A general method for the selective retrieval of surface labeled plasma membrane components has been devised. The basis of the technique is the covalent attachment of compounds containing 2-iminobiotin, the cyclic guanidino analog of biotin, onto the cell surface proteins and the use of immobilized avidin to recover the labeled components uncontaminated by other cytosolic and membrane components. The pH-dependent interaction of 2-iminobiotin with avidin makes recovery possible. At high pH the free base form of 2-iminobiotin retains the high affinity specific binding to avidin characteristic of biotin, whereas at acidic pH values, the salt form of the analog interacts poorly with avidin.

Model studies on the interaction of 2-iminobiotinylated proteins with avidin-Sepharose 4B show that for tight binding to the affinity matrix, the pH of the column must be 9.5 or higher, that a single 2-iminobiotin group is sufficient for binding, and that proteins with different extents of labeling behave similarly when the low pH buffer is applied. When intact human erythrocytes were sequentially labeled with periodate and 2-iminobiotin hydrazide and the Triton X-100-solubilized plasma membrane proteins were subjected to affinity isolation, the major sialoglycoproteins, periodic acid-Schiff (PAS) 1, PAS 2, and PAS 3, plus two proteins with apparent molecular weights higher than band 3 were retrieved. The recovery of these proteins is not due to a nonspecific adsorption to the affinity matrix.

The realization that the plasma membrane is intimately involved in a variety of biological processes has led to an increasing awareness of the need for developing techniques for the study of composition, organization, expression, and metabolic fate of cell surface glycoproteins. Technically it is not easy to investigate these proteins since, in many cases, they are minor membrane constituents, making their detection and isolation difficult. The classical approach has been directed toward the initial isolation and purification of the plasma membrane prior to its chemical/biochemical dissection (1). Although this approach is adequate for the study of the erythrocyte plasma membrane, membrane fractionation of more complex eukaryotic cells has proved difficult. To avoid problems inherent to membrane fractionation, alternative techniques have been developed for the analysis of the surface components of cells. Some of the commonly used procedures for labeling and identifying external membrane proteins have included (a) chemical labeling (2, 3), (b) enzymatic labeling (3, 4), and (c) lectin-binding studies (5). Methods a and b are limited to analytical use. Lectins have been used to isolate membrane glycoproteins (6), but it is desirable to purify the plasma membrane prior to affinity isolation since intracellular membranes may also contain glycoconjugates.

An analytical and preparative approach which could provide information concerning the organization and function of surface membrane components has been developed. The method is based on the introduction of a nonmembrane ligand onto the plasma membrane of intact cells by a variety of enzymatic and chemical reactions. The "tagged" species are then isolated by virtue of their unique interaction between the covalently attached ligand and its binding protein. This report documents that 2-iminobiotin, the cyclic guanidino analog of biotin, retains the tight interaction with avidin at high pH (>9) but interacts weakly at low pH values (<6). 2-Iminobiotinylated proteins are selectively retained by an immobilized avidin column at pH 9 to 11 and are specifically eluted from the affinity matrix at pH 4 or by the addition of biotin. We have demonstrated the potential of this technique for the study of cell surface proteins by labeling and selectively retrieving the periodate-sensitive components from the surface of intact human erythrocyte.

**EXPERIMENTAL PROCEDURES**

**Materials**

Alkaline phosphatase (calf mucosal), d-biotin, benzamidine, cyanogen bromide, PMSF, p-nitrophosphosphate, and Sepharose 4B were obtained from the Sigma Chemical Company. Dicyclohexylcarbodiimide and N-hydroxysuccinimide were from Aldrich Chemical Company. Avidin was purified from egg white by affinity chromatography on 2-iminobiotin-6-aminoxyhexyl Sepharose 4B. Fetuin was from the Grand Island Biological Company, and insulin was a generous gift from the Lilly Research Laboratories, Indianapolis, IN. Lactoperoxidase and glucose oxidase were obtained from Boehringer Mannheim Biochemicals. d-[carboxyl-14C]Biotin (56 mCi/ mmol) and NaB[15]H, (900 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA. [a-1H]Acetazolamide (2.5 mCi/mmol) was a generous gift from Dr. Robert Rando, Harvard Medical School, Boston, MA. [3H]Insulin was a generous gift from Dr. Charles Rubin, Albert Einstein College of Medicine, Bronx, NY. d-(3,4-Diaminohydroxyphenyl-2-yl)pentanoic acid, the diamino derivative of biotin, was prepared by the alkaline hydrolysis of biotin with Ba(OH)2 at 150°C for 20 h (7). 2-Iminobiotin was synthesized by reaction of the diamino derivative with cyanogen bromide (8) and the free base converted to the hydrobromide by treatment with 1% HBr (v/v).

**Syntheses**

N-Hydroxysuccinimide Ester of 2-Iminobiotin Hydrobromide—To a solution of 2-iminobiotin hydrobromide (324 mg; 1 mmol) and 1 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; PAS, periodic acid-Schiff; NaBH4, sodium borohydride; PBS, phosphate-buffered saline (10 mm sodium phosphate, pH adjusted to 7.4 or 8.0 with NaOH, containing 140 mm NaCl and 3 mm KCl). 2 G. Heney and G. A. Orr, manuscript in preparation.
N-hydroxysuccinimide (115 mg; 1 mmol) in 3 ml of N,N-dimethylformamide (distilled \textit{in vacuo} before use) at 4°C was added dicyclohexylcarbodiimide (206 mg; 1 mmol). The reaction was stirred at 4°C for 1 h and then at room temperature for an additional 24 h. The dicyclohexylurea was removed by filtration and the organic solvent taken off in vacuo. The residue was recrystallized from hot propan-2-ol yielding the N-hydroxysuccinimide ester in 58% yield (m.p. 160-161°C).

**C$_{18}$H$_{12}$N$_4$O$_7$SBr**

Calculated: C 39.91, H 5.02, N 13.30, S 7.61 %

Found: C 40.00, H 5.20, N 13.20, S 7.34 %

The reaction proceeded equally well in dry dimethylsulfoxide.

2-Iminobiotin Methyl Ester Hydrochloride—2-Iminobiotin was converted to its methyl ester hydrochloride by treatment with methanolic HCl at 4°C for 15 h. Removal of the solvent gave the desired compound in quantitative yield (m.p. 156-157°C).

**C$_{18}$H$_{13}$N$_4$O$_7$S**

Calculated: C 44.97, H 6.86, N 13.30, S 7.11 %

Found: C 44.81, H 6.73, N 13.21, S 7.06 %

2-Iminobiotin Hydrazide Hydrochloride—2-Iminobiotin methyl ester hydrochloride (0.5 g) was dissolved in methanol (15 ml) and hydrazine hydrate (1 ml) was added. After 5 h at room temperature, the reaction mixture was taken to dryness in vacuo, washed several times with diethyl ether, and the residue recrystallized from propan-2-ol/diethyl ether to give the hydrazide in 55% yield (m.p. 209.5-210°C).

**C$_{18}$H$_{15}$N$_5$O$_7$S**

Calculated: C 39.91, H 5.02, N 13.30, S 7.61 %

Found: C 40.00, H 5.20, N 13.20, S 7.34 %

Phosphatase activity was monitored spectrophotometrically using p-nitrophenolphosphate (1 mM) in 50 mM Tris-HCl, pH 8.0, containing 1 mM MgCl$_2$ and 1 mM PMSF. The reaction was stopped by the addition of 10 ml of ice-cold PBS (pH 7.4), and the absorbance changes were corrected for dilution during the titration. The concentration of avidin was in the range of 0.2 mg/ml at pH 10.0 and 0.5 mM NaCl. Optimal conditions for Schiff's base formation were established with [a-$^{3}$H]acetylhydrazide (12). The oxidized erythrocytes (1 ml of packed cells) were incubated with an equal volume of 10 mM 2-iminobiotin hydrazide in the pH 0.5 buffer for 3 h at 30°C. The cells were washed three times with PBS, respectively. Ghosts from 0.5 ml of intact cells were reacted with 200 pCi of $^{3}$H-Bolton Hunter reagent in 0.25 ml of 50 mM sodium borate, pH 8.0, for 60 min at 4°C. The reaction was terminated by the addition of 200 mM glycine in 50 mM sodium borate, pH 8.0, for 15 min at 4°C.

**Affinity Chromatography—**Ghosts were solubilized in 2% Triton X-100, 50 mM sodium borate, pH 8.0, containing 2 mM benzonamide and 1 mM PMSF, for 30 min at 4°C. After centrifugation at 30,000 x $g$ for 15 min, the supernatant was adjusted to pH 10, and NaCl (1 M) was added. Affinity chromatography (3-m1 column) was carried out as described for the model system except that the salt concentration of the column equilibrating and washing buffers was increased to 1 M and that of the elution buffer reduced to 0.1 M. In some instances, specific elution was carried out with 50 mM Tris, pH 6.8, containing 1 mM benzonamide. All chromatographic buffers contained 0.5% Triton X-100. Radioactivity of the eluted fractions was determined in an Intertechnique CG 4000.

**Polyacrylamide Gel Electrophoresis—**Solubilized ghosts and specifically eluted proteins were subjected to slab gel electrophoresis according to the method of Laemmli (13). The stacking and separating gels contained 3.5 and 8.0% acrylamide, respectively. The gels were stained with Coomassie blue, dried, and visualized by autoradiography on Kodak SB-5 x-ray film.

**RESULTS**

**Syntheses**

Since 2-iminobiotin contains a potentially reactive guanidino nitrogen, all syntheses involving the modification of the side chain carboxyl group were carried out with the guanidino group protected as the hydrobromide or hydrochloride salt.

**Interaction of 2-Iminobiotin with Avidin**

The cyclic guanidino group of 2-iminobiotin has a $pK_a$ in the region of 11.5 to 12, and it has been suggested that only the base form has the high affinity binding to avidin characteristic of avidin (14). We have studied the interaction of 2-iminobiotin with avidin as a function of pH by a spectral titration method (10) (see under “Experimental Procedures”). At pH 10.0 the sharp equivalence point indicates that stoichiometric binding of 2-iminobiotin to avidin is occurring (Fig. 1). As the pH is lowered, greater curvature is observed, indicating that the interaction of the analog with avidin is becoming increasingly weaker. If only the ionization of the guanidino group affects binding, the titration would become pH independent as the pH was lowered. The large decrease in binding observed below pH 6 suggests that another ionization is affecting the ligand-protein interaction. Since 2-iminobiotin and its methyl ester show similar binding curves at pH 5 (data not shown), it appears that the reduction in binding efficiency of 2-iminobiotin to avidin at low pH is a combination of the ionizations of the guanidino group of the analog and an undetermined group on avidin. At all pH values tested, 2-iminobiotin was displaced from avidin by the addition of benzonamide. This is observed as an increase in the value of $\Delta A_{278}/A_{207}$ from 0.75 to 0.9 upon the addition of benzonamide.
Selective Retrieval of Labeled Plasma Membrane Components

Fig. 1. Spectrophotometric titration of avidin with 2-iminobiotin at various pH values. Upper: ○, 50 mM sodium carbonate, pH 10.0; △, 50 mM sodium borate, pH 8.3; ▲, 50 mM potassium phosphate, pH 6.9. Lower: ○, 50 mM ammonium acetate, pH 5.0; △, 50 mM glycine-HCl, pH 2.8. See under "Experimental Procedures" for details.

Model Affinity Isolation Studies

The interaction of 2-iminobiotinylated fetuin with immobilized avidin was investigated to obtain information concerning the effect of pH, detergent, and the extent of 2-iminobiotinylation on the affinity isolation of labeled membrane components. 2-Iminobiotinylated [3H]fetuin was completely retained by the avidin-Sepharose 4B column at pH 11 and was eluted as a sharp peak when the low pH buffer was applied (Fig. 2). Specific elution could be achieved at any pH values if biotin (1 mM) was included in the elution buffer (data not shown). In contrast, [3H]fetuin passed straight through the affinity matrix. In both cases, the total radioactivity recovered from the column was more than 90%.

(a) pH of Binding—For efficient affinity isolation of the 2-iminobiotinylated proteins, the pH of sample application and column equilibration buffers must be >9 (Fig. 3).

(b) Detergent—The effects of several detergents, all at a concentration of 0.1% (w/v), on the interaction of 2-iminobiotinyl fetuin with immobilized avidin have been studied. At this concentration, Triton X-100, Tween 80, Ammonyx-LO, and deoxycholate had no effect on the affinity isolation. Due to the precipitation of deoxycholic acid at pH 4, specific elution in the presence of deoxycholate was carried out with biotin at a pH of 7 or greater. Sodium dodecyl sulfate completely destroyed all binding whereas Lubrol PX resulted in only partial binding of the derivatized fetuin. Columns have been used several times in the presence of Triton X-100 and Ammonyx-LO without loss of activity.

(c) Extent of 2-Iminobiotinylation—Although most of the model studies have been carried out with labeled fetuin, similar results have also been obtained with an insulin derivative containing a single 2-iminobiotin group (data not shown). ^125^I-Insulin was reacted first with citraconic anhydride under conditions known to block only the two a-amino groups (15) and then with the N-hydroxysuccinimide ester of 2-iminobiotin to label the single a-amino group. Since this derivative

Fig. 2. The interaction of [3H]fetuin and 2-iminobiotinyl-[3H]fetuin with avidin-Sepharose 4B. Avidin-Sepharose 4B (9 ml of gel), prepared by the method of Bodanszky and Bodanszky (11), was equilibrated with 50 mM ammonium carbonate buffer, pH 11, containing 0.5 M NaCl. [3H]Fetuin or 2-iminobiotinyl-[3H]fetuin (5 to 10 X 10^6 cpm, 0.5 to 1.0 mg in 1 ml of equilibrating buffer) was applied to the affinity column, and the column was washed with pH 11 buffer (15 ml/h) until the radioactivity returned to background levels. Specifically retained proteins were eluted with 50 mM ammonium acetate, pH 4.0, containing 0.5 M NaCl. Fractions of 1.3 ml were collected, and the radioactivity in the eluted fractions was determined in Aquasol using an Intertechnique LS 4000 scintillation counter. Protein was measured at 280 nm. All experiments were carried out at 4°C.

Fig. 3. The interaction of 2-iminobiotinyl-[3H]fetuin with avidin-Sepharose 4B as a function of the pH of the sample application buffer (○, 50 mM potassium phosphate, pH 7.5; △, 50 mM ammonium carbonate, pH 9.5; all buffers contained 0.5 M NaCl). In all cases, specifically retained protein was eluted with 50 mM ammonium acetate, pH 4, containing 0.5 M NaCl.
Selective Retrieval of Labeled Plasma Membrane Components

Intact human erythrocytes were oxidized with 0.5 mM periodate at 4°C. This concentration of periodate represents approximately a 2-fold molar excess relative to the surface sialic acid content (18). The formation of Schiff’s bases between the newly formed surface aldehydic groups and an aldehyde hydrazide was studied as a function of pH using [α-3H]acetohydrazide (12). Bond formation was maximal between pH 6 and 6.5. A specific Mn2+-catalyzed enhancement of a similar reaction has been reported (19). No enhancement of Schiff’s base formation was seen in the presence of Mn2+ with acetohydrazide. Rather, the addition of 5 mM Mn2+ to the incubation mixture at these pH values (6 to 6.5) caused aggregation of the cells. No visible aggregation occurred when the divalent cation was omitted from the reaction. Maximal labeling of the oxidized cells at pH 6.5 could be achieved with 5 mM acetohydrazide after a 3-h incubation. These conditions were used to label the periodate-treated cells with 2-iminobiotin hydrazide.

Membrane polypeptides were iodinated in order to increase the sensitivity of the detection system. Intact cells and ghosts were iodinated with lactoperoxidase. Two major proteins, band 3 and PAS 1, are labeled in the intact cell. The same proteins plus an increasing number of internal proteins are labeled in membrane ghosts. Ghosts were also iodinated with 125I-Bolton Hunter reagent. This reagent causes the acylation of primary amino groups and, therefore, can be considered as a general protein detection method. The reaction results in a labeling pattern qualitatively similar to that obtained after staining with Coomassie blue. In agreement with other workers (20), PAS 1 is labeled poorly by this reagent.

2-Iminobiotinylated components were isolated from the Triton X-100 solubilized ghosts by affinity chromatography on immobilized avidin (Fig. 4). Specific elution was achieved either by the addition of 50 mM ammonium acetate, pH 4, or by 50 mM Tris-HCl, pH 6.8, containing 1 mM biotin. In all cases, greater than 90% of the applied radioactivity was re-

(d) Function—The potential of this technique would be greatly increased if membrane proteins could be retrieved without significant impairment of their function. For this reason we have investigated the effect of 2-iminobiotinylation and subsequent affinity chromatography on the activity of mammalian alkaline phosphatase. This enzyme is known to be membrane bound (17). The enzyme was derivatized with the N-hydroxysuccinimide ester of 2-iminobiotin at two different molar ratios of ester to protein, i.e. 15 and 90. The activities of the native phosphatase and the two specifically eluted derivatives were compared by a spectrophotometric assay. The specific activities were found to be within 10% of each other (native, 75.8 units/mg; 150 nmol of activated ester, 72.8 units/mg; 900 nmol of activated ester, 79.6 units/mg). It appears, therefore, in the case of mammalian alkaline phosphatase, that the selective retrieval procedure using the activated ester method of derivatization had little effect on enzyme function.

Selective Retrieval of Periodate-sensitive Glycoproteins on Human Erythrocyte Membrane

FIG. 4. Chromatography of erythrocyte membrane polypeptides on avidin-Sepharose 4B (B) and biotin-treated avidin-Sepharose 4B (A) after the sequential periodate oxidation and 2-iminobiotin hydrazide labeling of intact human erythrocytes. Upper, erythrocyte ghosts radioiodinated with 125I-labeled Bolton-Hunter reagent; middle, erythrocyte ghosts radioiodinated with lactoperoxidase/glucose oxidase; lower, intact erythrocytes radioiodinated with lactoperoxidase/glucose oxidase. See under “Experimental Procedures” for details.

FIG. 5. Sodium dodecyl sulfate polyacrylamide gel (8%) electrophoresis of the erythrocyte polypeptides specifically eluted from the avidin-Sepharose 4B column (see Fig. 4). A, intact cells radioiodinated with lactoperoxidase/glucose oxidase; B, erythrocyte ghosts radioiodinated with lactoperoxidase/glucose oxidase; C, erythrocyte ghosts radioiodinated with 125I-labeled Bolton-Hunter reagent. Native erythrocyte polypeptides stained with Coomassie blue or periodic acid-Schiff were used as marker proteins.
covered from the column. The applied samples and the specifically eluted components were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The autoradiograms of the specifically eluted fractions from the three iodination procedures are qualitatively similar (Fig. 5). As expected, the major retrievable proteins are the sialoglycoproteins, PAS 1, PAS 2, and PAS 3. In addition, two higher molecular weight proteins are also isolated with electrophoretic mobilities between bands 2 and 3. The selective retrieval of all five proteins is not the result of nonspecific adsorption to the affinity matrix. No difference in elution or electrophoretic profiles is found with the two specific elution regimens employed in this study. In addition, pretreatment of the column with biotin prevented any radioactivity from being bound (Fig. 4). The omission of either the periodate oxidation or the 2-iminobiotin hydrazide labeling step also resulted in no radioactivity being retained by the affinity column (data not shown).

**DISCUSSION**

In principle the technique of selective retrieval of labeled membrane components could be used with any external labeling reagent which has its own high affinity binding protein.

In this study we have exploited the affinity of avidin for the low molecular weight compound, biotin \( K_D \sim 10^{-15} \) m \( (21) \). The tightness of the complex provides not only one of its greatest and most unique advantages, but also its greatest limitation; dissociation of the complex is impossible under mild and nondenaturing conditions. The conditions necessary for even partial dissociation of the complex are rigorous, requiring a combination of low pH, 6 M guanidine-HCl, and elevated temperatures \( (21) \). It is apparent that if one wishes to incorporate this interaction as part of a selective retrieval system, ways must be found to disrupt the complex under mild and, if maintenance of protein function is important, non-denaturing conditions.

In this report, we have shown that replacing biotin with its cyclic guanidine analog, 2-iminobiotin, results in the formation of an avidin-analog complex which retains the low dissociation properties characteristic of the parent complex at high pH but dissociates rapidly when the pH is lowered to mildly acidic values. The reduction in affinity appears to be the result of the combined ionizations of the guanidine group of 2-iminobiotin and some group(s) on avidin. If a protein is acid sensitive, specific elution can also be achieved at higher pH values by the addition of biotin.

The use of the 2-iminobiotin-avidin complex as the basis of a selective retrieval system for labeled membrane proteins has been demonstrated here. Model studies, involving the interaction of 2-iminobiotinylated fetuin and insulin with immobilized avidin, have been used to investigate the effect of pH, temperature, extent of labeling, and detergent on the affinity isolation of 2-iminobiotinylated components. Studies with alkaline phosphatase indicated that 2-iminobiotinylation followed by affinity isolation had little effect on enzymatic activity. Furthermore, we have shown that after the sequential oxidation and 2-iminobiotin hydrazide labeling of intact human erythrocytes, the "tagged" components can be isolated uncontaminated by other membrane components. PAS 1, PAS 2, PAS 3, plus two components with apparent molecular weights between band 2 and band 3, have been isolated. Under our conditions little, if any, band 3, the major component in the human erythrocyte membrane, is isolated. This is in agreement with other workers \( (18, 22) \) who observed low extents of labeling of band 3 after periodate-\( \text{Na}[\text{H}] \) treatment. Our studies indicate that the adsorption and elution of all five proteins is not due to some nonspecific interaction with the matrix. There have been reports of proteins of higher apparent molecular weight than band 3 being exposed on the external surface of the human erythrocyte plasma membrane. A protein with an apparent molecular weight of 170,000 was labeled using the impermeant chemical probe, isothionyl acetic acid \( (23) \). A photoaffinity label with limited permeability, \( N-(4-\text{azido-2-nitrophenyl})-2\text{-aminoethyl sulfonate (NAP-taurine}) \) was used to demonstrate labeling of several bands \( (2.1, 2.2, 2.4, 2.5, 2.6) \) with electrophoretic mobility between bands 2 and 3 \( (24) \). At least 20 glycoproteins in the human erythrocyte membrane were labeled with \( \text{Na}[\text{H}] \), after oxidation of terminal galactosyl and \( N\)-acyethylgalactosaminyl residues with galactose oxidase \( (25) \). Six of these proteins had apparent molecular weight in excess of band 3.

Other selective retrieval methodologies have been developed. For example trinitrobenzene sulfonate \( (26, 27) \) and diiodofluorescein isothiocyanate \( (28) \) have been used to label cell surfaces followed by recovery of the labeled components, either by affinity chromatography \( (26, 27) \) or by immunoprecipitation \( (28) \), using antibodies directed against the respective small molecular weight chemical probe. The major disadvantage of these methods is that they are limited in the specificity of their labeling reaction. Both reactions are directed toward primary amino groups, and it would require difficult chemical syntheses to modify these hapten for attachment to other sites on the membrane.

A selective retrieval system based on the 2-iminobiotin-avidin interaction offers several advantages. Avidin can be readily isolated in large quantities \( (50 \text{ to } 100 \text{ mg scale}) \) by affinity chromatography on a 2-iminobiotin-6-aminohexyl Sepharose 4B column. In addition, avidin can be readily conjugated with electron dense, fluorescent and isotopic labels, and, importantly, biologically active avidin derivatives can be purified by affinity chromatography. A major advantage of this interaction is the versatility in the labeling reaction. By modification to the valeric acid side chain, 2-iminobiotin can be used in a variety of chemical and enzymic labeling reactions. Some of the methods that we have developed include the attachment of 2-iminobiotin to \( (a) \) Gal/GalNAc by galactose oxidase 2-iminobiotin hydrazide; \( (b) \) sialic acid by periodate/2-iminobiotin hydrazide; \( (c) \) glutamine by transglutaminase; \( (d) \) and primary amino groups by the \( N\)-hydroxysuccinimide ester of 2-iminobiotin.

In summary, I have demonstrated the use of the pH-dependent interaction between 2-iminobiotin and avidin as the basis for the selective retrieval of labeled external membrane proteins. A major advantage of this general procedure is that it will allow us to study the surface composition of intact cells without the prior isolation and purification of the plasma membrane. This selective retrieval system will also serve as a powerful first step in the purification of surface proteins for structural/functional studies and for the preparation of antibodies.

**REFERENCES**

Selective Retrieval of Labeled Plasma Membrane Components