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EFFECT OF LOW LEVELS OF TRYPSIN ON ERYTHROCYTE MEMBRANES

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SUMMARY

The effects of trypsin at concentrations of 1 μg/ml on red blood cell membranes (ghosts) have been investigated. Tryptic digestion induced a gross morphologic change as observed by phase microscopy with the formation of small close vesicles by a process of exocytosis. These vesicles were more tightly sealed (less leaky) to hexoses than the parent ghosts. Coincident with vesiculation there occurred a decrease in particle density from 1.06 to 1.01 as determined by equilibrium density gradient centrifugation in Dextran. Since the equilibrium density in sucrose gradients was much less affected, the creation of new charge groups on the internal membrane surface was suggested. Continued accessibility of all sialic acid residues to attack by neuraminidase after tryptic digestion indicated that the exocytotic vesicles were in fact "right-side-out".

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed that two of the major membrane peptides (mol. wt of 89000 and 77500) were selectively attacked. Under these conditions, proteolysis did not cause the release of protein from the membrane. We suggest that the vesiculation and alteration in membrane permeability seen in association with the cleavage of these two peptides indicates that one or both are critical to the maintanence of erythrocyte membrane structure, probably through their association with lipid.

INTRODUCTION

In recent years proteolytic enzymes have been used to probe various aspects of cell surface structure and function. This technique, so useful in the delineation of structure-function relationships in soluble proteins, has not been fully exploited for membrane sytems. The bulk of reports have described the effects of proteolysis on an array of membrane functions including certain enzyme systems[1,2], the regulation of cell growth and division[3–5] hormonal stimulation[6,7] and nerve conduction[8,9]. These reports have not attempted to correlate the alterations in the function of the plasma membrane with its structure. Several investigations concerning proteolysis-induced changes in ATPase activity and ion transport have employed electron microscopy to assess concomitant structural changes in the membrane. In all these instances, however interpretation is difficult due to a lack of specific information concerning the nature of the peptides cleaved, the resultant changes in the biophysical

state of the membrane and way in which these changes are translated into functional effects.

In the course of studying glucose transport in human erythrocyte ghosts prepared by the method of Dodge *et al.*[10], we made the observation that unperturbed Dodge ghosts were leaky to hexoses and incapable of selective uptake of D-glucose. However, ghosts subjected to proteolysis were more tightly sealed and exhibited clearcut permselectivity to D-glucose. This was surprising, since a number of studies indicated that proteolysis of ghosts can yield cleavage of virtually all membrane peptides[11,12], a situation which, a priori, would not be expected to have salutory effects on either overall membrane permability or a specific transport function. In fact several workers have previously reported that proteolysis of microsomes from muscle abolished calcium transport and rendered the particles more leaky to Ca^{2+} ions[13–16]. This report will describe the effects of mild proteolysis of Dodge ghosts on their morphology, non-specific permeability (to hexoses), and buoyant densities and the concomitant alterations in membrane peptides. The characterization of the glucose transport system which is uncovered by proteolysis, and the mechanism by which it is uncovered will be examined elsewhere[17].

METHODS

Human erythrocyte ghosts were prepared from fresh blood by the method of Dodge *et al.*[10] as modified by Fairbanks *et al.*[18].

Trypsinization of erythrocyte ghosts was carried out at room temperature in 5 mM phosphate (pH 8.0) containing 1–2 mg/ml membrane protein in the presence or absence of 0.25 M sucrose. Except where noted, trypsin concentration was 1 μg/ml. The reaction was terminated by the addition of 5-fold excess of soybean trypsin inhibitor in the same buffer. Samples designated "o time" received soybean trypsin inhibitor prior to trypsin. All incubations were continued until soybean trypsin inhibitor had been added to the last time-point sample. When measurements of glucose flux were performed, sucrose was always present during the incubation and aliquots for this assay were removed after the addition of soybean trypsin inhibitor. These were mechanically vesiculated by several passages through a 27-gauge needle and glucose uptake then measured.

After addition of soybean trypsin inhibitor, the particles were sedimented at 30000 $\times$ *g* for 30 min and subsequently handled in one of two ways: (A) If sucrose had been omitted during the proteolysis the pellet was simply resuspended (Fraction A) in 0.5 mM phosphate (pH 8.0), 0.1 mM $MgSO_4$ ("dilute phosphate buffer") and used directly for further analyses. (B) If sucrose had been present during the trypsinization, the pellet was washed 3 times in "dilute phosphate buffer" and resuspended in this buffer before further use (Fraction B).

For density gradient studies, 10-ml linear gradients of two types were used: Dextra, ρ 1.0–1.07 or sucrose ρ 1.01–1.20, both containing 0.5 mM phosphate (pH 8.0), 0.1 mM $MgSO_4$. 2-ml aliquots of Fraction A or B were top loaded onto the gradients and these were centrifuged in a Beckman L2-65B preparative ultracentrifuge to equilibrium overnight in a SW 41 rotor at 25000–35000 rev./min. Fractions were collected by aspiration from above, diluted 30-fold into "dilute phosphate buffer", and sedimented at 30000 $\times$ *g* for 30 min. The pellet was resuspended in this buffer

and used for electrophoretic analysis. For experiments in which sialic acid was determined, the post gradient particles were washed 3 additional times in dilute phosphate buffer to remove Dextran. Prior to cholesterol and phospholipid determinations the samples were washed 3 times with 0.1 mM $MgSO_4$, 1.0 mM Tris–HCl (pH 8.0). For sialic acid assays aliquots of whole ghosts, Fraction A and B, and of post gradient particles, at concentrations of 0.1–0.6 mg/ml were used. Hydrolysis of glycopeptide sialic acid moieties were carried out for 1 h either in 0.075 M H_2SO_4 at 80°C or by 100 units/ml neuraminidase (*Vibrio cholerae*) in 1 mM $CaCl_2$, 0.1 M sodium acetate buffer (pH 5.4) at 23 or 37 °C (ref. 19). Portions of these incubations were taken either before or after removal of the membranes by centrifugation, and assayed for free sialic acid by the method of Warren[20], with minor modifications. Total sialic acid was taken as the amount released by acid hydrolysis; accessible sialic acid was that released by enzymatic treatment. Less than 2% of the total sialic acid was liberated in control incubations containing no enzyme.

Polyacrylamide gel electrophoresis and the staining of gels for peptides and glycopeptides was performed by the method of Fairbanks *et al.*[18]. Samples were prepared for electrophoresis by the addition of the following (final concentration): Sucrose (5–10%), dithiothreitol (40 mM), EDTA (1 mM), and sodium dodecyl sulfate (1%). The samples were then incubated at 37 °C for 30 min. Phospholipid was determined by the method of Babson *et al.*[21] and cholesterol by a modification[22] of the method of Bartlett[23].

Glucose uptake and washout were measured by the method of Carter *et al.*[24]. Protein was routinely determined by measurement of native fluorescence. Aliquots of membrane suspended on 0.1% sodium dodecyl sulfate were excited at 286 nm (slit = 4 nm) in a Hitachi–Perkin–Elmer MPF 2A spectrofluorimeter. Emission was recorded at 338 nm (Slit = 20 nm). Tryptophan served as standard, and the ratio of mg protein/mg tryptophan was 42.0, obtained from parallel protein estimations by the method of Lowry *et al.*[25] using bovine serum albumin as standard.

Trypsin, 2 times recrystallized was obtained from Sigma, soybean trypsin inhibitor is a product of Worthington Biochemicals. Neuraminidase was obtained from Behring Diagnostics.

RESULTS

The effects of proteolysis on the morphology of the ghosts was followed by phase microscopy. In incubations containing 1 μg/ml of trypsin, the ghosts initially appeared as biconcave discs. After 5 min, a population of ghosts showed loss of their characteristic shape, with conversion into spherical forms. Associated with this change, there was the onset of progressive exocytosis. The exocytotic buds, varying widely in size, eventually pinched off and appeared as free vesicles. By 30–60 min (occasionally up to 90 min) essentially all the ghosts had been converted to spherical vesicles. At no time was endocytosis seen. When sucrose was absent, the vesiculation was considerably accelerated. In incubations carried out at 10 and 100 μg/ml trypsin, breakdown to vesicles was extensive at the earliest times observed (<1 min).

Trypsinization was halted at various times by the addition of soybean trypsin inhibitor and aliquots of the entire incubation mixture were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The patterns obtained with the

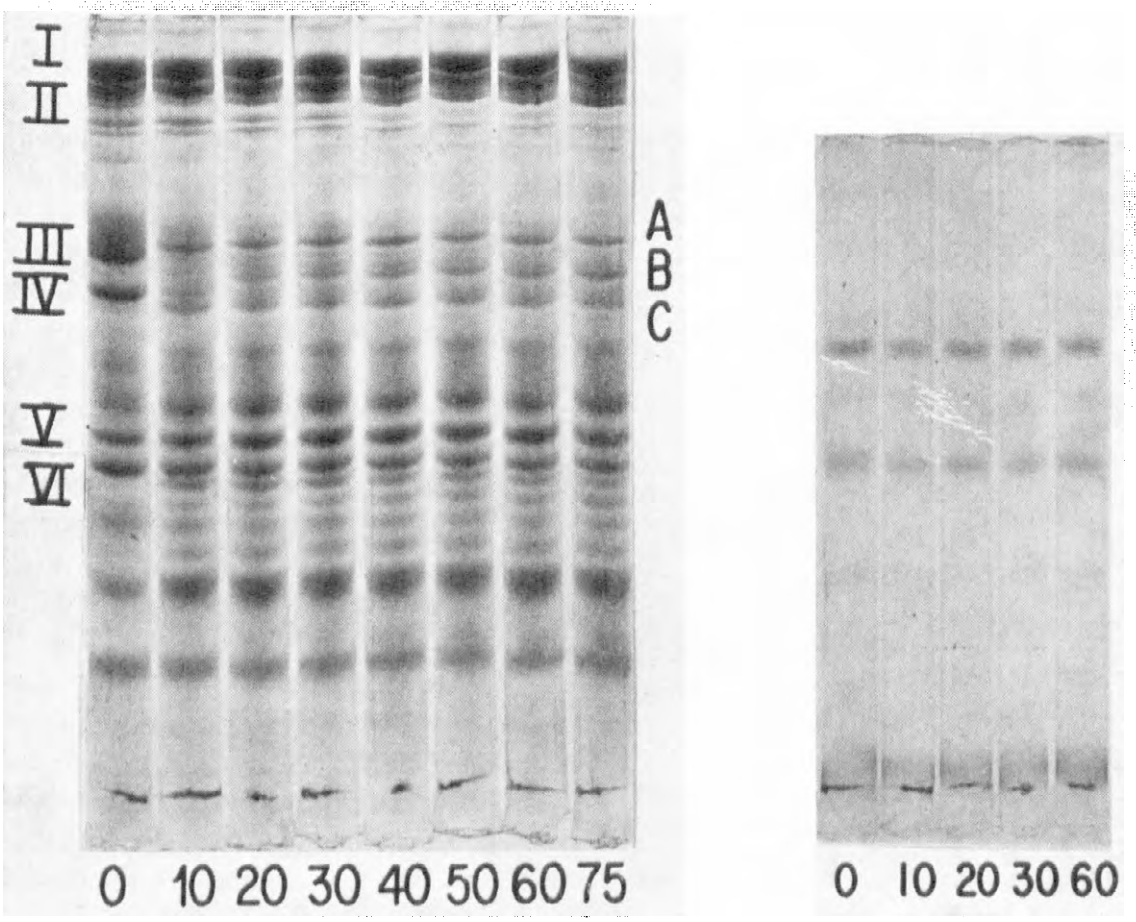

Fig. 1. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of erythrocyte membranes stained with Coomassie Blue. Pattern on far left represents unproteolyzed membranes. Roman numerals indicate the major intrinsic peptide species. Other gels represent membranes treated with trypsin for the times (in min) indicated. Letters identify some of the new species created and/or unmasked by trypsin-treatment.

Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of erythrocyte membranes from same experiment as Fig. 1, but stained with periodic acid–Schiff reagent. Time (in min) of trypsin-treatment is listed below each gel.

incubation control samples (Fig. 1, soybean trypsin inhibitor added at zero time, incubation continued for 75 min) are essentially identical to those previously reported for erythrocyte membranes by Lenard[26] and Fairbanks *et al.*[18]. To allow comparability we have adopted the nomenclature of the latter authors. Six major peptides and a number of minor components are identifiable by staining with Coomassie Blue (Fig. 1, far left). In addition, three major glycopeptides are selectively stained by periodic acid–Schiff reagent (Fig. 2). Two peptides (Components III and IV) appear to be exquisitely sensitive to proteolysis and disappear almost totally within 10 to 30 min concurrent with the appearance of several lower molecular weight proteolysis products (Fig. 1). The slight difference in mobility between Component III and Band A is reproducible. Band A may be a proteolysis product or a proteolysis resistant species normally obscured by the trailing portion of Component III. Similarly, the mobilities of Bands B and C have been consistently observed to differ from that of band IV. Later in the incubation, minor but reproducible cleavage of Component II is apparent. During this time span no other major peptide or glycopeptide (Fig. 2)

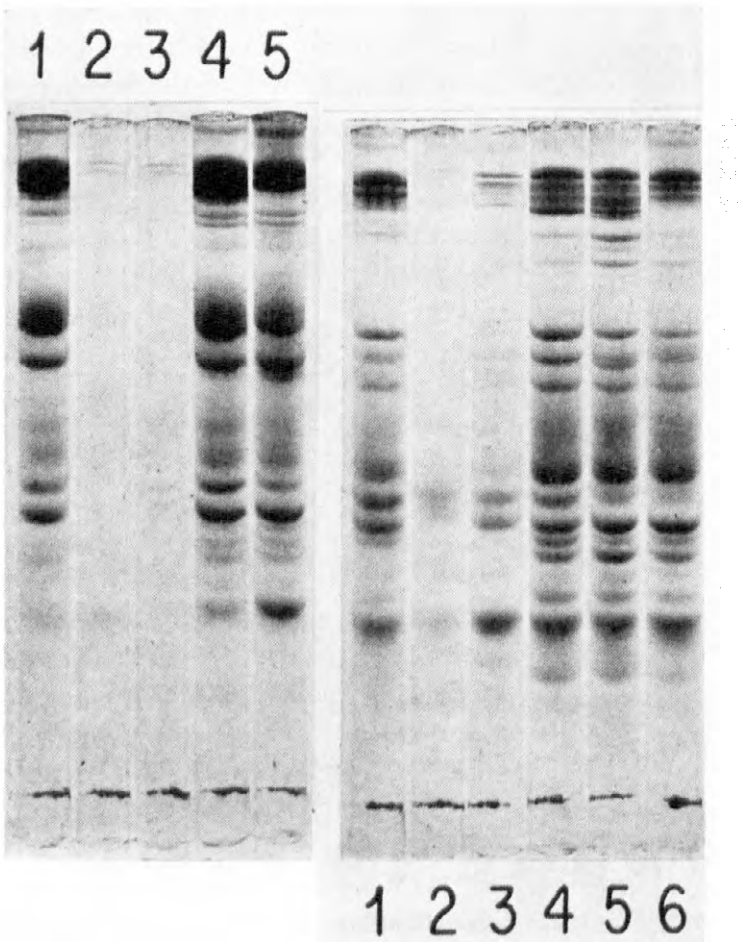

Fig. 3. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of erythrocyte membranes before (left panel) and after (right panel) partial proteolysis with trypsin, 1 μg/ml for 5 min. The particles were carried through washing and density gradient centrifugations as described in Methods, and aliquots for electrophoretic analysis were obtained at the following steps: Gel 1 = uncentrifuged ghost supension (incubation mixture); Gel 2 = supernatant of 48000 $\times$ g for 30 min centrifugation of incubation mixture; Gel 3 = supernatant of first washing in "dilute phosphate buffer"; Gel 4 = washed membranes prior to density gradient centrifugation (Fraction B); Gel 5 = membranes from Dextran gradient at ρ 1.06; Gel 6 = membrane from Dextran gradient at ρ 1.01.

is cleaved. Of the minor components, those immediately below Band II are cleaved with a time course similar to Band II. Changes in other minor components are largely obscured by the superimposition of proteolysis products. After 5 min incubation, by which time extensive cleavage of Bands III and IV had occurred (Fig. 3, Gel 1: compare right and left panels), the ghost suspensions were centrifuged and the supernatants were analyzed for protein content (in one experiment) and by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (in four experiments). The supernatant contained 70 μg total protein in the zero time incubation and 94 μg protein after 5 min of proteolysis. Since the original ghost suspension contained 2.50 $\pm$ 0.1 mg protein, and trypsin and soybean trypsin inhibitor contribute approximately 30 μg to the content of soluble protein, it can be seen that proteolysis under these conditions releases less than 3% of the membrane protein. Analysis of a 1/50 aliquot (0.1 ml) of these supernatants by polyacrylamide gel electrophoresis in sodium dodecyl

sulfate (Fig. 3, Gel 2, left panel, supernatant zero time; right panel, supernatant from ghosts subjected to proteolysis for 5 min) confirms the trivial loss of protein from both the control and proteolyzed ghosts. When, however these particles were washed once in 0.5 mM phosphate buffer (pH 8.0) containing 0.1 mM $MgSO_4$, 62 μg protein were released from the zero time ghosts, whereas 556 μg protein were removed from the proteolyzed ghosts into the wash solution. Analysis of a 1/50 aliquot of the wash solutions by polyacrylamide gel electrophoresis in sodium dodecyl sulfate confirms that negligible amounts of protein are removed from the control samples whereas significant portions of Bands I, II, V and VI are eluted from the proteolyzed ghosts along with a portion of a major proteolysis product of molecular weight of 18000 (Fig. 3, Gel 3, left panel wash of zero time; right panel, wash of ghost subjected to proteolysis for 5 min).

Untreated membranes contained 100 ± 5 nm sialic acid per mg protein and trypsinization (1 μg/ml) of erythrocyte membranes for up to 60 min did not alter these values. Furthermore, 95 to 105% of the glycopeptide sialic acid was accessible to neuraminidase in both control and proteolyzed samples.

The permeability of intact ghosts to sugars was compared before and after trypsinization. Ghosts were incubated with D- and L-glucose (5 mM each) and after equilibration, aliquots were subjected to millipore filtration followed by washes with single drops of buffer (Table I). Untreated ghosts lost essentially all radioactivity when washed with minimal amounts of buffer, indicating that the sugars were simply trapped in the pellet or contained within very leaky structures. After trypsin treatment the ghosts were strikingly resistant to complete washout of the labeled sugars by buffer, indicating that the sugars were contained in much more tightly resealed vesicles (further studies confirm that this retained sugar is within the intravesicular space rather than bound to the membrane)[17]. Mechanical disruption

TABLE I

WASHOUT OF LABELED SUGARS FROM PLAIN MECHANICALLY DISRUPTED, AND TRYPSIN-TREATED RED CELL GHOSTS

The preparation of erythrocyte ghosts and trypsinization ared described in Methods. Mechanical vesiculation was accomplished by 8–10 rapid passages through a fine bore needle. Membranes were incubated for 30 min at 20 °C with L-[^{14}C]glucose and D-[^{3}H]glucose (5 mM each). Aliquots were rapidly filtered and washed with the indicated number of drops of cold Krebs–Ringers phosphate buffer. Radioactivity remaining on the millipore filter was then determined by dual channel counting[24].

Washes (drops)	*Ghosts*		*Mechanically vesiculated ghosts*		*Trypsinized vesiculated ghosts*	
	L-*Glucose (pmoles)*	D-*Glucose (pmoles)*	L-*Glucose (pmoles)*	D-*Glucose (pmoles)*	L-*Glucose (pmoles)*	D-*Glucose (pmoles)*
1	1497	1313	1504	1374	2091	2313
2	177	118	512	487	1559	1767
3	169	137	243	206	1325	1599
5	76	83	320	330	1273	1488
7	53	39	255	248	1153	1362
10	76	63	244	240	1027	1207
20	37	18	185	162	891	1040

of untrypsinized ghosts produced vesicles whose resistance to the washout of glucose was slightly improved over the parent ghost, but still far less than the trypsinized vesicles.

Aliquots of red blood cell membranes taken at various times of proteolysis (Fractions A and B prepared as described in Methods) were subjected to density gradient centrifugation. In Dextran gradients, control preparations yielded a single broad band at a modal density of approximately 1.06. With progressive proteolysis, a band of much lighter density appears (ρ approx. 1.01) associated with a progressive decrease in the amount of membrane in the denser band. At 60 to 90 min, essentially all of the particles band near ρ 1.01 (Fig. 4). In sucrose gradients the particles show no such bimodal distribution (Fig. 5); however, the modal density does decrease slightly with proteolysis from 1.14 to 1.12.

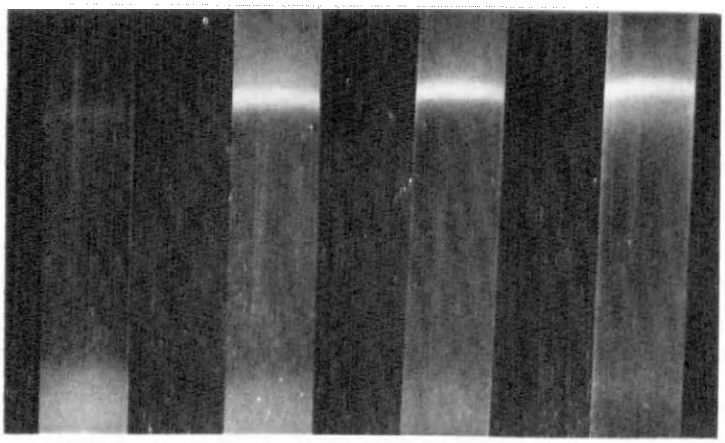

Fig. 4. Dextran gradients of erythrocyte membranes at various times after proteolysis. From left to right 0, 5, 30 and 60 min. ρ = 1.01–1.07.

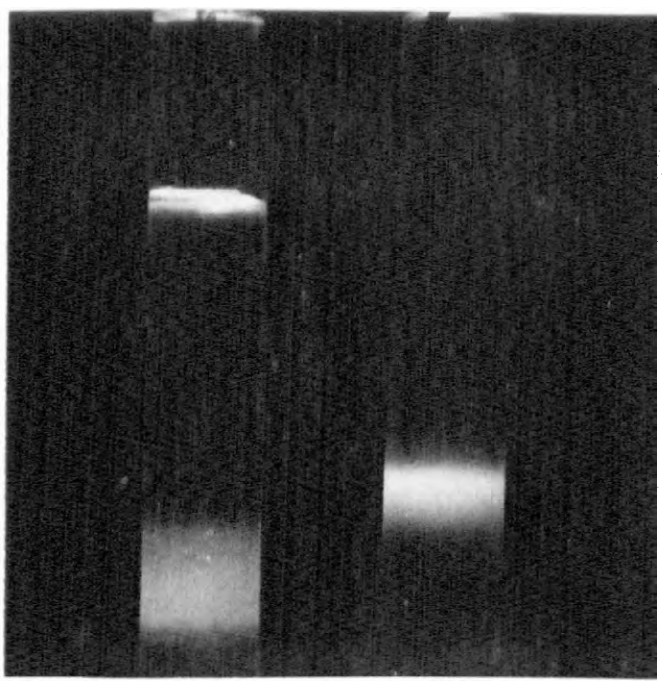

Fig. 5. Dextran gradient, ρ = 1.01–1.07, (left) and sucrose gradient, ρ = 1.01–1.20, (right) of erythrocyte membranes after 5 min of proteolysis.

Effect of proteolysis on the phospholipid, cholesterol, and protein content of red blood cell membranes is shown in Table II. The phospholipid/cholesterol ratio is insensitive to trypsin treatment, but both the phospholipid/protein and cholesterol/protein ratios in the proteolyzed particles after washing is consistent with the elution of protein into the wash solutions as demonstrated in Gel 3, Fig. 3, and roughly quantitates these losses at 18–20%. This figure is comparable to the estimate of 22% protein loss derived from direct measurement of protein, and this similarity indicates that negligible amounts of lipid are lost from the membrane during proteolysis.

TABLE II

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF TREATED AND CONTROL RED BLOOD CELL MEMBRANES

Values are the mean ± S.D. of 3 to 9 determinations. Trypsinization was carried out in he absence of sucrose and membranes were totally converted to the low density form by the 30-min trypsin-treatment. All samples were washed 3 times with 0.5 mM $MgSO_4$, 1 mM Tris–HCl (pH 8.0) before assay.

	Cholesterol (μmoles/mg protein)	*Phospholipid (μmoles/mg protein)*	*Phospholipid/ cholesterol*
Untreated membranes	0.88 ± 0.05	0.94 ± 0.02	0.94 ± 0.08
Trypsinized zero time	0.89 ± 0.12	1.00 ± 0.17	0.89 ± 0.13
Trypsinized 30 min	1.14 ± 0.13	1.22 ± 0.19	0.93 ± 0.7
Calculated protein loss due to trypsin-treatment	20%	18%	

Further characterization of the two vesicle populations found in the Dextran gradients was attempted. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the light and heavy fractions (Fig. 3) was performed. Due to the selective elution of certain components (noted above) during the washing and gradient steps, the electrophoretic patterns obtained after washing differ markedly from that seen in the uncentrifuged incubation mixture. Thus the peptides remaining associated with the membrane appear relatively enriched. The differences between the heavy and light vesicle population are relatively minor (Fig. 3, lower panel compare Gels 5 and 6) are confined to the relatively greater loss of high molecular weights components (in the region of Band II) in the light fraction. The heavy and light fractions contained essentially identical amounts of glycopeptide sialic acid which in both fractions was 95–105% accessible to neuraminidase.

Although intact erythrocytes transport glucose by facilitated diffusion[29], red blood cell ghosts or mechanically vesiculated ghosts cannot be shown to preferentially take up D-glucose over L-glucose. At a point in the proteolysis when Components III and IV have been selectively and extensively cleaved, preferential uptake of D-glucose becomes demonstrable. This phenomenon is explored in detail elsewhere[17].

DISCUSSION

The identification of the peptide components of the red blood cell membrane separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate has been

previously reported[18,26]. Several of these peptides have been partially characterized, especially those in the molecular weight region of 200000 (refs 28 and 29) (probably corresponding to the fraction called Spectrin by Marchesi and co-workers) and the sialoglycopeptides[30]. However, with the exception of the latter, which appear to contain blood group activities[30], the role of these peptide components in red blood cell membrane structure and function is unclear.

Proteolysis of Dodge ghosts with low concentrations of trypsin results in the extensive cleavage of only two of the major membrane peptides, Components III and IV. Concomitant with this and before significant proteolysis of other membrane peptides has occurred, the ghosts lose their biconcave shape by sphering and breakdown into vesicles by exocytosis. In addition to proteolysis[16], vesiculation of erythrocyte ghosts may be induced by a variety of poorly understood maneuvers, including alkaline pH, low ionic strength and chelating agents[11,19]. Depending largely on the presence of divalent cations, the process may occur by exo- or endocytosis[19]. While the mechanisms involved in vesiculation are unknown, it has been observed that conditions which foster vesiculation are associated with the loss of certain easily eluted peptides (Components I, II and V) from the membrane[11,19]. Selective proteolysis of components III and IV does not in itself result in release of other membrane peptides. However, the association of several peptides with the membrane is "labilized" in that significant portions of Components I, II, V, and VI can be eluted from proteolyzed ghosts by washing in a medium which cannot remove these components from unproteolyzed ghosts. Thus, whether proteolysis-induced vesiculation is directly related to cleavage of Components III and/or IV or to the consequent alterations in the association of other peptides with the membrane is uncertain. Nevertheless, the occurrence of this proteolysis-induced morphologic transition does suggest that Components III and/or IV (especially III) are important in the maintenance of the normal structure of the red blood cell membrane. Several observations support this hypothesis and indicate that the interaction of Component III with the membrane with membrane lipids is the critical aspect in this function.

The structure of the red blood cell membrane appears to be stabilized in large part by hydrophobic interactions[31,32]. The interaction of Component III with the membrane appears to be predominantly hydrophobic, as it cannot be released by manipulations of ionic strength pH and chelating agents such as will solubilize Components I, II, V and VI[18]. Furthermore, Component III is not released from membrane lipid by guanidine hydrochloride, whereas 50% of the membrane protein can be removed by this agent[33,34]. The association of Component III with the membrane is also resistant to urea treatment[35]. Component III appears to penetrate to both faces of the membrane[11,36] and is the most abundant single peptide species in the membrane by factor of 3-fold on a molar basis (see calculation, ref. 18). That this close association of Component III with membrane lipid is significant to the organization of these lipids is suggested by our observation on the non-specific permeability of vesicles from trypsinized ghosts. Such vesicles are much more tightly sealed (impermeable) than the parent ghosts or vesicles produced by mechanical means. Since the non-specific permeability of membranes is dependent largely on the organization of membrane lipids[37,38] we infer that the alteration in vesicle permeability accompanying the cleavage of Components III and IV reflects a change in membrane lipid structure. Proteolysis induced changes in erythrocyte membrane lipid organization

have been previously reported. Dried preparations of erythrocyte membranes have been studied by low angle X-ray diffraction. Dehydration of the membranes results in separation of part of the lipid to form independently diffracting phases, while a band indicative of residual lipoprotein structure remains. When erythrocyte membranes are trypsinized (conditions not specified) essentially all of the lipid crystallizes out during drying and the band arising from residual lipoprotein is abolished[39].

As to the nature of the change which permits resealing, we have no specific information. The density gradients studies, however, may be germane to this question. As first pointed out by Wallach and Kamat[40], and later eleborated by Steck *et al.*[41] into a formal model, the isopycnic density of semipermeable vesicles depends on their environment. This appears to be due to the Donnan osmotic forces created by non-diffusible species sequestered in the vesicle interior. These may consist in either fixed charges on the inner surface of the membrane or trapped solutes. In gradients of non-penetrating solutes, such as Dextran (molecular weight 110000) when the ambient concentration of permeant ionic species is low, trapped solutes as well as the fixed charges on the inner surface of the vesicles contribute significantly to the Donnan osmotic equilibrium. The vesicles retain a relatively large internal aqueous compartment and this accounts for the lower isopycnic density observed in such gradients.

In sucrose gradients, proteolyzed membranes show a slight decrease in density. This result is consistent with the reported effects of proteolysis on the density of liver smooth microsomal membranes in sucrose gradients[42], and probably reflects the increased lipid/protein ratios. Furthermore, in sucrose the proteolyzed red blood cell vesicles distribute in a single band. The isopycnic density distribution of the vesicles obtained in Dextran gradients differ in two respects. First, the equilibrium density is much lower in Dextran; and second in association with proteolysis a new population of particles of very low buoyant density is generated. The proteolysis induced change in density observed in the Dextran gradients is too large to be accounted for by the changes observed in the lipid/protein ratios. As proteolysis proceeds, the membranes are progessively converted to this much lower density form. In accord with the considerations outlined above, this would indicate that the intravesicular effective Donnan osmotic forces have increased. This is not due to entrapped solutes: homogenization of ghosts in the presence of sucrose or soybean trypsin inhibitor *plus* trypsin, does not diminish their buoyant density in Dextran gradients; proteolysis of ghosts in the presence of sucrose is likewise without effect on the buoyant density. In both these instances the sucrose probably leaks out during the washing and gradient steps. More likely, the lower buoyant density probably reflects an increase in effective charge density on the inner surface of the membrane. The nature of these new charges is unclear. The density of the proteolyzed vesicle is similar to that described for inside out red blood cell vesicles[19]. However, this overall inversion does not appear to occur in association with proteolysis, since the glycopeptide sialic acid residues, which are entirely on the outer surface of the intact ghosts and thus susceptible to neuraminidase[19], maintain this orientation in the proteolyzed vesicles. Comparison by polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the low and high density particles fractionated at a single time during proteolysis reveals largely similar patterns. Of importance is that both species have undergone proteolysis, and the major difference

is that the lighter particles are more depleted of components in the molecular weight region of Band II.

While the identity of the new charged groups on the inner surface of the membrane is unknown, we suggest that proteolysis may promote resealing by allowing a reorientation of charged groups, perhaps from the membrane interior to its inner surface. Although frankly speculative, this contention is consistent with other types of observations. For example, Lepke and Passow[43] have suggested that a reorientation of membrane charges may explain the effects of pH of the hemolysis medium on the sealing of erythrocyte ghosts.

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REFERENCES

1 S. Orrenius, A. Berg and L. Ernster, *Eur. J. Biochem.*, 11 (1969) 193.
2 B. Kuylenstierna, D. G. Nicholls, S. Hovmoller and L. Ernster, *Eur. J. Biochem.*, 12 (1970) 419.
3 M. M. Burger, *Nature*, 227 (1970) 170.
4 B. Ozanne and J. Sambrook, *Nature, New Biol.* 232 (1971) 156.
5 G. S. Hand, Jr, *Exp. Cell Res.*, 64 (1971) 204.
6 J. F. Kuo, I. K, Dill and C. E. Holmund, *J. Biol. Chem.*, 242 (1967) 3659.
7 T. Kono, *J. Biol. Chem.*, 244 (1969) 5777.
8 J. M. Tobias, *J. Gen. Physiol.*, 43 (1960) 515.
9 I. A. Tasaki and T. Takenaka, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 84.
10 J. T. Dodge, C. Mitchell and D. H. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
11 T. L. Steck, G. Fairbanks and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2617.
12 K. L. Carraway, D. Kobylka and R. B. Triplett, *Biochim. Biophys. Acta*, 241 (1971) 934.
13 R. Colman, J. B. Finean and J. E. Thompson, *Biochim. Biophys, Acta*, 173 (1969) 51.
14 G. Inesi and H. Asai, *Arch. Biochem. Biophys.*, 126 (1968) 469.
15 A. Martonosi, *J. Biol. Chem.*, 243 (1968) 71.
16 V. T. Marchesi and G. E. Palade, *Proc. Natl. Acad. Sci. U.S.*, 58 (1971) 991.
17 J. R. Carter, Jr, J. Avruch and D. B. Martin, *Biochim Biophys. Acta*, 291 (1973) 506.
18 G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2606.
19 T. L. Steck, R. S. Weinstein, J. H. Straus and D. F. H. Wallach, *Science*, 163 (1970) 255.
20 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971.
21 A. L. Babson, P. O. Shapiro and G. E. Phillips, *Clin. Chim. Acta*, 7 (1962) 800.
22 H. A. I. Newman, C. T. Liu and D. B. Zilversmit, *J. Lipid Res.*, 2 (1961) 403.
23 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466.
24 J. R. Carter, Jr, and D. B. Martin, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 1343.
25 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. H. Randall, *J. Biol. Chem.*, 193 (1951) 265.
26 J. Lenard, *Biochemistry*, 9 (1970) 1129.
27 F. Bowyer, *Int. Rev. Cytol.*, 6 (1957) 469.
28 V. T. Marchesi and E. Steers, Jr, *Science*, 159 (1968) 203.
29 S. L. Marchesi, E. Steers, Jr, V. T. Marchesi and T. W. Tillack, *Biochemistry*, 9 (1970) 50.
30 R. J. Winzler, in F. A. Jamieson and T. H. Greenawalt, *Red Cell Membrane, Structure and Function*, J. P. Lippincott Co., Philadelphis, Pa., 1969, p. 157.
31 D. F. H. Wallach, *J. Gen. Physiol.*, 54 (1969) 35.
32 M. Glaser, H. Simpkins, S. H. Singer, M. Sheetz and S. I. Chan, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 721.
33 J. T. Gwynne and C. Tanford, *J. Biol. Chem.*, 245 (1970) 3209.
34 T. L. Steck, *Biochim. Biophys. Acta*, 255 (1972) 553.

35 R. L. Juliano and A. Rothstein, *Biochim. Biophys. Acta*, 249 (1971) 227.
36 T. L. Steck, in C. F. Fox, *Proc. 1972 ICN–UCLA Symp. Biol.* Academic Press, New York, 1972.
37 H. Davson and J. F. Danielli, *The Permeability of Natural Membranes*, 2nd edn, Cambridge University Press, Cambridge and New York, 1952.
38 W. D. Stein, *The Movement of Molecules Across Cell Membranes*, Academic Press, New York, 1967.
39 J. B. Finean and R. Coleman, in J. R. Villanueva and F. Ponz, *Membranes, Structure and Function*, Academic Press, New York, 1970, p. 11.
40 D. F. H. Wallach and V. B. Kamat, *Proc. Natl. Acad. Sci. U.S.*, 52, (1964) 72.
41 T. L. Steck, J. H. Strauss and D. F. H. Wallach, *Biochim. Biophys. Acta*, 203 (1970) 385.
42 A. Ito and R. Sato, *J. Cell. Biol.*, 40 (1969) 179.
43 S. Lepke and H. Passow, *Biochim. Biophys. Acta*, 255 (1972) 696.