin up to 0.1 μg/ml (Fig. 2) slightly increased the rate of sulfate equilibrium exchange. This increase is possibly related to fragmentation which is indicated by the decrease of sulfate retention. At 25 μg/ml, internal trypsin decreases the rate of exchange (as expressed in terms of half-times, see Materials and Methods) by about 20–50 %. The well-known inhibitors of anion exchange, phlorizin, SITS, DAS and APMB are capable of further reducing the rate of sulfate exchange in the internally trypsinized ghosts (Table II, Fig. 6). However, the degree of inhibition is somewhat lower than in untreated ghosts. In three additional experiments trypsinized ghosts were exposed to 0.28 mM N_2ph-F at 37 °C, pH 7.2 for 30 min, subsequently washed at 0 °C to remove the unreacted modifier, and then subjected to flux measurements at 37 °C in the absence of modifier in the medium*. The degree of inhibition was 72, 74 and 37 %, respectively.

TABLE I

EFFECT OF EXTERNAL TRYPSIN ON THE INHIBITION OF SULFATE EQUILIBRIUM EXCHANGE BY DIACETAMIDOSTILBENE DISULFONIC ACID (DAS).

Prior to the flux measurements the resealed ghosts were exposed to the action of trypsin at the concentrations indicated below for 45 min at 37 °C, pH 7.4. After removal of the trypsin by washing at 0 °C, the equilibrium exchange was measured at 30 °C, pH 7.4. The inhibitor was only present during the flux measurements. $t_{\frac{1}{2}}$ represents the half-time of the equilibrium exchange as measured in the absence of DAS.

External trypsin (μg/ml)	$t_{\frac{1}{2}}$ (min)	External DAS (1.5 mM) (% inhibition)
0	65	73
2.5	69	78
25.0	69	83

* The inhibition of anion permeability as measured after termination of dinitrophenylation at 37 °C by washing, decreases with the temperature at which the flux determinations are made [9]. In order to obtain maximal effects, the flux was measured at 37 °C instead of at 30 °C as in the other experiments.

TABLE II

EFFECTS OF VARIOUS INHIBITORS ON SULFATE EQUILIBRIUM EXCHANGE IN INTERNALLY TRYPSINIZED AND UNTREATED RESEALED GHOSTS (30 °C)

Composition of the medium (mM): Na_2SO_4, 5.0; Sodium phosphate, 20.0; NaCl, 122.5 pH 7.1. Concentrations of inhibitors (mM): SITS, 0.25; DAS, 1.5; Phlorizin, 2.5.

Internal trypsin (μg/ml)	Control $t_{\frac{1}{2}}$	Inhibition (%)			SO_4 retention (%)
		SITS	DAS	Phlorizin	
–	85.5	79	60	60	40.9
25	107.5	73	52	52	
–	82	82	69	69	22.4
25	136	76	56	56	
–	132	92	66	64	31.2
25	149	70	44	40	
–	90	92	65	72	28.2
25	134	62	53	67	
–	60	87	64	67	29.1
25	130	76	23	26	

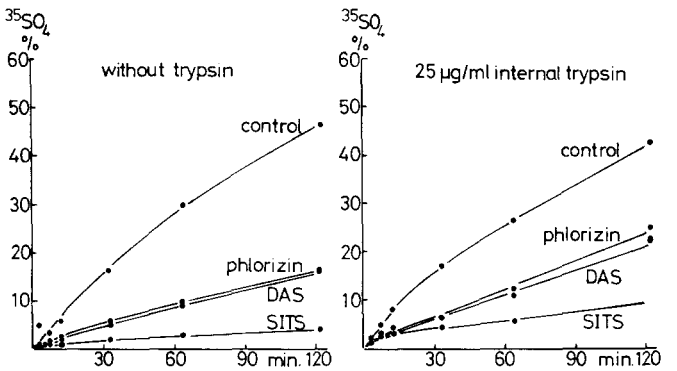


Fig. 6. Inhibition of $^{35}SO_4$ equilibrium exchange in untreated and internally trypsinized ghosts by phlorizin, DAS and SITS. The non-penetrating inhibitors were present in the external medium. Experimental conditions as described in legend to Table II. Ordinate: concentration of $^{35}SO_4$ in the medium as a percent of the equilibrium value. Abscissa: time in min.

In untreated ghosts the non-penetrating inhibitors DAS and phlorizin were shown to exert their inhibitory effect on sulfate equilibrium exchange at the outer but not at the inner surface of the membrane. In contrast, APMB was found to inhibit at either surface [12]. Fig. 7 shows that internal trypsin does not affect the capacity of internal APMB to inhibit sulfate exchange.

(5) *Cation permeability*

The cation permeability of the trypsinized ghosts has not been investigated in detail. However, it was found that ghosts become leaky for K^+ if exposed to external or internal trypsin. The susceptibility to external trypsin does not exist in intact cells [8]. This indicates that resealing under our experimental conditions does not lead to

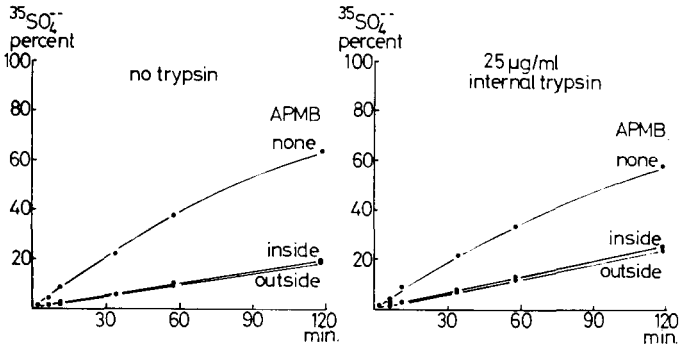


Fig. 7. Inhibition of $^{35}SO_4$ equilibrium exchange by external and internal APMB in untreated and internally trypsinized ghosts. The experimental conditions as described in the legend to Table II. Ordinate concentration of $^{35}SO_4$ in the medium, as a percent of the equilibrium value. Abscissa: time in minutes. APMB concentration 10 mM.

a complete reconstitution of the original organization of the proteins in the outer cell surface. The effect of trypsin on cation permeability does not represent the formation of a non-specific leak pathway. This is evident from the observed inhibition of anion movements.

DISCUSSION

The main result of the present paper consists of the finding that it is possible to measure anion transport in trypsin-containing ghosts and to demonstrate that the anion transport system, although partially inhibited by the enzyme, is still susceptible to powerful additional inhibition by typical modifiers of anion transport such as SITS, DAS, APMB, phlorizin and N_2ph-F. This result is noteworthy since it is obtained after a release from the the membrane of about 60 % of the total membrane protein and partial degradation of all of the remaining proteins whose split products continue to be attached to the membrane. Ghosts treated with internal trypsin should be useful, therefore, for attempts to identify the constituents of the anion transport system.

Among the three or four major peptides found to survive in the exhaustively trypsinized membrane only two carry common binding sites for two different inhibitors of anion transport, SITS and N_2ph-F, and hence are likely to be involved in anion transfer. These peptides with molecular weights of 58 000 and 48 000 are derived from the protein in band 3, the participation of which in anion transport has previously been suggested [17, 19]. Our findings are reminiscent of the observation that after exposure to low concentrations of pronase, the protein in band 3 is split into two moieties of 65 000 and 35 000 daltons without significant inhibition of anion exchange [6]. They would be compatible with the idea [6] that the protein is still able to carry out its transport function in whole or in part after enzymatic cleavage and that the susceptibility of the surviving transport mechanism to chemical modification remains, at least quantitatively, unaltered. It should be kept in mind, however, that the findings described could be explained alternatively on the assumption that the

protein in band 3 and its common binding sites for SITS and N_2ph-F do not participate in anion transport but that a much smaller number of as yet unidentified binding sites on a different protein are involved. The presence of traces of unidentified binding sites in exhaustively trypsinized ghosts is evident from the work with diazo[^{35}S]-sulfanilic acid (Fig. 5), an agent which is known to be an inhibitor of anion exchange. These results suggest that even after trypsination of the ghosts and removal of most of the major membrane proteins, membrane constistuents derived from the minor membrane proteins are still present. Thus, enzymatic "cleaning" of the membrane by trypsin is not complete.

The inhibition produced by chemical modifiers in trypsin-treated ghosts is expressed as a percent of a control of trypsin-treated ghosts which had not been exposed to the modifiers. This percentage is directly comparable to the corresponding percentage observed in ghosts which had not been treated with trypsin. Thus, it is easy and reasonably unequivocal to compare the effects of chemical modifiers on intact and trypsinized ghosts. In contrast, the absolute values of the rates of anion exchange in untreated and enzyme-treated ghosts are not directly comparable, and hence it is difficult to determine quantitatively the effect of trypsin alone on anion transport. In order to obtain comparable quantities, it would be necessary to calculate permeability coefficients. For this purpose, one would need to multiply the reciprocal half-times with the respective volume/surface ratios of the trypsinized and untreated ghosts. Since the trypsinized ghosts which are smaller than the untreated ghosts, form vesicles and "globules" of varying sizes it is not possible to obtain accurate estimates of the volume/surface ratio. Obviously, this ratio must be lower in the many small vesicles which are surrounded by the same total surface area as the smaller number of larger ghosts from which they are derived. Hence the actual inhibition is larger than indicated by the observed increase of the half times. A very crude calculation (unpublished) suggests that the maximal inhibition by trypsin alone as measured under conditions where the enzyme digested more than 95 % of the protein in band 3 may be around 60–70 %.

The difficulties associated with calculating permeability coefficients made it impossible to study quantitatively the relationship between the splitting of the protein in band 3 by trypsin and inhibition of anion exchange. Nevertheless, it is apparent that there is little if any inhibition of sulfate exchange at concentrations of internal trypsin which only degrade the protein in the bands above band 3 and leave that in band 3 itself largely unaffected (Fig. 2). Under these conditions there is actually a slight decrease of the half times (which are inversely proportional to the rate constants presented in Fig. 2). This does not necessarily indicate an increase of the permeability of the membrane but more likely reflects a decrease of the volume/surface ratio associated with increasing fragmentation and suggests that the permeability did not undergo major changes. This finding would suggest that the partial inhibition by higher concentrations of trypsin is related to the cleavage of protein in band 3.

External trypsin has no effect on sulfate exchange in intact cells [8] and ghosts. This is in contrast to the behavior of internal trypsin and suggests a structural asymmetry of the anion transfer system in the red blood cell membrane. Such an asymmetry was previously inferred from the observation that certain disulfonic acids [12, 17] and phlorizin [18] exert an inhibitory effect at the outer but not at the inner

surface of the red cell ghosts. In contrast to these agents APMB inhibits SO_4 equilibrium exchange at either membrane surface with similar strength [12, 17]. Interestingly enough, internal trypsination does not significantly reduce the ability of APMB to inhibit at the inner membrane surface. This finding is compatible with the view [12] that the anion transport system may be composed of a trypsin-insensitive mobile carrier which is capable of combining with internal or external APMB, and of additional immobile modifier sites in the outer membrane surface which are capable of combining with SITS or N_2ph-F and which may be associated with the protein in band 3.

The effects of internal trypsin are not only of interest in relation to anion permeation. So far as the protein in band 3 is concerned, they confirm that it extends all the way across the membrane (previous evidence is summarized in ref. 1). They are compatible with the assumption that the protein is composed of a single peptide species, and that the two SITS binding sites in the outer cell surface are located in different regions of the peptide chain. Thus, our findings support the conclusions drawn earlier by Jenkins and Tanner from their work about the effects of trypsin on iodinated red cell membranes [23]. It should be borne in mind, however, that our conclusions depend on the assumption that the 58 000 and 48 000 dalton fragments are the only products of enzymatic hydrolysis of band 3. As has already been stated this is suggested by the fact that the sum of the molecular weights of the two fragments is close to the molecular weight of the protein in band 3. Nevertheless, it cannot be excluded that water-soluble fragments had been formed which were lost during the washing procedure preceding the solubilization of the membrane in dodecyl sulfate and the subsequent electrophoresis. If such fragments would in fact be formed, it would not be necessary to postulate that the two SITS binding sites reside on the same peptide chain. This would imply that the protein in band 3 is not homogeneous. A non-uniform respense of that protein to externally applied enzymes had been demonstrated recently [24] and would make the assumption of a heterogeneity of the protein in band 3 not entirely implausible.

Our experiments showed that digestion by trypsin of spectrin and the protein in band 2,1 largely preceeds the degradation of the protein in band 3. Hence by a careful choice of the incubation conditions, it is possible to prepare ghosts which are nearly devoid of the larger membrane peptides and still contain much of the protein in band 3. It has been postulated that the membrane particles observed in freeze fractured red cell membranes contain most if not all of the proteins in band 3 [21]. There is also some evidence with suggests that spectrin controls the spatial arrangement of these particles in the membrane [20]. Our findings would be compatible with such a view if one assumes that the sequential attack on spectrin and band 3 protein is not simply due to differences of susceptibilities between the two molecular species to enzymatic attack but to a protection of the protein in band 3 by apposed spectrin.

The most obvious effect of internal trypsin consists of the fragmentation of the ghosts. This effect takes place when the enzymatic digestion is still largely confined to spectrin and the protein in band 2,1. Since spectrin is believed to control the rigidity of form of the red cell membrane (22) it is tempting to suspect a causal relationship between its degradation and the fragmentation of the ghosts. However, the photographs of the ghosts exposed to external trypsin also show some shape changes and suggest that other factors may also be involved in the control of the shape of the red cell membrane.

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