

THE ENZYMATIC IODINATION OF THE RED CELL MEMBRANE

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ABSTRACT

An enzymatic iodination procedure utilizing lactoperoxidase (LPO), radioactive iodide, and hydrogen peroxide generated by a glucose oxidase-glucose system has been described and utilized for a study of the red cell membrane. 97% of the incorporated isotope is in the erythrocyte ghost and 3% is associated with hemoglobin. No significant labeling of the red cell membrane occurs in the absence of LPO or by the deletion of any of the other reagents. A 6 million-fold excess of chloride ions inhibits iodination by no more than 50%. Incorporation of up to 1×10^6 iodide atoms into a single erythrocyte membrane results in no significant cell lysis. The incorporated label is exclusively in tyrosine residues as monoiodotyrosine. 10–15% of the trichloroacetic acid-precipitable radioactivity can be extracted with lipid solvents but is present as either labeled protein or ^{125}I . Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of solubilized membrane proteins reveals only two labeled protein bands out of the 15 present, and the presence of $50\text{--}1 \times 10^6$ iodide atoms per ghost does not alter this pattern. Component *a* has a molecular weight of 110,000, is carbohydrate poor, and represents 40% of the total label. Component *b* has an apparent molecular weight of 74,000, contains all of the demonstrable sialic acid, and accounts for 60% of the total label. Trypsinization of iodinated, intact red cells results in the disappearance of only component *b*, the appearance of labeled glycopeptides in the medium, and the absence of smaller, labeled peptides remaining in the membrane. Pronase treatment hydrolyzes component *b* in a similar fashion, but also cleaves component *a* to a 72,000 mol wt peptide which is retained in the membrane. A combination of protease treatment and double labeling with ^{125}I and ^{131}I does not reveal the appearance of previously unexposed proteins.

INTRODUCTION

Chemical reagents designed to react at cell surfaces have been used to label the external surface of the plasma membrane. Sulfhydryl reagents such as para-chloromercuribenzenesulfonic acid (1–4), 4-acetamido-4'-isothiocyanate-stilbene-2-2'-disulfonic acid (5), radiolabeled sulfanilic acid diazonium salt (6), and recently ^{35}S -formyl methionyl sulfone methyl phosphate (7) have been employed as membrane probes with varying degrees of success. Despite these efforts, a reagent which specifically and covalently labels the plasma membrane without damaging membrane function is still

required. This report describes an enzymatic iodination system which satisfies these requirements.

In the presence of hydrogen peroxide, iodide, and a nucleophilic acceptor, a number of peroxidases will catalyze the formation of a carbon-halogen bond (8, 9). Lactoperoxidase (LPO)¹

¹ *Abbreviations used:* A, acrylamide; 20 DB, 20 mosmols Na phosphate; 310 DB, 310 mosmols Na phosphate, Dodge buffer; DIT, diiodotyrosine; EDTA, ethylenediaminetetraacetic acid; GO, glucose

was selected because of its optimal activity at physiological hydrogen and chloride concentrations. The hydrogen peroxide generating system of glucose and glucose oxidase (GO) was used because it generated controlled, continuous, and small quantities of this reactive chemical. Another laboratory published an enzymatic iodination technique utilizing LPO while this work was in progress (10). The method differs from ours in a number of important aspects, as well as their results on the labeling of erythrocyte membrane proteins (11, 12). We are now presenting our technique and results on the specific labeling of surface proteins of human erythrocyte membranes in full detail. A brief account has been given elsewhere (13).

MATERIALS AND METHODS

Conditions for Iodination

Fresh human blood (type O) was drawn into 10 units/ml heparin and centrifuged at 1000 *g* for 45 min. The buffy coat and red cells immediately underlying were aspirated and the remaining cells resuspended in 310 mosmols Na phosphate, Dodge buffer (310 DB), pH 7.2 and centrifuged at 1000 *g* for 10 min. After two more rinses, the cells were iodinated. 1 ml of the incubation mixture contained $1-2 \times 10^9$ cells in phosphate-buffered saline (PBS) unless otherwise stated, 5 μ moles glucose, 3.6 munits glucose oxidase (Sigma Chemical Co., St. Louis, Mo., type V), 10-100 μ Ci carrier-free Na 125 I (New England Nuclear Corp., Boston, Mass.), and 3.6 munits lactoperoxidase (LPO kindly supplied by Dr. S. J. Klebanoff). Glucose oxidase activity was measured using a coupled peroxidase-*o*-dianisidine system with the following final concentrations of reagents: 0.05 M phosphate buffer, pH 7.2; 0.005% horseradish peroxidase; 0.5% glucose and 0.01% *o*-dianisidine. The change in absorbance with time at 460 nm was recorded and a molar extinction coefficient of 11,300 was used to establish the activity. 1 unit is that amount of enzyme producing 1 μ mole peroxide/min at 25°C. LPO activity was measured according to the Worthington Biochemical Catalogue (Worthington Biochemical Corp., Freehold, N. J.) assay for horseradish peroxidase (EC No. 1.11.17) at pH 7.2 instead of 6.0. The

oxidase; LPO, lactoperoxidase; MBA, *N,N'*-methylene bis acrylamide; MIT, monoiodotyrosine; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline, with divalent cations (Grand Island Biological Co.); PMSF, phenyl methane sulfonyl fluoride; SDS, sodium dodecyl sulfate; SP, 0.154 M NaCl, 0.01 M Na phosphate; TCA, trichloroacetic acid.

assay used *o*-dianisidine as substrate and the units are identical to glucose oxidase except that 1 unit of LPO decomposes 1 μ mole peroxide/min at 25°C.

To ascertain the specificity of the iodination reaction, controls were performed which consisted of deleting each reagent or combinations from the complete iodination system. To assess possible inhibition of the iodination reaction by chloride ions, erythrocytes were iodinated in the absence of Cl⁻ (310 DB) or in 0.154 M NaCl, 0.01 M Na phosphate, pH 7.2 (SP).

Cells were iodinated from 1 to 60 min at 37°C with constant agitation. The reaction was terminated by the addition of 10 vol cold 310 DB containing 10^{-5} M Na₂S₂O₃. The cells were rinsed five to seven times in 10 vol 310 DB and pelleted at 700 *g* for 10 min at 4°C. The procedure of Dodge et al. (14) for preparation of ghosts was followed with slight modification. After 1 hr in 40 vol of 20 mosmols Na phosphate (20 DB), pH 7.4, the lysed cells were centrifuged at 25,000 *g* for 45 min (Lourdes Instrument Corp., Old Bethpage, N. Y., 9RA) and the pellet was immediately taken up in a small volume of 20 DB (one-step ghosts) or rinsed three times in 10 or 20 DB (four-step ghosts). Four-step ghosts were free of hemoglobin and one-step ghosts contained approximately 3-5% of the original cell hemoglobin. Hemoglobin was prepared from the first lysis supernatant by centrifugation for 90 min at 100,000 *g* to remove ghost vesicles, the supernatant chromatographed on Sephadex G-100, and the hemoglobin peak separated.

Radioactivity Measurements

The radioactivity present in labeled erythrocytes, ghosts, or lysis supernatants was measured by precipitation of a suitable aliquot in 10% trichloroacetic acid (TCA) followed by centrifugation and three to four rinses in 10% TCA. The resulting pellet was solubilized in 0.2 N NaOH, and an aliquot was added to Bray's scintillation fluid and counted in a liquid scintillation spectrometer (Nuclear-Chicago, Des Plaines, Ill.) optimized for 125 I. Counting efficiency was 60%.

Gel Electrophoresis

Four-step ghosts were dialyzed against 500 vol of 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β -mercaptoethanol, pH 7.5 for 3 days at 4°C, with the dialyzing solution replenished every 24 hr (15). Care was taken to exclude any residual leukocytes. Sodium dodecyl sulfate (SDS) was added to the dialyzed ghosts (1-1.5 mg protein/ml) at a final concentration of 2% and the suspension heated for 3 min at 100°C. Sucrose (8%) and bromphenol blue (0.005%) were added and the sample was applied to SDS-polyacrylamide gels of 6 mm \times 100 mm. 0.1% SDS gels were prepared according to Silverstein et

al. (16) and 1.0% SDS gels according to Lenard (15). The ratio of acrylamide to *N,N'*-methylene bis acrylamide (A/MBA) was varied between 20/1 and 37/1, depending on the resolution desired. The percentage of acrylamide ranged from 5 to 10%. The gels were run at 4–6 mA/gel for 8–16 hr. Specific gel composition and electrophoresis conditions are found in the figure legends. After electrophoresis, gels were cut into 2-mm sections and counted directly in a gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) optimized for ^{125}I . Before slicing, the same gel or a duplicate was stained for protein with coomassie blue (17) or for carbohydrate with the periodic acid-Schiff reagent (PAS) (18). For the determination of sialic acid content, gels were sliced and 2-mm sections processed according to Bretscher (7). Molecular weight markers chymotrypsinogen, ovalbumin, bovine albumin, human gamma globulin (Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y.), and β -galactosidase (Sigma Chemical Co.) were prepared and run in parallel with membrane proteins.

Identification of the Iodinated Species

It was important to ascertain the nature of the iodinated species. Four-step dialyzed ghosts (approximately 0.5 mg protein) were TCA precipitated, washed three times in TCA, and the pellet was neutralized and dried. Since acid or alkaline hydrolysis caused extensive deiodination (19), enzymatic hydrolysis was used. 2.5 mg/ml crude trypsin (Grand Island Biological Co., Grand Island, N. Y.) or the same concentration of pancreatin (Calbiochem, Los Angeles, Calif.) in 0.05 M Tris-HCl, pH 8.0 and 1 drop toluene were added and the mixture was incubated for 24 hr at 37°C. An additional 2.5 mg trypsin was added after 24 hr and the mixture incubated another 24 hr. The success of crude trypsin presumably depended upon the presence of other proteases and peptidases which in concert degraded proteins to the level of amino acids.

Following the method of Roche et al. (19), the enzymatic digest was acidified with 1 N HCl to pH 1, cold monoiodotyrosine (MIT) was added as carrier and the aqueous phase was extracted five times with 3 vol of acidified *n*-butanol. When care was taken to completely extract the enzyme digest, 80–85% of the initial radioactivity was recovered. The digestion, acidification, and extraction procedures yielded complete recovery of added M^{125}IT or ^{125}I . The butanol extract was dried under nitrogen and taken up in a small aliquot of the same solvent and chromatographed on flexible gel plates (MN, Sil G, Brinkman Instruments Inc., Westbury, N. Y.) in a solvent system of *n*-butanol/acetone/1 N NH_4OH at 1/4/1 (20). After drying the plate, 0.5 cm strips were cut from the origin to solvent front (18 cm) and counted in toluene-

liquifluor. Standards of M^{125}IT and ^{125}I mixed with carrier were always run in lanes adjacent to the unknown samples, and peaks were localized by counting and visualization with ninhydrin. MIT of *R_f* 0.30 and iodide, *R_f* 0.85, were resolved by this solvent system; however, di- and monoiodotyrosine were not.

A more quantitative recovery of the protein-bound radioactivity was obtained using the Sephadex gel filtration method of Izzo et al. (21). The enzymatic digest was TCA precipitated, rinsed, and the pooled supernatants were applied directly to a Sephadex G-25 Fine column, 1.2 × 56 cm. The radioactivity was eluted with 1 M acetic acid, and fractions of 1.5 ml were collected at a rate of 10 ml/hr and counted directly in the gamma counter. The elution profiles of ^{125}I , M^{125}IT , and diiodotyrosine (DIT by OD^{280}) were determined for comparison to the unknown. MIT ($K_d = 2.3$) was well separated from DIT ($K_d = 3.65$) and iodide ($K_d = 1.3$).

To assess the amount of lipid labeling during the iodination reaction, TCA-precipitated ghosts were extracted with 3/1 ethanol/ether at -20°C for 2 hr (22), centrifuged 2.5 hr at 3000 rpm at -15°C, and the supernatant was dried under nitrogen. The protein content and radioactivity were determined and aliquots were chromatographed in solvent systems designed to separate neutral lipids (petroleum ether/diethyl ether/glacial acetic acid, 90/10/2) or phospholipids (chloroform/methanol/water, 65/25/4) along with suitable lipid standards. Radioactivity was measured in 1 cm strips and the standards were visualized with iodine vapor. In addition, the extract was digested with crude trypsin and the hydrolysate processed as described above for chromatography in the butanol/acetone/ammonia solvent system.

Alteration of the Membrane

To study the erythrocyte membrane structure, a combination of proteolytic enzymes and double labeling of intact cells was employed. Iodinated cells were incubated with 250 or 1000 $\mu\text{g}/\text{ml}$ trypsin (Worthington Biochemical Corp., twice crystallized) in PBS, 250 $\mu\text{g}/\text{ml}$ pronase (Calbiochem) in PBS, 100 $\mu\text{g}/\text{ml}$ neuraminidase (*Cl. perfringens*, Sigma Chemical Co.) in SP, or a combination of neuraminidase and trypsin at the above concentrations. There was no measurable proteolytic activity in the neuraminidase preparations using azocoll (Calbiochem) as substrate. The rate of hydrolysis was measured by withdrawing 1 ml aliquots and immediately pelleting the cells. In the case of trypsin, 0.1 vol soybean trypsin inhibitor (Sigma Chemical Co.) at 5 mg/ml was present during the centrifugation. The supernatant was aspirated, counted, TCA precipitated, and the soluble radioactivity measured. The cell pellet was lysed in water, counted, TCA precipitated, and the pellet solubilized and counted. For gel elec-

trophoresis, iodinated intact cells (approximately 1×10^9 /ml) were incubated with 250 μ g/ml trypsin for 30 min at 37°C. Trypsin inhibitor was added and the cells were centrifuged. The supernatant was saved for further analysis of released peptides and the cells were rinsed three to four times as described above. The trypsinized ghosts were processed for gel electrophoresis as described above. Trypsin inhibitor was present throughout the entire procedure.

In double-labeling experiments, iodinated (^{125}I) cells ($1-3 \times 10^9$ cells/ml) were trypsinized, rinsed, and the intact cells reiodinated with ^{131}I and the LPO system at identical substrate concentrations. The cells were again rinsed six to seven times, lysed, and processed for gel electrophoresis. The gel slices were counted for the presence of both ^{131}I and ^{125}I .

In experiments where pronase was employed, no inhibitor was added until the cells were lysed, at which point phenyl methane sulfonyl fluoride (PMSF, Eastman Organic Chemicals, Rochester, N. Y.) at a final concentration of 0.03% was added and present throughout the subsequent rinses and dialysis. Double iodination was performed in a slightly different manner. Iodinated red cells ($1-3 \times 10^9$ /ml) were exposed to 250 μ g/ml pronase for 30 min at 37°C, ghosts prepared, and gels run of the solubilized protein. Unlabeled cells were exposed to pronase, rinsed, then iodinated, rinsed, lysed, and ghosts prepared for electrophoresis.

The labeled membrane fragments released by exposure of intact cells to the two proteases were studied. Cells were incubated with trypsin or pronase, centrifuged, and the supernatant of the first centrifugation was precipitated with 10% TCA for 1 hr at 4°C. The acid-soluble peptides were neutralized with 5 N NaOH, dialyzed 12 hr against twice-distilled water, and lyophilized. The lyophilized sample was taken up in 10^{-5} M $\text{Na}_2\text{S}_2\text{O}_8$, applied to a Sephadex G-100 column (2.5×50 cm) equilibrated with the same solution, and 5-ml fractions were collected at a rate of 15 ml/hr. Aliquots were counted in Bray's. The lyophilized sample was also solubilized in 2% β -mercaptoethanol, 2% SDS and electrophoresed in 5 or 7.5% gels with A/MBA of 37/1. Gels were counted as described. Membrane fragments released by the combined trypsin-neuraminidase action were treated in a similar way.

Other Procedures

Hemoglobin was determined by the pyridine-hemochromogen method (14), protein according to Lowry et al. (23) using lysozyme as a standard, and sialic acid by the Warren thiobarbiturate method (24) after hydrolysis of the sample for 1 hr in 0.1 N H_2SO_4 at 80°C.

RESULTS

Characteristics of the Iodination Reaction

The incorporation of the iodide label into red cell ghosts was linear during a 30 min incubation, with 96% of the total radioactivity associated with the ghosts (Fig. 1). There was very little label in the lysis supernatant, which was predominantly hemoglobin. There was a significant but variable inhibition of iodination in the presence of chloride. The inhibition was less than 50% after 30 min of incubation, yet the chloride to iodide ratio was 6×10^6 to 1, indicating that chloride was an extremely poor inhibitor of the iodination reaction catalyzed by LPO. Recently it was reported that halide ions inhibit glucose oxidase (25). In either the presence or absence of chloride, the incorporation of radioactivity into red cell ghosts was 96% of the total after 30 min. During the incubation there was less than 0.5% lysis as measured by the amount of hemoglobin in the first rinse. A total of 500-1000 atoms of iodide were incorporated into each ghost after 30 min of incubation in 310 DB.

In an experiment designed to saturate all labeling sites, 2.5×10^8 cells were incubated with 10 $\mu\text{Ci/ml}$ Na^{125}I (0.004 nmoles/ml), the enzyme system and increasing amounts of Na^{127}I (or K^{127}I) for 2 hr (Fig. 2). With increasing amounts of

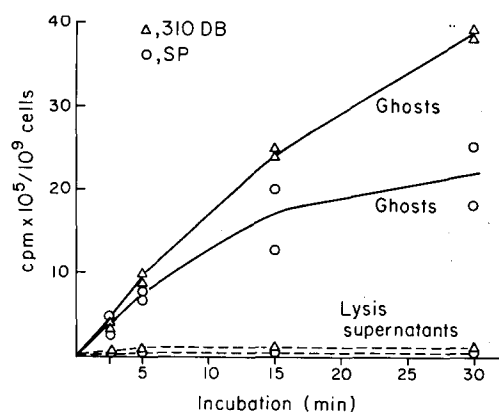


FIGURE 1 The iodination of erythrocytes. 1 ml of the reaction mixture contained 1×10^9 washed cells in the appropriate buffer, 5 μ moles glucose, 3 munits LPO, 6 munits GO, and 50 μCi carrier-free Na^{125}I . One-step ghosts were prepared with 3-5% residual hemoglobin. \triangle — \triangle , ghosts from cells iodinated in 310 DB (no chloride); \circ — \circ , ghosts from cells iodinated in SP (containing chloride); \triangle --- \triangle , lysis supernatant, DB; \circ --- \circ , lysis supernatant, SP.

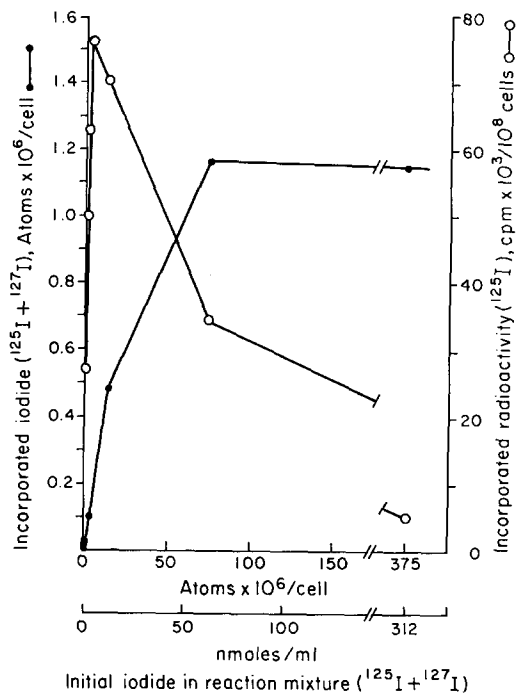


FIGURE 2 Effect of iodide concentration on incorporation of ^{125}I and ^{127}I into intact red blood cells. 1 ml of the reaction mixture contained 2.5×10^8 cells in PBS, 3.6 munits LPO, 7.2 munits GO, 5 μmoles glucose, 10 μCi Na^{125}I (0.004 nmoles), and varying concentrations of Na^{127}I from 0 to 300 nmoles. Incubation for 120 min at 37°C . Number of iodide atoms incorporated per cell calculated from 1.3×10^5 atoms/cpm for carrier-free Na^{125}I at day 0 (half-life of 60 days) and known dilution of Na^{125}I with Na^{127}I .

iodide, there was a decrease in the efficiency of incorporation, suggesting saturation of the available iodination sites. These results indicate that approximately 1×10^6 sites per cell can be iodinated. At 60 and 300 nmole/ml iodide, both GO and LPO concentrations were in excess with a total of 1530 nmoles/ml H_2O_2 produced (GO) and 760 nmoles/ml consumed (LPO), so substrate limitation could not explain the data. An anomalous result was the increased incorporation of radiolabeled iodide with increasing amounts of ^{127}I added (Fig. 2) up to 2.5 nmoles/ml ($^{127}\text{I}/^{125}\text{I}$ of 600/1) before any significant competition occurred. The complex relationship of the various reactants and their effect on this reproducible anomaly were not investigated further.

Table I establishes the specificity of the LPO-catalyzed iodination. The deletion of any one reagent reduced the incorporation into ghosts by

approximately 99%. Even the deletion of exogenous glucose (Table I, *e*) inhibited iodination to 1.3% of the complete system. This suggested that sufficient endogenous glucose did not diffuse across the membrane to react with exogenous GO. Preincubation of the cells with either LPO or GO for 30 min followed by two rinses and subsequent incubation of the cells with the complete system minus the preincubated enzyme resulted in similar low levels of iodination. These controls eliminated the possibility of adsorption of either LPO or GO to specific proteins on the surface and localized iodination. The relative amount of incorporated label in the first lysis supernatant was much larger in all the controls than in the complete system. This may reflect the adsorption of unreacted isotope during precipitation of large amounts of protein.

A control (control *b*, Table I) was always performed in subsequent experiments. A high level of nonspecific labeling in the absence of LPO indicated the presence of a contaminant or altered reagent in the system. It was particularly important to use fresh isotope that had not been exposed to air for long periods of time, since oxidized isotope resulted in a high level of incorporation even in the absence of LPO. The amount of cell lysis

TABLE I
Controls of the Iodination Reaction

Reagent deleted	Radioactivity (cpm/10 ⁸ cells)	
	Ghosts	1st supernatant (Hb)
<i>a.</i> None	390×10^4	20×10^4
<i>b.</i> LPO (in DB)*	3×10^4	6×10^4
<i>c.</i> LPO (in SP)*	3×10^4	4×10^4
<i>d.</i> GO (in DB)	2×10^4	5×10^4
<i>e.</i> Glucose (in DB)	5×10^4	7×10^4
<i>f.</i> GO, LPO (in DB)	3×10^4	5×10^4
<i>g.</i> GO, LPO, glucose (in DB)	4×10^4	5×10^4

Iodination conditions for the complete system (*a*) per milliliter of final solution: 1×10^8 washed red blood cells, 5 μmoles glucose, 7.2 munits glucose oxidase, 3.6 munits lactoperoxidase, 50 μCi Na^{125}I . Appropriate volumes of buffer replaced the specific reagent deleted. Incubation was carried out for 30 min at 37°C .

* Iodination performed in 310 mosmols Na phosphate pH 7.2 (DB) or 0.154 M NaCl, 0.01 M Na phosphate, pH 7.2 (SP).

TABLE II
Distribution of Label after Iodination of Intact Red Blood Cells

Fraction	Protein (mg)	Radioactivity (cpm)	Distribution of label (%)	Specific activity (cpm/mg protein)	Relative enrichment
Total RBC	422	5.1×10^7	100	0.12×10^6	1
Ghosts	5.2	4.94×10^7	97	9.6×10^6	80
Hemoglobin	420	0.14×10^7	3	0.0033×10^6	0.03

Iodination conditions per milliliter (total of 10 ml): 1×10^9 washed cells in PBS, 5 μ moles glucose, 3.6 munits LPO, 7.2 munits GO, 50 μ Ci carrier-free Na 125 I. Incubation was carried out at 37°C for 30 min. Four-step ghosts and hemoglobin were prepared as described in Methods.

due to the hydrogen peroxide levels generated by GO was also assessed by this control.

Table II lists the typical distribution of label found in one of 12 experiments. Four-step ghosts were prepared which contained no measurable hemoglobin. 97% of the incorporated label was in ghosts. Since ghost protein represented 1.3% of the total protein, this resulted in an 80-fold increase in specific activity of ghosts over the total erythrocyte and a 2600-fold increase of ghosts over hemoglobin. If hemoglobin was directly exposed to the LPO system, it was heavily iodinated (data not shown). These results clearly indicate that the membrane is serving as a barrier to the iodination of intracellular hemoglobin by LPO. Light and electron microscope radioautography have confirmed these findings.²

Identification of Iodinated Species

Having established the specificity and distribution of the TCA-precipitable radioactivity, we next evaluated the nature of the labeled moiety (Tables III and IV). Greater than 95% of the radioactivity recovered in the *n*-butanol extract (80% of initial counts) migrated with an *R_f* identical to that of both chemical and radiolabeled MIT in a solvent system designed to resolve MIT from iodide (Table III). The identity of the unknown with MIT was established by corunning a radioactive standard with the sample. Analysis of the tryptic digest on a Sephadex column yielded similar results. No label was found to be associated with DIT or histidine.

Approximately 15% of the TCA-precipitable label could be extracted with 10% H₂O in acetone or 3/1 ethanol/ether (Table IV). These solvents

² Hubbard, A. L., and Z. Cohn. Unpublished observations.

have been reported to extract neutral lipids as well as phospholipids (21, 26). The radioactive material did not migrate with any known phospholipid or neutral lipid standard chromatographed in two different solvent systems. A variable percentage (10–30%) ran with 125 I. When the lipid extract was digested with trypsin, greater than 50% of the label chromatographed with an M 125 IT standard. In addition, about 10–15% of the total protein could be extracted with either organic solvent mixture. This suggested that the majority of the labeled material extracted into lipid solvents was protein or 125 I.

TABLE III
Identification of the Iodinated Species

Method	%* MIT	% I- <i>idide</i>	% DIT
A. Thin layer chromatography† (<i>n</i> -butanol/acetone/1 N NH ₄ OH, 1/4/1)	95	<2	§
B. Gel filtration Sephadex G-25 Fine (1 M acetic acid)	80	15	0

* Amount of each species expressed as per cent of recovered radioactivity (total radioactivity in the thin layer chromatography or column run).

† TCA-precipitated ghost protein first extracted with 3/1 ethanol/ether and then digested as described in Methods. 80% of radioactivity was hydrolyzed and extracted into acidified *n*-butanol. § MIT and DIT not well separated in this solvent system.

|| TCA-precipitated ghost protein neutralized, dried, and then digested as described in Methods. Trypsin digest TCA precipitated and supernatant (85% of original radioactivity) applied to column with 96% recovery.

TABLE IV
Characterization of Iodinated Species Soluble in Organic Solvents

Experiment	Organic solvent	Organic solvent phase		Protein residue	
		cpm	Protein (μg)	cpm	Protein (μg)
1	3/1 ethanol/ether	1,244	13.7	6,805	80
2	a. None	—	—	309,000	100
	b. 10% H ₂ O in acetone	49,500	8.4	248,000	—
	c. 3/1 ethanol/ether	34,000	11.4	182,000	—

Four-step ghosts TCA precipitated, rinsed four times in 10% TCA, dried, and the above solvents added for 1 hr at -20°C . Radioactivity and protein determined as described in Methods.

Electrophoretic Identification of Labeled Proteins

Solubilized ghost protein was electrophoresed and the gels were processed for radioactivity, protein, carbohydrate, or sialic acid (Fig. 3). Only two proteins were labeled with ^{125}I out of the 15 present. 95% of the applied radioactivity was recovered in these two peaks and greater than 95% was retained in the gel after coomassie blue staining in 50% methanol and destaining in acetic acid. This is additional evidence for the protein character of the incorporated label. In gels electrophoresed for shorter times than normally employed, 3–5% of the label coincided with the SDS front which Lenard presumed to be the region of lipid migration. The first labeled peak (component *a*) has an apparent molecular weight of 110,000, stains intensely with coomassie blue, and only slightly with PAS. It has no detectable sialic acid. This component represents approximately 40% of the total label. The second peak (*b*) migrates at 74,000 mol wt, stains for protein when a large quantity is applied, but heavily with the PAS stain. It also contains all of the measurable sialic acid and represents about 60% of the total label. In this experiment, 400 atoms of iodide were incorporated per ghost; however, the amount of label was increased to 1×10^6 atoms per ghost with no change in the pattern of labeling and no change in the relative amounts of labeling in the two peaks. No additional PAS-positive bands at molecular weight smaller than component *b*, as reported by others (21), were observed. This may reflect the amount of membrane material applied or the sensitivity of the methods used.

The anomalous behavior of glycoproteins (27) was observed when 1% SDS gels (5.0% A/MBA

of 37/1) were used to analyze the labeling pattern of membrane protein from iodinated cells (Fig. 4). The glycoprotein migrated at 100–105,000 mol wt under these conditions and coincided with component *a* to give a broad peak of radioactivity. The

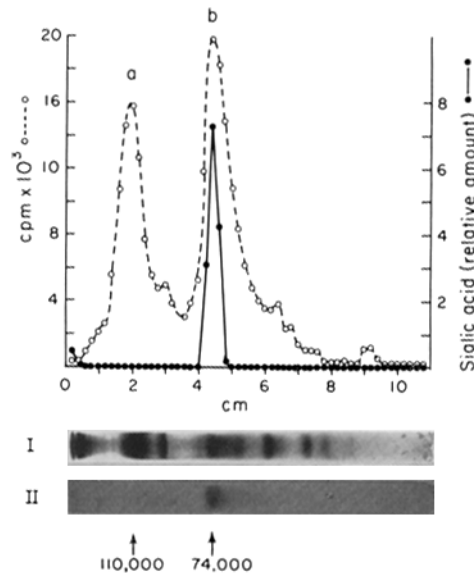


FIGURE 3 SDS-polyacrylamide gel electrophoresis patterns of labeled proteins from human red cell membrane. 0.1% SDS gels consisted of 7.5% monomer with A/MBA of 20/1. Electrophoresis 16 hr at 6 mA/gel. Bromphenol blue ran off at approximately 12 hr. After electrophoresis, parallel gels were treated in the following ways: Gel I (circa 70 μg protein) stained for protein; Gel II (circa 150 μg protein) stained for carbohydrate; \circ --- \circ , cut into 2-mm sections, counted, and radioactivity distribution plotted (98% recovery of radioactivity applied); \bullet — \bullet , cut into 2-mm sections and sialic acid content measured. All procedures given in Methods.

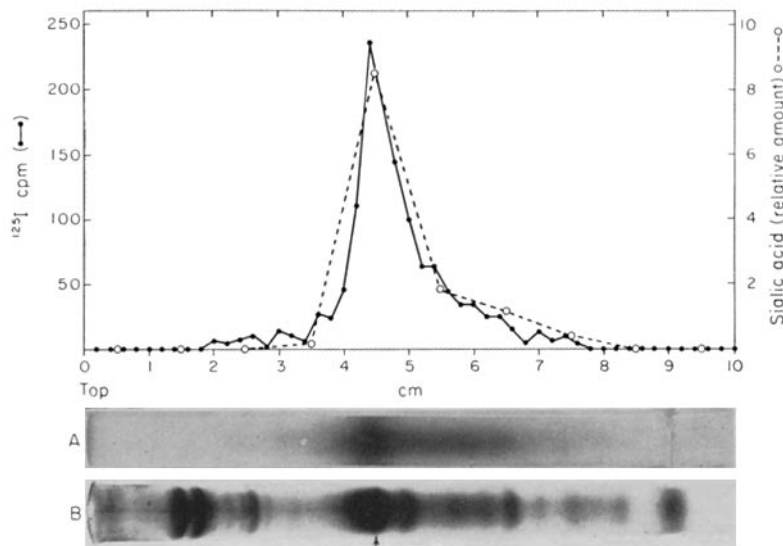


FIGURE 4 SDS-polyacrylamide gel electrophoresis patterns of labeled proteins from human red cell membrane. 1.0% SDS gels consisted of 5% acrylamide with A/MBA of 37/1. Electrophoresis 4 hr at 10 mA/gel. After electrophoresis, parallel gels were treated in the following ways: Gel A (circa 150 μg protein) stained for carbohydrate; Gel B (circa 50 μg protein) stained for protein; ●—●, cut into 2-mm sections, counted, and radioactivity distribution plotted (>95% recovery); ○---○, cut into 10-mm sections and sialic acid content measured. All procedures given in Methods.

enzymatic iodination of twice frozen and thawed ghosts resulted in the labeling of all membrane proteins (data not shown).

Results of Proteolytic Action on Intact Cells

Having established that only two protein species in the red cell membrane are normally exposed to the exterior, it was of interest to see whether proteolytic enzyme treatment would release either of these labeled proteins and whether unlabeled proteins would become exposed after such treatment. In Table V the results of such a study are shown. After 60 min of incubation with trypsin at 250 to 1000 $\mu\text{g}/\text{ml}$, 40–45% of the total label could be released from intact red cells. Prolonging the incubation for up to 240 min released only 5% more of the label. There was approximately 1% lysis during trypsinization. Preincubating the cells with neuraminidase had no effect on the amount of label subsequently released. Pronase released 45% of the total label. There was 5% lysis of cells during pronase digestion.

The time course of trypsinization was measured using labeled, intact cells (Fig. 5 A). The release of labeled components was 50% complete in the first 5 min. Cells labeled with 5–200 atoms of iodide gave identical results. The kinetics of sialic acid

release were somewhat different from that of iodide-labeled material (Fig. 5 B).

The nature of the labeled components remain-

TABLE V
Effect of Enzymes on Labeled Red Blood Cells

Enzyme	Concentration ($\mu\text{g}/\text{ml}$)	% of total counts released*
Trypsin	1000	45 (1)‡
Trypsin	250	40 (5)
Neuraminidase,§	100,	
Trypsin	250	46 (1)
Pronase	250	45 (3)

Prelabeled intact cells (cells allowed to stand 24 hr at 4°C before protease treatment) incubated with enzyme 60 min at 37°C, sedimented, and supernatant and cells analyzed for radioactivity.

* Per cent values obtained from TCA-soluble counts in supernatant and sum of TCA-soluble supernatant counts and TCA-insoluble cell-associated counts. (5% of $t = 0$ supernatant counts were TCA insoluble and counts remained constant; 10% of $t = 0$ cell counts were TCA soluble and counts remained constant.)

‡ Number of experiments.

§ Neuraminidase preincubated with cells for 10 min then trypsin added.

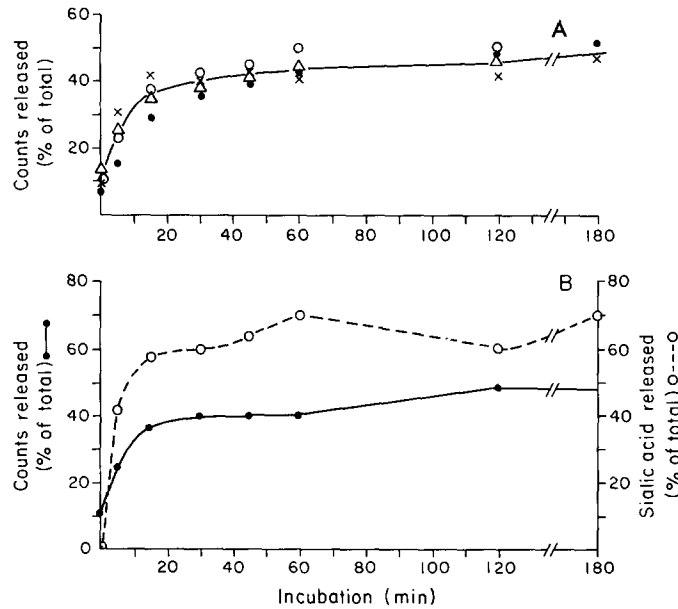


FIGURE 5 A The effect of trypsin on the release of ^{125}I material from labeled intact erythrocytes. Approximately 5×10^8 iodinated cells/ml incubated with $250 \mu\text{g/ml}$ trypsin at 37°C with constant agitation. Reaction stopped by addition of 0.1 vol 5 mg/ml cold inhibitor and cells sedimented. See Table V for details of measurement. Atoms of iodide per cell at time zero: x, 5-10; ●, 25-75; △, 100-150; ○, 150-200.

FIGURE 5 B The effect of trypsin on the release of sialic acid and ^{125}I material from labeled erythrocytes. 5×10^8 cells/ml incubated and processed as above. Only cell pellet was assayed for presence of sialic acid. Total sialic acid measured in triplicate from cells before enzyme exposure.

ing in the membrane was next examined. The gel patterns of ghost proteins from control and trypsinized cells are shown in Fig. 6. One component, *b*, was attacked by trypsin. This corresponded to the sialic acid-rich glycoprotein as is evident from the labeling pattern as well as the coomassie blue- and PAS-stained gels. After trypsinization, the diffuse protein stain of the glycoprotein was absent and a *nonradioactive* band appeared with an approximate molecular weight of 40,000. The PAS-positive band also disappeared and no smaller product was present. In contrast, the carbohydrate-poor protein (peak *a*) was unaffected by trypsinization. The per cent of total label released by trypsin could be completely accounted for by the loss of label in the glycoprotein (peak *b*). Experiments with neuraminidase and trypsin treatment gave gel patterns identical to those obtained when trypsin alone was used. Evidently the trypsin-sensitive site(s) is proximal to the iodinated sites, otherwise a labeled protein of smaller size would have appeared in the gels of trypsinized cells. The residual 10-20% of the

label in peak *b* migrated with a molecular weight similar to that of the intact glycoprotein (in 5, 7.5, and 10% gels), but its exact amount varied. On the basis of the coomassie blue patterns of control and trypsinized cells in Fig. 6, it would seem that no other proteins in the membrane of intact cells were susceptible to trypsin. This result depended upon the proper control of enzymatic hydrolysis. When trypsin inhibitor was not present during the 12 rinses and lysis steps, the resulting gel patterns were much different. Both labeled proteins were hydrolyzed to peptides of 30-60,000 mol wt, and the coomassie blue staining pattern showed no large proteins of 250,000 mol wt. This suggested continued action of trypsin on membrane proteins exposed after cell lysis.

Double-labeling techniques were used to investigate the possible exposure of new proteins after trypsinization of the external surface (Fig. 7). No new species were revealed. If a new protein was exposed on the trypsinized cell surface, it would have been labeled with ^{131}I only and there was clearly no such protein. The distributions of

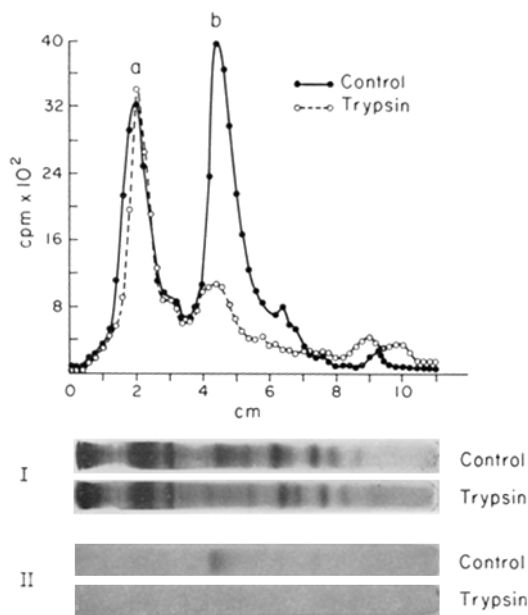


FIGURE 6 SDS-polyacrylamide gels of membrane protein from control and trypsinized ^{125}I -labeled red cells. Gel composition 7.5% acrylamide, A/MBA of 20/1, 0.1% SDS. Electrophoresis for 12 hr at 5 mA/gel. Graph represents distribution of ^{125}I in 2-mm sections with >95% recovery in each gel; Gels I stained for protein and Gels II for carbohydrate.

radioactivity of ^{125}I and ^{131}I were identical. This does not rule out the possibility that previously unavailable sites on the same protein become available for iodination after trypsin. The peak occurring at mol wt 26,000 (third peak in Fig. 7) is often seen in control runs and represents less than 5% of the total label.

Exposure of intact iodinated erythrocytes to pronase released slightly more label in a 60 min incubation (Table V) than with trypsin, and gel electrophoretic analysis of solubilized ghost proteins revealed differences in the proteins hydrolyzed (Fig. 8). As with trypsin, the glycoprotein was attacked by pronase as evidenced by disappearance of the PAS-positive band (not shown) and radioactivity in the area of peak *b*.³ However, pronase also hydrolyzed peak *a* to yield a 72,000 mol wt cleavage product which remained in the

³ These 5.5%, 20/1 gels were prepared so that the glycoprotein would run at 90,000 mol wt, otherwise the pronase cleavage product at 72,000 and the glycoprotein at 74,000 in 7.5% gels, would have had the same mobility and obscured analysis.

membrane and retained 85% of the radioactivity of the original peak. This suggests that the pronase-sensitive sites are distal to most of the iodinated sites. As seen in the coomassie blue-staining pattern (Fig. 8), the band at 110,000 mol wt disappeared and one at 72,000 mol wt appeared. No other proteins of the membrane appeared to be digested by pronase treatment of intact cells.

Direct confirmation of the conversion of peak *a* to the 72,000 mol wt peak was obtained by sequential trypsin and pronase digestion. Iodinated erythrocytes (Fig. 9 A) were first trypsinized to yield an intact cell containing primarily component *a* exposed on the membrane (Fig. 9 B), and then pronase treated. Component *a* at 110,000 mol wt disappeared and a peak at 72,000 mol wt appeared (Fig. 9 C). 93% of the original radioactivity in peak *a* was retained in the membrane after pronase with no evidence of smaller radioactive peptides either in the membrane or in the medium (see below).

As in the case with trypsin, no new protein species were revealed by pronase hydrolysis of the intact cell (Fig. 10). The patterns of radioactivity were identical whether pronase was incubated with intact cells before or after the LPO iodination. The 72,000 mol wt cleavage product of component *a* was still on the external surface of

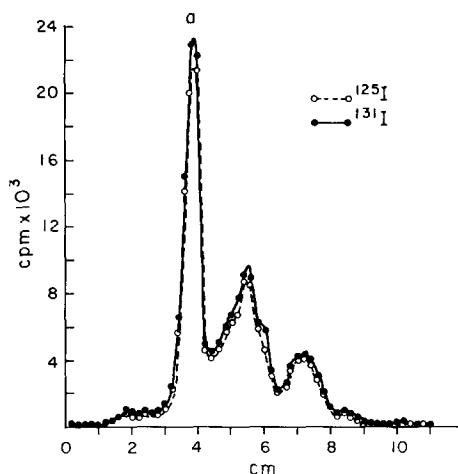


FIGURE 7 SDS-polyacrylamide gel of membrane proteins from iodinated (^{125}I), trypsinized, and reiodinated (^{131}I) intact, red cells. Gel composition 5% acrylamide, A/MBA of 37/1, 1.0% SDS. Electrophoresis for 7.5 hr at 5 mA/gel. Graph represents distribution of ^{125}I and ^{131}I in a single gel, with 95% and 80% recovery, respectively. See Methods for details.

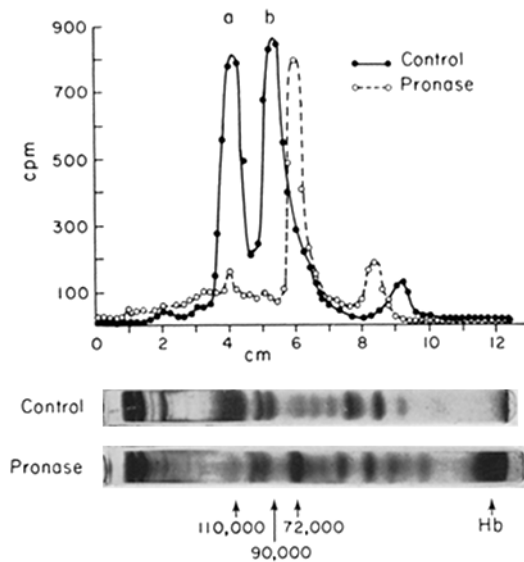


FIGURE 8 SDS-polyacrylamide gels of membrane proteins from control and pronase-treated, ^{125}I labeled, red cells. Gel composition 5% acrylamide and 20/1 A/MBA, 0.1% SDS. Electrophoresis for 10 hr at 5 mA/gel. Graph represents distribution of ^{125}I (>95% recovery in each gel); gels stained for protein.

the cell membrane since it could still be labeled after pronase treatment.

Analysis of Label Released

An analysis of the labeled material released by enzyme digestion of the external surface of erythrocyte membrane (Table VI) showed that 98–99% of the label was soluble in 10% TCA which suggested small peptides. However, when the fragments were chromatographed on a Sephadex G-100 column (Fig. 11 A) most of the label (peak I) appeared slightly before albumin, suggesting a peptide of 70,000 mol wt. A smaller, variable peak (peak II) appeared slightly ahead of myoglobin. When fragments from neuraminidase-trypsin experiments were chromatographed, peak II contained all of the protein-bound nondialyzable radioactivity. To test whether the presence of sialic acid residues on the glycopeptide fragments was causing the artifactual shift from high to low molecular weight, the isolated tryptic fragments were exposed to neuraminidase and the digest was chromatographed (Fig. 11 B). The results were similar to those of the trypsin-neuraminidase experiment. These data suggest that the mobility of the sialopeptides in Sephadex was influenced by

the negatively charged sialic acid residues and that their removal resulted in migration behavior based on size rather than charge.

Similar results were obtained with labeled fragments from pronase-treated cells. No additional

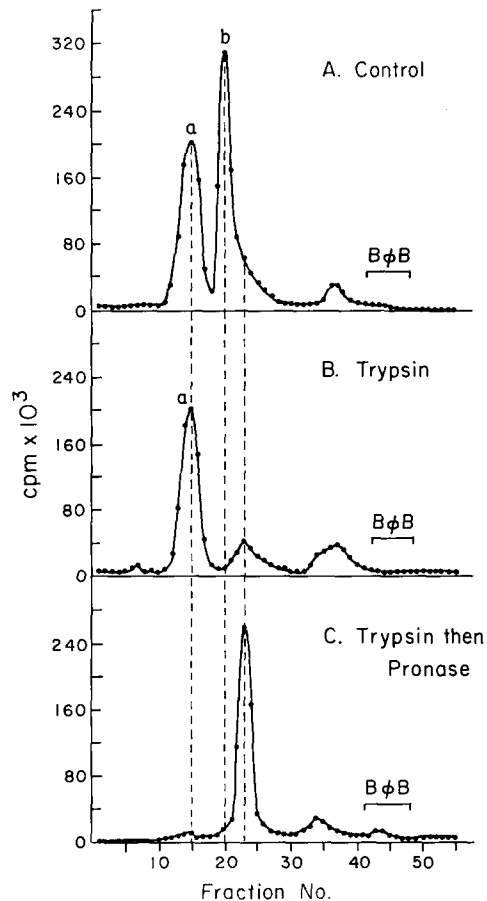


FIGURE 9 SDS-polyacrylamide gels of membrane proteins from ^{125}I -labeled red blood cells exposed sequentially to trypsin and pronase. Graphs represent distribution of ^{125}I in unstained gels to which *circa* 80 μg undialyzed membrane protein was applied. 3×10^{10} intact cells were iodinated together in SP and then treated as follows: A, no protease; B, 250 $\mu\text{g}/\text{ml}$ trypsin, 45 min at 37°C, cells rinsed eight times in 310 DB with trypsin inhibitor present before and during preparation of ghosts; C, 250 $\mu\text{g}/\text{ml}$ trypsin as in B, cells rinsed four times in 310 DB then 250 $\mu\text{g}/\text{ml}$ pronase added for 45 min at 37°C, cells rinsed four times in 310 DB with trypsin inhibitor present and lysed in the presence of 0.035% PMSF. Gel composition 5% acrylamide with 20/1 of A/MBA, 0.1% SDS. Electrophoresis 10 hr at 4.5 mA/gel. BφB indicates bromphenol blue position in gel.

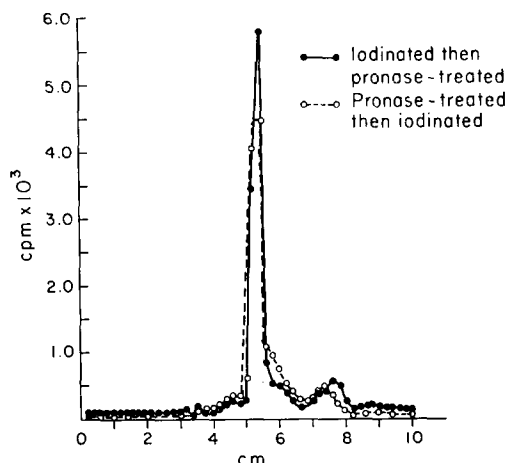


FIGURE 10 SDS-polyacrylamide gels of membrane proteins from pronase-treated cells. Cells either pronase treated and then iodinated or iodinated and then pronase treated. Gel composition 5.5% acrylamide, A/MBA of 20/1. Electrophoresis for 9.5 hr at 5 mA/gel, >95% recovery of radioactivity in each gel.

peaks of radioactivity corresponding to the 30,000 mol wt fragment from the hydrolysis of component *a* were detected. Since this fragment contains little of the radioactivity from component *a*, its fate has not been established. Gel electrophoresis in 5% gels (not shown) of both trypsin and pronase fragments gave only one radioactive peak of 100,000 mol wt.

DISCUSSION

General Technique

In this study the extent to which the lactoperoxidase iodination system satisfied the requirements of a membrane label was examined. Human erythrocytes were used because of their availability, the ease in isolation of the plasma membrane, and the presence of a highly colored intracellular protein whose release was a quick and accurate measure of membrane damage. In this study 96–97% of the total incorporated radioactivity was recovered in the membrane, with only 3–4% in the hemoglobin fraction and without apparent cell damage. The large sizes of the two enzymes, LPO of 78,000 and GO of 115,000 mol wt, preclude their penetration into the cell. However, hydrogen peroxide and iodide could cross the membrane and might react nonenzymatically with intracellular constituents. The low labeling

of intracellular hemoglobin would seem to rule this out. In addition, a control consisting of the iodination system lacking LPO was always performed to eliminate such a possibility. The low labeling of ghosts and hemoglobin in such a control demonstrates the strict requirement for lactoperoxidase. The large, impermeable reagent (LPO) which is required for reactivity combined with the small size of the label actually incorporated (^{125}I of 3 A) makes the LPO iodination system particularly useful. Another advantage is the absence of cell damage as a result of exposure to peroxide and iodide. There was less than 0.5% lysis of red cells during a 60 min incubation in which from 100 to 500,000 atoms of iodide were incorporated into the membrane of a single erythrocyte. These labeled cells were stable for over 48 hr at 4°C.

Similar studies have been performed with mouse fibroblasts.² Iodinated L cells continue to phagocytose, divide, and spread on a surface to the same extent as nonlabeled cells. We feel that the glucose-glucose oxidase couple used to generate a continuous micromolar supply of hydrogen peroxide avoids possible peroxidation of lipids or proteins and permanent cell damage. Others employing the lactoperoxidase labeling system have reported the periodic addition of 0.10 mM quantities of hydrogen peroxide (28, 29) which we have found lyses cells. The reaction mixture contains 0.3 μg protein as GO, 3 μg protein as LPO, compared to about 600 μg of erythrocyte membrane protein. GO and LPO are undoubtedly iodinated, but controls have demonstrated that neither enzyme adsorbs to the membrane, so the results of gel electrophoresis reflect labeling of only the membrane proteins.

The mild incubation conditions are clearly advantageous when one is interested in labeling and studying living cells. The iodinating capacity of LPO at physiological pH and in the presence of a 6 million-fold excess of chloride ions is sufficient to label cells in a short time. In addition, the specificity of the label for tyrosine residues in protein combined with the nonreutilizable nature of monoiodotyrosine is an advantage when studying the turnover of the labeled protein.

Erythrocyte Proteins Labeled

When intact erythrocytes are iodinated using the LPO system, only two membrane proteins are labeled out of 15 bands present as detected by

TABLE VI
*Characterization of Labeled Material Released from Iodinated, Intact Erythrocytes after Exposure to Proteases**

Fraction	Trypsin [‡] cpm × 10 ⁶	Pronase [‡] cpm × 10 ⁶	Neuraminidase + Trypsin [§] cpm × 10 ⁶
A. Ghosts (before enzyme)	2.8	2.8	18.0
Ghosts (after enzyme)	1.8	1.6	12.0
B. Total released material	1.0	1.25	7.83
TCA soluble	0.98	1.20	7.35
TCA precipitable	0.001	0.005	0.004
C. TCA-soluble material	0.98	1.20	7.35
Dialyzable	0.30	0.25	1.8
Nondialyzable	0.70	0.90	6.0
D. Sephadex G-100 analysis			
Dialyzable counts	100% ¹²⁵ I	100% ¹²⁵ I	25% ¹²⁵ I ¶
Nondialyzable counts: (see Fig. 10)			
In peak I	75%	80%	—
In peak II	25%	20%	75% ¶

* Procedures for characterization found in Methods and Materials.

‡ 10¹⁰ cells iodinated in 10 ml with 1 ml of reaction mixture containing 5 μmoles glucose, 3 munits each LPO and GO, 50 μCi Na ¹²⁵I, all in PBS.

§ 5 × 10⁹ cells iodinated in 10 ml with 1 ml containing 5 μmoles glucose, 6 munits each LPO and GO, 100 μCi Na ¹²⁵I, all in PBS.

¶ TCA-precipitable radioactivity.

¶¶ Fragments chromatographed after neutralization of TCA supernatant. No dialysis.

coomassie blue staining of gels (Fig. 3). These findings agree with those of Bretscher (7), who found two proteins of 105,000 and 90,000 mol wt labeled using the ³⁵S-sulfone of formyl methionyl methyl phosphate, which was said to acylate amino groups. The conditions under which the cells were labeled resulted in 16% of the incorporated label reacting with hemoglobin, a much higher value than the 3% found in this study. However, the observation that the same protein components in the red cell membrane are labeled using two entirely different reagents suggests that only these two components are exposed on the external surface of red cells and available for labeling or proteolytic attack. In addition, it is further evidence that the results of the iodination procedure are not dependent upon the tyrosine content of the membrane proteins. Both reagents can label all membrane protein components when procedures are taken to expose these proteins.

The nature of each of these two labeled proteins suggests that they are distinct moieties. Component

a, a protein of 110,000 mol wt, is carbohydrate poor, has no known function, and yet comprises approximately 25% of the total membrane protein (30). The number of chains per erythrocyte membrane would be approximately 1 × 10⁶. The other labeled component is a glycoprotein which contains all the measurable sialic acid present in the ghost (Fig. 3). Exposure of intact red cells to neuraminidase results in the removal of all the bound sialic acid (31), a finding consistent with the localization of the iodinated glycoprotein to the external surface. This glycoprotein has been isolated from red cell ghosts by a variety of techniques (31, 34, 35 for review) and possesses the MN blood group activity and the myxovirus receptor site. The isolated glycoprotein has a molecular weight of 31,000 or 55,000, depending on the analytical method used (32-34). In this study, using SDS-polyacrylamide gel electrophoresis, it was found that the amount of acrylamide monomer as well as the ratio of monomer to cross-linker (A/MBA) influenced the migration

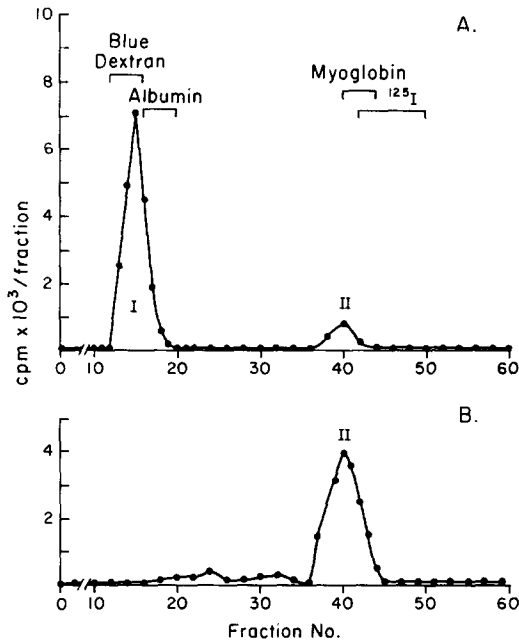


FIGURE 11 Sephadex G-100 elution profiles of labeled material released from intact, iodinated red cells exposed to trypsin. Details of trypsinization and processing of released material given in Methods. (A) Iodinated tryptic peptides eluted with 10^{-5} M $\text{Na}_2\text{S}_2\text{O}_3$. 5-ml fractions collected. (B) Iodinated tryptic peptides treated for 1 hr at 37°C with $100 \mu\text{g}/\text{ml}$ neuraminidase before chromatography.

behavior of this labeled component (Figs. 3, 4, 8). It has been estimated that this component comprises 3% of the membrane protein so that 3×10^5 copies are present in each ghost (21). Together, components *a* and *b* are represented by about 1.5×10^6 chains per ghost. Assuming that each has molecular dimensions $50 \times 50 \times 100 \text{ \AA}$ (7) (an overestimate for the glycoprotein), this amounts to only 20% of the red cell surface. The remaining 80% is as yet unaccounted for.

Reports in which intact red cells were exposed to protein labeling reagents and only one molecular weight species was found labeled must be reconsidered. In both studies (10, 36) proteins were analyzed on 5% SDS-polyacrylamide gels, which we and others (7, 27) have shown do not resolve the two labeled proteins. The study using ^{35}S -sulfanilic acid diazonium salt (36) presents a distribution of radioactivity having a peak at 125,000 mol wt with a distinct shoulder at the leading edge, which is most likely the glycoprotein.

Proteases

A number of reports have dealt with the effects of proteolytic enzymes on the red cell membrane (36-40). In this study we have been able to combine double labeling techniques with protease treatment to examine both the material released and the residual membrane protein of the treated cell.

Trypsin has been used extensively to modify the erythrocyte membrane, and the unique finding of others has been the lack of apparent change in the membrane protein-staining patterns after trypsinization of intact cells (30, 40). Others, studying the nature of the released material, demonstrated that sialopeptides were removed by trypsin (41-45). We have confirmed both findings. This apparent discrepancy becomes explicable when it is seen in the LPO iodination experiments (Fig. 6) that only the labeled glycoprotein is affected by trypsin. It stains very lightly with coomassie blue, presumably because of its negative charge, but when the PAS stain is used, its presence is obvious in untreated cells as is its absence after trypsin treatment. No other proteins in the intact cell are attacked by trypsin. The other labeled protein, component *a*, is not affected by trypsin except when the enzyme is allowed to act on ghosts or solubilized protein. Then this and many other proteins are hydrolyzed. Such a uniquely specific action indicates that component *a* is arranged in the membrane such that no lysine or arginine residues are accessible to trypsin. Jackson et al. (44) have shown that the glycoprotein extractable with lithium diiodosalicylate has two trypsin-sensitive sites, but in the intact cell only one of these is accessible. After trypsinization, no labeled component of smaller molecular weight was found in the membrane, suggesting that the labeling sites were distal to the trypsin-sensitive site and thus released into the medium. The nature of the residual label in the region of the intact glycoprotein has not been established. The use of freeze-etch techniques has revealed a change in the distribution of the 85 \AA intramembranous particles after trypsin (46). These particles appear to represent the hydrophobic segment of the surface glycoprotein. The externally disposed portions of these particles still retain some viral and phytohemagglutinin receptor activity which is in accord with our finding that only 60% of the total sialic acid could be released with trypsin. Others have obtained similar results (41, 45). The finding that

after trypsinization no new protein species become accessible to the LPO iodination system indicates that layers of protein do not exist on the external surface of the membrane. In addition, prolonged trypsinization (3 hr) does not lead to the hydrolysis of proteins other than the glycoprotein and suggests that no radical rearrangement of internal proteins has occurred.

The action of pronase on iodinated, intact erythrocytes provides further evidence that only two proteins are exposed on the surface of the cell. The same two proteins which can be iodinated are also accessible to pronase. Component *a* is cleaved to a 72,000 mol wt product. Direct evidence that the pronase cleavage product is derived from the 110,000 mol wt component of untreated cells comes from the sequential use of trypsin and pronase to iodinated cells. By first removing component *b* with trypsin, we were able to show that pronase treatment of intact cells converted component *a* of 110,000 mol wt to a 72,000 mol wt product which remained in the membrane. In addition, Berg (36) has followed the time course of pronase action and found an approximate one-to-one relation between the disappearance of component *a* (mol wt 125,000 in his study) and the appearance of material at 50–100,000 mol wt. We have found that most (85%) of the label initially present in component *a* is retained after pronase has cleaved a 30–40,000 mol wt fragment from the original protein. Bretscher's peptide maps of the intact and pronase-treated protein are almost identical, suggesting that the 30,000 mol wt peptide either is not accessible to label or has no tyrosine or lysine residues—yet it is susceptible to pronase attack. In these two studies, as well as our own, no other major proteins are hydrolyzed by pronase. As with trypsin, no new protein species are uncovered by hydrolysis of the two surface proteins. The 72,000 mol wt cleavage product is still on the outer surface and remains accessible to the LPO iodination system.

Concluding Remarks

There are numerous uses for a chemical reagent which, when added to the exterior of a cell, will specifically react with membrane constituents. We have successfully employed an enzymatic iodination system to probe the membrane structure of the erythrocyte. Currently this technique is being used to study the turnover of membrane proteins in cells with a more complicated system

of cytomembranes as well as the flow of surface membrane as it is interiorized. Other obvious uses of the label include an examination of the membrane at different stages of the cell cycle and in comparing normal and transformed cells.

We wish to express our thanks to Miss Tien-ling Chang for her excellent technical assistance and to Dr. Samuel C. Silverstein for careful reading of the manuscript and helpful suggestions.

This investigation was supported by grants AI 07012 and AI 01831 from the Public Health Service and a National Science Foundation Predoctoral Graduate Fellowship.

Received for publication 21 April 1972, and in revised form 2 June 1972.

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