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Disposition of the Major Proteins in the Isolated Erythrocyte Membrane. Proteolytic Dissection*

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ABSTRACT: Vesicles derived from human erythrocyte membranes were separated into two fractions of normal and inside-out orientation. By treating each species with proteolytic enzymes, the two faces of the red cell membrane were selectively digested. The course of proteolysis was monitored by the consumption of alkali and by polyacrylamide gel electrophoresis of the membrane proteins dissolved in sodium dodecyl sulfate. By both criteria, whole ghosts and normally oriented vesicles were much more susceptible to proteolysis than inside-out vesicles. One protein was intrinsically resistant to proteolysis. All of the other major components that could be stained with coomassie blue were extensively digested at the outer surface. Only one of these, a predominant component of mol wt 89,000, was attacked by digestion of inside-out vesicles. The three sialoglycoproteins, which were detectable only by carbohydrate staining, were digested by proteolytic attack at either surface. The data are not consistent with a symmetrical membrane organization, but rather suggest a highly asymmetric arrangement of oriented proteins, at least some of which appear to span the thickness of the membrane.

The proteins of the human erythrocyte membrane have recently been analyzed and partially characterized in several investigations (see Fairbanks *et al.*, 1971). The molecular architecture of this plasma membrane has likewise been intensively studied, but remains more elusive. The present study sought to determine how the major component proteins were represented at the two faces of the isolated erythrocyte membrane. We asked: "Does each protein type equally and symmetrically populate each surface; do certain proteins exist at one surface and not the other; do any major proteins penetrate from the outer to the inner surface; and if there are penetrating proteins, are all molecules of one type oriented in the same direction or are they symmetrically or randomly disposed?"

Our study was made feasible by the development of methods for the preparation and purification of inside-out membrane vesicles, which bare the membrane's cytoplasmic face to selective attack, just as intact ghosts and right-side-out vesicles proffer the normal outer surface. It was assumed (see Discussion) that the proteins in these vesicles retained the orientation they held in the parent membrane. By means of gentle digestion with impermeable proteolytic enzymes, each of the membrane's two faces were probed separately; the consequences were assessed by polyacrylamide gel electrophoresis of the membrane proteins dissolved in SDS.[1]

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; IO, inside out; RO, right-side out; PAS, periodic acid–Schiff; TD, tracking dye; I–VI, numerals designating the six major protein components demonstrated by electrophoresis; PAS-1–3, the three major glycoprotein components demonstrated by electrophoresis (Fairbanks *et al.*, 1971).

Experimental Section

Red blood cell ghost membranes were prepared according to Fairbanks *et al.* (1971).

Inside-out (IO) and right-side-out (RO) vesicles were prepared as in Steck *et al.* (1970) with slight modification. Ghost pellets (3–4 mg of protein in 1 ml) were mixed with 30–40 ml of cold 5×10^{-4} M sodium phosphate buffer (pH 8.0) in Sorvall SS-34 tubes. After 1 hr or more on ice the suspensions were made 10^{-4} M in $MgSO_4$. The magnesium ion promoted the retention of polypeptide components I, II, and V which were otherwise eluted by the incubation at very low ionic strength (Fairbanks *et al.*, 1971) and stabilized the membranes against untoward vesiculation during the subsequent fractionation steps. The membranes were pelleted at 15,000 rpm for 30 min. The ghosts underwent spontaneous vesiculation by endocytosis at this time. The pellets were resuspended to about 3 mg of protein/ml in 5×10^{-4} M sodium phosphate (pH 8.0)–10^{-4} M $MgSO_4$, and vesiculation was completed by five passes through a 27-gauge needle.

Aliquots (2 ml) of the vesicle mixture were layered over 10.5 ml of a Dextran-110 (Pharmacia) density gradient (1.01–1.07 g/cm^3) in 5×10^{-4} M sodium phosphate (pH 8.0)–10^{-4} M $MgSO_4$. After centrifugation for 15–16 hr at 30,000 rpm in a Spinco SW-41 rotor, two major zones formed. The zone at 1.01–1.03 g/cm^3 was composed of inside-out vesicles, while the 1.05–1.065-g/cm^3 band contained normally oriented vesicles (Steck *et al.*, 1970). There was no pellet. The vesicles were freed of dextran by one or two washes in the same buffer or 5×10^{-4} M NaCl–10^{-4} M $MgSO_4$ and were stored at 4° for up to 5 days.

Assays. Protein was determined fluorometrically as described by Fairbanks *et al.* (1971), except that no SDS was present.

Cholesterol was assayed according to Leffler and McDougald (1963) following extraction of lipids by the method of Albrink (1959).

Phospholipid phosphorus was assayed according to Ernster *et al.* (1950). The extracted lipids were first dried under nitrogen and ashed according to Lowry *et al.* (1954).

Sialic acid was assayed according to Warren (1959), following hydrolysis in 0.1 N H_2SO_4 at 80° for 1 hr.

Proteolytic Digestion. TITRATION WITH ALKALI. Stock enzyme solutions (1 mg/ml in water) were brought to pH 8.500 with NaOH. Intact IO or RO vesicles (1 mg of protein) were mixed with 0.1 mmole of KCl and 0.1 μmole $MgSO_4$ in 0.9 ml of H_2O. An Instrumentation Laboratory No. 14150 combination pH electrode filled with 0.1 M KCl and attached to an Instrumentation Laboratory Model 205 digital pH meter was immersed in the sample, which was stirred under a stream of water-saturated, CO_2-free nitrogen. The pH was adjusted to 8.500 and maintained there by the addition of 0.01 N NaOH from a microburet. After a 10-min control period of monitoring the rate of NaOH consumption (which was very small and not corrected for), 0.10 ml (100 μg) of trypsin (twice crystallized, Worthington), α-chymotrypsin (Worthington CDS), or pronase (Calbiochem) was added to initiate the reaction.

ELECTROPHORESIS. Washed ghosts, inside-out vesicles, or right-side-out vesicles (1 mg of protein/ml) were incubated with 10 μg/ml of trypsin or α-chymotrypsin in 0.1 ml of 2.5 mM sodium phosphate buffer (pH 8.0) for designated intervals at room temperature. Digestions with papain (twice crystallized, Sigma) were performed in 2.5 mM sodium phosphate buffer (pH 7.0), containing 0.5 mM cysteine, 0.5 mM Na_2EDTA, and 0.5 mM $MgSO_4$. To terminate the reactions, an equal volume (0.1 ml) of soybean trypsin inhibitor (50 μg/ml), phenylmethylsulfonyl fluoride (4 mM in 20% 2-propanol), or iodoacetamide (0.1 M) was added to the trypsin, chymotrypsin, and papain samples, respectively.[2] After 10 min at room temperature the samples were made ready for electrophoresis by a 20-min incubation at 37° with 0.05 ml of the following fivefold concentrate: SDS, 5%; dithiothreitol, 20 mM; EDTA, 5 mM; Tris-HCl (pH 8.0), 50 mM; pyronin Y, 50 μg/ml; sucrose, 25% (w/w). This treatment converts the membrane proteins into monomeric polypeptides (Fairbanks *et al.*, 1971); however, the term *proteins* is retained in referring to the gel bands.

Parallel experiments were performed on intact erythrocytes by digesting that concentration of cells possessing 1 mg/ml of membrane protein (about 1.75×10^9 cells/ml) with 10 μg/ml of each enzyme. Proteolysis was terminated with the appropriate inhibitors and the cells were washed once. Ghosts were then prepared and readied for electrophoresis as outlined above.

Electrophoresis was performed according to Fairbanks *et al.* (1971). Polyacrylamide gels (5.6% containing 1% SDS) were loaded with 40 μg of membrane protein. A field of about 12 V/cm (9 mA/gel tube) was applied until the pyronin Y tracking dye had migrated exactly 75 mm (75–90 min). The dye band was marked with drafting ink and the gels were fixed and stained with either coomassie brilliant blue or PAS (Fairbanks *et al.*, 1971).

Results

Characterization of the Vesicles. Inside-out vesicles are created when treatment with very dilute, alkaline buffer induces endocytosis in purified ghosts. The portion of the membrane not undergoing this inversion is recovered as sealed vesicles of normal (RO) orientation (Steck *et al.*, 1970).

To establish that the IO and RO vesicle preparations were altered only in orientation and were otherwise like the parent membrane, several of their chemical constituents were compared to ghosts (Table I). The values for ghost cholesterol, phospholipid, and sialic acid, as well as the cholesterol/phospholipid ratio, were in accord with previous studies. Both vesicle types showed enrichment of each constituent with respect to protein content, particularly the right-side-out fraction. It seems that protein has been lost selectively from these vesicles. (The apparent loss of phospholipid needs to be further substantiated.)

Similar levels of acetylcholinesterase and Na^+,K^+-dependent ATPase activity were found in the ghosts and the two vesicle fractions (T. L. Steck, 1970, unpublished data). Precise comparisons are made difficult by the variable loss of nonenzyme protein and the membrane barrier to the free diffusion of substrates.

The protein patterns obtained by polyacrylamide gel electrophoresis provided a more stringent and relevant means of evaluating vesicle composition. Figure 1 shows electrophorograms of ghost, IO, and RO vesicle proteins. The profiles are superimposable, except for the selective depletion of three components, I, II, and V. (In the scan of RO vesicles, the depletion of II exposes some minor bands it usually obscures.)

[2] If enzyme inhibitors are not added, the proteins become very susceptible to spurious proteolysis when the membranes are dispersed in SDS (*e.g.*, see Figure 2). Pronase is particularly troublesome in this respect.

TABLE I: Chemical Composition of Ghosts and Vesicles.[a]

Components	Ghost		IO Vesicles	RO Vesicles
	Literature[b]	This Study		
Cholesterol (nmoles/mg of protein)	552[c] 562[d]	564 ± 16	657 ± 13	748 ± 5
Phospholipid (nmoles/mg of protein)	653[c] 702[d]	674 ± 19	722 ± 26	790 ± 42
Sialic acid (nmoles/mg of protein)	78[e] 105[f]	89 ± 2	107 ± 2	117 ± 0.5
Cholesterol/phospholipid (mole/mole)	0.84[c] 0.80[d]	0.84	0.91	0.95
Sialic acid/phospholipid (mole/mole)		0.13	0.15	0.15
Sialic acid/cholesterol (mole/mole)		0.16	0.16	0.16

[a] Values are the mean plus and minus standard deviation from triplicate determinations in a representative experiment. [b] Values from references *c* and *d* were calculated by assuming a value of 5.7×10^{-10} mg of protein/ghost (Fairbanks *et al.*, 1971). [c] From Ways and Hanahan (1964). [d] From Sweeley and Dawson (1969). [e] From Rega *et al.* (1967). [f] From I. Hagen (1970, personal communication).

Polypeptides I, II, and V can be recovered in solution in the 5×10^{-4} M phosphate incubation buffer (Fairbanks *et al.*, 1971). Some loss of these components was invariable in our endocytosis protocol; those levels of pH, ionic strength, and divalent cation which blocked their release also suppressed endocytosis.

The right-side-out vesicles are usually more impoverished of bands I, II, and V than their inverted counterparts. We relate this to the localization of these components on the membrane's outer surface (see below); inside-out vesicles are thought to sequester the released proteins within their lumens while the normal vesicles lose the material to the bulk solution.

Proteolysis. TITRATION WITH ALKALI. The vesicle preparations appeared similar enough to each other and to the parent ghosts to serve as suitable subjects for studying the sidedness of isolated red blood cell membranes. The various species were therefore subjected to mild proteolysis, the effects of which were taken to reflect the reactivity of each externally oriented face.

Figure 2 depicts the effect of digesting RO and IO vesicles with three enzymes of differing specificity. The inverted membranes were far less susceptible to proteolysis than their normally oriented counterparts. Intact ghosts, not depicted here, were as vigorously digested as the RO vesicles. These particular experiments were designed to demonstrate some additional points. (1) The rate of proteolysis was limited by steric factors (organizational and/or conformational) in that disruption with SDS stimulated the reaction markedly and to a similar degree in both vesicle types (Figure 2A). (2) The inside-out vesicles did not contain a proteolysis inhibitor, since (a) right-side-out vesicles added following incubation of trypsin with inside-out vesicles were rapidly digested (Figure 2B); and (b) disrupting the inverted membranes with SDS permitted their digestion to proceed vigorously (Figure 2A,C).

Parallel studies at one-tenth the enzyme concentration (*i.e.*, 10 μg/ml) gave similar results. Furthermore, vesicle mixtures which were not fractionated on dextran gradients gave intermediate proteolysis profiles which reflected the relative proportions of the two vesicle species present. This indicates that the gradient separation procedure did not artifactually induce the observed differential reactivity.

Proteolysis. ELECTROPHORETIC ANALYSIS. Polyacrylamide gel electrophoresis in SDS was used to evaluate the effects of enzymatic digestion on the individual polypeptides of the red cell membrane when intact and when isolated in the form of ghosts, RO vesicles, and IO vesicles.

Intact erythrocytes were subjected to proteolytic digestion and their membranes purified, dissolved, and electrophoresed. None of the major components stained with coomassie blue was significantly affected by trypsin, chymotrypsin, or papain digestion, provided that the enzymes were completely inhibited prior to hemolysis. However, the major PAS-stained sialoglycoproteins were readily digested by all three enzymes.[3] These studies will be fully reported elsewhere; it is important to note here that the resistance of the intact erythrocyte membrane to proteolysis contrasts sharply with the susceptibility of isolated membranes.

Ghosts were likewise incubated with trypsin, chymotrypsin, and papain (Figure 3). Each enzyme generated a different pattern of proteolysis products, but all three enzymes digested all of the major polypeptide components except band VI. Insoluble, resin-bound derivatives of these enzymes (Enzite-trypsin, chymotrypsin, and papain; Miles-Seravac) were also employed to ensure that the enzymes were not permeating the ghost membrane. They, too, digested all of the major bands except VI; proteolysis was, however, less extensive than with the soluble enzymes, perhaps because of the unfavorable kinetics of interaction using the solid-phase enzymes.

To test whether proteolytic digestion rendered the ghosts permeable to macromolecules, we centrifuged trypsin-treated preparations to equilibrium in dextran gradients, as outlined in the Experimental Section. We found, as did Ito and Sato (1969) for microsomal vesicles, that the digested membranes still excluded dextran and, indeed, equilibrated at a somewhat lighter density than the untreated controls. Inside-out and right-side-out vesicles performed similarly.

The one resistant band, VI, has been shown to be selectively eluted from ghost membranes by 0.15 M NaCl washes (Fair-

[3] This observation provides further evidence that the PAS-positive components are glycoproteins which do not correspond to any of the coomassie blue bands (Fairbanks *et al.*, 1971).

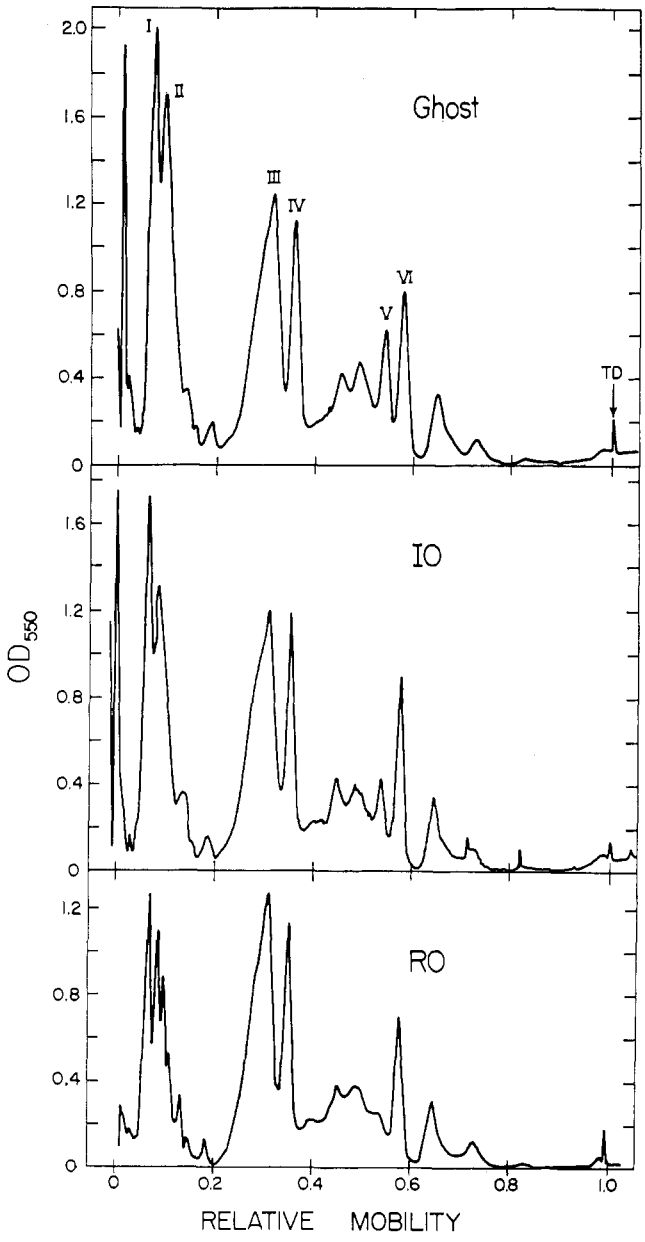

FIGURE 1: Electrophorograms of ghost, IO, and RO membrane proteins. Membrane samples were prepared and electrophoresed as described in the Experimental Section. Gels were stained with coomassie brilliant blue and scanned at 550 nm. To facilitate comparison, the absorbance scale was adjusted in the IO and RO tracings to give about the same peak height for band III (*i.e.*, 1.2) as in the ghost scan.

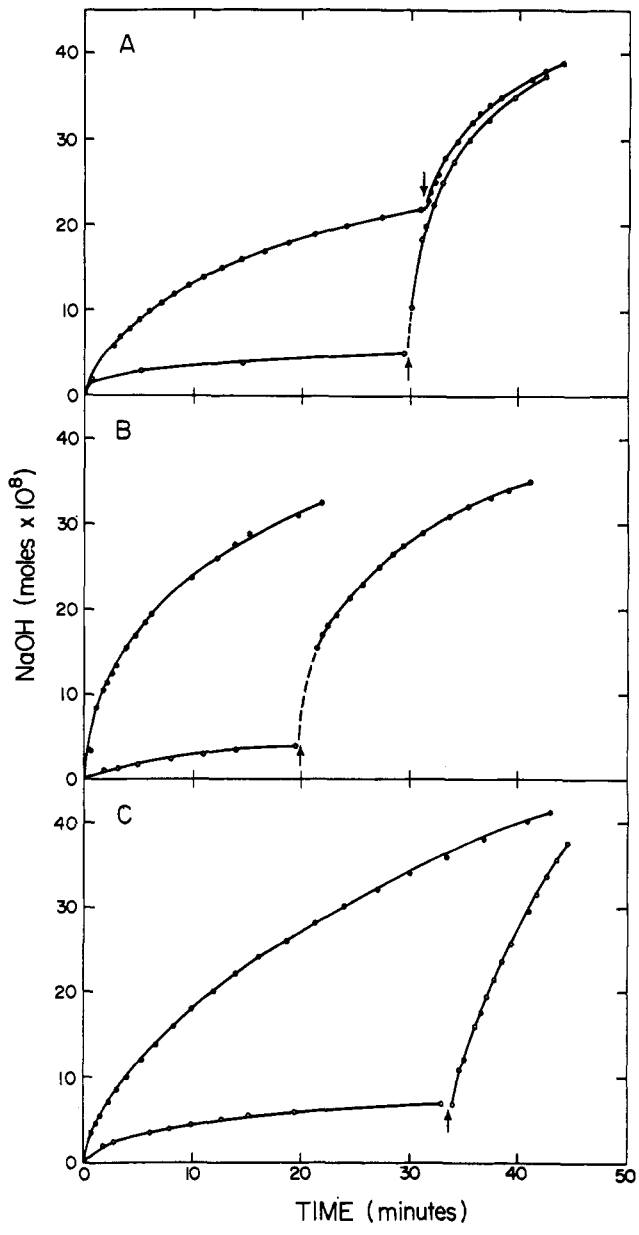

FIGURE 2: Time course of proteolysis. RO (●—●) and IO (○—○) vesicles (1 mg of protein/ml) were incubated with enzymes (100 μg/ml) at room temperature. The generation of acid during proteolysis was monitored by maintaining the pH at 8.500 with measured volumes of 0.01 N NaOH. (A) Chymotrypsin digestion. At the arrows, the reaction mixtures were made 0.2% in SDS. (B) Trypsin digestion. At the arrow, an equal amount of RO vesicles was added to the IO vesicle reaction mixture. (C) Pronase digestion. At the arrow, the IO reaction mixture was made 0.2% in SDS.

banks *et al.*, 1971). This polypeptide was as resistant to the three proteolytic enzymes in its soluble, isolated state as when membrane bound. Furthermore, proteolytic digestion of the membranes selectively released this undigested protein into the medium. Finally, treatment with 1% SDS made this component quite sensitive to proteolysis. We have concluded that the resistance of band VI to proteolysis is intrinsic rather than a result of steric protection afforded by its position in the membrane.

Inside-out and right-side-out vesicles were digested, dissolved, and electrophoresed in parallel (Figure 4). As in the case of the intact ghosts, the RO vesicles exhibited complete susceptibility of all their major polypeptide components except band VI. The proteolysis patterns varied according to the enzyme employed and in general corresponded to the results found with whole ghosts (Figure 3).

The digestion pattern of IO vesicles was strikingly different. Trypsin treatment showed a very circumscribed effect. Band III progressively diminished over the 3-hr incubation, while a discrete band grew up reciprocally just ahead of band IV. Band III was quite sensitive to chymotrypsin and papain, even after 5-min digestion. A single, major proteolysis product appeared with all three enzymes: a sharp, heavy band between IV and V with an apparent molecular weight of about 65,000. Its integrated optical absorbance and mobility indicated that, at least initially, 1 mole of this fragment was gen-

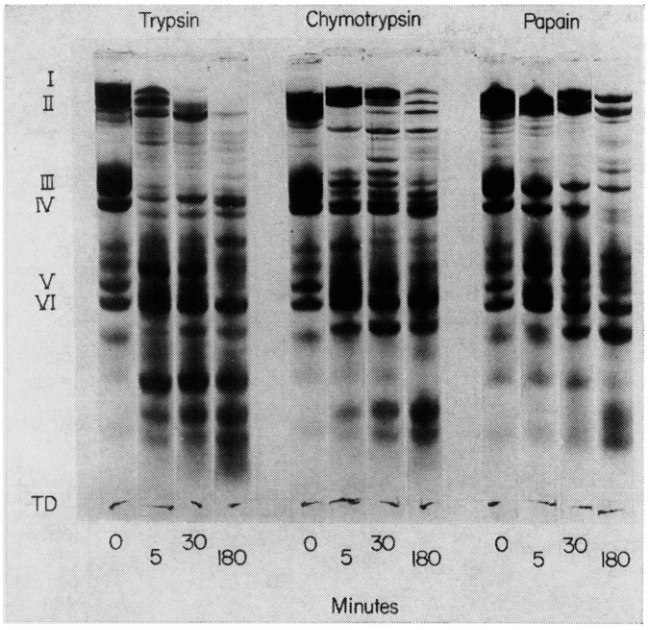

FIGURE 3: Proteolysis of ghosts. Purified ghosts (1 mg of protein/ml) were incubated at room temperature for 0–180 min with trypsin, chymotrypsin, and papain (10 μg/ml). The membranes were dissolved and electrophoresed as described in the Experimental Section. The gels were stained with coomassie brilliant blue. In these and subsequent electrophorograms, the proteolytic enzyme proteins are not detected.

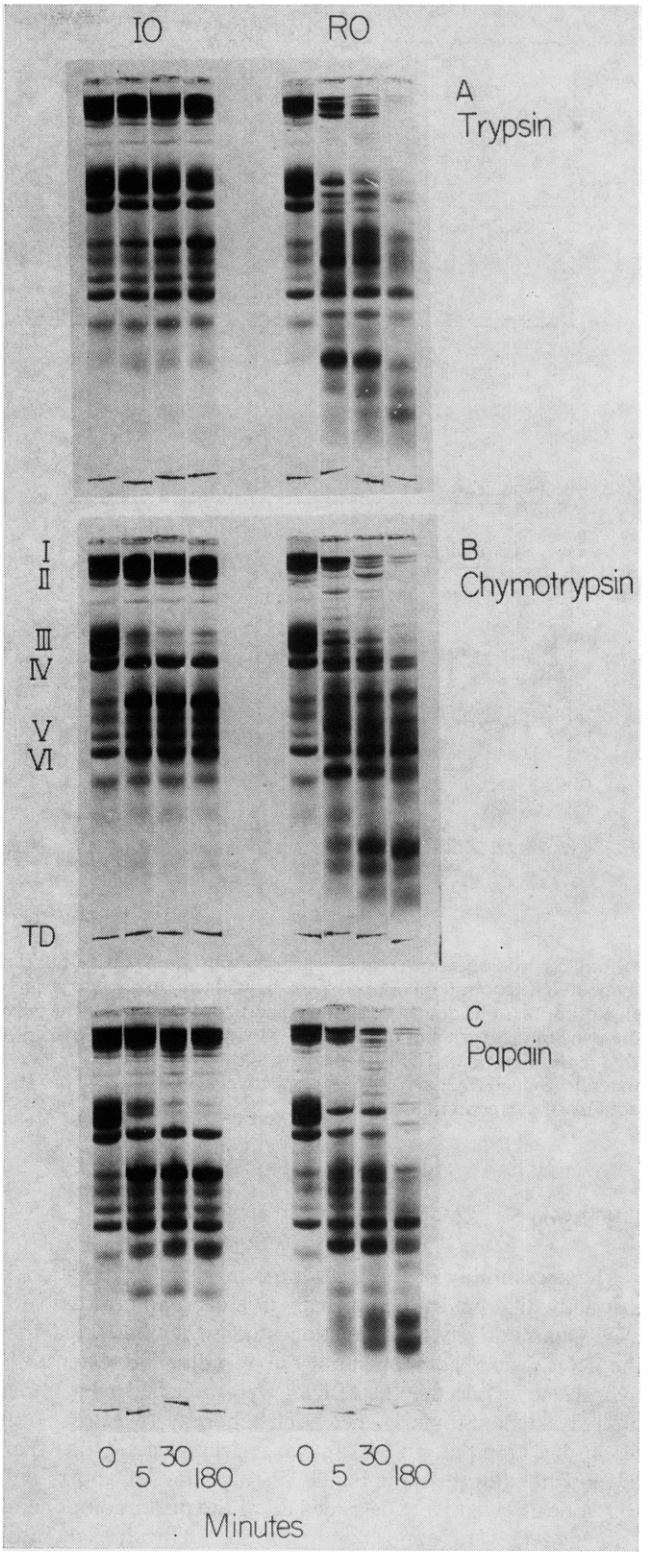

FIGURE 4: Proteolysis of vesicles: protein stain. Inside-out (left series) and right-side-out (right series) vesicles (1 mg of protein/ml) were incubated at room temperature for 0–180 min with trypsin (A), chymotrypsin (B), and papain (C) (10 μg/ml). The membranes were dissolved and electrophoresed as described in the Experimental Section. The gels were stained with coomassie brilliant blue.

erated for each mole lost from component III. In the papain series, three smaller cleavage products are also evident.

No component other than band III appeared to be significantly perturbed by digestion of the IO vesicles, except for a slight thinning of bands I and II after the 3-hr papain incubation. No loss of the integrated stain intensity resulted from even the 3-hr digestion of the IO vesicle species. In contrast, RO vesicles lost 39, 16, and 20% of the total stain absorbance of their gel electrophorograms during their 3-hr incubation with trypsin, chymotrypsin, and papain, respectively.

Digestion of Glycoproteins. Parallel studies were performed to elucidate the disposition of the major membrane glycoproteins. These gels were stained by the periodic acid–Schiff method which reveals three sialoglycoprotein bands not visualized by conventional protein stains (Fairbanks *et al.*, 1971).

Figure 5 shows the densitometer tracings of IO and RO vesicles treated with no enzyme, trypsin, chymotrypsin, and papain. The salient features of these experiments are: (1) the untreated IO and RO preparations exhibited the same PAS patterns as whole ghosts (Fairbanks *et al.*, 1971); (2) papain digestion destroyed most of the glycoprotein band intensities; discrete cleavage products were not discernible; (3) trypsin was somewhat less vigorous than papain but diminished each band significantly; (4) chymotrypsin reduced the intensity of the PAS bands the least and generated a discrete new band of slightly faster mobility than PAS-1; the integrated optical absorbance of the chymotrypsin gel scans (corrected for background) was more than 80% of their respective undigested controls; (5) with none of the enzymes was a significant difference observed between the RO and IO vesicle digestion patterns; (6) the susceptibility of PAS-1–3 to these proteolytic enzymes confirms that they contain protein, even though they do not take up conventional protein stains. The band moving just behind the dye marker is not digested and probably contains glycolipid and/or plasmalogen (Fairbanks *et al.*, 1971).

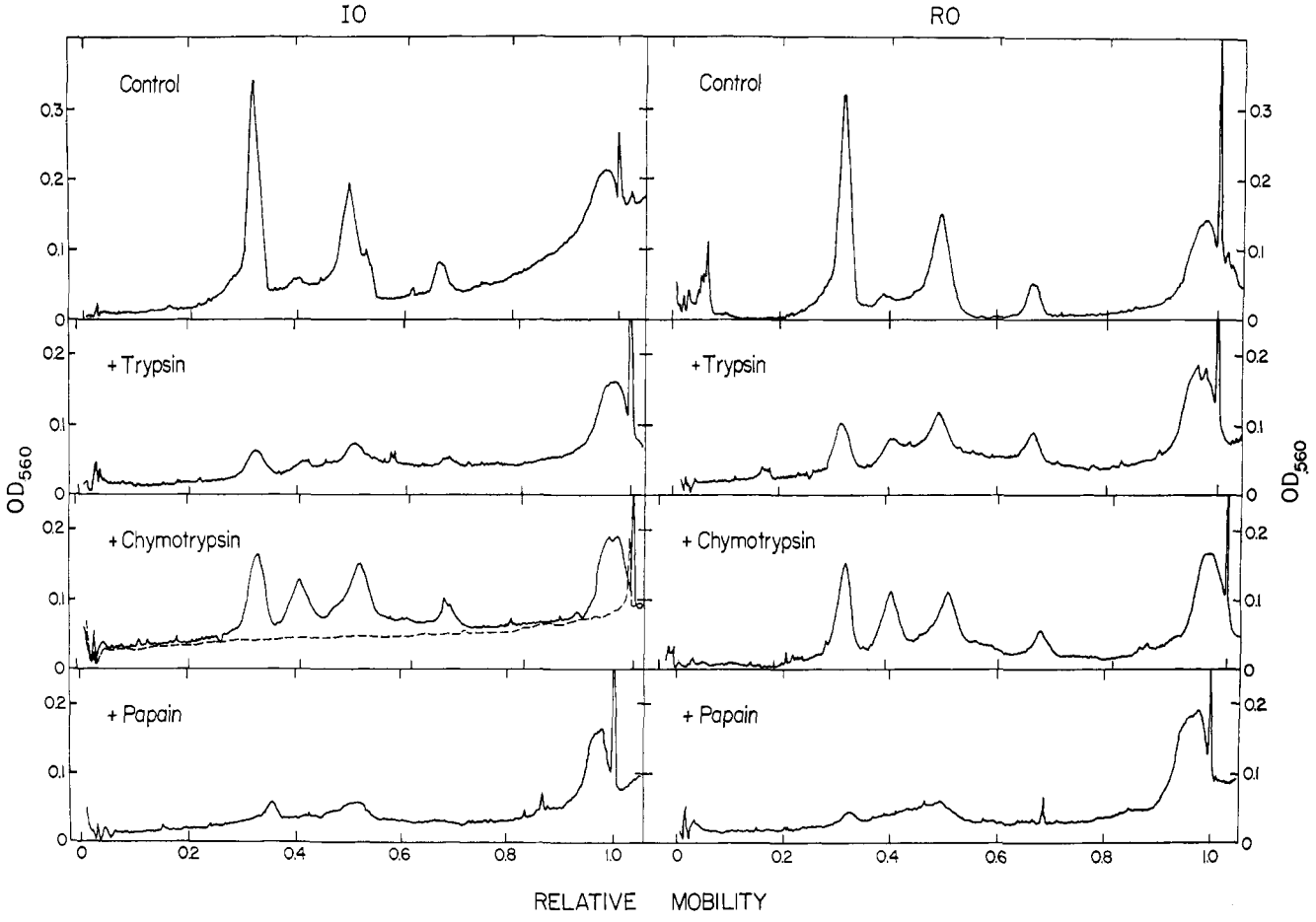

FIGURE 5: Proteolysis of vesicles: carbohydrate stain. Inside-out (left series) and right-side-out (right series) vesicles (1 mg of protein/ml) were incubated at room temperature for 1 hr with no enzyme, trypsin, chymotrypsin, or papain (10 μg/ml). The membranes were dissolved and electrophoresed as described in the Experimental Section. The gels were stained by PAS treatment and scanned at 560 nm. Superimposed on the densitometer tracing of the IO + chymotrypsin gel is a scan of a stained, blank gel (dashed line) indicating the rising base-line artifact frequently observed. The PAS-positive sialoglycoproteins (peaks with mobilities 0.31, 0.49, and 0.66 relative to the tracking dye) were designated PAS-1, PAS-2, and PAS-3, respectively (Fairbanks *et al.*, 1971). The broad peak just behind the tracking dye reflects the Schiff-positive activity of the membrane lipids.

Discussion

The accessibility of the membrane proteins to digestion is distinctly different in the normally oriented and inverted vesicles. Quantitatively, the titration studies demonstrated that the RO vesicle components were rapidly and extensively hydrolyzed, while the IO vesicles were attacked to a rather limited extent. Similarly, gel electrophoresis revealed a different digestion pattern for the two vesicle species. Other than the intrinsically resistant band, VI, digestion of ghosts and RO vesicles extensively degraded all of the major components evaluated. (Several minor bands, although difficult to monitor in the presence of multiple proteolysis products, also appeared to be susceptible to attack at the outer surface). In contrast, only band III and the PAS-positive sialoglycoproteins were vulnerable in inside-out vesicles. The other major and minor bands persisted despite extensive treatment.

While the digestion patterns varied somewhat with the enzyme employed, susceptibility to proteolysis appeared primarily to be a function of the membrane architecture. Disruption of the membrane with small amounts of SDS led to extensive, indiscriminate proteolysis. Furthermore, the proteins in the vesicles do not appear mobile or random, but rather specifically disposed.

Topographic deductions from these data require the assumption that no significant alteration in the organization of the membrane proteins attended the preparation of the vesicles. Verification of this premise is under study. At present, there is no evidence for such rearrangement. Right-side-out vesicles exhibit the "sidedness" of the ghost membranes in all respects thus far tested. Inside-out vesicles show the anticipated internalization of surface sialoglycoproteins (Steck *et al.*, 1970) and acetylcholinesterase (T. L. Steck, unpublished data). We therefore feel that the assumption is justified in this context; furthermore, it affords many interesting deductions which should be verifiable independently.

To facilitate the interpretation of our findings, we drew several possible arrangements of proteins about a functional barrier to proteolytic penetration (Figure 6A–G). Only a few of these were found to be consistent with the present data. Consider models A, B, and C. These call for the double representation of the same polypeptide species on both sides of the membrane barrier. In that case, attack from either side should yield partial digestion of each gel band but leave a resistant fraction remaining. The electrophorograms reveal that the opposite is true: each band is digested in an all-or-none fashion in both RO and IO incubations. No protein component appears to doubly populate both sides of the proteolysis barrier.

Among the coomassie blue stained polypeptides, bands I, II, IV and V are most compatible with the arrangement seen in model G, in that they all appear to be completely accessible at the outer and not the inner aspect of the membrane. Alternatively, they could be positioned as in model F, provided that the portions penetrating to the inner surface were intrinsically resistant to attack by the three test enzymes. In any case, none of the symmetrical models (A–E) are consistent with these results.

Band III appeared to be both penetrating and asymmetrical (as in model F). It was completely digested in both types of vesicles but gave different proteolysis products in the two cases (Figure 4). The polypeptide appeared to be more extensively digested from the outer surface; attack at the cytoplasmic face yielded a single major product with all three test enzymes. Its differential susceptibility could derive from intrinsic conformational factors and/or steric protection by the membrane.

Band VI resisted digestion in both RO and IO vesicle preparations, consistent with model D. However, this component was just as resistant to proteolysis when solubilized in saline. This suggests that intrinsic conformation, rather than membrane architecture, protected the molecule; therefore, its localization cannot be analyzed by proteolysis. It may be adsorbed to both membrane faces, since it was partially eluted from both RO and IO vesicles by incubation in 0.15 M NaCl and was released intact from ghosts and IO and RO vesicles during the course of proteolytic digestion.

The sialoglycoprotein components, PAS-1–3, were readily attacked by trypsin and papain digestion of both RO and IO vesicles, and in both vesicle types showed the same partial resistance to chymotrypsin attack. No consistent difference in the proteolysis products from the two vesicle types was apparent. These data are most compatible with model E. On the other hand, the sialic acid moiety is known to be localized on the outer surface of the erythrocyte membrane (Eylar *et al.*, 1962; Cook and Eylar, 1965; Steck *et al.*, 1970), suggesting that model F would be more appropriate.

Figure 6H summarizes schematically our findings in this and the preceding paper (Fairbanks *et al.*, 1971). None of these assignments is considered proven; however, each constitutes a specific, verifiable hypothesis. In essence, all the major membrane proteins appear asymmetrically oriented; furthermore, at least some of them span the thickness of the proteolysis barrier. It is conceivable that all are penetrating proteins, but that components I, II, IV, and V are intrinsically resistant to proteolysis at the cytoplasmic face. It is perhaps more likely that only components III and PAS-1–3 are penetrating proteins. They may correspond to the particles shown by freeze-cleave electron microscopy to traverse the membrane interior (Weinstein and Koo, 1968). Recent freeze-etch electron microscopic studies on the membrane glycoproteins support this suggestion (Pinto da Silva *et al.*, 1970; Tillack *et al.*, 1970).

We have found a striking discrepancy between the resistance to proteolysis of the surface of intact erythrocytes and the susceptibility of the isolated membranes. Rosenberg and Guidotti (1969) reported a similar observation. In addition, the lipids of the intact membrane are much more resistant to phospholipase digestion than those of the isolated membrane (Ibrahim and Thompson, 1965). Furthermore, Mitchell and Hanahan (1966) and Fairbanks *et al.* (1971) have demonstrated the release of membrane components from ghosts by hypertonic saline washes which leave the membrane of the intact erythrocyte unperturbed. Thus, isolation of the erythrocyte membrane increases its reactivity to a variety of agents. The nature of this alteration is under investigation.

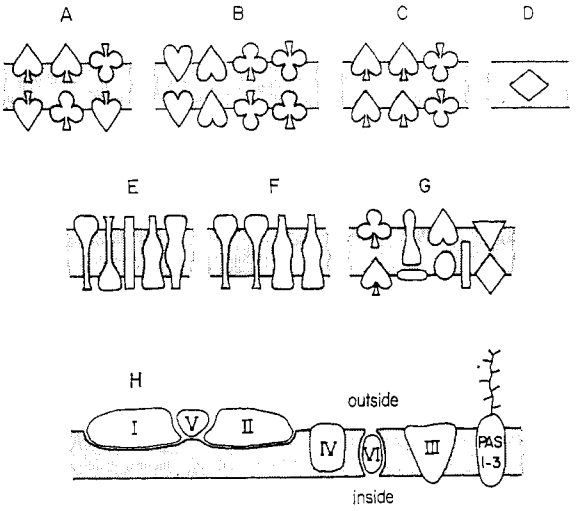

FIGURE 6: Possible arrangements of protein components in the membrane cross section. Each unshaded form denotes a protein species in a particular distribution and orientation with respect to a hypothetical barrier to penetration of the enzyme probes (shaded zone). Models A–C all represent a double distribution of protein species which have symmetrical (A), random or mobile (B), or asymmetrical (C) orientations. In model D, the protein is buried within the barrier and is therefore inaccessible. Model E shows penetrating proteins of symmetrical, random, or mobile orientation. Asymmetrically oriented, penetrating proteins are depicted in model F. Model G shows an asymmetrical distribution of components across the barrier which could have any orientation. Model H illustrates a possible disposition for each of the major protein and glycoprotein components considered in this study. Based on their behavior in elution studies (Fairbanks *et al.*, 1971), components I, II, and V have been pictured as being rather superficial in location and physically associated *in situ*, while component VI has been assigned an indeterminate position.

In two studies of the labeling of intact erythrocytes by nonpenetrating reagents, only one membrane protein was extensively labeled (Berg, 1969; Phillips and Morrison, 1970). It appears to be the same component in both cases and to have a molecular weight of around 90,000 (our estimate). Because PAS-1 falls in this size range and is the major component to be digested during the proteolysis of intact erythrocytes, we suggest that this is the reactive component in the labeling studies. The presumed projection of sialoglycoproteins into the extracellular space may be responsible for this enhanced accessibility. Since they contribute no more than 5% of the membrane protein (Winzler, 1969), the sialoglycoproteins are unlikely to be the sole protein constituent of the outer membrane surface.

The surface of the intact erythrocyte fails to react with antisera directed against components I and II while isolated stroma bind these antibodies (Marchesi *et al.*, 1969; Furthmayr and Timpl, 1970). These results are in accord with our proteolysis experiments; *i.e.*, bands I and II are not digested in intact cells but are susceptible in ghosts. The conclusion of these authors that components I and II must be confined to the inner surface of the membrane is not supported by direct evidence. In contrast, we have shown these bands to be digested only at the outer surface of isolated membranes; we do not know, as yet, why they are not attacked in the intact cell.

Proteolytic digestion could have perturbed membrane organization even while revealing it. Complementary studies with nonpenetrating, covalent labels are in progress. However,

a labeling technique might not discern between models A and B, C and F, C and G, E and F, etc. Since no qualitative differences were found between 5- and 180-min digestion or between soluble and solid-phase enzyme digestion, and because of the distinctive membrane asymmetry clearly indicated by the data, we infer that the enzyme probes revealed rather than obscured intrinsic membrane organizational features.

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Periodate Oxidation of Carbohydrate Moiety of Stem Bromelain without Much Alteration in Enzymatic Activity*

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ABSTRACT: A possible noninvolvement of the carbohydrate moiety of stem bromelain in the mechanism of catalysis was examined by oxidizing neutral sugar residues of the glycoenzyme with periodate. In 0.1 M sodium metaperiodate stem bromelain consumed 26–27 moles of the oxidant after 3–5 hr at 0° in the dark. The reactive SH group of the enzyme was found to be intact, because this group has been protected with tetrathionate before and during the oxidation with periodate. Total neutral sugars remaining after 1-, 3-, and 5-hr oxidation were 20, 5, and 2%, respectively, while the enzymatic activity on casein was lost only partially, remaining 83, 72, and 64%, respectively. The activity on α-*N*-benzoyl-L-arginine ethyl ester and *N*-benzoylglycine ethyl ester and the milk clotting activity also decreased only to similar extents.

These results suggest that neutral sugars of stem bromelain may not be essential participants in catalysis. The amino acid composition of periodate-oxidized stem bromelain did not show appreciable change from that of the unoxidized enzyme, except that some of methionine residues were oxidized to form methionine sulfoxide. No significant conformational change was detected by the measurements of optical rotatory dispersion and circular dichroism spectra after the periodate oxidation. The true mechanism for the observed partial inactivation of stem bromelain by periodate is still undetermined.

A pineapple thiol protease, stem bromelain, is a glycoenzyme with a molecular weight of 33,000 (Murachi *et al.*, 1964), which contains a single heterooligosaccharide unit per molecule. Being a glycoprotein is one of the marked differences in the structure of stem bromelain and papain (Murachi and Takahashi, 1970). The composition and structure of the carbohydrate moiety were studied using

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