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Proteolytic Digestion of Erythrocytes, Resealed Ghosts, and Isolated Membranes†

Richard B. Triplett and Kermit L. Carraway*

ABSTRACT: The organization of the major proteins of the erythrocyte membrane was studied by proteolysis of intact red cells, resealed ghosts, and isolated membranes followed by electrophoretic analysis of membranes from the treated samples using acrylamide gel electrophoresis in sodium dodecyl sulfate. Trypsin digestion of intact human erythrocytes or resealed ghosts cleaves only the glycoprotein of the major membrane proteins. Pronase or chymotryptic digestion results in cleavage of a second component of mol wt 108,000 to yield apparently identical fragments of mol wt 70,000. Treatment of isolated erythrocyte membranes with proteolytic enzymes results in extensive degradation of the membrane proteins. The differences in the digestibility between the membrane of the intact cell and that of the isolated ghost appears to be primarily due to the ability of the protease to penetrate into the ghost, as shown by the permeability of the ghost to inactivated, labeled chymotrypsin. Neither tryptic nor chymotryptic digestion gives any discernible cleavage of the major membrane proteins (exclusive of the glycoproteins) for intact erythrocytes of several species (bovine, dog, sheep, and horse) under the same conditions used for digestion of human cells. Bovine erythrocytes are also quite resistant to Pronase digestion, but cleavage of the 108,000 molecular weight component of dog cells is noted with Pronase.

In a previous study of bovine and human red blood cells by chemical modification and proteolytic digestion we have shown that the membrane glycoprotein(s) is the most readily accessible of the major proteins of the erythrocyte membranes (Carraway *et al.*, 1971). A second protein with a molecular weight near 100,000 was also shown to be present at the cell surface, but less readily accessible. Similar results have been obtained with intact cells using different modification (Bretscher, 1971a; Phillips and Morrison, 1971b) or proteolytic digestion (Bender *et al.*, 1971; Phillips and Morrison, 1971c) methods. However, a different orientation of the membrane proteins has been proposed by Steck *et al.* (1971), based on the proteolytic digestion of right-side-out and inside-out vesicles. A major problem in the interpretation of protease studies arises from the difference in the digestibility of the membrane proteins in the intact cell when compared to the isolated membrane. In the current study we have shown that this difference results from the increased permeability of the ghost to protease. In addition variations in the digestibility of membrane proteins of intact erythrocytes have been shown to be dependent on both the type of protease used and species of animal from which the erythrocytes were obtained.

Experimental Section

Materials. Chemicals for electrophoresis were obtained from Eastman (highest purity grade) or Canalco. PMSF,[1]

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[1] Abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; mosm, milliosmolar (ideal).

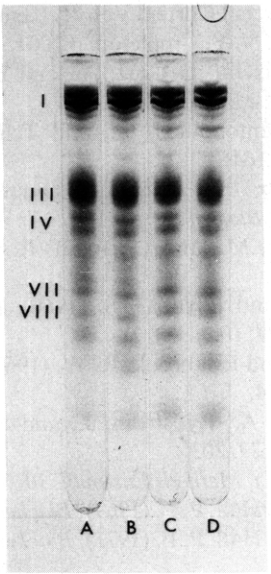

FIGURE 1: Concentration dependence of trypsin digestion of human red blood cells. Washed red cells (1.2 mg/ml of membrane protein) were incubated at room temperature for 1 hr with trypsin (method A). Trypsin concentrations were: gel A, untreated control; gel B, 0.05 mg/ml; gel C, 0.5 mg/ml; gel D, 2.0 mg/ml. Gels were run, stained, and destained as described in Experimental section.

sodium dodecyl sulfate, and trypsin (twice crystallized) were purchased from Sigma, α-chymotrypsin (three-times crystallized) from Worthington, and [^{3}H]acetic anhydride from Amersham-Searle. All other chemicals were reagent grade or highest purity available.

Membrane Preparation. Human blood was obtained from the Dallas Community Blood Bank in acid–citrate–dextrose solution and used within 1 week of the withdrawal date. Animal bloods were collected in citrate or acid–citrate–dextrose solution and used within 2 days of collection. Red cells were washed four to six times in isotonic phosphate, Tris, or sodium chloride at pH 7.4. Washed erythrocyte membranes were prepared by the procedure of Dodge *et al.* (1963) with 20 mosm phosphate or 10 mM Tris (pH 7.4). For the preparation of resealed ghosts washed red cells were hemolyzed in 10 mM Tris (in the presence or absence of 1 mM $MgCl_2$ or 1 mM $CaCl_2$) at pH 7.4. After 10 min sufficient 3.0 M sodium chloride was added to bring the final concentration of salt to 0.17 M, and the suspension was incubated at 37° for 30 min (Redman, 1971).

Proteolytic Digestion. METHOD A. Washed erythrocytes and resealed ghosts (25% suspension) were digested 1.0 hr with trypsin or chymotrypsin at appropriate concentrations in isotonic phosphate–saline or Tris–saline at pH 7.4. PMSF was added to inhibit proteolysis and the cells were washed with isotonic buffer. Membranes were prepared, solubilized in sodium dodecyl sulfate, and subjected to electrophoresis.

METHOD B. Pronase digestion of human erythrocytes was performed according to the method of Bender *et al.* (1971). Pronase digestion of erythrocytes from other species was performed by the same procedure except that the membranes were prepared by hemolysis in Ca^{2+}–Veronal buffer (pH 7.4) (Burger *et al.*, 1968; Kobylka *et al.*, 1972) to ensure membrane integrity.

METHOD C. Digestion of isolated membranes (2.5 mg/ml) was performed in isotonic phosphate–saline or Tris–saline (pH 7.4). The proteolysis was stopped by the addition of hot sodium dodecyl sulfate and a 3-min incubation at 100° in 1% sodium dodecyl sulfate. Mercaptoethanol was added to 1%, and samples were incubated overnight at room temperature and subjected to electrophoresis.

Electrophoresis. Acrylamide electrophoresis was performed as described previously (Kobylka *et al.*, 1972) on 10-cm 5% gels containing 0.1% sodium dodecyl sulfate. Samples for electrophoresis were solubilized by incubation in 1.3% sodium dodecyl sulfate and 1% mercaptoethanol in 0.1 M phosphate at pH 7.8 at room temperature overnight or at 100° for 3 min. Protein was determined by the method of Lowry *et al.* (1951). The gels were stained with Coomassie Blue according to the procedure of Fairbanks *et al.* (1971) with the elimination of the third staining step and were destained in 7% acetic acid. Stained gels were scanned at 550 nm on a Gilford 2000 spectrophotometer equipped with a Model 2410 linear transport assembly. Band designations are given as previously described (Kobylka *et al.*, 1972; Carraway and Shin, 1972).

Permeability of Erythrocyte Membrane to Chymotrypsin. Inactive α-chymotrypsin was prepared by treating with PMSF (Fahrney and Gold, 1963). The inactive enzyme (2 mg/ml) was labeled by incubating with [^{3}H]acetic anhydride (10 mM, 0.5 mCi) in buffer (pH 8.0) at room temperature for 1 hr. For permeability studies the labeled enzyme at three concentrations (0.05, 0.10, and 1.0 mg per ml) was incubated with 30, 50, and 70% suspensions of packed ghosts or erythrocytes for 15 min. The suspensions were centrifuged and aliquots of 0.1 ml were taken for counting in Bray's solution (Bray, 1960). Control samples of labeled enzyme were prepared which contained no erythrocytes or ghosts.

Results

Proteolytic Digestion of Intact Human Erythrocytes. Trypsin digestion of intact human erythrocytes has little effect on the major proteins of erythrocyte membranes (Carraway *et al.*, 1971, and Figure 1). The protein[2] bands of Figure 1 have been labeled according to Kobylka *et al.* (1972) and Carraway and Shin (1972). The designated components gave the following average molecular weight values in this study: I, 220,000 (average of the two bands); III, 108,000; IV, 84,000 (average of the two bands); VII, 42,000; and VIII, 37,000. Only minor changes in protein patterns result from trypsin treatment of the intact erythrocyte, such as an increase in hemoglobin (the fast-running band), which is less readily released from membranes of trypsin-treated cells. The significance of these minor changes is not known. The glycoprotein molecules of the erythrocyte membrane were essentially completely cleaved (90–100%) for both bovine and human erythrocytes, even though the other major membrane proteins were not affected. The addition of Ca^{2+} (0.1–1.0 mM) had no significant effect on the digestibility of the proteins of the erythrocyte membranes when intact erythrocytes were subjected to trypsin.

It was of interest to compare the effects of different proteases on membrane proteins derived from the digestion of intact erythrocytes for two primary reasons. First, Bender

[2] In this paper the term protein will be used to describe those species which stain with Coomassie Blue on acrylamide gels in sodium dodecyl sulfate. Since glycoproteins do not stain readily with Coomassie Blue, they will be excluded from this designation. No differentiation is made between the terms protein and polypeptide, since we currently have no information about the arrangement of polypeptide subunits into proteins in the membrane.

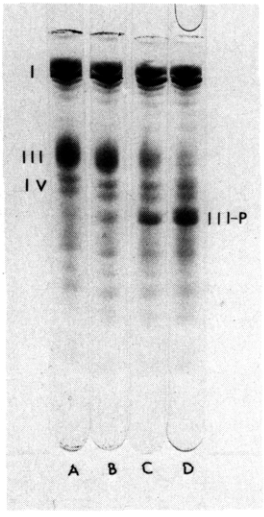

FIGURE 2: Concentration dependence of α-chymotrypsin digestion of human red cells. Experimental conditions are described in Figure 1. Chymotrypsin concentrations: gel A, untreated control; gel B, 0.05 mg/ml; gel C, 0.5 mg/ml; gel D, 2.0 mg/ml.

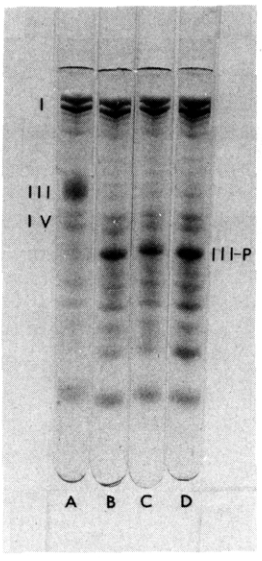

FIGURE 3: Concentration dependence of Pronase digestion of human erythrocytes. Washed red cells (1.2 mg/ml of membrane protein) were incubated at room temperature for 1 hr with Pronase (method B). The enzymes were inhibited with PMSF and the cells were washed two to five times before preparation of membranes. Pronase concentrations were: gel A, untreated control; gels B and D, 0.05 mg/ml; gel C, 0.1 mg/ml.)

et al. (1971) had shown that the nonspecific protease mixture Pronase cleaves component III (our designation) to yield a fragment, whose molecular weight was reported as 73,000. This component is apparently identical to the protein which we partially labeled with diazotized sulfanilic acid (Carraway *et al.*, 1971). Second, the protein patterns derived after digestion of inside-out or right-side-out vesicles (Steck *et al.*, 1971) suggested that a different specificity of digestion was shown by chymotrypsin and trypsin. We therefore sought to determine if this specificity could be useful in examining cell surface differences and comparing the orientations of the membrane proteins in the membrane of intact cells with those of ghosts or inside-out vesicles.

When human erythrocytes are digested with chymotrypsin under conditions similar to those used for trypsin, a marked decrease in the amount of component III is observed on the gels in addition to the loss of glycoprotein. The concentration dependence of the chymotrypsin digestion is shown in Figure 2. The disappearance of component III (mol wt 108,000) is paralleled by an increase in a proteolytic fragment (designated III-P) with a molecular weight of 70,000. Component III is apparently quantitatively converted to III-P, since the protein present in bands III-P plus III is equivalent to that of III for membranes of undigested cells after correction is made for the difference in molecular weight. This indicates that component III must be present as a single polypeptide chain or a group of closely related polypeptide chains, all of which must be oriented in the same manner in the membrane. For comparison human erythrocytes were digested with Pronase according to the procedure of Bender *et al.* (1971). Figure 3 shows the electrophoresis patterns obtained from membranes of these cells. There is a remarkable similarity between the membrane protein patterns from chymotrypsin- and Pronase-treated cells. The molecular weights of the fragments from the two digestion procedures are indistinguishable by our experimental methods. This indicates that component III has a very limited number of sites that are accessible to proteolytic attack from the outside of the cell.

Proteolytic Digestion of Erythrocytes from Other Species. Experiments were performed with erythrocytes from other species to determine if any differences in the surface properties could be shown. We had previously noted that the glycoprotein of each species which was examined has a different molecular weight (Kobylka *et al.*, 1972) and some variability in carbohydrate composition (Khettry *et al.*, 1971; A. Khettry, K. L. Carraway, and B. G. Hudson, unpublished observations). It was of interest to determine whether the variations in glycoproteins might affect the accessibility of the major membrane proteins to proteolytic digestion. When erythrocytes from a number of different species (bovine, horse, sheep, and dog) were subjected to trypsin treatment, the results were the same as obtained with human erythrocytes. None of the major proteins were discernibly digested. However, the results obtained by chymotryptic digestion of human erythrocytes differed from those obtained with all other species examined. No digestion of component III was noted for any of the other species under conditions which resulted in essentially complete digestion of this component for human erythrocytes (Figure 4).

The accessibility of component III in erythrocytes from other species was studied further by Pronase digestion. In bovine erythrocytes this component is resistant to digestion even by this nonselective protease mixture. Evidence that some digestion may be occurring is indicated by the formation of a new minor band (labeled P, Figure 5) with a molecular weight of about 60,000. Pronase digestion of component III of dog erythrocytes produced three new bands with mol wt 80,000, 72,000, and 49,000 (labeled P_1, P_2, and P_3 in Figure 5). Whether all of these arise from component III cannot be ascertained at present. It is possible that the Pronase digestion alters the membrane properties such that some additional components are not removed by the hemolysis process. An additional interesting finding is that the remaining component III of the dog membrane appears as a doublet in the gel.

Proteolysis of Resealed Ghosts. The proteins of the erythrocyte membrane are much more susceptible to proteolytic digestion in the isolated membrane than in the intact cell (Carraway *et al.*, 1971; Carraway and Triplett, 1971; Bender *et al.*,

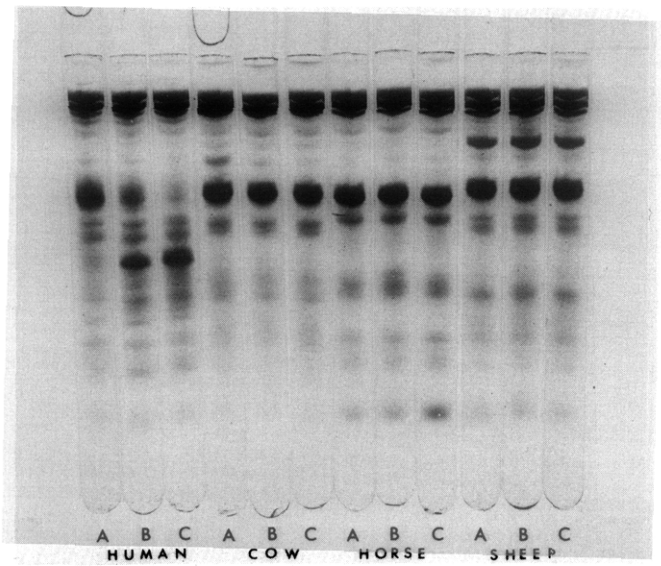

FIGURE 4: Effect of chymotrypsin digestion on erythrocyte membrane proteins of different species. Digestion was performed as described in Figure 1. Gel A of each set is untreated control; gel B, 0.5 mg/ml of chymotrypsin; gel C, 2.0 mg/ml of chymotrypsin. The fastest moving band in each gel is hemoglobin.

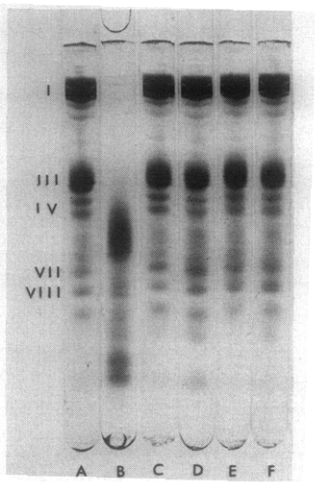

FIGURE 6: Effect of trypsin digestion (method A) on proteins of resealed ghosts of human erythrocytes. Preparation of resealed ghosts is described in Experimental Section. Gels A, C, and E are untreated control samples of isolated washed ghosts, resealed ghosts, and resealed ghosts prepared in the presence of Mg^{2+}, respectively. Gels B, D, and F are from samples treated with 100 μg/ml of trypsin. Gel B, washed ghosts; gel D, resealed ghosts; gel F, resealed ghosts prepared in presence of Mg^{2+}.

1971). This observation suggests that the ghosts must be permeable to the protease or that the protein components of the membrane must be reorganized during the processes of hemolysis and washing. The effects of proteolysis on the membrane proteins of the resealed ghost were studied to determine if any significant reorganization of the membrane protein components occurs as a result of the hemolysis process. Erythrocytes were hemolyzed, then resealed in the presence and absence of Mg^{2+}. The resealed ghosts were washed, treated with trypsin, and hemolyzed a second time to obtain hemoglobin-free membranes. Figure 6 shows the electrophoresis patterns obtained from the trypsin-treated resealed ghosts plus the pattern from a sample of isolated membrane which was treated with trypsin. The electrophoretic results show that none of the major membrane proteins are cleaved in the resealed ghost. This result contrasts sharply with the extensive cleavage of proteins of the isolated membranes, and indicates that the hemolysis process does not irreversibly alter membrane structure to the extent that additional membrane proteins are made accessible to trypsin at the cell surface. If any such alterations do occur, they must be reversed by the resealing procedure. Additional studies showed that the protein profiles of human erythrocyte ghosts resealed in the presence of Ca^{2+} were not altered by trypsin treatment. Resealed membranes can also be prepared under appropriate conditions from ghosts which are virtually hemoglobin free and these are resistant to proteolysis by trypsin.

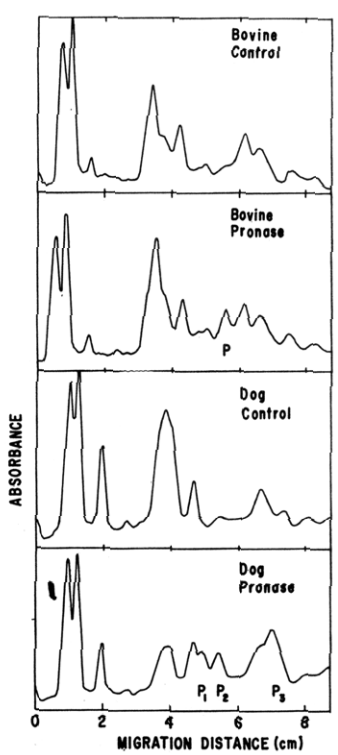

FIGURE 5: Effect of Pronase digestion on bovine and dog erythrocytes. Digestion and membrane isolation were performed as described in Experimental Section (method B). Pronase concentration for digestion was 0.1 mg/ml.

Proteolysis of Isolated Erythrocyte Membranes. In an earlier study (Carraway *et al.*, 1971) we showed that trypsin treatment of hemoglobin-free human erythrocyte membranes causes extensive degradation of the erythrocyte membrane proteins. All of the major membrane proteins were degraded except VIII, which was released into the medium (Steck *et al.*, 1971). It was therefore of interest to perform a study of the time course of digestion of the membranes at low protease concentrations to see if an order of accessibility could be established for the various polypeptides which might reflect their organization in the isolated membrane. Steck *et al.* (1971) performed a similar study, but it was not detailed enough to determine which polypeptides were digested first. Figures 7 and 8 show the gel scans for the proteins from human erythrocyte ghosts which have been treated with chymotrypsin and trypsin, respectively, at concentrations of 10 μg/ml for 0–20 min. Several interesting points should be

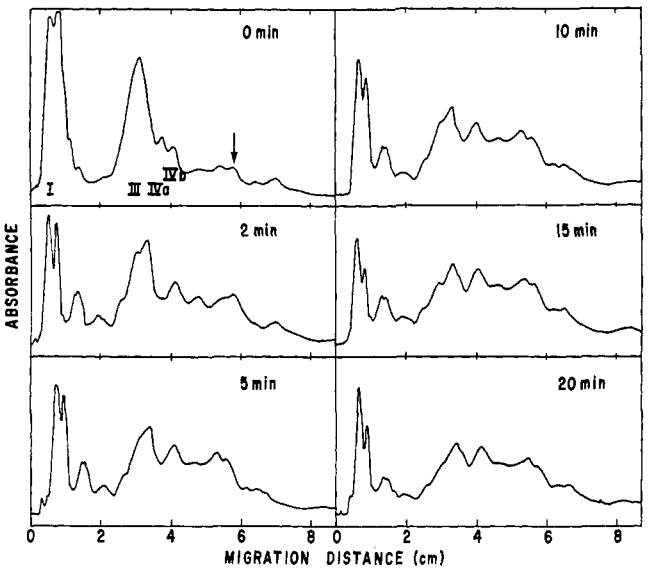

FIGURE 7: Time course of α-chymotrypsin digestion of human erythrocyte membranes. Membranes (1.2 mg/ml of protein) were digested with 10 μg/ml of α-chymotrypsin (method C). Reactions were stopped at indicated intervals by the addition of hot sodium dodecyl sulfate. The absorbance is 0–3 units full scale in each scan. The position of component VIII is indicated by the arrow.

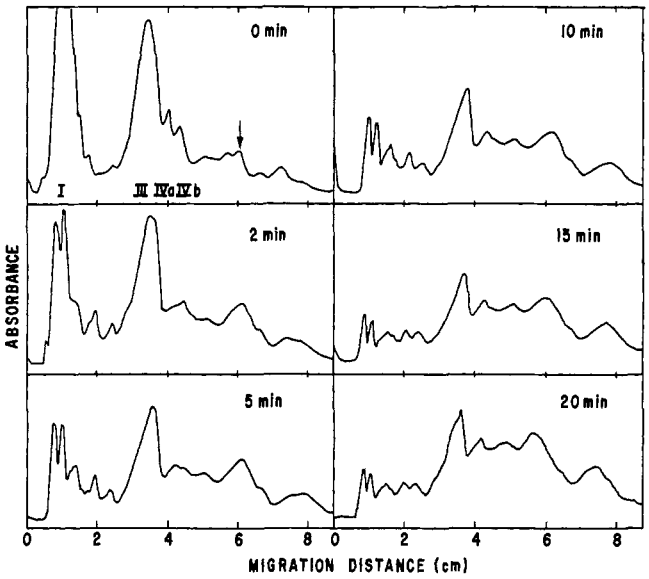

FIGURE 8: Time course of trypsin digestion of human erythrocyte membranes. Experimental conditions are described in Figure 7.

noted from this study. (1) Band IVa is the first to be completely removed from the pattern by each enzyme. (2) There is a specificity of digestion for the two enzymes. Trypsin appears to act more readily on component I than III, while chymotrypsin performs in the opposite manner. (3) Component VIII does not appear to be digested, as noted by Steck *et al.* (1971), although overlap by digestion products from higher molecular weight polypeptides may affect this observation. (4) Component III appears as a doublet after a short-term digestion by chymotrypsin. (5) There is no product of mol wt 70,000 which appears during the course of digestion of component III by chymotrypsin. (6) These current studies differ in some respects from those of Steck *et al.* (1971), but this may arise in part from the fact that our studies were performed in isotonic solutions at pH 7.4, while theirs were performed in hypotonic buffers at pH 8.0.

One factor which must be considered in all of the proteolysis studies is that the results are affected by the release or activation of a membrane-bound red cell protease (Moore *et al.*, 1970). Although this possibility cannot be completely ruled out, its contribution must be small, since the activity of membrane-bound protease is quite low in erythrocyte membranes which are free of white cells (R. B. Triplett, unpublished results) and since the proteolysis effects noted can be stopped in their course by rather specific inhibitors (*e.g.*, soybean trypsin inhibitor, PMSF).

Permeability of the Erythrocyte Ghost to Proteases. Evidence for the permeability of the isolated membrane to proteases was obtained by measuring the exclusion of a sample of inactivated labeled chymotrypsin from erythrocytes and hemoglobin-free ghosts. Samples of the labeled enzyme at three concentrations were incubated with suspensions containing 30, 50, and 70% (v/v) packed erythrocytes or ghosts. The suspensions were centrifuged and aliquots of the supernatant were counted to determine the amount of enzyme excluded (nonpermeable). Table I shows an example of the results obtained with 50% suspensions of erythrocytes and ghosts. No corrections were made for the volume of interstitial space between the packed membranes, the internal volume of the membrane or the adsorption of protein to the membrane. Some adsorption does occur as indicated in the decreased number of counts in the solution incubated with ghosts (column 2) when compared to the number of counts in the control, which was free of erythrocytes or ghosts (column 4). The results clearly indicate that the erythrocyte excludes the enzyme while the ghost does not. Therefore, proteases are apparently able to penetrate into the ghost and digest proteins inside as well as outside of the membrane.

Discussion

The objective of this study was to gain knowledge of the organization of the proteins within the erythrocyte membrane by proteolytic digestion experiments. A significant source of controversy concerning the organization of these proteins has been the difference in accessibility between the proteins of the membrane of the intact cell when compared to the isolated ghost. The present results indicate that this difference can be

TABLE I: Permeability of Erythrocytes and Ghosts to Labeled Chymotrypsin.

Chymotrypsin Concn (mg/ml)	Cpm Determined		Cpm Predicted	
	RBC	Ghost	Impermeable[a]	Permeable[b]
0.05	1,150	530	1,480	740
0.10	2,410	1,210	2,420	1,210
1.0	22,200	11,900	26,900	13,500

[a] Calculated from value for total permeability by assuming that 50% of the volume of the suspension is inaccessible. [b] Determined experimentally by adding aliquots of labeled chymotrypsin to control buffer samples containing no cells or ghosts.

explained in part by the permeability of the ghost to proteins. In this fashion we can readily account for the altered digestibility and the accessibility to iodination by lactoperoxidase (Phillips and Morrison, 1971a). These results do not rule out the possibility that other organizational differences may exist between the isolated membrane and the membrane of the intact erythrocyte. Such differences have been suggested for the lipids of the erythrocyte membrane based on chemical modification (Carraway *et al.*, 1972) and lipolytic enzyme (Zwaal *et al.*, 1971) studies. No significant protein differences have been found, although the methods may be too insensitive to detect them. The present results also do not rule out the possibility that conformational changes of individual membrane proteins may occur and affect their apparent accessibility to protease.

With regard to the organization of the proteins in the membrane it is of interest to compare the present studies to results obtained previously by others. A strong parallel has been noted between the digestion of the inside-out vesicles of Steck *et al.* (1971) and the intact erythrocytes. Proteins of both of these membranes show strong resistance to digestion. In both cases trypsin treatment has almost no effect on the major proteins, while chymotrypsin digests component III to yield III-P, the 70,000 molecular weight fragment. A similar parallel has been noted between the right-side-out vesicles and the isolated ghosts in that both of these show extensive degradation by proteases. These similarities suggest that the difference between the inside-out and right-side-out vesicles may be the same as between intact erythrocytes and ghosts, *i.e.*, a difference in permeability to the enzymes. It does not seem likely that two membrane forms (erythrocyte and inside-out vesicle) which show such similar digestion patterns represent inverted orientations. However, the possibility that protein organizational or conformational changes occur in the transition from erythrocyte to washed ghost or from ghost to inside-out vesicle should not be ignored. Examples of such protein mobility within membranes have been noted as a result of protease treatment of membranes and viral transformation of cultured cells (Nicolson, 1971).

The localization of several of the proteins can be predicted. The present study confirms the presence of two major proteins at the membrane surface for the human erythrocyte, the glycoprotein, and component III. A significant proportion of III may be within the membrane, since it had a limited number of sites accessible from the outside of the cell, even though it is obviously accessible in the ghost. Bretscher (1971b,c) has suggested that III and the glycoprotein extend completely through the membranes on the basis of chemical labeling studies with an impermeable reagent. His studies make two assumptions: (1) the isolated membrane does not differ from the membrane of the intact cell in its protein organization; and (2) the reagent can react only at the outside and inside surfaces of the membrane. Since the reagent must travel through channels within the membrane (presumably aqueous) in order to get inside, it seems equally likely that reaction might occur within the membrane. Any protein which bounded a membrane channel would be accessible to reagent in the ghost even if it were not present at either membrane surface. Therefore, the question of whether any components extend completely through the membrane has not yet been unequivocally answered.

Component I, which has variously been called spectrin (Marchesi and Steers, 1968) or tektin A (Clarke, 1971), has been localized at the inner surface of the ghost membrane by the ferritin-labeled antibody technique (Nicolson and Singer, 1971). The similarity in the accessibility to digestion of components I and IVa suggests that they may be proximally located, probably at the membrane inner surface. Components IVa and IVb do not appear to be related functionally or in location, but the two polypeptides of I apparently are related. The localization of the other polypeptide chains is more difficult. Component VIII contains in part the subunit of glyceraldehyde-3-phosphate dehydrogenase (B. C. Shin, unpublished results), which is probably located at the inner surface of the membrane if it is bound to the membrane in the intact cell. The glycoprotein extends from the outer surface of the membrane and into the lipid bilayer. It must be remembered that this structure is based on the assumption that there are no major organizational changes of membrane proteins in going from the intact erythrocyte to the ghost.

Two further questions are also important to an understanding of membrane structure. (1) How far do the proteins at the surface of the bilayer penetrate into the bilayer? (2) Are there proteins of the cell interior such as hemoglobin and enzymes which are normally associated with the membrane of the intact cell? Further investigations are needed to clarify these points and others, which are pertinent to an understanding of the functioning of not only red cell membranes but also membranes of more complex cells.

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^{13}C and ^{1}H Nuclear Magnetic Resonance Relaxation Measurements of the Lipids of Sarcoplasmic Reticulum Membranes†

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ABSTRACT: ^{13}C nuclear magnetic resonance spectra of sarcoplasmic reticular membranes yield several well-defined resonances from the membrane lipids, identified as the terminal methyl, $(CH_2)_n$, and olefinic carbons of the fatty acid chains and the N^+Me_3 of the choline head group of lecithins. The spectra correspond in intensity to 60–90% of the membrane lipids which is substantially more than is observed in the sharp components of the ^{1}H spectrum. The proton spectra are very similar to those described by Davis and Inesi (Davis, D. G., and Inesi, G. (1971), *Biochim. Biophys. Acta 241*, 1) in which, unlike the ^{13}C spectra, the N^+Me_3 resonance is very weak at 30°. The spin–lattice (T_1) relaxation times have been measured for the assigned resonances in both the ^{13}C and ^{1}H spectra and are compared with the relaxation times of sonicated vesicles of the lipids extracted from the membrane. The ^{13}C chain resonances have very similar T_1 values in both the membrane and vesicle preparations, but the N^+Me_3 T_1 value is significantly shorter in the membrane. This is tentatively attributed to interaction with membrane protein. The relaxation of the $(CH_2)_n$ proton resonance is not characterized by a single T_1 relaxation time in the membrane, and the relaxation of the $(CH_2)_n$, CH_3, and N^+Me_3 groups are all complex in the extracted lipid preparation, unlike the relaxation observed in vesicles of homogeneous lecithins. The results are used to define the problems of relating relaxation measurements to the organization of the lipids in the membrane. An independent estimate of the extent of the bilayer in the membranes from measurement of the binding of the spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl is consistent with the ^{13}C intensity and relaxation data.

The proton nuclear magnetic resonance (nmr) spectrum of the sarcoplasmic reticulum membrane recently described by Davis and Inesi (1971) is the first high-resolution spectrum from a functionally intact membrane. These spectra contain sharp well-defined resonances corresponding in intensity to about 20% of the protons in the fatty acid chains of the phospholipids ($(CH_2)_n$ and CH_3), while the remaining fatty acid protons appear to be included in a much broader peak under these sharp resonances. In addition, although about 75% of the membrane phospholipids are lecithins (Fiehn and Hasselbach, 1970; Meissner and Fleischer, 1971), the intensity of the choline N^+Me_3 resonance was very weak, and at higher temperatures where the resonance became more fully developed there was a simultaneous decline in the ability of the vesicles to accumulate calcium (Davis and Inesi, 1971).

These spectral features raise several issues regarding the organization of the lipids within the membrane. The appearance of only 20% of the fatty acid chain protons in the sharp components of the membrane spectra might correspond to that fraction of the lipids in a simple bilayer structure unperturbed by the presence of membrane protein, with the major fraction of the lipid resonances broadened either as a result of some more tightly packed structural organization, or by direct intermolecular interaction with membrane proteins. Alternatively, the sharp resonances might represent a "superfluid" fraction of the lipids with motional characteristics that reflect the functional environment of the calcium-transport system, which involves a major portion of the membrane protein (Ikemoto *et al.*, 1971). Clearly the possibility of heterogeneous regions of lipid composition or organization is of considerable interest. Similarly the diminished N^+Me_3 resonance, apparently related to functional integrity, suggests intimate intermolecular interactions with other membrane components, since in sonicated lipid vesicles of synthetic lecithins and natural lecithin mixtures, the N^+Me_3 proton resonance is fully developed (Sheard, 1969; Birdsall *et al.*, 1971; Lee *et al.*, 1972).

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