Biochimica et Biophysica Acta, 512 (1978) 54—71

BBA 78091

APPLICATION OF A NEW SURFACE LABELING REAGENT, EDTA DERIVATIVE, ON ERYTHROCYTES AND PLATELETS

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(Received March 29th, 1977)

Summary

The modes of binding of a new class of impermeant metal-chelating probe, the complex of $^{111}In^{3+}$ to 1-(*p*-benzenediazonium) ethylenediamine tetraacetic acid (azo-phenyl-EDTA), to human and rabbit erythrocyte membranes and the effect of binding on the function of rabbit platelets have been studied. The metal chelate, azo-phenyl-EDTA · [$^{111}In^{3+}$] bound covalently to membrane proteins following reaction with intact erythrocytes. The amount and the pattern of labeling was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide disc and slab gels for radioactivity. The pattern of labeling of intact human erythrocytes by azo-phenyl-EDTA · [$^{111}In^{3+}$], by pyridoxal phosphate-NaB^3H_4 and by galactose oxidase-NaB^3H_4 was also compared. The following results were obtained:

(a) The pattern of labeling of intact human erythrocyte by azo-phenyl-EDTA · [$^{111}In^{3+}$] differed from other commonly used probes for labeling external membrane surfaces. Five polypeptides were labeled by the metal chelates. In addition to the known major proteins (protein band III, PAS-1, PAS-2 and PAS-3 of Fairbanks et al. (1971) Biochemistry 10, 2606—2617) a protein (radioactive band 4) which migrated slightly slower than PAS-3 in SDS gel was labeled heavily by the metal chelate. This protein has an apparent molecular weight of 37 500 in 8.4% acrylamide-SDS gel. About 40% of bound radioactivity was found in this protein. The diazo linkage of the metal chelate to this protein was found to be especially unstable to heat.

(b) In rabbit erythrocyte membranes, the metal chelate bound to three polypeptides with apparent molecular weights of 96 000, 43 000 and 33 000 in 8.4% acrylamide gel. They are probably glycoproteins in nature.

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Abbreviations: SDS, sodium dodecyl sulfate; azo-phenyl-EDTA, 1-(*p*-benzenediazonium) ethylenediamine tetraacetic acid; TEMED, *N,N,N',N'*-tetraethylenemethylenediamine.

(c) The binding of the probe to platelets did not affect the platelet aggregability induced by adenosine diphosphate. In vivo studies indicated that the labeled platelets accumulated at the plague of atherosclerotic rabbits.

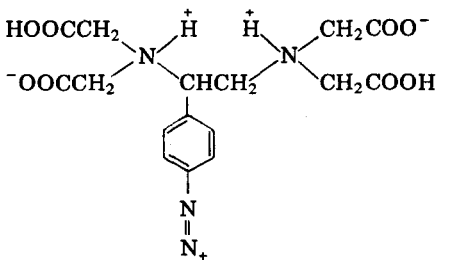
(d) The bifunctional analog of EDTA may permit new applications of metals with useful physical properties for studies of cell membranes.

Introduction

One important aspect of membrane structure is the location of proteins in membranes. A number of probes to label the surface of cells or their isolated membranes have given us considerable insight into the structure of membranes in general and that of human erythrocytes in particular. The subject of labeling membranes has been reviewed recently [1]. The probes employed in the study of the disposition of external proteins in human erythrocyte membranes can be classified into two general categories. One is specific for the polypeptide part of membrane proteins [2—12], the other is specific for the carbohydrate part of membrane proteins [13—15]. The pattern and the amount of labeling to proteins on the external suurface by these probes have been shown to be different. The differences may result from the difference in the accessibility and specificity of the probes to proteins on the outer cell-surface.

The ions of many heavy metals exhibit a wide range of useful spectral and radioactive properties, such as scattering of electrons and X-rays, electron paramagnetic resonance spectra, fluorescence spectra, the production of line-broadening and chemical shifts in nuclear magnetic resonance spectra, and the emission of correlated γ-ray cascades. However, due to their lack of specificity to many biologically interesting molecules, the applications of metal ions as probes are very limited. By using a chelating agent whose complex with metals can bind covalently to macromolecules in a controlled manner, new applications are possible.

The present report describes the modes of binding of a new class of impermeant probe, the complex of $^{111}In^{3+}$ to 1-(*p*-benzenediazonium) ethylenediaminetetraacetic acid (compound I), to human and rabbit erythrocyte membranes and the effect of binding on rabbit platelets, with respect to the volume, oxygen uptake, ADP-induced aggregation, and in vivo aggregation. The pattern of the labeling to proteins on the outer surface of erythrocyte membranes by the metal chelate has been compared with results obtained using pyridoxal phosphate-sodium borohydride, galactose oxidase-sodium borohydride, as cell-surface probes for amino groups in membrane polypeptides and galactose moieties in the glycoproteins, respectively.



(I)

Methods and Materials

All procedures involving platelets were performed at room temperature using plastic vessels. White male New Zealand rabbits, weighing 2—3 kg, were employed for all the experiments. Atherosclerosis in rabbits was accomplished by feeding ad libitum (for 29 days) a cholesterol (0.25% diet containing a specific necrotizing agent for the aortic endothelium. The details of this new procedure will be described elsewhere.

Preparation of azo-phenyl-EDTA · [$^{111}In^{3+}$]. $^{111}InCl_3$ (Medi + Physics, Glendale, Calif.) was purified by eluting with 0.1 M HCl from a 0.7 × 10 cm column of AG1-X8 anion-exchange resin and evaporating the fractions in nalgene beakers by a heating lamp. The synthesis of azo-phenyl-EDTA was according to Sudberg et al. [16]. The diazonium salt was stored at −20°C and the concentration of diazonium ions [17] was measured prior to each labeling experiment. The $^{111}In^{3+}$ was complexed to azo-phenyl-EDTA in a sodium bicarbonate buffer (pH 6.0) or in a 3.8% sodium citrate/HCl (pH 5.3). The formation of a stable complex of azo-phenyl-EDTA · [$^{111}In^{3+}$] was determined by a 0.5 × 7 cm column of chelex-100 which can only retain free $^{111}In^{3+}$ and not $^{111}In^{3+}$ chelated by EDTA.

The ionic strength of the solution of azo-phenyl-EDTA · [$^{111}In^{3+}$] was adjusted by addition of solid NaCl or 1 M sodium citrate to a final concentration of 0.9% NaCl or 3.8% sodium citrate.

Erythrocyte and ghost preparation. Blood was obtained from normal donors or rabbits using 3.8% sodium citrate as an anticoagulant. The erythrocytes were washed with phosphate-buffered saline (pH 7.4) and centrifuged at 1000 × *g* for 10 min four times to remove the buffy coat and serum proteins. Erythrocyte ghosts were prepared according to Dodge et al. [18].

Labeling of erythrocytes with azo-phenyl-EDTA · [$^{111}In^{3+}$]. Prior to labeling, washed cells were washed again in a carbonate buffer containing 50 mM each of NaCl, Na_2CO_3, and $NaHCO_3$ (pH 9.8). The same buffer was diluted 5-fold to wash ghosts. Washed cells or ghosts were suspended in 3 vols. of the carbonate buffer solution. Aliquots of cold isotonic solution of azo-phenyl-EDTA · [$^{111}In^{3+}$] were added to the suspensions of cells or ghosts. The labeling mixtures which contained about 20% cell or ghost suspension and 20 μM azo-phenyl-EDTA · [$^{111}In^{3+}$] (0.5 mCi) were stirred slowly in a rotary shaker at 22°C for 15 min. The reactions were stopped by adding 40 vols. of cold phosphate-buffered saline containing 5 mM sodium phosphate (pH 7.4) to the cell suspension. Cells were separated by centrifugation and washed four times with 40 vols. of the phosphate-buffered saline. A phosphate buffer containing 5 mM sodium phosphate (pH 7.4) was used to stop the labeling reaction of ghosts and to wash out the unbound reagent by centrifugation. The membrane and hemoglobin fractions of the labeled cells were isolated as described above. The hemoglobin fraction of the labeled cells was further purified by eluting with the phosphate buffer from a 0.5 × 25 cm column of Sephadex G-25 (Pharmacia, N.J.).

For the study of the permeability of azo-phenyl-EDTA · [$^{111}In^{3+}$] to erythrocyte membrane, erythrocytes were also labeled in the phosphate-buffered saline (pH 7.4) at 4°C for 12 h. As a control, fresh hemoglobin

(1 mg/ml) was labeled at pH 9.8 for 15 min or at pH 7.4 for 12 h. The reaction was stopped by passage of the reaction mixture of hemoglobin over a 0.5 × 25 cm column of Sephadex G-25 that had previously been equilibrated with the phosphate buffer. Any remaining azo-phenyl-EDTA · [$^{111}In^{3+}$] was removed by subsequent dialysis of the hemoglobin fraction at 4°C against the phosphate buffer.

Labeling of erythrocytes with pyridoxal phosphate-NaB^3H_4. Erythrocytes were labeled by a modification of the method of Rifkin et al. [19]. A 30% suspension of red cells in phosphate-buffered saline (pH 9.0) and 0.5 mM pyridoxal phosphate was incubated at 37°C for 30 min. The unbound pyridoxal phosphate was removed by centrifugation and washed three times with 40 vols. of cold phosphate-buffered saline (pH 9.0). The cells were tritiated by incubating 1.5 ml of 30% cell suspension with 0.5 mCi NaB^3H_4 in phosphate-buffered saline (pH 9.0) at 0°C for 30 min. At the end of reaction, 40 vols. of phosphate-buffered saline (pH 7.4) containing 1 mg $NaBH_4$ was added to the reaction mixture. Cells were separated by centrifugation and washed three times with 40 vols. of phosphate-buffered saline (pH 7.4). The membrane and hemoglobin fractions of the labeled cells were isolated as described above.

Labeling of erythrocytes with galactose oxidase-NaB^3H_4. Erythrocytes were labeled by a slight modification of method of Gahmberg [13]. A volume of 1 ml of 50% suspension of washed cells were incubated with 5 units of galactose oxidase in phosphate-buffered saline (pH 7.0) at room temperature for 1 h. The cells were then washed three times in phosphate-buffered saline (pH 7.4) and tritiated by incubating 1.5 ml of 30% cell suspension with 0.5 mCi NaB^3H_4 in phosphate-buffered saline (pH 7.4) at room temperature for 30 min. At the end of reduction, 40 vols. of phosphate-buffered saline (pH 7.4) containing 1 mg $NaBH_4$ was added to the reaction mixture. Cells were separated by centrifugation and washed three times with 40 vols. of phosphate-buffered saline (pH 7.4). The membrane and hemoglobin fractions of the labeled cells were isolated as described above.

The concentration of proteins was determined by the Lowry method [20], using human serum albumin as a standard. For measurement of radioactivity, membranes or protein were dissolved in 2—3 vols. of a solubilizing solution containing 45% protosol (New England Nuclear), a 5% water and 50% toluene. Radioactivity was measured with toluene scintillation mixture of omnifluor (New England Nuclear) in a liquid scintillation counter (Beckman) at room temperature.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed according to Weber and Osborn [21] in 0.1% SDS gel (4.2% or 8.2% acrylamide, 0.13% or 0.26% methylenebisacrylamide and 0.05% of 0.025% TEMED, respectively) and Fairbanks et al. [22] in 1% SDS gel (5.5% acrylamide, 0.20% methylenebisacrylamide and 0.05% TEMED). Ammonium persulfate (Fisher Scientific Co., Fairlawn, N.J.) at a final concentration of 0.15% was used to catalyze the polymerization of disc gels (10 × 0.5 cm) or slab gels (14 × 10.5 × 0.25 cm). Slab gel electrophoresis was performed in a modified apparatus of Studier [23]. Two slab gels can be run simultaneously. Each slab gel contained 20 sample wells 4 mm wide, 10 mm deep and 3 mm apart. Membranes or

proteins to be subjected to electrophoresis were dissolved in a solubilizing solution, containing 2% SDS, 3.3 M urea, 100 mM 2-mercaptoethanol, 25 μg/ml pyronin Y (tracking dye), 10% sucrose and 10 mM sodium phosphate (pH 7.0) (for 0.1% of SDS gels) or 10 mM Tris · HCl (pH 8), 1 mM EDTA (pH 8.0) (for 1% SDS gels). Except where otherwise noted, the denatured membranes or proteins were incubated at 100°C for 3 min and then at room temperature for 30 min. The loaded disc gels were run at 2—4 mA per tube until the pyronin Y tracking dye band reached a mark of 75 mm from the origin. The two loaded slab gels were run with the voltage gradient of 30 V and the current at 150 mA. Gels were calibrated with human serum albumin (Schwarz/Mann), creatine kinase (Worthington), carbonic anhydrase (Calbiochem) hemoglobin and the cross-linking products of both human serum albumin and creatine kinase by glutaraldehyde.

Gels were stained and destained according to Fairbanks et al. [24]. Destained disc gels (up to four) were aligned, cut to 1.25-mm slices and counted on a Nuclear-Chicago well-type gamma counter. Gel densitometry was done on a Gilford spectrophotometer and Model 2400 linear transport accessory. Destained slab gels were photographed, treated with dimethylsulfoxide/2.5-diphenyloxazole according to Bonner and Lasky [24] and vacuum dried. The dried gels were covered with Kodak RP X-Omat film, wrapped in aluminum foil, and stored at −70°C for 1—12 days until developed. Film densitometry was done on a double-beam recording microdensitometer (Joyce, Loebel and Co. Ltd.).

Labeling of platelets. Blood was drawn by cardiac puncture and mixed with sodium citrate (9 vols. blood : 1 vol. citrate). Platelet-rich plasma was obtained by centrifuging the citrated blood for 4 min at 375 × *g*. Platelets were collected by centrifuging the platelet-rich plasma for 15 min at 2000 × *g*. The platelet-poor plasma was decanted and saved. A modified Ringer solution containing 0.107 M NaCl, 0.004 M KCl, 0.002 M Na_2SO_4, and 1 mg/ml glucose was used to wash the platelets by centrifugation at 2000 × *g* for 10 min twice. The concentrations and the size distribution curves of platelets were determined by means of a Coulter counter. Washed platelets were incubated with either the isotonic solution of 20—25 μM azo-phenyl-EDTA · [$^{111}In^{3+}$] (1mCi) or the modified Ringer solution as a control in a concentration of $3 \cdot 10^8$ platelets per ml for 1 h at room temperature. To stop the reaction, 20 ml of platelet-poor plasma were added, and the platelets were collected by centrifugation at 2000 × *g* for 10 min. The platelets were further washed by 15 ml of platelet-poor plasma twice. The washed platelets were suspended in fresh platelet-poor plasma for further characterization.

Characterization of labeled platelets. Aggregation of platelets was monitored by measuring the change in turbidity. The mixture of 0.4 ml of platelet-rich plasma and 0.1 ml 15 mM $CaCl_2$ in 0.9% NaCl was used to set an arbitrary intensity of the transmitted light (650 nm). The ADP-induced aggregation was initiated by adding 0.1 ml 1 mM ADP in 0.9% NaCl to the mixture of 0.4 ml of platelet-rich plasma and 0.1 ml 15 mM $CaCl_2$ and the change in the light transmission with time was recorded at 37°C. The oxygen consumption of platelets ($2—3 \cdot 10^8$ cells) was measured in 2 ml of platelet-rich plasma by an oxygen electrode at 22°C. The morphology of platelet were examined by

electron microscopy. Washed platelets were fixed in 3% glutaraldehyde. The fixed cells were further washed in cacodylate buffer, postfixed in 1% OsO_4 for 1 h at 4°C before dehydration in ethanol solutions, and embedded in Epon. Sections were collected on copper grids, stained with uranyl acetate and lead citrate and examined in a Carl Zeiss (Elektronenmikroskop EM 9S-2) electron microscope. For in vivo study, labeled platelets ($1.5 \cdot 10^8$ cells) were suspended in 0.5 ml platelet-poor plasma and injected into either a normal or a atherosclerotic rabbit through the ear vein. 20 h after the transfusion the sample blood was collected, the rabbits were killed and samples of major organs were taken, washed by 0.9% NaCl, weighed and counted in a gamma counter.

Results

Impermeability of cell membranes to azo-phenyl-EDTA · [$^{111}In^{3+}$]

The binding of azo-phenyl-EDTA · [$^{111}In^{3+}$] to hemoglobin, membranes of intact cells and ghosts is given in Table I. As can be seen, the present label did not bind to hemoglobin in intact erythrocytes. In contrast, hemoglobin alone was labeled heavily. Bretscher [25] has pointed out that when the pH of the reaction is high a low incorporation of cell-surface labels into hemoglobin in intact cells may sometimes result from the pH gradient across the membrane bilayer. Labeling of erythrocytes by azo-phenyl-EDTA · [$^{111}In^{3+}$] at physiological pH was tried. A low level of label bound to hemoglobin in intact erythrocytes and an extensive labeling of hemoglobin alone were observed, when the reactions were at pH 7.4 for 12 h. Similarly, erythrocyte ghosts were labeled heavily, when the ghost membranes were labeled from both sides. These results suggest that the membrane is reasonably impermeable to the label. It was also found that other metal ions, such as $^{59}Fe^{3+}$, can also form a complex with azo-phenyl-EDTA to serve as an impermeant probe of the red cell membrane (Hwang, K.J., unpublished).

Comparison of labeling of human erythrocyte by azo-phenyl-EDTA · [$^{111}In^{3+}$] and by two other surface probes

When intact normal human erythrocytes were treated with azo-phenyl-EDTA · [$^{111}In^{3+}$] at pH 9.8 and the isolated membranes analyzed by SDS-polyacrylamide gel electrophoresis, the staining patterns of Coomassie Blue

TABLE I

INCORPORATION OF AZO-PHENYL-EDTA · [$^{111}In^{+3}$] INTO HEMOGLOBIN AND MEMBRANE PROTEINS

	Membrane fraction (cpm/μg)	Hemoglobin (cpm/μg)
1. Rabbit intact cells, pH 9.8, 15 min	124	0
2. Rabbit hemoglobin, pH 9.8, 15 min		399
3. Rabbit ghost, pH 9.9, 15 min	1912	
4. Rabbit intact cells, pH 7.4, 12 h	105	0.44
5. Rabbit hemoglobin, pH 7.4, 12 h		550
6. Human intact cells, pH 9.8, 15 min	250	0.88

and periodic acid-Schiff were the same as those from membranes without the labeling (Fig. 1). This suggested the mobilities of membrane proteins in SDS-polyacrylamide gel were not affected by the binding of metal chelates. The amount and the pattern of labelings were assessed by fluorography of dried gels for radioactivity. Three radioactive components were resolved in 5.5% acrylamide gel system of Fairbanks et al. [22]. These had apparent molecular weights of 96 000, 52 000, and 30 000 (Figs. 2f—2h). Simultaneous electrophoresis on a single slab gel of membranes from intact normal human erythrocytes labeled with ^3H by pyridoxal phosphate-NaB^3H_4, and by galactose-NaB^3H_4 permitted direct comparison of the labeling patterns (Figs. 2a—e). The results in Fig. 2 suggested that radioactive band with 96 000 daltons move as protein band III and PAS-1, and radioactive bands with 52 000 and 30 000 daltons migrated with PAS-2 and PAS-3, respectively.

It was noted that in addition to the labeling of protein band III, PAS-1, PAS-2, and PAS-3 by pyridoxal phosphate-NaB^3H_4 [10], a weak band of

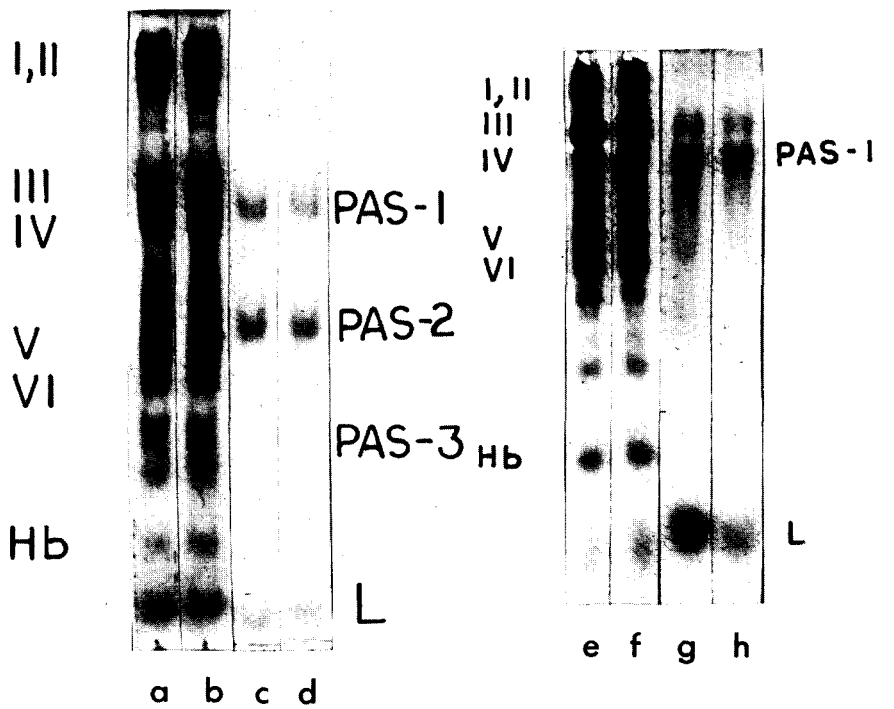


Fig. 1. The staining patterns of Coomassie Blue and periodic acid-Schiff's reagent of human erythrocyte membranes in (a—d) four dried 5.5% acrylamide gel (Tris · HCl buffer) and in (e—h) four dried 8.4% acrylamide gel (phosphate buffer). Gels a and c (or e and g) are, respecively, the stain of Coomassie Blue and periodic acid Schiff's reagent of controlled human erythrocyte membrane. Gels b and d (or f and h) are, respectively, the Coomassie Blue and periodic acid-Schiff's stain of normal human erythrocyte membrane labeled with azo-phenyl-EDTA · [$^{111}In^{3+}$]. The nomenclature of the proteins is according to Fairbanks et al. [22]. Hb is hemoglobin band, L is the periodic acid-Schiff's reagent stain of glycolipid. Due to the contamination of Schiff reagent from gels c, d, g and h the glycolipid bands also appeared in gels stained with Coomassie Blue during the drying process.

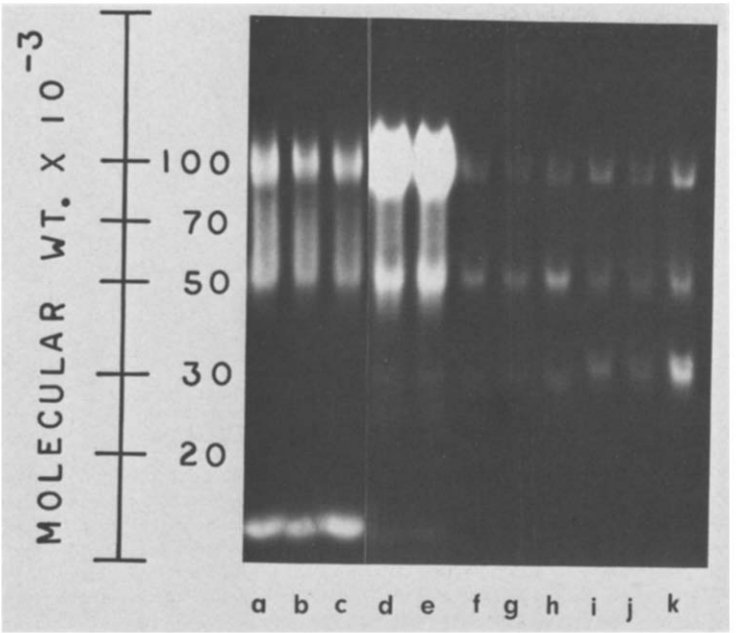


Fig. 2. The fluorogram of 5.5% acrylamide gel (Tris · HCl buffer). Erythrocytes from three healthy normal donors were labeled (a—c) by galactose oxidase-NaB^{3}H$_4$, (d, e) by pyridoxal phosphate-NaB^{3}H$_4$, (f—h) by azo-phenyl-EDTA · [^{111}In^{3+}], and (i—k) by azo-phenyl-EDTA · [^{111}In^{3+}] (without the heating process prior to the gel electrophoresis). (a—c) was a fluorogram from an exposure of 8 h to the dried slab gel. (d—k) was a fluorogram from an exposure of 11 days to the same dried slab gel. Erythrocyte membranes for gel a, f and i were from the first donor; membranes for gels b, d, g and j were from the second donor; and membranes for gels c, e, h and k were from the third donor. The fluorogram from the first donor's membrane labeled with pyridoxal phosphate-NaB^{3}H$_4$ was not shown because of the shadow from the overexposure of gel c.

26 000 daltons and several other proteins with molecular weight ranged from 52 000 to 96 000 were detected by the sensitive fluorogram. The pattern of the labeling of proteins from 52 000 to 96 000 daltons by pyridoxal phosphate-NaB^{3}H$_4$ were quite similar to that labeled by galactose oxidase-NaB^{3}H$_4$. A radioactive band of about 26 000 daltons from human erythrocyte membranes labeled by galactose oxidase-NaB^{3}H$_4$ can also be detected, when the exposure times of the fluorogram was increased to 15 days (data not shown).

It has been shown that in the presence of SDS and Tris buffer, (not phosphate buffer), PAS-1 and PAS-2 are interconvertible favoring PAS-2 at higher temperature [26—28]. The conversion of radioactive band with 96 000 daltons to 52 000 daltons after a brief treatment of heat (Figs. 2 and 3) suggested that the metal chelates bound to both PAS-1 and PAS-2. Furthermore, an extra radioactive band, which ran as 31 000 daltons was detected in the 5.5% acrylamide gel when the erythrocyte membrane was not heated. Since PAS-3 does not participate in the aggergation/disaggregation equilibrium between PAS-1 and PAS-2 [28], the disappearance of the radioactive band with 31 000 daltons upon the treatment of heat could be due to diazo linkages unstable to heat rather than a result of the interconversion between human erythrocyte membrane glycoproteins. A similar observation of azoproteins containing

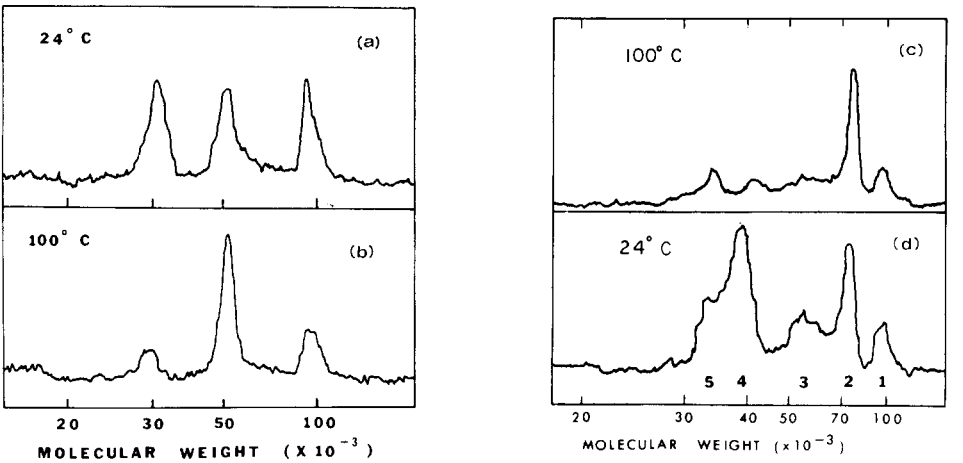


Fig. 3. The effect of heat on the aggregation/disaggregation of glycoproteins and the stability of diazo linkages of azo-phenyl-EDTA · [$^{111}In^{3+}$] to the proteins on the external surface of human erythrocyte membranes. Labeled membranes of same amount of radioactivity of ^{111}In were analyzed in (a, b) 5.5% acrylamide Tris · HCl-buffered gel and in (c, d) 8.4% acrylamide phosphate-buffered gel. Samples for a and d were incubated with Tris · HCl-buffered and phosphate-buffered SDS-solubilizing solutions at 24°C, respectively, whereas samples for b and c were heated at 100°C for 3 min before the electrophoresis. These are the film densitometries of fluorogram from the slab gels. The numbers underneath the tracing of d correspond to the five radioactive bands observed in the fluorogram of Fig. 4.

linkages unstable to heat has been reported [29]. The origin of such heat-sensitive bonds is not clear.

Observation of a new protein on the external surface of human erythrocyte membrane

To eliminate the ambiguity due to the interconversion of glycoproteins and to resolve the overlap of PAS-1 and protein band III, a phosphate-buffered 8.4% acrylamide gel system of Weber and Osborn [21] was adopted. As shown in Fig. 1, the protein band III and PAS-1 were separated quite well in such a gel system. The staining of carbohydrates for PAS-2 and PAS-3, however, was very diffuse and broad. Thus, the assignment of PAS-2 and PAS-3 was difficult. A diffuse periodic acid-Schiff's reagent stain move in front of PAS-1 was also observed when human erythrocyte membrane was separated in a similar phosphate-buffered gel system [12]. Five radioactive components were resolved in 8.4% acrylamide gel (Figs. 3 and 4). These had apparent molecular weights of 96 000 (band 1), 74 000 (band 2), 59 000 (band 3), 37 500 (band 4), and 32 500 (band 5). Radioactive band 1 corresponds to a faint periodic acid-Schiff's reagent-positive band and to protein band III (see Fig. 1). Radioactive band 2 migrated with the major glycoprotein (PAS-1). This suggested that the radioactive band with 96 000 daltons in 5.5% acrylamide gel resulted from the metal chelates bound to protein band III and PAS-1. Among all the labeled polypeptides, radioactive band 4 appeared to be most sensitive to heat (Figs. 3 and 4). This suggested that radioactive band 4 in 8.4% acrylamide gel corresponded to the radioactive band with 31 000 daltons in 5.5% acrylamide gel (Fig. 2i—k). Furthermore, radioactive band 3 and band 5 may correspond

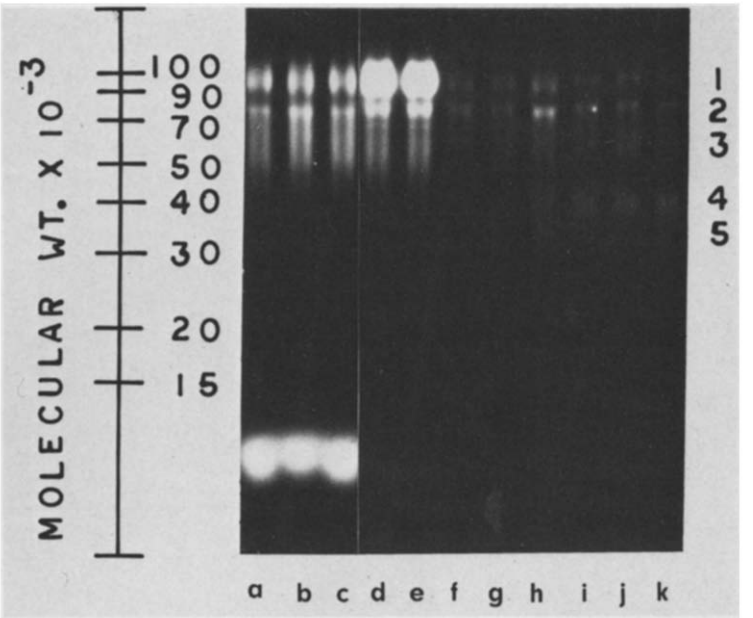


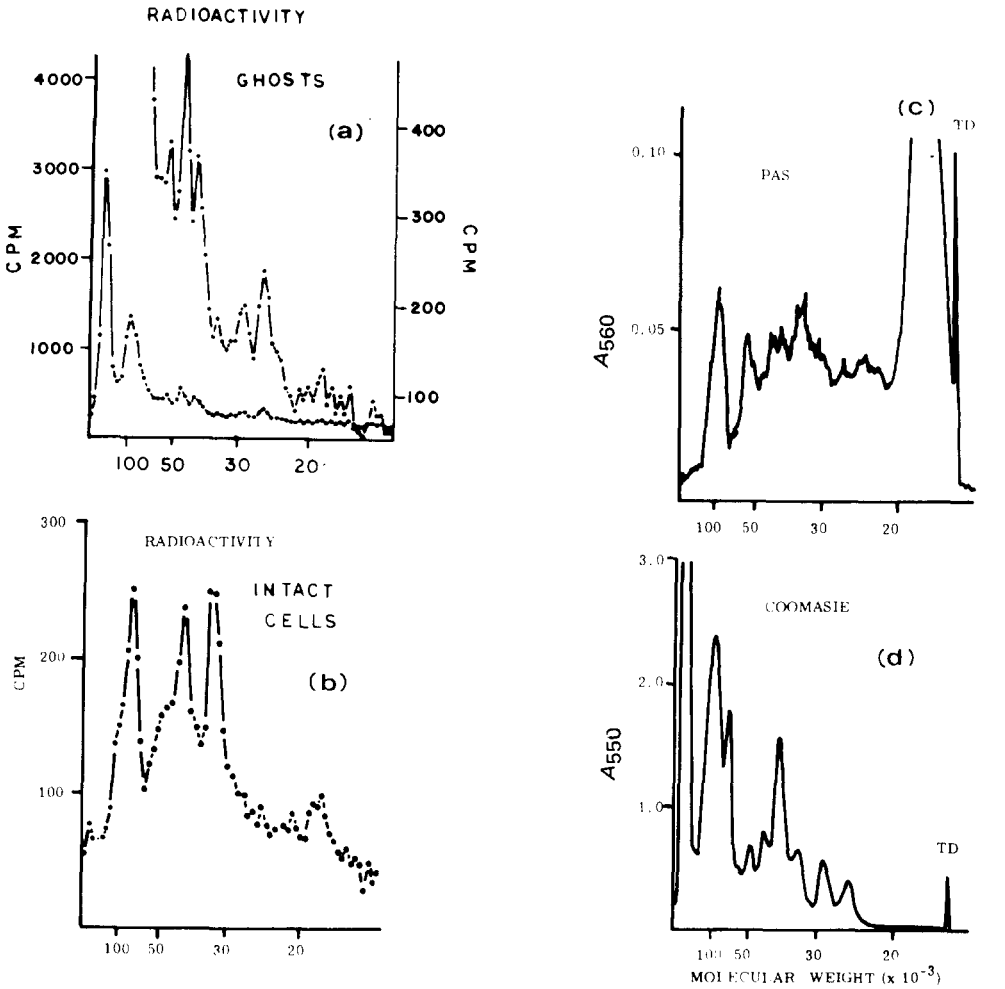
Fig. 4. The fluorogram of 8.4% acrylamide gel (phosphate buffer). The labeled erythrocyte membranes were the same as those in Fig. 2, except that SDS-polyacrylamide gel and buffer were different (Methods and Materials). The numbers on the right correspond to the five radioactive bands discussed in the text.

to PAS-2 band and PAS-3 band in Tris-buffered gel, respectively.

Three lines of evidence suggested that radioactive band 4 (Figs. 3 and 4) was not a results of proteolytic degradation from proteins of high molecular weight. The first evidence was the failure to detect any increase of radioactivity in region from 40 000 to 100 000 daltons when the membrane was heated at 100°C for 3 min immediately after it was dissolved in SDS-urea solution. The heating process was supposed to quench any possible proteolytic degradatin. The second evidence came from the fact that the mobilities of radioactive bands 1, 2 and 3 were not changed, when the membrane was heated prior to the SDS gel analysis (Figs. 3 and 4). Finally, radioactive band 4 was consistently observed in five different normal healthy individuals in different experiments. Thus, the radioactive band 4 appears to be a new surface protein labeled by the present metal-chelating probe. The labeling patterns of human erythrocyte by azo-phenyl-EDTA · [$^{111}In^{3+}$], by pyridoxal phosphate-NaB^3H_4 and by galactose oxidase-NaB^3H_4 were further compared in a phosphate-buffered slab gel (Fig. 4). Again, the most distinct difference between the metal-chelating probe and the other two surface probes was in the labeling of radioactive band 4.

Labeling of rabbit erythrocyte by azo-phenyl-EDTA · [$^{111}In^{3+}$]

When intact rabbit erythryocytes were labeled with the metal chelates at pH 9.8, three proteins of the cell membranes were found to carry the label from the SDS gel analysis (Fig. 5). These had apparent molecular weights of



96 000, 43 000 and 33 000 in 8.4% acrylamide gel (phosphate-buffered gel), and 96 000, 68 000, 45 000 in 4.2% acrylamide gel (phosphate-buffered gel). In contrast, when rabbit erythrocyte ghosts were treated with azo-phenyl-EDTA · [$^{111}In^{3+}$] at pH 9.8, virtually all proteins were labeled (Fig. 5). Unlike the human erythrocyte membrane, a much fainter periodic acid-Schiff's reagent staining pattern was seen corresponding to all the glycoproteins in rabbit erythrocyte membrane (Fig. 5c and g). A radioactive peak with 96 000 daltons corresponded well with a periodic acid-Schiff's reagent peak and a Coomassie Blue peak. The molecular weight of the other two radioactive peaks was found to depend on the acrylamide concentration. It has been shown that the migration rate of glycoproteins depends on the acrylamide concentration in the SDS gel [30,31]. Other labeling studies [1,32] have also shown the presence of glycoproteins on the outer surface of rabbit erythrocyte membranes. The present finding suggests that there are at least three proteins located on the outer cell surface of rabbit erythrocyte. They are probably glycoproteins in nature.

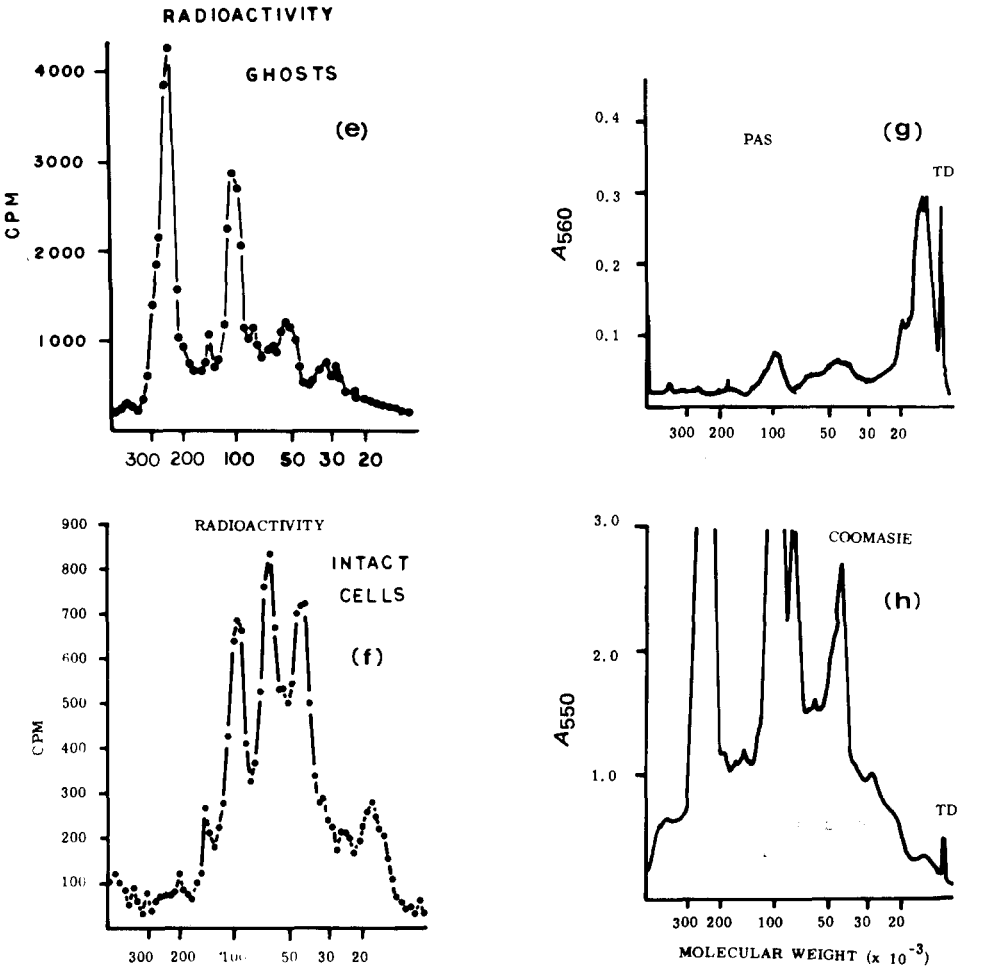


Fig. 5. The labeling patterns of intact rabbit erythrocytes and rabbit erythrocyte ghosts with azo-phenyl-EDTA · [$^{111}In^{3+}$]. 8.4% acrylamide disc gel (a—d) and 4.2% acrylamide disc gels (e—h) were used to assess the labeled rabbit erythrocyte ghosts (a, e) and intact cell membranes (b, f) for radioactivity, for carbohydrates by periodic acid-Schiff's reagent stain (c, g) (PAS) and for polypeptide by Coomassie Blue stain (d, h). (Details in Methods and Materials.)

Effect of labeling on rabbit platelets

The binding of the metal-chelating probe does not appear to induce any swelling of the platelets as indicated by the identical size distribution of labeled platelets and the unlabeled control (Fig. 6). Changes in platelet morphology with the binding of the probe were examined by electron microscopy. Fig. 7 shows electron micrographs of thin sections of the labeled platelets and the control. Organelles within the labeled platelets remained randomly distributed as in the normal control platelets. There were, however, some changes in the surface contours in the labeled platelets. The labeled platelets exhibited some activity of the oxygen uptake. The rate of the oxygen consumption of labeled platelets, however, was 62% of that of the control. The results of ADP-induced aggregation of the labeled platelets in platelet-poor plasma is shown in Fig. 8.

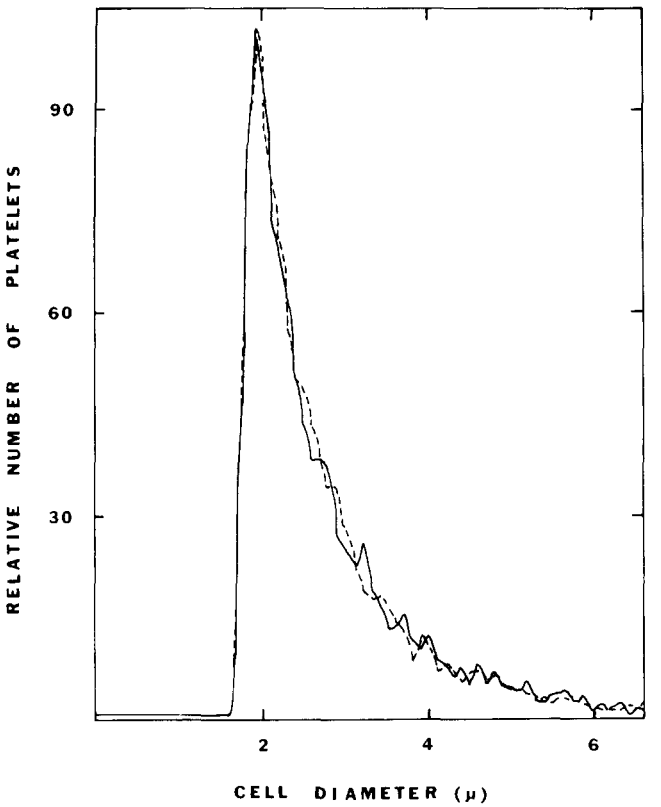


Fig. 6. Volume distribution curve of platelets. The solid line refers to unlabeled control, and the interrupted line to labeled platelets.

Both the rate and the extent of aggregation of the labeled platelets were identical to those of the control, indicating that the probe does not interfere with the normal function of platelet aggregation. Labeled platelets were transfused into two normal and two atherosclerotic rabbits to study the in vivo behavior of the labeled platelets. The distribution of the radioactivity of ^{111}In in some of the major organs is listed in Table II. Spleen appears to be the major site of the radioactivity of ^{111}In. This is consistent with the observation that spleen

TABLE II

DISTRIBUTION OF LABELED PLATELETS IN ATHEROSCLEROTIC AND NORMAL RABBITS

The numbers are expressed in terms of cpm per mg of tissue. The cpm has been corrected for the background and decay.

Organ	Atherosclerotic rabbit		Normal rabbit	
	No. 1	No. 2	No. 1	No. 2
Aorta	72	60	0	0
Kidney	2 368	2 484	1 940	2 108
Spleen	16 976	9 920	19 684	15 384
Liver	3 180	2 704	2 192	2 612

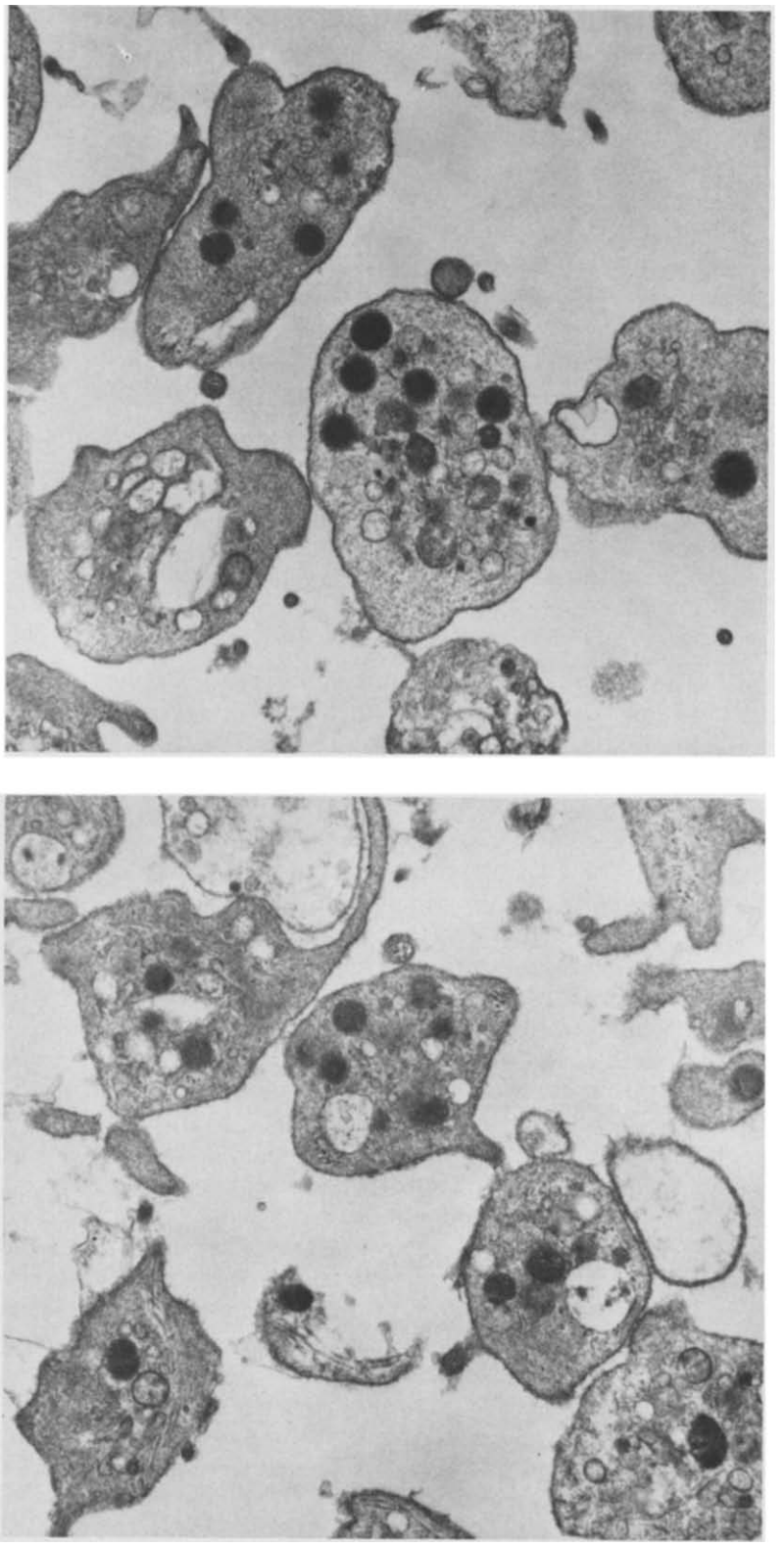


Fig. 7. Electron micrographs of platelets (×4750). (A) (top) Unlabeled control; (B) (bottom) labeled platelets.

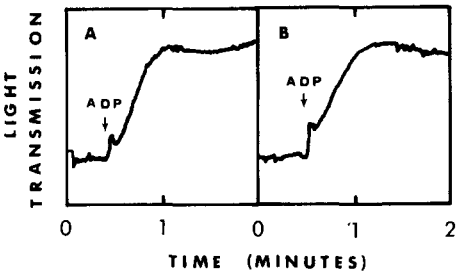


Fig. 8. ADP-induced aggregation curve of platelets. (A) Unlabeled control; (B) labeled platelets. The arrow indicates the addition of ADP.

is the major sequestering organ of platelets [34]. Furthermore, the aorta of the two atherosclerotic rabbits contained a low but significant amount of radioactivity of ^{111}In, whereas the aorta of the two normal rabbits contained virtually no radioactivity from ^{111}In. These results indicated that the labeled platelets can deposit on the preformed plaque in atherosclerotic lesions of the aorta.

Discussion

The data presented here indicate that azo-phenyl-EDTA · [$^{111}In^{3+}$] binds with high selectivity to the proteins on the outside of intact erythrocytes. When the metal chelate is reacted with ghosts, all the membrane proteins carry label (Fig. 8). The absence of radioactivity in spectrin (Figs. 2 and 4) and hemoglobin (Table I) from labeled intact erythrocytes demonstrates that the metal chelate satisfies the requirements of membrane impermeant probes. The negative charge in the EDTA group of the metal chelate may be responsible for membrane impermeability. It is conceivable that any di- or trivalent metal ions which can be chelated strongly by EDTA may be complexed to azo-phenyl-EDTA to form an impermeant probe.

The pattern of reaction of azo-phenyl-EDTA · [$^{111}In^{3+}$] with normal intact human erythrocyte membrane differs quantitatively and qualitatively from that of other impermeant probes. In addition to the known major proteins (protein band III, PAS-1, PAS-2, PAS-3), a protein (radioactive band 4 of Figs. 3 and 4) migrating slightly slower than PAS-3 in SDS gel was labeled heavily (Figs. 2, 3, and 4). The labeling of this polypeptide was not detected by other impermeant probes. In contrast to pyridoxal phosphate or galactose oxidase, protein band III was only labeled slightly by azo-phenyl EDTA · [$^{111}In^{2+}$]. The reactions of azo-phenyl-EDTA · [$^{111}In^{3+}$] can potentially react with tyrosine, histidine and amino groups, whereas the reaction of pyridoxal phosphate is highly specific with amino groups. At high pH, amino groups become very reactive to diazonium ions. The differences in labeling pattern between pyridoxal phosphate and azo-phenyl-EDTA is attributed to chemical specificity and the accessibility of the probes to proteins on the outer cell surface.

It is of interest that PAS-1 of intact human erythrocyte membrane is not labeled by diazo[^{35}S]sulfanilic acid [2] and ^{125}I-labeled 4,4′-diazonium-2,2′-disulfone stilbene [8], and is only labeled slightly by ^{125}I-labeled diazodiiodo-

sulfanilic acid [12]. In contrast, PAS-1 was labeled heavily by azo-phenyl-EDTA · [$^{111}In^{3+}$] (Figs. 3 and 4). These four impermeant probes all have a common reactive group, diazonium ion. Thus, accessibility factors related to steric hindrance and charge of probes and the conformation of proteins on the outer surface are responsible for the difference in labeling patterns.

Although sodium dodecyl sulfate gels have proven to be a valuable and simple way to analyze the molecular weights of many common proteins, the anomalous behavior of some glycoproteins in SDS gels have been demonstrated repeatedly. The aggregation/disaggregation of PAS-1 and PAS-2 in SDS can be modulated by the concentration of SDS, the use of Tris buffer, the protein concentration, and the temperature of incubation before electrophoresis [18]. The present study shows that there is a marked difference in the distribution in the periodic acid-Schiff's reagent stain and radioactivity, when the same membrane is analyzed in SDS gels with Tris buffer and phosphate buffer. It is clear that the comparison of labeling patter of human erythrocyte by various probes in SDS gels must be cautious, when the buffer systems are different.

The reagent used here has the ability to chelate a short half-life radioactive metal ($t_{1/2} \simeq 2.8$ days for ^{111}In) with an extremely high specific activity. Only a very small amount of the reagent is needed in a labeling experiment. A recent study on the loss in indium ions from human serum albumin which has been labeled by the probe has shown that less than 3% of the total ^{111}In is transferred to transferrin in definibrated human serum over a period of 168 h at 37°C [33]. The low concentration needed together with the slow exchange rate of $^{111}In^{3+}$ chelated by this reagent makes it ideal for labeling proteins or cells for studies related to their functions. Blood platelets are known to be extremely sensitive to in vitro manipulation. Swelling [35,36] and ultrastructural changes [37] can be caused by a number of agents or by changing the temperature. The present study has demonstrated that the binding of the probe to platelets does not interfere with the normal function of platelet aggregation, and that the labeled platelets can compete effectively with the large excess of host platelets in their accumulation atherosclerotic lesions. Furthermore, the strong binding constants of the EDTA group to many metals [38], for example, $K_d \simeq 10^{-25}$ for both In^{3+} and Fe^{3+}, allows the multiple labeling techniques. Moreover, the use of the metal chelate to preferentially attach heavy metals, Fe^{3+} or Hg^{2+}, for example, to one side of a membrane for the study of membrane structure by low-angle X-ray diffraction may be feasible.

It is also possible that azo-phenyl-EDTA can be converted to an analog which contains a primary amine as a reactive group. The labeling of the gamma probe to the outer surface of cells through membrane glycoproteins as described by Itaya et al. [14] will be possible. In the present study, the specific activity of incorporation of ^3H to membrane proteins and lipids on the outer surface of human erythrocyte by the galactose oxidase-NaB^3H_4 was about 150 times higher than that by pyridoxal phosphate-NaB^3H_4 (data not shown). This suggests that the carbohydrate moieties on the human erythrocyte membranes or perhaps other blood cells membrane are more readily available for labeling than polypeptides moieties on the outer cell surface. It is conceivable that the approach of labeling of a suitable γ-ray probe to carbohydrate moieties would turn out to be a useful way of making radiopharmaceuticals of blood

cells. Finally, azo-phenyl-EDTA can chelate metals with useful physical properties and can be used as a reporter group for studies of molecular motion by perturbed angular correlations of gamma radiation [39] and for measurement of distance by fluorescence spectroscopy [40].

Acknowledgements

One of us (K.J.H.) gratefully acknowledges the encouragement and support of Professor John D. Baldeschwieler. This research was supported by the National Institutes of Health Under Grant No. GM211111-03 and the National Science Foundation under Grant No. GP-38855x-3. This is Contribution No. 5300 from Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91125, U.S.A.

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