THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 36, Issue of December 25, pp. 17497-17503, 1987
Printed in U.S.A.

Purification and Partial Characterization of the M_r 30,000 Integral Membrane Protein Associated with the Erythrocyte Rh(D) Antigen*

(Received for publication, April 29, 1987)

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Erythrocytes bearing the Rh(D) antigen have an M_r 30,000 integral membrane protein which can be surface-labeled with ^{125}I and can be quantitatively immunoprecipitated from Triton X-100-solubilized spectrin-depleted membrane vesicles. The ^{125}I-labeled Rh(D)-associated protein was purified to radiochemical homogeneity from membrane skeletons solubilized in sodium dodecyl sulfate and urea by hydroxylapatite chromatography, gel filtration, and preparative polyacrylamide gel electrophoresis. The Rh(D)-associated protein was purified nearly 200-fold from 2 units of erythrocytes from *DD* individuals by employing similar methods on a large scale using the purified ^{125}I-labeled Rh(D)-associated protein as a tracer. The product appeared to be >95% pure and migrated as a diffuse band of M_r approximately 30,000–32,000 on silver-stained sodium dodecyl sulfate electrophoresis gels poured from 12% acrylamide. It is estimated that the Rh(D)-associated protein makes up approximately 0.5% of the original membrane protein. When concentrated, partially purified Rh(D)-associated protein forms dimers and larger oligomers which are stable in sodium dodecyl sulfate and urea. The Rh(D)-associated protein was protected from degradation when intact erythrocytes or inside out membrane vesicles were enzymatically digested. These studies indicate that the M_r 30,000 protein associated with the Rh(D) antigen is linked to the membrane skeleton, resides within the lipid bilayer with minimal extra- or intracellular protrusions, exists normally as an oligomer, and can be purified in denatured form.

The Rh blood group system is of major importance in transfusion medicine yet is poorly understood at a molecular level (reviewed in Ref. 2). Rh(D) is the major antigen, but its hypothetical partner, Rh(d), has never been identified; hence the terms "Rh positive" and "Rh negative". Associated Rh alleles *E/e* and *C/c* are inherited together with *D* or *d* without recombinations. However, it remains uncertain whether these are the products of a single gene (3) or multiple closely linked genes (4). The Rh antigens may play a role in membrane structure, since rare individuals have erythrocytes which are misshapen, fragile, and lack all Rh phenotypes, "Rh-null" (5).

The Rh(D) antigen was shown to be a membrane protein with surface sulfhydryls (6) and phospholipid required for immunoreactivity (7). There are only 20,000–30,000 Rh(D) antigens/cell, and these reside at fixed intervals on the cell surface (8, 9). A protein of M_r approximately 28,000–33,000 was recently identified which can be surface ^{125}I-labeled on Rh(D) bearing erythrocytes and specifically precipitated with Rh(D) immune globulin (10–12). This Rh-associated protein contains no carbohydrate (13) and is linked to the membrane skeleton (14, 15). Purification and additional characterization of this Rh-associated protein was considered a direct approach to better understanding of this important and complex blood group system.

EXPERIMENTAL PROCEDURES AND RESULTS[1]

Purification of Rh-IMP—Since ^{125}I-labeled Rh-IMP can be isolated by immunoprecipitation in only very small amounts, traditional preparative methods were employed. Efforts to solubilize Rh-IMP in nonionic detergents met with little success (see Miniprint). Therefore, membrane skeletons were prepared from surface-^{125}I-labeled erythrocytes and were solubilized in Rh-purification buffer containing SDS and urea, and loaded onto an hydroxylapatite column (Fig. 3). The glycophorins eluted with low concentrations of sodium phosphate, while the ^{125}I-labeled Rh-IMP as well as band 3 eluted at higher phosphate concentrations and were separated by gel filtration. Fractions greatly enriched for ^{125}I-labeled Rh-IMP were then electrophoresed through preparative SDS-PAGE slabs and eluted in radiochemically pure form (Fig. 3, *lane 20*).

Large scale purifications were undertaken employing a similar purification scheme. Results of a typical purification are shown in Figs. 4 and 5. However, 20 other purifications of varying scales were performed with similar results. Membrane skeletons prepared from two units of erythrocytes from *DD* homozygotes were solubilized in the Rh purification buffer to which a tracer of ^{125}I-labeled Rh-IMP was added. This material was loaded onto a large hydroxylapatite column. The major peak of ^{125}I-labeled Rh-IMP was eluted in the 0.4 M step (Fig. 4*A*, *pool E*), subjected to gel filtration (Fig. 4*B*, *pool*

* This work was supported by National Institutes of Health Grants RO1 HL33991 and KO8 HL00883, March of Dimes Basil O'Connor Award 5-490, and by a grant from the Baltimore Rh Laboratory. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1, 2, 4, 6, and 7, Tables I and II, and Refs. 24–32) are presented in miniprint at the end of this paper. The abbreviations used are: Rh-IMP, M_r 30,000 *Rh*(D)-associated *i*ntegral *m*embrane *p*rotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, 7.5 mM sodium phosphate (pH 7.4), 0.15 M NaCl; band 3, anion transporter (nomenclature of Steck, Ref. 1); TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87 M-1395, cite the authors, and include a check or money order for $7.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

H), and then loaded onto a smaller hydroxylapatite column which was eluted with a sodium phosphate gradient (Fig. 4*C*). The ^{125}I-labeled Rh-IMP peak (*pools K–M*) overlapped and ran beyond the trailing side of the major protein peak. Silver-stained SDS-PAGE slabs (Fig. 5) showed a somewhat diffuse band of M_r approximately 30,000 on gels poured from 12% acrylamide monomer, and this band comigrated exactly with the surface-^{125}I-labeled Rh-IMP on autoradiographs of the gel (not shown). The same band was not visualized when an identical slab gel was stained with Coomassie Blue, although several of the contaminating proteins stained well (Fig. 5, *lanes K–M*). Pooled fractions were electrophoresed through preparative SDS-PAGE slabs and eluted. The principal contaminants migrated slightly faster on SDS-PAGE slabs and also eluted before the Rh-IMP from a final hydroxylapatite column. The final product was a diffuse band migrating at M_r 30,000–32,000 on 12% gels which appeared >95% pure when silver-stained slab gels were analyzed by densitometric scanning. The principal contaminants are an M_r 60,000 protein, which may be a dimer of Rh-IMP, and a small amount of aggregated material which barely entered the gel. Curiously, the major Rh-IMP band migrated at M_r approximately 32,000–34,000 when analyzed on slabs poured from 15% acrylamide, and identical aberrant behavior was reported for immunoprecipitated surface-^{125}I-labeled Rh-IMP (13).

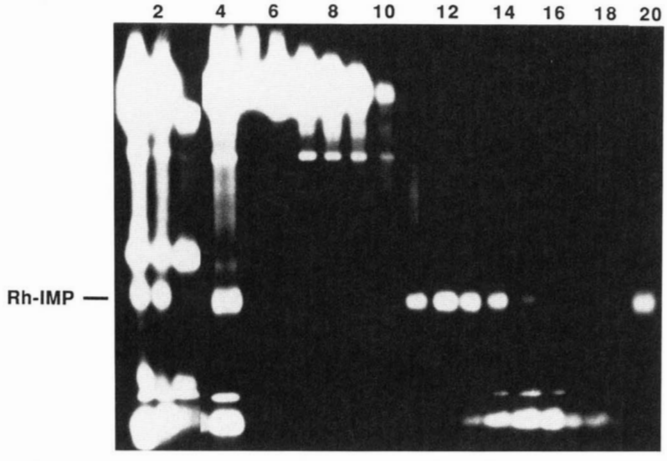

FIG. 3. **SDS-PAGE autoradiograph of small-scale isolation of ^{125}I-labeled Rh-IMP.** Membranes were prepared from 2 ml of ^{125}I-labeled erythrocytes from a *DD* individual (*lane 1*), and membrane skeletons were dissolved in Rh purification buffer (*lane 2*), loaded onto a small hydroxylapatite column and eluted with 0.2 M (*lane 3*) and 0.4 M sodium phosphate steps (*lane 4*). The 0.4 M peak was chromatographed by filtration through an Ultrogel AcA 22 column (*lanes 5–19*), and the peak Rh-IMP fractions were concentrated, electrophoresed, and eluted from preparative SDS-PAGE slab (*lane 20*).

The bookkeeping for different steps of a typical purification is shown in Table I. The final yield was 0.15 mg, representing recovery of 2.6% of the original pure ^{125}I-labeled Rh-IMP tracer. Major losses occurred at each step and resulted from the need to select the portions of the Rh-IMP peaks which overlapped the contaminants to smaller degrees. Nevertheless, a purification of nearly 200-fold was accomplished. The Rh-IMP content was estimated by back-calculation to be approximately 0.5% of the original membrane protein, approximately 60,000 copies/erythrocyte. This number is consistent with the Rh-IMP existing as an oligomer on the membrane, since the number of surface Rh(D) sites on a single erythrocyte membrane has been calculated to be approximately 20,000–30,000 (8, 16). The purified Rh-IMP was suitable for amino acid analysis, and the composition of the purified Rh-IMP is listed in Table II. Like other integral membrane proteins it is notable for a significant amount of hydrophobic amino acids, 42% of total.

DISCUSSION

This report describes a new method for purification and partial characterization of the M_r 30,000–32,000 protein present on Rh(D) bearing erythrocytes which can be surface-^{125}I-labeled and specifically precipitated with Rh(D) immune globulin. This protein is referred to in this text as "Rh-IMP," an acronym for *Rh*-associated *i*ntegral *m*embrane *p*rotein or *Rh im*uno*p*recipitate.

Several observations indicate that the relationship between the surface Rh(D) antigen and the Rh-IMP is complex. Rh-IMP has an extracellular domain containing tyrosine(s) which can be surface-^{125}I-labeled (Fig. 1). However, digestion of intact erythrocytes with neuraminidase or proteases failed to clip any detectable extracellular protrusions from the Rh-IMP and failed to quantitatively reduce the immunoprecipitable ^{125}I-labeled-Rh-IMP (Fig. 7). An earlier report noted the absence of of any identifiable surface glycosylation on Rh-IMP (13). It is not intuitively obvious why a nonglycosylated surface protein with minimal extracellular mass should be exceedingly immunogenic. While Rh(D) immune globulin reacted specifically with intact erythrocyte membranes bearing the Rh(D) antigen and permitted immunoprecipitation of the

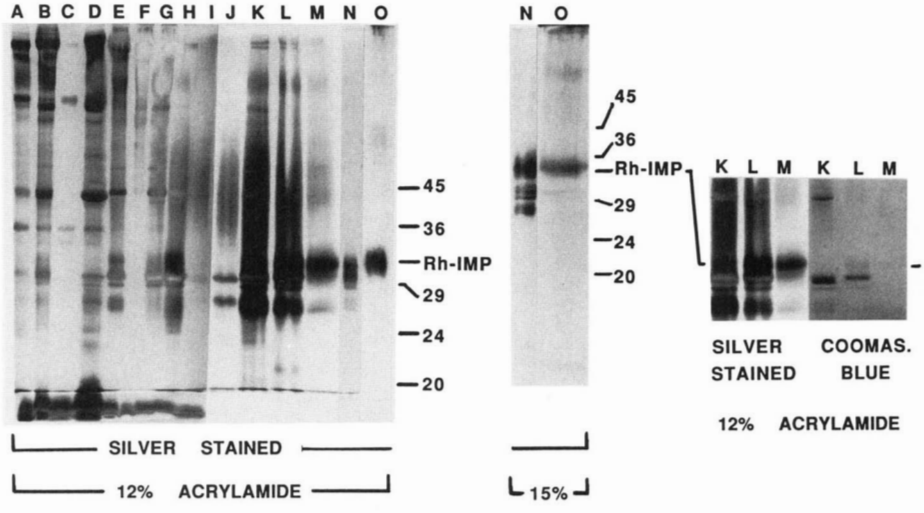

FIG. 5. **Large-scale Rh-IMP purification analyzed with SDS-PAGE.** Samples from the various stages of purification were electrophoresed through SDS-PAGE slabs (12 or 15% acrylamide as indicated) and were stained with silver reagent or Coomassie Blue. *Lane A*, erythrocyte membranes; *lane B*, membrane skeletons; *Other lanes* refer to fraction pools in Fig. 4: *lanes C–E*, pools from the hydroxylapatite elution steps; *lanes F–H*, pools from gel filtration chromatography; *lanes I—M*, pools from the hydroxylapatite gradient elution; *lane N*, Rh-IMP eluted from preparative SDS-PAGE slab; and *lane O*, >95% pure Rh-IMP eluted from a final hydroxylapatite gradient. The band identified as Rh-IMP comigrates exactly with the ^{125}I-labeled Rh-IMP as seen by autoradiography. *Numerals* between *panels* indicate mass standards in kilodaltons.

Rh-IMP (Fig. 2), the same immune globulin consistently failed to react with purified Rh-IMP or with other erythrocyte membrane proteins on Western blots. Furthermore, Rh(D) immune globulin failed to precipitate Rh-IMP from Triton X-100-solubilized membrane vesicles when added after the solubilization. Possible explanations for this include: 1) loss of an additional cofactor which is necessary for Rh(D) antigenicity; or 2) specific conformational requirements which are satisfied only if the Rh-IMP is embedded in the bilayer in a highly specific configuration or as a specific oligomer.

It is likely that both explanations are involved. Band 3 has been purported to be the membrane site of the Rh(D) antigen (17, 18), but band 3 can be precipitated with Rh(D) immune globulin from Rh(D)-negative cells and with nonimmune human globulin (Fig. 2). Phospholipid is an essential component of the Rh(D) antigen, and Rh(D) antigenicity can be abolished by extensive extraction with alcohol, but it can be restored by addition of exogenous phospholipid (7). Furthermore, several of the observations reported herein indicate that the Rh-IMP may reside as a dimer or higher order oligomer on the membrane, and oligomerization is known to increase the affinity of certain antibodies for surface antigens by several thousandfold (19).

The Rh(D) is also unlike most other erythrocyte antigens in several other important ways. The Rh(D) antigen is one of the most immunogenic of all erythrocyte antigens, despite a relatively small number of Rh(D) antigens/cell. Interaction of Rh(D)-specific IgG with antigens on the erythrocyte surface is notable for the failure of this reaction to lead to complement activation, probably due to inability of the immobile Rh(D) antigens to cluster on the cell surface at locations close enough for IgG molecules to permit complement activation. Recent identification of the association of the Rh-IMP with the membrane skeleton provides an important clue to this curious nature of the Rh(D) antigen (14, 15).

The sites of interaction of the Rh(D) antigen with elements of the erythrocyte membrane skeleton remain to be identified. Like band 3, the Rh-IMP could have a cytoplasmic tail which attaches directly to the underlying skeleton. However, digestion of inside out membrane vesicles failed to identify such a cytoplasmic domain. Alternatively, the Rh-IMP could interact with another membrane skeleton-linked integral protein by a side-to-side association. The number of Rh(D) antigens is far below the number of band 3 monomers, approximately 10^6, or even the number known to be linked to ankyrin, approximately 10^5 (20). The number of Rh(D) antigens (20,000–30,000) is similar to the number of spectrin-actin-protein 4.1 complexes, and association of the Rh(D) antigen and complex could conceivably involve a side-to-side interaction of the Rh-IMP with a membrane skeleton-linked glycophorin molecule (21, 22).

After submission of the manuscript for this paper, an article appeared describing the isolation of the Rh-IMP from certain types of blood by monoclonal immunoprecipitation (23).

REFERENCES

1. Steck, T. L. (1974) *J. Cell Biol.* **62,** 1–19
2. Rosse, W. F. (1987) *Basic and Clinical Immunohematology*, Blackwell, Boston, in press
3. Wiener, A. S. (1944) *Science* **100,** 595–597
4. Race, R. R. (1944) *Nature* **153,** 771–772
5. Sturgeon, P. (1970) *Blood* **36,** 310–319
6. Green, F. A. (1965) *Vox Sang.* **10,** 32–53
7. Green, F. A. (1972) *J. Biol. Chem.* **247,** 881–887
8. Nicolson, G. L., Masouredis, S. P., and Singer, S. J. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 1416–1420
9. James, N. T., and James, V. (1978) *Br. J. Haem.* **40,** 657–659
10. Moore, S., Woodrow, C. F., and McClelland, D. B. L. (1982) *Nature* **295,** 529–531
11. Gahmberg, C. G. (1982) *FEBS Lett.* **140,** 93–97
12. Ridgwell, K., Roberts, S. J., Tanner, M. J. A., and Anstee, D. J. (1983) *Biochem. J.* **213,** 267–269
13. Gahmberg, C. G. (1983) *EMBO J.* **2,** 223–227
14. Gahmberg, C. G., and Karhi, K. K. (1984) *J. Immunol.* **133,** 334–337
15. Ridgwell, K., Tanner, M. J. A., and Anstee, D. J. (1984) *FEBS Lett.* **174,** 7–10
16. Masouredis, S. P., Sudora, E. J., Mahan, L., and Victoria, E. J. (1976) *Transfusion (Phila.)* **16,** 94–106
17. Victoria, E. J., Mahan, L. C., and Masouredis, S. P. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 2898–2902
18. Yokoi, T., Iwasa, M., and Sagisaka, K. (1983) *Tohoku J. Exp. Med.* **141,** 143–154
19. Low, P. S., Waugh, S. M., Zinke, K., and Drenkahn, D. (1985) *Science* **227,** 531–533
20. Bennett, V. (1981) *Biochim. Biophys. Acta* **689,** 475–484
21. Mueller, T., and Morrison, M. (1981) in *Erythrocyte Membranes* (Kruckeberg, W., Eaton, J., and Brewer, G. eds) Vol. 2, pp. 95–112, Alan R. Liss, Inc., New York
22. Anderson, R. A., and Lovrien, R. E. (1984) *Nature* **307,** 655–658
23. Bloy, C., Blanchard, D., Lambin, P., Goossens, D., Rouger, P., Salmon, C., and Cartron, J. P. (1987) *Blood* **69,** 1491–1497

Additional references are found on p. 17503.

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SUPPLEMENTARY MATERIAL TO

Purification and Partial Characterization of the 30,000 M_r Integral Membrane Protein associated with the Erythrocyte R(D) Antigen

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EXPERIMENTAL PROCEDURES

Materials--Human blood of defined Rh (D,C,E,c & e) antigen types was obtained from the Johns Hopkins Hospital Blood Bank and from other volunteers. Rh genotypes were determined from the phenotypes by inference from the frequencies of the Rh gene complexes. LeukoPak leukocyte filters were from Fenwal Laboratories. Nonimmune human globulin was prepared from the serum of an Rh(D) positive individual by affinity chromatography employing protein A-Sepharose obtained from Pharmacia. Rh(D) immune globulin (Connaught), alpha-chymotrypsin, and TPCK-trypsin were from Cooper Biomedical. Carrier free $Na^{125}I$ was from Amersham. Iodogen was from Pierce. Ultrogel AcA 22 and 34 were from LKB. High resolution hydroxylapatite and protein A bearing Staphylococci were from Calbiochem. Phenylmethylsulfonyl fluoride, dithiothreitol, EDTA, Folin reagent, low molecular weight protein standards, papain, neuraminidase, and Triton X-100 were from Sigma. Polyethylene glycol (Carbowax 8000) was from Fisher. Pentex fraction V pure albumin was from Miles. Ultra pure urea was from Schwarz/Mann. Silver stain kits and electrophoresis reagents including pure SDS and acrylamide were from Biorad. X-Omat AR autoradiographic film and developing reagents were from Kodak. Cronex intensifier screens were from Dupont.

Methods--SDS-PAGE slabs were prepared using the buffer system of Laemmli (24), and acrylamide and urea concentrations are noted in the figure legends. All samples were reduced by incubation in 0.2M dithiothreitol at 60°C for 10 minutes prior to electrophoresis. Protein determinations were performed by the method of Lowry et al (25) employing the appropriate buffer controls and bovine serum albumin as a standard. Final stages of the purification were evaluated by densitometrically scanning silver stained gels and back-calculating from amino acid analyses. Samples for amino acid analysis (approximately 3 ug protein) were precipitated with acetone at -20°C, hydrolyzed with 6N HCl vapors at 110°C for 24 hours, evaporated to dryness, and derivatized with phenyl isothiocyanate using standard procedures (26). The preparations were analyzed using the Pico-Tag amino acid analysis system (Waters-Millipore) in the Peptide Laboratory in the JHU Department of Biological Chemistry.

Surface ^{125}I-labeling of erythrocytes--The method employing Iodogen and $Na^{125}I$ was modified (27). Whole blood was washed 4 times in chilled PBS, 1mM NaEDTA; plasma and buffy coat were removed by suction, and the erythrocytes were suspended to a hematocrit of 10%. Five mls of suspension and 2 mCi of $Na^{125}I$ were shaken for 30 minutes at room temperature in a glass scintillation vial coated with 1.0 mg of Iodogen. Five vials were used in 10 mCi preparations. Unincorporated ^{125}I was removed by washing the cells several times in chilled PBS, 1mM NaEDTA, 5mM KI, 1mM dithiothreitol. The extent of ^{125}I incorporation into membrane proteins was usually 1-3% (see Fig.1).

Preparation of erythrocyte membranes--Methods for each step were derived from those described by Bennett (28). Erythrocytes were obtained from Rh type specific blood stored up to 5 weeks at 4°C in citrate phosphate dextrose adenine blood bank preservative bags. The plasma proteins, leukocytes, and platelets were removed by 3-4 washes in 10 volumes of ice cold PBS, 1mM NaEDTA with 5 minute 700 x g centrifugations. Residual leukocytes were eliminated from large scale preparations by filtration through Leuko-Pak filters. Membranes were prepared by lysing the erythrocytes and washing repeatedly until the membranes were white with 7.5mM sodium phosphate (pH 7.4), 1mM NaEDTA, 0.2mM phenylmethylsulfonyl fluoride and once more in the same buffer also containing 50mM NaCl. Membrane-skeletons were prepared by extracting membranes with 1% (v/v) Triton X-100, 0.1M NaCl, 10mM sodium phosphate (pH 7.4), 4mM dithiothreitol, 0.2mM phenylmethylsulfonyl fluoride for 30 minutes at 0°C, and the insoluble membrane-skeletons were pelleted by centrifugation at 44,000x g for 30 minutes. This was repeated once. Spectrin and actin stripped inside-out membrane vesicles were prepared by incubating membranes with >10 volumes 0.2mM NaEDTA (pH 8.0), 0.2mM phenylmethylsulfonyl fluoride for 30 minutes at 37°C and the vesicles were collected by centrifugation at 44,000 x g for 30 minutes. These vesicles were sometimes further stripped of all peripheral proteins by incubating in >10 volumes of 1M KI, 7.5mM sodium phosphate (pH 7.4), 1mM NaEDTA, 1mM dithiothreitol, 0.2mM phenylmethylsulfonyl fluoride for 30 minutes at 37°C. The KI vesicles contain only integral membrane proteins and were collected by centrifugation at 44,000 x g for 30 minutes, resuspended in 7.5 mM sodium phosphate, and centrifugation was repeated.

Immunoprecipitation of ^{125}I-labeled Rh-IMP--Washed surface ^{125}I-labeled erythrocytes were suspended to a hematocrit of 20% in PBS, 1mM NaEDTA containing 1mM NaN_3, 5mM D-glucose, 2% (w/v) bovine serum albumin, and incubated with Rh(D) immune globulin overnight while shaking at 22°C. The concentration of immune globulin was varied, but in experiments where quantitative immunoprecipitations were desired, 50 ul of immune globulin was added to each 1.0 ml of erythrocyte suspension, since preliminary experiments demonstrated that this is in excess of the globulin required to reproducibly immunoprecipitate >95% of surface ^{125}I-labeled Rh-IMP. Unadsorbed immune globulin was removed by washing the erythrocytes several times with 50 volumes of chilled PBS. Membranes were prepared and stripped of spectrin as described above. The resulting spectrin-depleted membrane vesicles were solubilized in 1 volume of 1% (v/v) Triton X-100 in 7.5mM sodium phosphate (pH 7.4) at 0°C and pelleted by centrifugation at 44,000 x g for 30 minutes. The supernatant was removed, combined with 1 volume of 1% (v/v) protein A bearing Staphylococci (previously extracted in SDS and prepared as described, ref. 29) and shaken at 4°C for 2 hours. The Staphylococci-immune complexes were washed twice in 25 volumes of 2M urea, 1% Triton X-100, 0.1M glycine with centrifugations at 2,500 x g, and the immunoprecipitated ^{125}I-Rh-IMP was eluted by incubating the pelleted Staphylococci in 0.1 volume of 2% (w/v) SDS at 60°C for 10 minutes (Fig. 2).

Isolation of pure Rh-IMP--Purifications were conducted in two stages: (1) small scale isolation of radiochemically pure surface labeled ^{125}I-Rh-IMP, and (2) large scale isolation of Rh-IMP utilizing the ^{125}I-Rh-IMP as a tracer. The column chromatography steps were all conducted at room temperature, and the standard Rh purification buffer contained 0.2% (w/v) SDS, 4M urea, 7.5mM sodium phosphate (pH 7.4), 4mM dithiothreitol, 1mM NaN_3 with variations in SDS or sodium phosphate concentrations as indicated.

Small scale purifications of ^{125}I-Rh-IMP were undertaken either by immunoprecipitating surface ^{125}I-labeled Rh-IMP or purifying it by column chromatography. In both methods, 10 mCi surface labelings were prepared utilizing erythrocytes from DD homozygotes. Immunoprecipitations were performed as described above, and co-precipitating ^{125}I-labeled band 3 was eliminated by eluting the ^{125}I-Rh-IMP from a preparative SDS-PAGE slab. In the column chromatography preparations, the labeled erythrocytes were washed free of unbound ^{125}I. Membranes were prepared and extracted with Triton X-100. The insoluble membrane-skeletons were solubilized in 4 volumes of Rh purification buffer by incubating at room temperature for 30 minutes. The insoluble material was removed by centrifugation at 44,000 x g for 30 minutes, and the supernatant was loaded onto a 1 x 12 cm hydroxylapatite column (high resolution) at a flow rate of 10 ml/hour at room temperature and eluted with two steps of Rh purification buffer containing 0.2M and 0.4M sodium phosphate (pH 7.4). The 0.4M step contained the ^{125}I-Rh-IMP peak and was loaded onto a 2.5 x 90 cm column packed with Ultrogel AcA 22 equilibrated with Rh purification buffer at a flow rate 15 ml/hour. The fractions were analyzed by SDS-PAGE and autoradiography (Fig. 3). The peak ^{125}I-Rh-IMP fractions were combined, electrophoresed through a 0.3 x 14 x 14 cm preparative SDS-PAGE slab. The slab gel was cut into 3mm sections, and the ^{125}I-labeled Rh-IMP peak was eluted by shaking the slice for 24-48 hours at 24°C in 1-2 mls of Rh purification buffer.

Large scale purifications started with two units of blood from DD homozygotes, and each step was monitored by SDS-PAGE stained with silver reagent (Fig. 5) and exposed to autoradiograph film. Erythrocyte membrane-skeletons were prepared as described (28) and solubilized in 200 mls of Rh purification buffer containing 1% (w/v) SDS by incubating for 60 minutes at room temperature. Approximately 100,000 cpm of radiochemically pure ^{125}I-Rh-IMP (from the small scale purification) was added as a tracer. After centrifugation at 44,000 x g for 30 minutes, the supernatant was passed through a 0.2 micron membrane filter, and the material was loaded onto a 2.5 x 80 cm column packed with high resolution hydroxylapatite at a flow rate of 50 mls per hour while 5 ml fractions were collected. The column was sequentially eluted with steps of 0.15M and 0.4M sodium phosphate (pH 7.4) in Rh purification buffer (Fig. 4A). The 0.4M step contained the ^{125}I-Rh-IMP and was concentrated to 180 mls in a dialysis bag shaken with a covering of solid polyethylene glycol and then dialyzed until clear against Rh purification buffer. The contents of the bag were chromatographed through two 5 x 90 cm columns packed with Ultrogel AcA 34, connected in sequence, and equilibrated with Rh purification buffer at a flow rate of 65 mls/hour while 8 ml fractions were collected (Fig. 4B). The peak ^{125}I-Rh-IMP fractions were loaded onto a 1.5 x 18 cm hydroxylapatite column equilibrated with Rh purification buffer and running at 10 mls/hour while 5 ml fractions were collected. The column was eluted with a 100 ml step of 0.15M sodium phosphate and thereafter with a 300 ml linear gradient of 0.15 to 0.30M sodium phosphate (pH 7.4) in Rh purification buffer (Fig. 4C). The ^{125}I-Rh-IMP partially overlapped the major protein peak, and fractions from the trailing edge were combined, concentrated against polyethylene glycol and electrophoresed through and eluted from 0.3 x 14 x 14 cm preparative SDS-PAGE slabs. The major contaminants at this stage possess slightly faster mobility than the ^{125}I-Rh-IMP and were best separated by employing slabs poured from 15% acrylamide. Generally, detectable amounts of contaminating proteins of faster migration were still present, but these could be eliminated by rechromatographing the eluate on a 0.8 x 4 cm hydroxylapatite column which was eluted with a 0.15 to 0.3M sodium phosphate gradient.

RESULTS

Surface ^{125}I-labeling of erythrocytes of defined Rh types--It has been demonstrated that erythrocytes bearing the Rh(D) antigen have a 30,000 M_r protein which can be surface ^{125}I-labeled, and immunoprecipitated with Rh(D) specific immune globulin (10,11). Proteins with extracellular domains containing a tyrosine may incorporate ^{125}I during lactoperoxidase or Iodogen catalyzed ^{125}I-labelings (27,30,31) and autoradiographs of labeled erythrocyte membranes demonstrated incorporation of ^{125}I into several recognized integral membrane proteins: band 3 (the anion transporter) and glycophorins A and B in their monomeric and dimeric forms. In addition, a diffuse, labeled band was detectable at approximately 30,000 M_r which corresponds to the previously identified Rh(D) protein and is referred to in this text as "Rh-IMP" (Fig. 1). Labeling of the Rh-IMP was most intense in erythrocytes from DD homozygotes where it accounted for 1 to 2% of the total membrane-incorporated ^{125}I, when determined by densitometric analysis of the autoradiographs (Fig. 1, lane 1). The Rh-IMP on erythrocytes from Dd heterozygotes was labeled less intensely (lane 2), and the labeling was still detectable but much lighter on erythrocytes from individuals lacking D antigen (dd homozygotes, lane 3). The 30,000 M_r band failed to label with ^{125}I-Bolton Hunter reagent, a lysine specific ligand (not shown). As expected, spectrin-depleted membrane vesicles from these preparations contained all of the surface ^{125}I-labeled proteins even after all peripheral proteins were stripped with 1M KI (lanes 4-6). However, when untethered integral membrane proteins were extracted from erythrocyte membrane with nonionic detergents such as Triton X-100, most of the integral proteins appeared in the extract (lanes 7-9), but the ^{125}I-labeled Rh-IMP was extracted only minimally with >90% remaining with the insoluble membrane-skeletons (lanes 10-12).

Immunoprecipitation of ^{125}I-labeled Rh-IMP--The Rh-IMP can be immunoprecipitated from Triton X-100 solubilized erythrocyte membranes with Rh immune globulin (10,11). Since the Rh-IMP is predominantly linked to the Triton-insoluble membrane-skeleton (14,15), a spectrin extraction step was included in order to quantitatively immunoprecipitate the Rh-IMP (see methods). As expected, Triton solubilized membrane vesicles prepared from surface ^{125}I-labeled erythrocytes were found to include ^{125}I-labeled band 3, glycophorins, Rh-IMP, and some low molecular weight components (Fig. 2, lane 5'). When complexed with Rh immune globulin, the Triton X-100 solubilized ^{125}I-labeled Rh-IMP was dramatically immunoprecipitated from erythrocytes of DD individuals. The immunoprecipitate of erythrocytes from Dd individuals was less prominent, while none was immunoprecipitated from dd (Fig. 2, lanes 1-3). Furthermore, a band of approximately 60,000 Mr (probably a dimer of ^{125}I-labeled Rh-IMP) was seen in the precipitate prepared from the erythrocytes of DD individuals, while a band appearing to be band 3 was noted in all preparations. It is likely that the latter was precipitated by contaminating autoantibodies reacting with the extracellular domain of band 3, since band 3, but not ^{125}I-labeled Rh-IMP, was precipitated when nonimmune human globulin was substituted (Fig. 2, lane 5). Furthermore, it is unlikely that immunoprecipitation of the ^{125}I-labeled Rh-IMP was nonspecific, since similar immunoprecipitations were obtained after wash in increasing urea concentrations or if precipitated with protein A sepharose (Fig. 2, lanes 6-13).

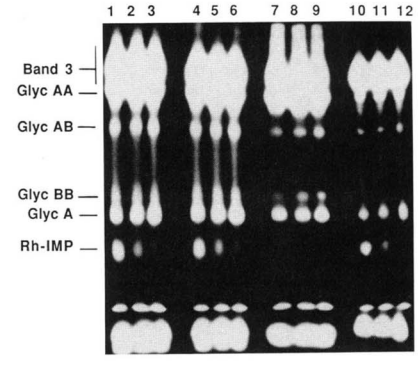


FIG. 1. SDS-PAGE autoradiograph of membrane preparations from surface ^{125}I-labeled erythrocytes of known Rh types. Blood samples were obtained from individuals of specific Rh genotypes: DD (R^2R^2, lanes 1,4,7,10), Dd (R^1r, lanes 2,5,8,11), and dd (rr, lanes 3,6,9,12). Intact erythrocytes were labeled with ^{125}I, and whole erythrocyte membranes (lanes 1-3), KI-extracted spectrin-depleted membrane vesicles (lanes 4-6), Triton X-100 soluble extracts from whole membranes (lanes 7-9), and insoluble membrane-skeletons (lanes 10-12) were prepared, electrophoresed through an SDS-PAGE slab (10% acrylamide) and exposed to autoradiograph film. "Glyc AA", "BB", "AB", and "A" refer to the dimers, heterodimers and monomers of glycophorins A and B.

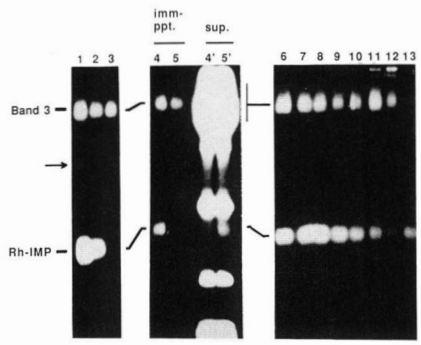


FIG. 2. **SDS-PAGE autoradiographs of Rh(D) immunoprecipitations of ^{125}I-labeled erythrocytes.**

Left panel: Rh(D) immunoprecipitated membrane proteins from ^{125}I-labeled erythrocytes from DD (lane 1), Dd (lane 2), and dd individuals (lane 3). The arrow identifies a 60,000 M_r band which may represent a dimer of ^{125}I-labeled Rh-IMP.

Middle panel: ^{125}I labeled erythrocytes from a DD homozygote incubated with human Rh(D) immune globulin (lanes 4 and 4'), or nonimmune human globulin (lanes 5 and 5'); "immppt." = immunoprecipitate, 100% of sample; "sup." = unprecipitated supernatant, 30% of sample.

Right panel: Immunoprecipitated membrane proteins similar to lane 1 were washed in buffers of different stringencies prior to electrophoresis. Precipitates in lanes 6 and 13 were washed with PBS, 1mM NaEDTA, 1% Triton X-100. Precipitates in lanes 7-12 were washed with 0.1M glycine, 1% (v/v) Triton X-100, and increasing urea concentrations: lane 7 = 1M urea, lane 8 = 2M, lane 9 = 3M, lane 10 = 4M, lane 11 = 5M, and lane 12 = 6M.

Protein A-bearing Staphylococci were used to pellet the immunoprecipitate in lanes 1-5, 6-12, and protein A-Sepharose was employed to pellet the immunoprecipitate in lane 13. Samples from the different preparations were electrophoresed through SDS-PAGE slabs (12% acrylamide) and exposed to autoradiograph film.

Purification of Rh-IMP, detergent solubilizations--While the Rh-IMP contained in spectrin-stripped membrane vesicles could be solubilized to varying degrees in nonionic detergents, this was generally not useful on a preparative scale, since the ^{125}I-labeled Rh-IMP aggregated to a higher molecular weight form which remained at the tops of the SDS-PAGE slabs. Whether these aggregates include only Rh-IMP or also contain other constituents is unknown. Efforts both to prevent and to break up the aggregate with urea, dithiothreitol, and with other detergents were of not successful. Curiously, the behavior of the nonionic detergent solubilized Rh-IMP was less ideal than the anti-D--Rh-IMP complex which was stable when solubilized in Triton X-100 and failed to aggregate (see Fig. 2).

During the preparation of membrane-skeletons, the majority of band 3 and glycophorins A and B are extracted in the detergent soluble phase, and the ^{125}I-Rh-IMP is a notable component of the insoluble membrane-skeleton pellet (Fig. 1). Efforts to selectively extract the ^{125}I-labeled Rh-IMP from the membrane-skeletons by a variety of salt and detergent combinations were not successful, so solubilization of the total membrane-skeleton pellet in Rh-purification buffer containing SDS and urea was deemed necessary (see Results).

Oligomerization of Rh-IMP--Integral membrane proteins frequently exist as dimers or higher order oligomers on the plasma membrane, and certain proteins such as the glycophorins remain dimers even when electrophoretically separated in the presence of SDS. Several observations indicate that this may also be true for the Rh-IMP. In addition to the notable band at 30,000 M_r the immunoprecipitrations frequently showed a small amount of ^{125}I-labeled protein which migrated at approximately 60,000 M_r and is likely a dimer of Rh-IMP (Fig. 2, arrow). When calculated from the purification data, the number of copies of Rh-IMP per erythrocyte is approximately twice the anticipated number. Material from the late stages of the purification migrated as a single band of 30,000 M_r when electrophoresed through slab gels containing SDS and 4M urea. However, when larger amounts of the same material are concentrated and electrophoresed similarly, a smear of higher molecular weight oligomers was observed (Fig. 6).

Rh-IMP is protected from enzymatic digestion while within the lipid bilayer--Extracellular and cytoplasmic domains of integral membrane proteins are frequently identified by selective proteolytic degradation at the appropriate face of the lipid bilayer, and most integral membrane proteins have domains on both sides of the bilayer. The Rh-IMP is surface labeled with ^{125}I, precipitable with specific antibodies, yet linked to the underlying membrane-skeleton. It was therefore considered likely that the Rh-IMP would span the lipid bilayer. Nevertheless, efforts to degrade putative extracellular

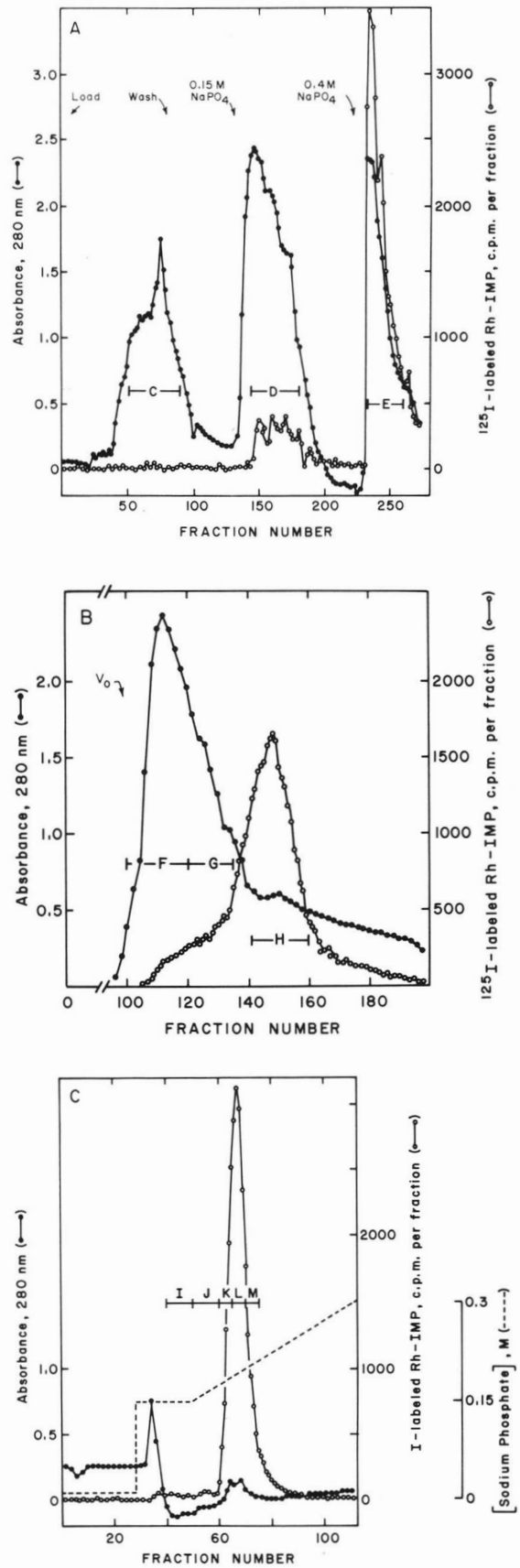


FIG. 4. **Large scale isolation of Rh-IMP from two units of erythrocytes from a DD homozygote.**

Panel A: membrane-skeletons were dissolved in Rh purification buffer, loaded onto a hydroxylapatite column, and eluted in steps: Rh purification buffer (pool C), 0.15M (pool D), and 0.4M sodium phosphate (pool E).

Panel B: the ^{125}I-labeled Rh-IMP peak (pool E) was filtered through Ultrogel AcA 34 columns.

Panel C: the ^{125}I-labeled Rh-IMP peak (pool H) was loaded onto a smaller hydroxylapatite column and step-eluted with 0.15M sodium phosphate followed by a linear gradient of 0.15 to 0.3M sodium phosphate.

and cytoplasmic domains were unsuccessful when enzymes were employed at concentrations which entirely degraded the other erythrocyte integral membrane proteins (Fig. 7).

Intact surface ^{125}I-labeled erythrocytes were digested with high concentrations of neuraminidase, chymotrypsin, trypsin, or papain, before the ^{125}I-labeled Rh-IMP was quantitatively immunoprecipitated. There was no significant reduction in the quantity of precipitated ^{125}I-labeled Rh-IMP, change in the appearance of the diffuse band, nor truncation to a smaller molecular weight (on 12% acrylamide gels, Fig. 7, lanes 1-5, or on 15% gels-not shown). This indicates that little digestion of the ^{125}I-Rh-IMP had occurred despite marked digestion of the other integral proteins which remained in the unprecipitated supernatant (lanes 1'-5').

Spectrin-depleted membrane vesicles prepared as described in "Methods" are known to be >85% inverted with the cytoplasmic face of the bilayer exposed. Inside-out membrane vesicles were prepared from surface ^{125}I-labeled erythrocytes and digested with concentrations of chymotrypsin and trypsin which totally digested the cytoplasmic domain of band 3 (Fig. 7, lanes 7-12). Nevertheless, these preparations showed minimal degradation of ^{125}I-labeled Rh-IMP until employment of protease concentrations high enough to disrupt the bilayer (lane 12).

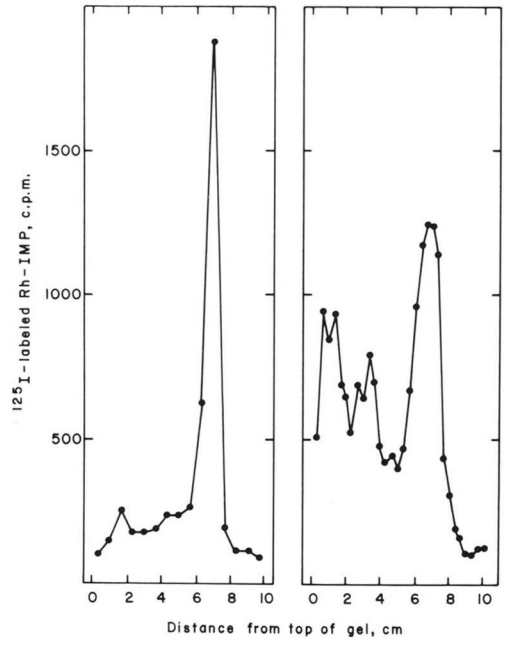
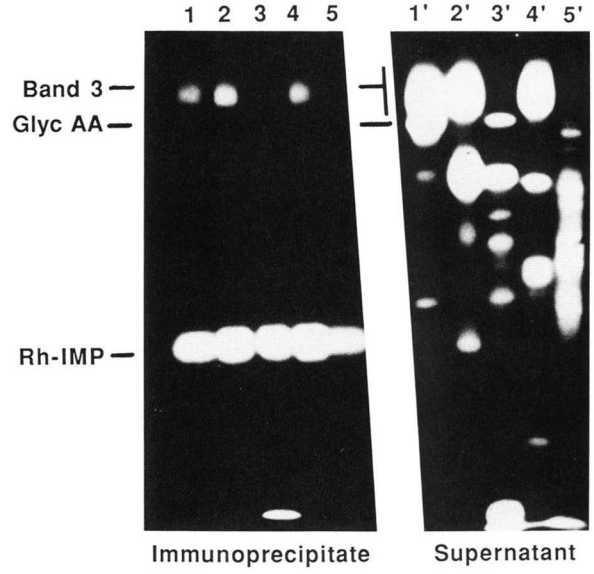


FIG. 6. **Concentration-dependent oligomerization of purified Rh-IMP in denaturing conditions.** A tracer of ^{125}I-labeled Rh-IMP was added to Rh-IMP purified from 50 ml of blood (left panel) or two units of blood (right panel). The Rh-IMP peaks from the corresponding hydroxylapatite gradients were added to dialysis membranes which were covered with solid polyethylene glycol and concentrated to 5 mls. The phosphate precipitates were redissolved by dialyzing the sample against Rh purification buffer, and the contents of the bags were layered onto the tops of 0.3 x 14 x 14cm preparative SDS gels (12% acrylamide, 4M urea) and electrophoresed overnight at 45 volts. The gels were cut into parallel slices and counted for ^{125}I. The sample applications were estimated to be 0.5mg, 30% pure Rh-IMP (left panel) and 8mg, 24% pure (right panel).



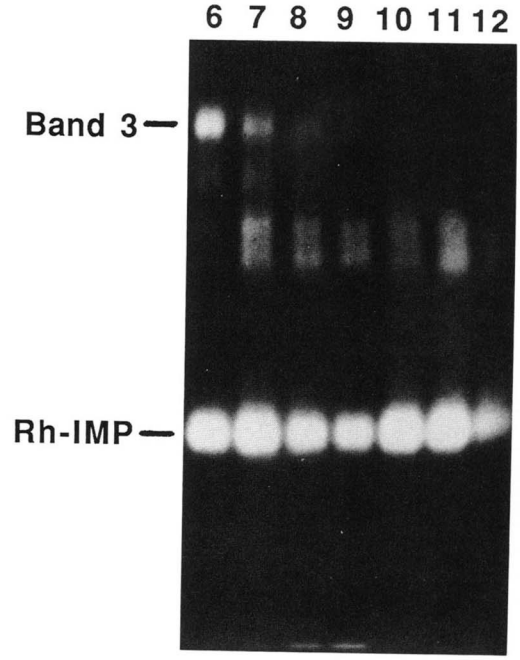


FIG. 7. **The plasma membrane protects Rh-IMP from enzymatic digestion at the extracellular and intracellular faces of the bilayer.** In the upper panels, intact erythrocytes from a DD homozygote were surface ^{125}I-labeled, suspended to a hematocrit of 10% in PBS, 1mM NaEDTA and incubated at 37°C for 60 minutes with no addition (lanes 1 and 1') or 250ug/ml of selected enzymes: neuraminidase (lanes 2 and 2'), alpha-chymotrypsin (lanes 3 and 3'), trypsin (lanes 4 and 4'), or papain (lanes 5 and 5'). The cells were then washed thoroughly to remove all traces of the enzymes, and Rh(D)-IMP was quantitatively immunoprecipitated (lanes 1-5) from the other membrane proteins (lanes 1'-5'). The samples were electrophoresed through an SDS-PAGE slab (12% acrylamide) and exposed to autoradiograph film (lanes 1-5 for 70 hours and lanes 1'-5' for 8 hours). Densitometric scanning of the immunoprecipitate demonstrated no significant reductions of ^{125}I-Rh-IMP (lanes 2-4 > 90% of control, lane 5 = 78%). The low molecular weight material at the bottom of lane 3 is believed to be derived from band 3. Analysis of the nonprecipitated proteins (lanes 1'-5') demonstrated complete degradation of the glycophorins (lanes 2' and 4'), band 3 (lane 3'), or both (lane 5').

In the lower panel, inside-out membrane vesicles were prepared from surface ^{125}I-labeled erythrocytes from a DD homozygote which had been saturated with Rh(D) immune globulin. The vesicles were suspended to 200ug/ml in PBS, 1mM NaEDTA containing no addition (lane 6), or containing alpha-chymotrypsin at 2, 8, or 40ug/ml (lanes 7-9) or trypsin at the same concentrations (lanes 10-12) and incubated at 0°C for 45 minutes. Phenylmethylsulfonyl fluoride was added to 2mM, and all traces of the proteases were removed by thoroughly washing the vesicles. ^{125}I-labeled Rh-IMP was immunoprecipitated from the Triton X-100 solubilized vesicles and electrophoresed through an SDS-PAGE slab (12% acrylamide) which was exposed to autoradiograph film and assessed by densitometric scanning of the autoradiographs. Band 3 was >80% degraded (lanes 7 and 8) and completely degraded (lanes 9-12) at enzyme concentrations which produced only modest reductions in the amount of immunoprecipitated ^{125}I-labeled Rh-IMP (lanes 7,8,10, and 11 >90% of control; lane 9 = 79% and lane 12 = 60%).

TABLE I

PURIFICATION OF Rh-IMP

STEP	PROTEIN mg	^{125}I RECOVERY PERCENT[a]	FOLD PURIFIED	RELATIVE PURITY PERCENT[b]
1. MEMBRANES	1126	--	1.0	
2. SKELETONS	476	95	2.4	
3. H.A. STEP	165	60	4.4	
4. GEL FILT.	21	31	16.8	<10
5. H.A. GRAD.	8.0	24	33.2	24
6. GEL ELUT.	0.62	7.1	132	38
7. H.A. GRAD.	0.15	2.6	184	>95

[a]Recovery of radiochemically pure ^{125}I-labeled Rh-IMP tracer.

[b]Determined from scanning silver stained SDS-PAGE slab with laser densitometer.

TABLE II

AMINO ACID COMPOSITION OF Rh-IMP

AMINO ACID	MOLES PERCENT
ASX	5.0
GLX	6.3
SER	10.9
GLY	15.9[a]
HIS	2.8
ARG	4.5
THR	4.6
ALA	8.9
PRO	5.1
TYR	3.4
VAL	5.8
MET	1.8
CYS	0.7
ILE	3.9
LEU	12.2
PHE	3.9
LYS	4.5

[a]Possibly an overestimate due to Tris glycine electrophoresis buffer.

MINIPRINT REFERENCES

24. Laemmli, U.K. (1970) Nature **227**, 680-685
25. Lowry, O.H., Rosebrough, N.J., Farr, A.C., and Randall, R.J. (1951) J.Biol. Chem. **193**, 265-275
26. Bidlingmeyer, B.A., Cohen, S.A., and Tarvin, T.L. (1984) J. Chromatography **336**, 93-104
27. Fraker, P.J., and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. **80**, 849-857
28. Bennett, V. (1983) Methods in Enzymol. **96**, 313-324
29. O'Keefe, E., and Bennett, V. (1980) J. Biol. Chem. **255**, 561-568
30. Phillips, D.R., and Morrison, M. (1971) Biochemistry **10**, 1766-1771
31. Hubbard, A.L., and Cohn, Z.A. (1972) J. Cell Biol. **55**, 390-405
32. Bennett, V., and Branton, D. (1977) J. Biol. Chem. **252**, 2753-2763.