A thiolation reagent for cell surface carbohydrate.

Yung Chuan. Wu

University of Windsor

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RÉCU

Ottawa, Canada
K1A 0N4
A THIOLATION REAGENT
FOR CELL SURFACE CARBOHYDRATE

BY
YUNG CHUAN WU

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario
1979
ABSTRACT

2-Acetamino-4-mercaptopbutyric acid hydrazide containing carbon-14 in the acetyl group has been prepared through hydrazinolysis of the synthetic intermediate $[^{14}C]$-N-acetyl-DL-homocysteine thiolactone. The compound has been characterized and evaluated as a hydrazone-forming reagent for sugar-derived aldehydes on the human erythrocyte membrane, where sodium periodate has been used for oxidation of carbohydrate moieties of the cell surface. Periodate-dependent incorporation was found to be stable to physiological conditions and to greatly exceed incorporation via thiol-disulfide interchange. Approximately 80% of the label incorporated into whole cell membranes could be precipitated by trichloroacetic acid from the solubilized membrane fraction. Molar levels of incorporation comparable to those of the periodate-borotritide technique were achieved. The bifunctional reagent is presented as a means of introducing thiol "handle" into the oligosaccharide segment of surface-exposed membrane glycoproteins.
ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. K. E. Taylor, for his patient direction and encouragement during the course of this work.

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To my colleagues who have rendered any assistance in this program I will also be indebted.

Also, I want to thank the members of my family who supported me with their encouragement, patience, and understanding.
To my dear parents
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ABBREVIATIONS

THAM tris (hydroxymethyl) aminomethane
EDTA ethylenediamine tetraacetate
TCA trichloroacetic acid
SDS sodium dodecyl sulfate
DOC sodium deoxycholate
TMS tetramethyilsilane
TSP sodium-3-trimethylsilylpropionate-2,2,3,3-d₄
tlc thin layer chromatography
EI electron impact
FI field ionization
homocysteine thiolactone hydrochloride  DL-homocysteine thiolactone hydrochloride
N-acetylhomocysteine thiolactone  N-acetyl-DL-homocysteine thiolactone
thiol hydrazide  2-acetamino-4-mercaptobutyric acid hydrazide
Rf relative mobility
E extinction coefficient
AgOAc silver acetate
HOAc acetic acid
AcCl acetyl chloride
\[ \text{Et}_3\text{N} \quad \text{triethyl amine} \]
\[ \text{EQ.} \quad \text{equivalent} \]
\[ \text{EtOAc} \quad \text{ethyl acetate} \]
\[ \text{MeOH} \quad \text{methanol} \]
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CHAPTER I

INTRODUCTION

The chemistry of the plasma membrane and the cell surface is a subject which has been of considerable interest to workers in many facets of biology for a number of years. This is understandable since a detailed knowledge of the biochemistry of this component is essential for explaining a wide variety of biological phenomena. In this respect the carbohydrate constituents of animal cell membranes are now considered to be especially important. Increasing evidence indicates that control of growth, behavior and proliferation of animal cells depends on the profile of surface glycoproteins and glycolipids (1-4). It is likely that the tumorigenicity of animal cells could be directly correlated with the organization and assemblage of glycoproteins and glycolipids in the membrane (5). Also, there is increasing evidence that cell surface glycoproteins are involved in a variety of surface-mediated processes such as ion transport (6,7), cell recognition (8), intercellular adhesion (9), and lectin agglutinability (10,11).

Thus, interest in these areas or in studying structure and function relationships of membrane proteins has led to
the introduction of several different types of cell surface specific labels. The labels can be classified into two categories: those effecting chemical modification of cell surface groups such as isethionyl acetimidate (IAI) (12), 4,4'-diisothiocyanostilbene disulfonate (DIDS) (13) for the reactive amino groups in the membrane, and those effecting enzymatic modification such as lactoperoxidase iodination (14) of tyrosine residues.

Most of the labelling reagents have been designed to be impermeant (e.g., diazobenzene sulfonate (DABS) (15), [35S]-N-formylmethionine sulfone methyl phosphate (35S-FMNMP) (16)) and hence to label exclusively the external surface of the plasma membrane. In some cases permeant reagents (e.g., acetic anhydride (17), ethyl acetimidate (IAI) (12), 1-fluoro-2,4-dinitro benzene (FDNB) (18)) have been used to probe a membrane function or to compare results with those obtained using impermeant reagents. Labelling reagents may also classified into ones that interact non-covalently with membrane constituents (e.g., para-chloromercuribenzenesulfonate (PCMB) (19)) and those that form covalent bonds (e.g., pyridoxal phosphate plus borohydride (20)).

Maddy was the first to outline several requirements for cell surface specific reagents (21). They should (a) be non-permeant and yet small, for accessibility; (b) react under physiological conditions of pH and temperature; (c) form a
stable covalent linkage with membrane constituents; and (d) be detectable in small amounts. An additional requirement, that introduction of the label should cause little or no perturbation of membrane structure or function, is relevant. It was found that certain criteria have been difficult to fulfil in practice (22).

The detection of label is dependent on the type of reagent used. Chromophoric and fluorescent groups are often employed for obvious reasons. Radioactive labels are quantitated by gamma counting, scintillation counting or autoradiography, depending on the isotope and system used.

As a result of the important role of glycoproteins in membranes, a number of procedures have recently been described for chemical and enzymatic modification of, and introduction of various labels into, glycoproteins of the cell surface. Sugar-binding proteins, termed lectins, which agglutinate cells through surface carbohydrate determinants, have been used extensively to investigate membrane structure and function and to isolate specific constituents (23,24). Also, membrane glycoproteins have been derivatized or labelled in the carbohydrate portion following generation of sugar aldehydes with galactose oxidase (25-27) or periodate (28,29). The former oxidizes the C6 hydroxyl groups of D-galactose and N-acetyl-D-galactosamine.
residues in cell surface glycoproteins and glycolipids. Several investigators have demonstrated labelling of the resultant aldehydic groups of human erythrocytes by reduction with tritiated sodium borohydride (Scheme 1). Liao, et al. (28) used sodium periodate under conditions which were suggested (29) to preferentially oxidize sialic acid, thus achieving selective modification of erythrocyte membrane sialoglycopeptides. Subsequent reduction with tritiated sodium borohydride resulted in labelling as depicted in Scheme 2.

Carbonyl groups reaction with primary amines to form Schiff's bases has been widely used in affinity chromatography (30), immobilized enzymes (31) and also applied to cell surface labelling, as exemplified by the following hydrazide labelling experiments.

Weber and Hof (32) and Eckhardt et al. (33) introduced dansyl hydrazine for condensation with aldehydic groups generated by periodate oxidation of protein-bound carbohydrate to form fluorescent hydrazones (Scheme 3). Heitzmann and Richards (34) analogously used biotin hydrazide to serve as a general reagent for detection of aldehydes. In that case the attached biotin was visualized (in the electron microscope) with ferritin-linked avidin (Scheme 4). Another cell surface labelling was presented by Itaya et al. (35). \(^{[35S]}\)-Methionine sulfone hydrazide was coupled to 6-aldehydosugars of galactose oxidase-
SCHEME 1. STRATEGY OF CELL SURFACE LABELLING WITH GALACTOSE OXIDASE / $^{3}_{\text{H}}$-BOROHYDRIDE
Scheme 2: Strategy for cell surface labelling with periodate / $[^3H]^{-}$ borohydride
SCHEME 3. INCORPORATION OF DANSYL HYDRAZINE INTO GLYCOCONJUGATE ALDEHYDES
SCHEME 4. INCORPORATION OF BIOTIN HYDRAZIDE INTO CELL SURFACE

SUGAR ALDEHYDES
treated erythrocytes (Scheme 5).

Based on the foregoing examples, the work of this thesis focuses on a bifunctional molecule (2-acetamino-4-mercaptobutyric acid hydrazide), as a reagent capable of hydrazone formation. It is different from the above labels in leading to introduction of a thiol "handle" into cell surface carbohydrate moieties. The thiol "handle" of the bifunctional reagent could subsequently react with some other reagents for purposes such as in situ cross-linking, cell immobilization or isolation of membrane constituents. The thiol hydrazide containing $^{14}$C in the acetyl group has been prepared and tested for labelling of periodate-generated aldehydes on the human erythrocyte membrane (Scheme 6).
SCHEME 5. INCORPORATION OF $[^{35}S]$-METHIONINE SULFONE HYDRAZIDE INTO CELL SURFACE SUGAR ALDEHYDES.
SCHEME 6. INCORPORATION OF $^{[14C]}$-2-ACETAMINO-4-MERCAPTOBUTYRIC ACID HYDRAZIDE INTO CELL SURFACE SUGAR ALDEHYDES
CHAPTER II

EXPERIMENTAL

A. General

The infrared (IR) spectrum was recorded on a Beckman IR-12 instrument. Proton nuclear magnetic resonance (NMR) spectra were obtained on a Jeolco C60 HL spectrometer or a Varian EM-360 spectrometer and reported in parts per million (δ). The following code was utilized: δ (multiplicity, number of protons, coupling constant). The mass spectrum (MS) was obtained on a Varian CH-5 DF mass spectrometer. The mass spectrum, EI, was obtained at an electron beam energy of 70 eV (electron volts). The source temperature of mass spectra (EI and FI) was 135°. The probe temperature of the EI spectrum was 195°, and that of the FI spectrum was 170°. The data was expressed in m/e (% relative abundance). All pH measurements were obtained on a Radiometer number 26 pH meter, equipped with a Radiometer semi-micro combination electrode number GK 2301-C. A Fisher-Johns melting point apparatus was employed to determine melting points. All aqueous solutions were prepared using distilled deionized water. All spectrophotometric measurements were performed on a Gilford Model 2000 absorbance recorder attached to a Beckman DU monochromator. Centrifugation was
performed on a Sorvall centrifuge RC-2B in rotor SS-34. Scintillation counting was performed on a Nuclear Chicago Unilux II scintillation counter. Counting efficiency was determined by the sample channels ratio method. A quench correction curve for $^{14}\text{C}$ (Appendix 1) was determined by linear regression analysis using quenched standards purchased from G. D. Searle (formerly Nuclear Chicago Amersham Searle). Attenuator and discriminator settings in each channel were determined as described in Nuclear Chicago Unilux II operating manual. Counting fluid was a mixture of Omnifluor (98% 2,5-diphenyloxazole (PPO), 2% p-bis-(o-methylstyryl)-benzene (bis MSB)) at a concentration of 4g/l in toluene.

B. Materials

Sodium phosphate monobasic and dibasic, phosphorus pentoxide, sodium acetate, silver nitrate, sodium sulfate, tris (hydroxymethyl) aminomethane (THAM), pyridine, ethylenediamine tetraacetate (EDTA), sodium periodate, sodium potassium tartrate, Folin-Ciocalteu phenol reagent, copper sulfate, trichloroacetic acid (TCA), hydrogen peroxide, and toluene (Scintanalyzed) were from Fisher Scientific Company (Fair Lawn, NJ). N-acetyl-DL-homocysteine thiolactone, DL-homocysteine thiolactone hydrochloride, iodoacetamide, sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC), bovine serum
albumin (BSA, fatty acid free), 2,2'-dithiobis-(5-nitropyridine) were from Sigma Chemical Company (St. Louis, MO). Triethylamine, sodium cyanoborohydride, acethyldrazide, Aldrithiol-2 (PyS-SPy), acrylamide, and N,N'-methylene-bisacrylamide (electrophoresis grade) were from Aldrich Chemical Company (Milwaukee, WI). Ethyl chloroformate was from BDH (Poole, England). Silica gel 60 (230-400 mesh) was from BDH (Toronto, Ont.). Hydrazine was from Eastman (Rochester, NY). Acetyl chloride was from Allied Chemicals Ltd. (Morristown, NJ). Omnifluor, Aquasol, Protosol, and $[1^{14}C]$-acetic acid (5.4 mCi/mmol) were from New England Nuclear (Boston, Mass). Deuterium oxide (D$_2$O) and sodium-3-tri-methylsilylpropionate-2,2,3,3-d$_4$ (TSP) were from Merck-Sharpe and Dohme of Canada Ltd. (Montreal, Que). Deuteriochloroform and tetramethyldisilane (TMS) were from Stohler Isotope Chemicals (Rutherford, NY, and Waltham, MA). Silica gel GF (2000 microns) preparative thin layer chromatography (tlc) plates were from Analtech Inc. (New Park, NY). Analytical silica gel tlc plates (0.25 mm thickness with fluorescent indicator) were from EM laboratory Inc. (Elmsford, NY) or from Brinkmann (Westbury, NY). Tlc solvent systems used were: A. ether: methanol, 7:3 (v/v) B. methylene chloride: acetone: formic acid, 100:25:0.5 (v/v/v). Visualization of chromatograms was achieved using a uv lamp or iodine vapor. The thiol present was checked by spraying with 1 mM 2,2'-dithiobis-(5-nitropyridine) in acetone. Human blood was obtained from Windsor Red Cross (not more than one week old).
C. Methods

**Synthesis of 2-Acetamin-4-mercaptobutyric Acid Hydrazide**

*(Thiol Hydrazide)*

To a solution of N-acetyllhomocysteine thiolactone (4.80g., 30 mmol) in methanol (12 ml) was added hydrazine (95%, 1.0g., 30 mmol), and the solution was stirred for two hours at room temperature. The reaction mixture was warmed and diluted with hot ethyl acetate (60 ml) and allowed to stand at 4°C. The resulting white crystals were filtered, washed with methanol:ethyl acetate (1:5, v/v) and dried in a dessicator overnight to give 4.9 g (85.4%) of white needles. The material was recrystallized from methanol:ethyl acetate (1:5, v/v) to give 3.4 g (59.2%) which was characterized by melting point, tlc (system A), nmr, ir, and ms.

**Synthesis of N-Acetyllhomocysteine Thiolactone with Silver Acetate**

To a solution of silver nitrate (11.2 g) in water (5 ml) was added saturated aqueous sodium acetate dropwise. The silver acetate was precipitated, filtered and dried under a vacuum. A solution of homocysteine thiolactone hydrochloride (1.536 g., 10 mmol) and silver acetate (1.669 g., 10 mmol) in acetic acid (100 ml) was stirred 30 minutes at room temperature. Silver chloride was filtered out and the filtrate was evaporated
to dryness, stirred with pyridine (18 ml), and treated with acetyl chloride (1.18 ml, 20 mmol), and stirred for 2 h. The mixture was mixed with 6 N HCl (50 ml) and extracted with three portions (10-15 ml) of methylene chloride. The combined methylene chloride extracts were dried over Na₂SO₄ and evaporated to give an oil which was characterized by tlc (system A).

**Synthesis of N-Acetylhomocysteine Thiolactone Using Acetyl Chloride**

To a suspension of homocysteine thiolactone hydrochloride (153.5 mg, 1 mmol) in chloroform (3 ml) was added triethylamine (202.4 mg, 2 mmol) at -20°C. To the solution was added acetyl chloride (78.5 mg, 1 mmol) in chloroform (2 ml). The mixture was allowed to warm to room temperature, stirred overnight, then mixed with 0.4 mM HCl (10 ml), and immediately extracted with three portions (10 ml) of methylene chloride. The combined extracts were dried over Na₂SO₄ and evaporated to give 102.1 mg (65%). The compound was recrystallized from toluene (2 ml) to give 47.8 mg (30.5%). The compound was characterized by tlc (system A), melting point, and nmr.

**Synthesis of N-Acetylhomocysteine Thiolactone via the Mixed Anhydride**

Acetic acid (60 mg, 1.0 mmol) and triethylamine (101.2 mg, 1 mmol) in chloroform (3 ml) were mixed and cooled
to 0°. To the solution, ethyl chloroformate (108.5 mg., 1 mmol) was added and stirred in the cold for 20 minutes. A cool solution of homocysteine thiolactone hydrochloride (145 mg., 0.94 mmol) and triethylamine (202.4 mg., 2 mmol) in chloroform (2.7 ml) was added. Stirring in the cold was continued for 2 h and the mixture was left overnight at room temperature. Solvent was evaporated, the product was dissolved in ethyl acetate (4 ml) and triethylamine hydrochloride was filtered off. Ethyl acetate was removed under a vacuum and the residue was recrystallized from toluene as above to give 77.2 mg (51%). The compound was checked by tlc (system A), nmr, and melting point. The compound was recrystallized again and melting point was checked.

Preparation of 2-Acetamino-4-mercaptobutyric Acid Hydrazide from Homocysteine Thiolactone Hydrochloride (One Pot Reaction)

The synthesis was formulated on a 0.94 mmol scale with respect to homocysteine thiolactone hydrochloride. The procedure was the same as above described except the acylation reaction time was cut down to 2 h, at which time N-acetylhomocysteine thiolactone was checked by tlc (system A) and the reaction mixture was directly treated with hydrazine (31.7 mg., 0.94 mmol) in the cold, and stirred for 2 h at room temperature. The solution was milky first, then turned to transparent, no
solid was precipitated. The product was checked by tlc (system A).

Two additional experiments were initiated as in the foregoing but they were subjected to ethyl acetate extraction. For one experiment, the ethyl acetate extract was treated directly with hydrazine (31.7 mg, 0.94 mmol) in the cold. For the other one, the ethyl acetate extract was evaporated, the residue was dissolved in methanol (0.36 ml) and hydrazine (30.4 mg, 0.91 mmol) was added. For both trials, the mixture was stirred for 2 h at room temperature. Neither reaction mixture showed any solid nor was a precipitate formed when the mixture in methanol was diluted with ethyl acetate (1.8 ml) and stirred. The product was checked by tlc (system A).

One further variation of this synthesis was attempted using equivalent molar amounts of starting materials through the ethyl acetate extraction. Volume of the extract was adjusted to 5 ml, the solution was treated with hydrazine (30.3 mg, 0.9 mmol) in the cold, and stirred for 2 h at room temperature. Solid formed and was filtered to give 83.5 mg (43.6%). The product was checked by tlc (system A), melting point.

N-Acetylhomocysteine Thiolactone Purification by Column Chromatography (conditions)

Authentic N-acetylhomocysteine thiolactone (130 mg, 0.82 mmol) was dissolved in ethyl acetate saturated with
triethylamine hydrochloride. Ethyl acetate was blown off under nitrogen, and chloroform (1 ml) was added, mixed well and the solution was applied to a column (1x10 cm) packed with silica gel 60 (3 g) in ether. The column was eluted with ether: methanol (7:3, v/v). Fractions of 1 ml were collected and checked for N-acetylhomocysteine thiolactone by tlc (system A). Fractions 5 to 10, containing N-acetylhomocysteine thiolactone, were combined and the solvent was blown off with nitrogen. The product was dried under a vacuum to give 119 mg (92%), the melting point of which was checked. Hydrazinolysis (25.3 mg., 0.75 mmol) of this material in methanol (0.3 ml) as above gave a crude yield of 112.8 mg (79%). The product was checked by tlc (system A) and melting point.

Preparation of 2-Acetamino-4-mercaptopbutyric Acid Hydrazide (with Purification of Intermediate N-Acetylhomocysteine Thiolactone)

The mixed anhydride procedure for N-acetylhomocysteine thiolactone was as described above through the ethyl acetate extraction step. The extract was dissolved in chloroform (1.2 ml) and chromatographed on silica gel 60 (3 g) as above. Fractions 6 to 11 (1.2 ml each), containing N-acetylhomocysteine thiolactone, were combined, solvent was blown off and the residue was dried in a vacuum to give 123.5 mg (84%). The melting point
was checked. The crude N-acetylhomocysteine thiolactone was dissolved in methanol (0.4 ml) and treated with hydrazine (25.2 mg., 0.75 mmol) in the cold. After stirring 2 h at room temperature, the solution was heated up and hot ethyl acetate (2.0 ml) was added. From the resultant two-phase system, the upper part was separated, cooled to -10°C and stored at -10°C for one day. Some solid formed (62.2 mg., 0.33 mmol) (42%), which was checked by tlc (system A) and melting point.

Synthesis of $^{[14}$C$]^{-2}$-Acetamino-4-mercaptobutyric Acid Hydrazide (Thiol Hydrazide)

Triethylamine (19.2 mg., 0.19 mmol) in chloroform (0.5 ml) was added to $[1^{-14}$C$]$-acetic acid (5.4 mCi/mmol., 0.19 mmol) at 0°C. The mixture was treated with ethyl chloroformate (20.6 mg., 0.19 mmol) and stirred in the cold for 20 minutes. The procedure was continued using equivalent amounts of reagents as described previously through the ethyl acetate extraction and column chromatography on silica gel (3 g). Fractions (1.2 ml) were monitored by tlc (system A) and by scintillation counting of 10 μl aliquots in scintillant cocktail (10 ml) (Omnifluor/toluene). Fractions 4 to 10 were combined, evaporated, dissolved in ethyl acetate (0.8 ml), and treated with excess hydrazine (10 μl) in the cold. The mixture was stirred 10-15 minutes in the cold, 2 h at room temperature and then evaporated under nitrogen and dried under a vacuum.
The product was stored in methanol (1 ml) at -20°. This solution was checked by tlc (systems A and B), scintillation counting and thiol titration.

**Thiol Titration on [14C]-Acetamino-4-mercaptopbutyric Acid Hydrazide (Thiol Hydrazide)**

Five microliters of synthetic [14C]-thiol hydrazide stock (1 ml in methanol) was diluted with methanol (195 μl). Ten microliters of the diluted solution were mixed with 0.5 mM Aldrithiol-2 (PyS-PF) (1 ml) in sodium phosphate buffer pH 8 in a cuvette (1.0 cm path, 1.0 ml volume). After 15 minutes at room temperature, the absorbance was read at wavelength 343 nm and concentration was calculated using $E_{343} = 8.08 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (36).

**Scintillation Counting for Different Spots on TLC Plates**

[14C]-thiol hydrazide (around 5/6 μl) was spotted on tlc plastic plate (0.25 mm thickness) along with authentic non-radioactive compound. The chromatogram was run in the solvent system B. Spots parallel to those shown by the uv lamp and/or detected by the thiol spray were cut into 1 cm² units. Each square was added to methanol (1 ml) in a counting vial for extraction, scintillation cocktail (10 ml) was added and vials were stored in the scintillation counter overnight.
Preparation of Erythrocyte Ghosts

The procedure here generally follows that of Fairbanks et al. (37). All this was done at 4°. Human blood was washed by suspension in phosphate buffered saline (PBS, 0.15M NaCl, 0.01M Na phosphate, pH 7.4) and centrifuged in a Sorvall centrifuge at 6,000 rpm for 10 seconds. The supernatnat and "buffy coat" were aspirated. The pellet was washed twice by resuspension and centrifugation in PBS. The resulting whole cells were lysed by suspension in 10 volumes of lysis buffer (0.1 mM EDTA made to pH 7.4 with THAM) and spun at 12,000 rpm for 20 minutes. The deep red supernatants were discarded, the tube was tilted and rotated to allow the loose ghost pellet to slide off the tightly packed button, which could be sucked up with little loss of ghosts. The pellets (ghosts) were resuspended in phosphate wash buffer (7-15 mM sodium phosphate, pH 7.4, 15 mM NaCl, 0.1 mM EDTA) and spun at 12,000 rpm for 10 minutes again, the supernatant was discarded. The washing was repeated until the pellet was white.

Surface Labelling of Whole Cells and Ghosts

The labelling procedure was similar to that of Heitzmann and Richards (34). Human erythrocytes from 1 ml of
blood were washed three times by suspension and centrifugation in PBS, then resuspended in PBS (1.25 ml). To the suspension was added 0.02 M sodium periodate (0.05 ml, 1 µmol) in PBS. After incubation at room temperature for 15 minutes, the mixture was diluted with PBS (5 ml), and centrifuged. The cells were washed twice in buffer, hemolysis was slight. The cells were resuspended in PBS (0.5 ml), and treated with a buffer solution (0.2 ml) containing non-radioactive thiol hydrazide (2 µmol), iodoacetamide (2.5 µmol) and 2.8 µCi (0.45 µmol) of $^{14}\text{C}$-thiol hydrazide. After a 2-hour incubation at room temperature, the mixture was diluted with PBS (5 ml), and centrifuged. The cells were washed twice in the same buffer and ghosts were prepared as described above. For ghost labelling, the cells were lysed and isolated first, then treated in PBS with sodium periodate and reacted with thiol hydrazide as for the whole cell experiments described above.

**Scintillation Counting on Ghosts**

Labelled ghosts (0.15 ml-0.20 ml) in a counting vial were mixed with Protosol (1.2 ml) and heated at 55°C until clear (around 2 h). To the solution was added 30% hydrogen peroxide (0.1 ml) to decolorize, and the sample was heated again for 30 minutes at 55°C, cooled, and diluted with scintillation cocktail (10 ml). Alternatively, the packed ghosts were solubilized with 2-3.2% SDS solution and made up to 0.5 ml
with buffer. Aliquots of solubilized ghosts were mixed with Protosol (1.0-1.2 ml) and then with cocktail as described.

Protein Determination on Ghosts

The procedure for the protein standard curve and sample determination was derived from Peterson's modification of the Lowry et al. assay (38,39). Bovine serum albumin (BSA, fatty acid free) (0.1 mg/ml) was used as the protein standard. The standard curve (Appendix 2) was obtained from least squares analysis on the log-log data for 10 different concentrations. Samples containing 5-100 μg of protein were brought up to a total volume 1 ml with distilled water. Reagent A (1.0 ml) was mixed, and the solution was allowed to stand for 10 minutes at room temperature. Then, reagent B (0.5 ml) was added, mixed immediately, and absorbance was read at 750 nm after 30 minutes. Reagent A was prepared from equal parts of stock CTC (0.1% copper sulfate, 0.2% sodium potassium tartrate, 10% sodium carbonate), 0.8 N NaOH, 10% SDS and water. Reagent B was prepared by mixing one volume of Folin-Ciocalteu phenol reagent with 5 volumes of water.

Protein Precipitation for Counting and Protein Determination

Protein precipitation is similar to Peterson's (38). A solubilized ghost sample (0.2 ml in 2% SDS) was mixed with
water (0.73 ml) and 0.15% DOC (0.1 ml), and allowed to stand for 10 minutes at room temperature. The mixture was treated with 72% TCA (0.17 ml, to give final concentration of 10% TCA), and centrifuged at 3,000 g for 15 minutes. The supernatants were removed by immediately decanting and turning the tube upside down over absorbant paper. The precipitate was saved for protein determination by the procedure described above. After determining protein concentration, 1 ml of the residue was mixed with 30% hydrogen peroxide (0.1 ml) in a counting vial. This mixture was incubated at 50°C until color disappeared, allowed to cool, diluted with Aquasol (10 ml) and counted.

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) of Membrane Samples**

Packed ghosts or TCA-precipitated membrane proteins (above) were taken up in 10% SDS solution to a final SDS concentration of 2%. These samples with diluted 1:1 with the sample buffer concentrate for the Laemml system (40) heated 3 minutes at 90°C and applied to tube gels (3% spacer gel, 8% separating gel) of the discontinuous system of the same author by K. E. Taylor. Staining and destaining with Coomassie blue followed the protocol of Fairbanks et al. (37).

For scintillation counting the gels were cut into 1-4 mm slices with each slice being digested by 30% hydrogen...
peroxide (0.1-0.2 ml) at 50° until clear (3-5 h). The resultant solutions were either (a) mixed with Protosol (1.0 ml) and then Omniflour/toluene cocktail (10 ml) or (b) mixed directly with Aquasol (15 ml).
CHAPTER III

RESULTS

Synthesis and Characterization of 2-Acetamino-4-mercaptobutyric Acid Hydrazide (Thiol Hydrazide)

Thiol hydrazide was synthesized by hydrazinolysis of N-acetylaminothiocyansilene thiolactone (Scheme 7). The reaction could be carried out in different solvents such as methanol, ethyl acetate or chloroform with comparable yields of pure compound. The characterization of thiol hydrazide is shown in Table 1. Since the compound contained a thiol group, it could be identified on the tlc plate by spraying with a thiol reagent (2,2'-dithiobis-(5-nitropyridine)) to show a yellow spot. The nmr, ir and ms spectra also gave proof of structure. The interpretation of ir spectrum is: asymmetric and symmetric N-H stretching respectively at 3280, 3065 cm\(^{-1}\), aliphatic C-H stretching at 2925 cm\(^{-1}\), two C=O stretching at 1655 cm\(^{-1}\), 1550 cm\(^{-1}\) respectively, broad N-H out of plane bend at 700 cm\(^{-1}\). The resonance at \(\delta 4.45\) ppm in the nmr represents the CH between NH and C=O. It was split into a triplet by an adjacent CH\(_2\). The triplet at \(\delta 2.6\) ppm which was split by an adjacent CH\(_2\) represents CH\(_2\) next to SH. The signal at \(\delta 2.06\) ppm represents two overlapping resonances of CH\(_3\) next to C=O and CH\(_2\) between
Scheme 7. Synthesis of thiol hydrazide
Table 1. Characterization of 2-Acetamino-4-mercaptobutyric Acid Hydrazide

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>76-85%</td>
</tr>
<tr>
<td>mp</td>
<td>131-132°C</td>
</tr>
<tr>
<td>tlc Mobility Rf</td>
<td>0.45(yellow) The Rf was not absolute, yellow spot was given by thiol reagent.</td>
</tr>
<tr>
<td>nmr</td>
<td>4.45(t,2,J=7) Deuterium oxide was employed as solvent. TSP was used as internal standard.</td>
</tr>
<tr>
<td></td>
<td>2.6 (t,1,J=7)</td>
</tr>
<tr>
<td></td>
<td>2.06(t,5)</td>
</tr>
<tr>
<td>ir</td>
<td>3280 cm⁻¹ Potassium bromide (KBr 1%) was used as the matrix.</td>
</tr>
<tr>
<td></td>
<td>3065 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td>2925 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td>1665 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td>1550 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td>700 cm⁻¹</td>
</tr>
<tr>
<td>ms(EI)</td>
<td>191(M⁺, 0.59) Data expressed in m/e (% relative abundance).</td>
</tr>
<tr>
<td></td>
<td>160(100)</td>
</tr>
<tr>
<td></td>
<td>131(25.0)</td>
</tr>
<tr>
<td></td>
<td>118(42.8)</td>
</tr>
<tr>
<td></td>
<td>90(82.3)</td>
</tr>
<tr>
<td>ms(FI)</td>
<td>382(2M,9.8)</td>
</tr>
<tr>
<td></td>
<td>192(M⁺1,21.5)</td>
</tr>
<tr>
<td></td>
<td>191(M⁺,100)</td>
</tr>
<tr>
<td></td>
<td>159(13.6)</td>
</tr>
</tbody>
</table>
CH and CH₂ with the central sharp singlet representing the CH₃ split by adjacent groups and appearing almost at the same position. The spectrum did not show NH and SH groups which were exchanged with solvent D₂O. Although the EI mass spectrum shows a trace of the molecular ions, the FI spectrum shows the molecular ions, 191(M, 100) as the predominant ions, which corresponds to the structure of the compound.

Synthesis of N-Acetylhomocysteine Thiolactone

A synthesis of the known compound, N-acetylhomocysteine thiolactone, was sought which could be carried out on a sub-millimole scale using procedures suitable for introduction of [¹⁴C]-acetate in some form. Scheme 8 shows the first of several pathways attempted on a 0.94 mmol scale. The method did not work well as judged by tlc which showed a yellow spot upon visualization with the thiol reagent. It was inferred that the ring structure had been opened to generate thiol under these conditions. This proposed reaction was similar to that of Benesch and Benesch (41) except that the thiolactone hydrochloride instead the corresponding hydroiodide was used, and acetyl chloride in pyridine was substituted for acetic anhydride in acetic acid.

Scheme 9 shows another attempt for this synthesis. As judged by the characterization shown in Table 2, the method
SCHEME 8. PROPOSED SYNTHESIS OF N-ACETYLP-DL-HOMOCYSTEINE THIOLACTONE USING SILVER ACETATE
SCHEME 9. PROPOSED SYNTHESIS OF N-ACETYL-DL-HOMOCYSTEINE THIOLACTONE USING ACETYLR CHLORIDE
Table 2. Characterization of N-Acetylhomocysteine Thiolactone

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Using Acetyl Chloride (Scheme 9)</th>
<th>From Mixed Anhydride (Scheme 10)</th>
<th>Authentic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>30%</td>
<td>46%</td>
<td>—</td>
</tr>
<tr>
<td>mp</td>
<td>108.0-108.5°C</td>
<td>109.0-112°C</td>
<td>109.5-111°C</td>
</tr>
<tr>
<td>Rf Ratio</td>
<td>0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(1.0)</td>
</tr>
<tr>
<td>&lt;sup&gt;b&lt;/sup&gt;nmr</td>
<td>6.33(s,1)</td>
<td>6.43(s,1)</td>
<td>6.28(s,1)</td>
</tr>
<tr>
<td></td>
<td>4.54(m,1)</td>
<td>4.52(m,1)</td>
<td>4.54(m,1)</td>
</tr>
<tr>
<td></td>
<td>2.9-3.3(m,4)</td>
<td>2.8-3.2(m,4)</td>
<td>2.8-3.3(m,4)</td>
</tr>
<tr>
<td></td>
<td>2.0(s,3)</td>
<td>2.0(s,3)</td>
<td>2.0(s,3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The ratio was obtained in comparison with authentic N-acetylhomocysteine thiolactone.

<sup>b</sup> Deuteriochloroform was employed as solvent using TMS as internal standard.

<sup>c</sup> Literature data from references (41,42).
seemed to work out. In this case as well, tlc showed a yellow spot with the thiol spray reagent before recrystallization, thus implying a degree of ring opening had occurred. However, pure material was obtained upon recrystallization but the low yield and the large volume of extraction solvent used especially for small scale were factors discouraging this pathway.

Scheme 10 shows a better way for making N-acetylhomo-
cysteine thiolactone through the mixed anhydride. The idea of the method is from Laliberte et al. (42)*. The results are shown in Table 2. Recrystallization of the compound from toluene had to be very slowly and carefully carried out in order to get pure crystals.

Preparation of Thiol Hydrazide without Intermediate
N-Acetylhomocysteine Thiolactone Purification

In the foregoing preparation of N-acetylhomocysteine thiolactone, almost half of the compound was lost in recrystallization, which was not good for synthesis on a still smaller scale, especially for radiochemical synthesis. Here we tried to do the hydrazinolysis directly in the N-acetylhomo-

*These authors actually made N-acetylhomocysteine thiolactone but not by the route adapted here; rather, the procedure used here is modelled after one they reported for other acylhomocysteine thiolactones.
SCHEME 10. SYNTHESIS OF N-ACETYL-DL-HOMOCYSTEINE THIODACTONE VIA THE MIXED ANHYDRIDE
cysteine thiolactone synthetic mixture in chloroform or with the crude N-acetylhomocysteine thiolactone which had been extracted from triethylamine hydrochloride with ethyl acetate. In the latter case, ring opening was attempted in ethyl acetate or methanol. In no case was solid formed despite evidence of product formation from tlc as shown in Appendix 3. In an attempt to find the factors interfering with solid formation, various additions were made to separate millimole-scale reactions starting from authentic N-acetylhomocysteine thiolactone. However, it was found that interference from triethylamine, acetic acid, or ethyl chloroformate was only observed when amounts of these in huge excess over those normally taken, were used. The possibility of cutting down the small excess of acetic acid and ethyl chloroformate used for the usual N-acetylhomocysteine thiolactone synthesis to an equivalent amount to the homocysteine thiolactone hydrochloride. In several trials, the yield was always low (crude yield 30-40%), the melting point was low (mp 116-122°C), and tlc showed the product along with extra spots of Rf 0.34 after mixed anhydride synthesis and of Rf 0.7 after hydrazinolysis. These were not seen when the small excess of mixed anhydride precursors were employed. This suggested that the acetylation reaction was not complete and homocysteine thiolactone was present.

Preparation of 2-Acetamino-4-mercaptobutyric Acid Hydrazide

with Intermediate N-Acetylhomocysteine Thiolactone Purification

The results of the foregoing demonstrated that it
was not possible to obtain pure thiol hydrazide from homocysteine thiolactone by avoiding purification of the intermediate thiolactone. Column chromatographic purification of that intermediate was next attempted after conditions of chromatography were determined for authentic N-acetylhomocysteine thiolactone. The recovery and purity were satisfactory for authentic compound, but not for the synthetic one (Table 3). For the latter, the tlc showed an extra spot above thiol hydrazide. In several attempts at this two-step scheme, the final product was variously an oil or a solid with the same tlc pattern. Preparative thin layer chromatography was attempted on the thiol hydrazide without improvement. In addition, a chloroform-methanol re-crystallization technique, in which sample was taken up in methanol and gradually transferred to chloroform with boiling, was found occasionally to furnish pure compound, but conditions seemed impossible to standardize and thus this approach was abandoned.

A model procedure was carried out as shown in Scheme 11 on a 0.2 mmol scale. The compound made in this way was not pure as tlc still showed a minor component faster moving than authentic thiol hydrazide. However, the nmr spectrum was virtually identical with that of authentic thiol hydrazide.
Table 3. Synthesis of Thiol Hydrazide through Intermediate N-Acetylhomocysteine Thiolactone Purified by Column Chromatography

<table>
<thead>
<tr>
<th>Intermediate N-Acetylhomocysteine Thiolactone</th>
<th>Recovery from Column</th>
<th>mp of the Recovered N-Acetylhomocysteine Thiolactone</th>
<th>Crude Yield of Subsequent Reaction for Thiol Hydrazide</th>
<th>mp of Crude Thiol Hydrazide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic</td>
<td>92%</td>
<td>109-112°C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79%</td>
<td>126.5-129.5°C&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Synthetic&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80%</td>
<td>100-105°C</td>
<td>46.5%</td>
<td>123-126°C&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a) The melting point from literature (41, 42) is 109.5-111°C.

b) The melting point of standard thiol hydrazide is 131-132°C (see earlier results).

c) Sometimes there was no solid forming.

d) Melting point was the same even after recrystallization.
SCHEME II. MODEL PROCEDURE FOR $[^{14}C]$-THIOL HYDRAZIDE SYNTHESIS
Synthesis and Characterization of $^{14}$C-2-Acetamino-4-mercaptobutyric Acid Hydrazide (Thiol Hydrazide)

$^{14}$C-Thiol hydrazide had been synthesized following the strategy of Scheme 11 and on almost the same scale as the synthesis described immediately above. The degree of acetylation with $^{1-14}$C-acetic acid and the thiol group content in the sample were determined by scintillation counting and by PyS-SPy thiol titration (36), respectively. The results are shown in Table 4. The purity of synthetic $^{14}$C-thiol hydrazide was examined by tlc (Appendix 4). One spot on the origin seemed clearer than that appearing from the standard thiol hydrazide in the same position. Comparison with the standard thiol hydrazide showed there was an extra yellow spot above thiol hydrazide. It was found (Appendix 4) that thiol hydrazide had the same mobility as acetyldrazide in the solvent system A. However, they could be differentiated in the solvent system B (Appendix 5). There it is apparent that the spot on the origin was neither hydrazine nor acetyldrazide. Determination of counts in each of the tlc zones indicated 290% of the counts associated with the desired thiol hydrazide (Appendix 5).

Cell Surface Labelling with $^{14}$C-Thiol Hydrazide

Oxidation with sodium periodate followed by reaction with $^{14}$C-thiol hydrazide should result in incorporation of
Table 4. Characterization of $[1^{14}C]$-2-Acetamino-4-mercaptopbutyric Acid Hydrazide (Thiol Hydrazide)

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[1^{14}C]$-Acetic Acid Used</td>
<td>0.185 mmol (1.0 mCi)</td>
</tr>
<tr>
<td>Labelled Acetyl Groups in Product by Scintillation Counting</td>
<td>0.104 mmol</td>
</tr>
<tr>
<td>Thiol Presenting in Sample from Thiol Titration</td>
<td>0.11 mmol</td>
</tr>
<tr>
<td>Yield</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Purity Checked by Tlc</td>
<td>≥90%</td>
</tr>
</tbody>
</table>
the label into cell surface sugars of the erythrocyte. The results given in Table 5 represent a set of experiments designed to characterize the labelling. Incorporation of thiol hydrazide occurred to the same extent in whole cells or in ghosts (compare experiments 1 and 3). In most experiments, equimolar iodoacetamide was used for blocking thiol groups on the reagent, thus preventing reagent incorporation via thiol-disulfide interchange (Scheme 12). The latter was checked in experiment 8 where the possibility for hydrazide formation was blocked, by omitting periodate oxidation, while thiol-disulfide interchange was encouraged, by omitting iodoacetamide in the second stage (Scheme 13). Increasing the amount of labelled reagent had no dramatic effect on the molar incorporation of label as shown by experiments 5 and 7. The results of experiment 3 can not be used to extend the range of reagent concentrations compared because a protein determination was not done on that membrane sample. It was of interest to check for hydrazide-hydrazide exchange reactions as a possible complicating factor. To this end a ghost sample was treated with $^{14}$C-thiol hydrazide for two hours then followed by adding a four-fold amount of non-radioactive thiol hydrazide for another two hours' reaction. It was found that the radioactivity incorporated did not change much (experiment 6) compared with that of an identical ghost sample treated only with $^{14}$C-thiol hydrazide (experiment 5).
Table 5. Red Cell Labelling with Thiol Hydrazide after Periodate Oxidation

<table>
<thead>
<tr>
<th>Ex. Cell Form</th>
<th>Periodate ( \mu \text{mol} )</th>
<th>Thiol Hydrazide ( \mu \text{mol} (\text{Ci/mol}) )</th>
<th>Iodoacetamide ( \mu \text{mol} )</th>
<th>Membrane Radioactivity ( \text{dpm} )</th>
<th>Reagent Incorporated ( \text{nmol} (\text{nmol/mg}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 whole</td>
<td>1</td>
<td>2.5(1.1)</td>
<td>2.5</td>
<td>27,000</td>
<td>10.9 e</td>
</tr>
<tr>
<td>2 whole</td>
<td>0</td>
<td>2.5(1.1)</td>
<td>2.5</td>
<td>744</td>
<td>0.3 e</td>
</tr>
<tr>
<td>3 ghost</td>
<td>1</td>
<td>2.5(1.1)</td>
<td>2.5</td>
<td>27,600</td>
<td>11.1 e</td>
</tr>
<tr>
<td>4 ghost</td>
<td>0</td>
<td>2.5(1.1)</td>
<td>2.5</td>
<td>754</td>
<td>0.3 e</td>
</tr>
<tr>
<td>5 ghost</td>
<td>1</td>
<td>0.5(5.4)</td>
<td>0.5</td>
<td>232,000</td>
<td>19.4(36.3)</td>
</tr>
<tr>
<td>6 ghost</td>
<td>1</td>
<td>0.5(5.4) then 2.0(0)</td>
<td>0.5</td>
<td>205,000</td>
<td>17.3(29.0)</td>
</tr>
<tr>
<td>7 ghost</td>
<td>1</td>
<td>1.0(5.4)</td>
<td>1.0</td>
<td>331,000</td>
<td>27.6(41.6)</td>
</tr>
<tr>
<td>8 ghost</td>
<td>0</td>
<td>1.0(5.4)</td>
<td>0</td>
<td>42,900</td>
<td>3.6(4.7)</td>
</tr>
</tbody>
</table>

a) All experiments based on cells or membranes from 1 ml blood (0.55-0.65 mg protein). In all experiments, final washed ghost sample solubilized directly in Protosol or after SDS treatment and counted in Omnifluor/toluene.

b) 15 minutes, 23° C
Table 5. (continued)
c) 2 hours, 23°C.
d) When present, premixed with thiol hydrazide.
e) Protein determination was not done in experiments 1 to 4.
SCHEME 12. IODOACETAMIDE AS A THIOL BLOCKING REAGENT
SCHEME 13. THIOL-DISULFIDE INTERCHANGE ON MEMBRANE PROTEINS

(a) No iodoacetamide
    Present
(b) No Periodate
    Oxidation
This indicated that hydrazide-hydrazone interchange was not important under the conditions employed.

**Label Incorporated into Sialoglycoproteins of Erythrocytes**

Intact whole cells or ghosts were oxidized with sodium periodate and coupled with $^{14}$C-thiol hydrazide to form hydrazone. These then were reduced with sodium cyanoborohydride (43) to form stable hydrazine derivatives (Scheme 14).

The label incorporated into membrane was determined, then protein precipitation as described by Peterson (38) was carried out to determine the label incorporated into sialoglycoproteins of the membrane. The results are shown in Table 6. It may be inferred that 89% of $^{14}$C label from the whole cell experiment was on the sialoglycoprotein while 58% was on the same component from the ghosts. This suggested that the label on sialoglycoproteins was less extensive due, most probably, to the partial incorporation of label into membrane lipids.

**SDS-Polyacrylamide Gel Electrophoresis of Labelled Membrane Proteins**

Membrane samples which had been treated with sodium cyanoborohydride from whole cell or ghost experiments and with or without TCA precipitation as in the foregoing were
SCHEME 14. REDUCTION OF HYDRAZONE WITH CYANOBOROHYDRIDE
Table 6. Comparison of Labelling in Cell Membranes and Sialoglycoproteins of Erythrocytes

<table>
<thead>
<tr>
<th>Cell Form</th>
<th>Periodate μmol</th>
<th>Thiol Hydrazide μmol (Ci/mol)</th>
<th>Membrane Radioactivity dpm</th>
<th>Reagent Incorporated in Membranes nmol (nmol/mg)</th>
<th>Reagent Incorporated in Protein ppt. nmol/mg</th>
<th>% Radioactivity in Membrane Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell (from 3 ml of blood)</td>
<td>3</td>
<td>3.0 (5.4)</td>
<td>632300</td>
<td>52.7 (22.7)</td>
<td>18.9</td>
<td>83 %</td>
</tr>
<tr>
<td>Ghost (from 1.5 ml of blood)</td>
<td>1.4</td>
<td>1.0 (5.4)</td>
<td>500159</td>
<td>41.7 (46.4)</td>
<td>26.9</td>
<td>58 %</td>
</tr>
</tbody>
</table>

a) Radioactivity determinations and protein precipitation for both experiments were carried out starting with washed ghosts. Thus for the whole cell experiments, the cells were converted to washed ghosts after oxidation and labelling.

b) Thiol hydrazide samples were pre-mixed with equimolar amounts of iodoacetamide.
subjected to SDS-polyacrylamide gel electrophoresis under conditions often used for membrane proteins (40). With loads per gel of 35,000-60,000 dpm (100-150 µg of protein) and Coomassie blue staining, the standard pattern of erythrocyte membrane bands was seen for the whole cell experiments. The ghost experiment samples showed some higher molecular weight aggregates were present. Indeed, some of these barely entered the spacer or "stacking" gel. The latter observations confirm those of Gahmberg et al. (44) who have suggested that periodate oxidation of ghosts leads to spectrin-containing aggregates. However, when the gels were sliced, digested in hydrogen peroxide and counted, in no case was the total recovery of radioactivity greater than 8% of the applied and in all cases there were no discernible differences in the distribution of radioactivity.
CHAPTER IV

DISCUSSION

Several hydrazide derivatives mentioned previously have been used for labelling glycoproteins in membranes. In this work an attempt has been made to utilize this hydrazide-hydrazone chemistry to introduce a second reactive group, the thiol, for subsequent derivatization (Scheme 7):

Thiol hydrazide of high purity has been prepared in high yield by hydrazinolysis of N-acetylhomocysteine thiolactone. Nmr, ir, tlc and ms data are in accord with the structure assigned (Table 1). It was also necessary to develop a small scale synthesis of N-acetylhomocysteine thiolactone which would be amenable to introduction of a $[^{14}\text{C}]$-acetyl group onto the skeleton.

The literature presents two methods for the synthesis of N-acetylhomocysteine thiolactone. Benesch and Benesch (41) have shown that treatment of homocysteine thiolactone hydroiodide with silver acetate followed by reaction with acetic anhydride in acetic acid gave the desired product. The second method given by Laliberte et al. (42) involves sodium acetate and acetic anhydride reacting with homocysteine thiolactone hydrochloride in dioxane. One
drawback of both of the methods is the undesirable loss of half amount of acetyl group during reaction using acetic anhydride as a reagent. From consideration of the expense of carrying out such a synthesis with a $^{14}$C-acetyl group source, neither of these two methods was chosen.

We have investigated several reactions for synthesis of N-acetylhomocysteine thiolactone starting with homocysteine thiolactone hydrochloride. Two worked out, but the rest did not, possibly because of the intermediate free base's participation in other ring opening reactions. One of the two successful reactions, that using acetyl chloride, was abandoned because of the low yield, the high volatility of acetyl chloride and large amount of extraction solvent that could not be satisfactorily extended to the sub-millimole scale synthesis intended (Scheme 9). The procedure chosen was adapted from Laliberte et al. (42) by substituting the corresponding acylation reagent where they had used an aminoacylation reagent. Coupling of homocysteine thiolactone with acetic acid by the mixed anhydride method proceeded smoothly without affecting the thiolactone structure (Scheme 10). The yield was moderate (Table 2). Due to the scale of the intended radiochemical synthesis, recrystallization of the N-acetylhomocysteine in toluene was not practical.
Based on the above considerations, initial attempts aimed at synthesizing the thiol hydrazide without purification of intermediate N-acetylaminothiocyasteine thiolactone. These attempts were all unsuccessful, although the desired product apparently was present in the mixtures obtained (Appendix 3). Column chromatographic purification of the intermediate N-acetylaminothiocyasteine thiolactone was attempted next. Conditions for silica gel chromatography were determined using doped (with triethylammonium hydrochloride) authentic N-acetylaminothiocyasteine thiolactone. In addition, some of the "purified" acetyl thiolactone was converted to thiol hydrazide of high purity in good yield (Table 3). When a synthetic sample of N-acetylaminothiocyasteine thiolactone was similarly treated, material after chromatography had a slightly depressed melting point and, upon hydrazinolysis, thiol hydrazide was shown to be the major product (by tlc) but there were traces of other compounds present. Nmr spectra of the product(s) at this stage were almost indistinguishable from those of authentic material.

Despite the uncertainties it seemed that the material in hand was of fairly high purity. This and considerations of the availability of starting materials and the cost of necessary materials lead to selection of this way (Scheme 11) for synthesis of [14C]-thiol hydrazide.
Carbon-14 thiol hydrazide was synthesized following the strategy shown in Scheme 11. Tlc showed the compound was >90% pure by scintillation counting. The yield was about 50%. The amount of $^{14}C$ calculated from scintillation counting was almost the same as the thiol content determined by spectrophotometric titration (Table 4). The latter correspondence shows that the degree of disulfide bond formation was slight in the freshly synthesized compound.

A number of chemical reagents have been used and described for modification of and introduction of various labels into cell surfaces (22). Most of the reagents, as electrophiles, show reactivity toward one or more functional side chains of protein components and suffer from lack of selectivity for a particular group of proteins and, thus, in most instances, use of these does not allow one easily to demonstrate that modification is specific for the functional groups of a particular proteins.

In this study, we introduce a label with a selectivity for membrane proteins containing oxidized sialyl residues. The major sialoglycoprotein of the human erythrocyte has been the most extensively studied and is known to have the sugar containing portion located externally (45). Mild peridate oxidation of several proteins results in selective modification of their sialyl residues and leads to the formation of $\alpha$- or
8-carbon aldehydic derivatives (46). Oxidation of sialyl residues of the isolated sialoglycoproteins requires only two equivalents of sodium periodate (28) per sialyl residue. Thus periodate oxidation following these conditions was used in the present study for generation of the cell surface aldehydes with which the hydrazide under study could form hydrazones.

Selectivity of the various techniques for cell surface carbohydrate has been studied. For example, Gahmberg and Hakomori (26) and Steck and Dawson (25) used galactose oxidase for oxidation followed by $[^3\text{H}]$-borohydride reduction. Gahmberg and Hakomori found 30% non-specific labelling of ghosts in absence of galactose oxidase and 14% with intact erythrocytes, while the data of Steck and Dawson (25) indicated 6-10% non-specific labelling of ghosts. In contrast, we found only 2.7% non-specific labelling of ghosts or whole cells by $[^{14}\text{C}]$-thiol hydrazide in the absence of sodium periodate (from Table 5).

There is no apparent difference in whole cell versus ghost labelling in the present study (Table 5) although strict comparison can not be made as protein determination was not carried out on those samples. This result is consistent with localization of sugar residues on the external surface. In apparent contrast, labelling with $[^3\text{H}]$-NaBH$_4$ following galactose oxidase oxidation by Gahmberg (27) showed a two-fold higher
level of radioactivity in ghosts as opposed to whole cells. However, it is not clear from that paper that excess reducing agent was present.

The importance of two different exchange reactions as possible complicating factors in use of the thiol hydrazide was studied. Hydrazide-hydrazone exchange was found to occur only to a small extent under the experimental conditions (Table 5). From this result, it might be anticipated that the hydrazones formed would be reasonably stable with respect to hydrolysis. Thiol-disulfide interchange would be another pathway for incorporation of thiol hydrazide, even in cells not pre-treated with periodate. In such an experiment (Table 5), the level of incorporation was about 11% of that for hydrazone formation. Thus it may be tentatively concluded that the thiol "handle" of the reagent could be left unblocked during hydrazone formation with sugar aldehydes and thus be available for subsequent manipulations.

It was of interest to determine the proportion of the radioactivity incorporated into membrane protein. From trichloroacetic acid precipitation, the radioactivity incorporated into sialoglycoproteins of membrane was 83% of the total membrane-bound label in whole cells and 58% in ghosts (Table 6). Liao et al. (28) reported 55-73% of the total radioactivity was in the sialoglycoprotein of periodate/boroetriiide-
modified membranes and they showed that some membrane lipids had also incorporated label. With the galactose oxidase-tritiated borohydride labelling technique, Steck and Dawson (25) found 15% of the total membrane-bound label to be in lipids of ghosts while Gahmberg and Hakomori (26) reported 40%.

Attempts to localize the labelled proteins by SDS-polyacrylamide gel electrophoresis have so far been unsuccessful. Radioactivity was low on each sliced gel after electrophoresis even when samples had been pre-treated with cyanoborohydride. It is thus inferred that the majority of counts were lost through the staining and destaining procedure. Liao et al. (28) mentioned that modified sialic acid was labile in the acetic acid solutions used for staining and destaining. Fairbanks et al. (27) also demonstrated that approximately 20% of the sialic acid was lost during gel fixing, staining and destaining. Weber and Hof (32) also reported that mild acid hydrolysis (0.2 N trifluoroacetic acid, 100°C) for 10 and 20 minutes removed 52% and 83%, respectively, of the label from a soluble sialoglycoprotein.

The Coomassie blue staining pattern on gels for the labelled membrane samples, however, were instructive in that they showed a different distribution of bands depending on whether whole cells or washed ghosts had been subjected to the periodate/thiol hydrazide procedure. Samples from labelled
whole cells showed the standard membrane protein pattern (37) whereas those from labelled ghosts showed a shift of staining intensity from the spectrin bands (bands 1 and 2 (37)) to higher molecular weight material. In fact, some of the stain intensity had barely entered the Laemmli system (40) spacer gel and, therefore, must have represented material of molecular weight in the millions of Daltons. This phenomenon was observed by Gahmberg et al. (44) in studies of periodate treatment of ghosts and attributed to cross-linking of spectrin chains (44). The spectrin polypeptides are known to be localized on the cytoplasmic face of the erythrocyte membrane (47) and have been the subject of other cross-linking studies (48).

In our study, incorporation of label was 35-40 nmol/mg of membrane protein while the data of Liao et al. (28) has been used to calculate (see Appendix 6) that 60-66 nmol/mg was incorporated using periodate/tritium borohydride. Use of $^3\text{H}$-borohydride for labelling the cell surface can cause problems. It can reduce not only C$_7$ aldehyde of oxidized sialic acid to alcohol, but also endogenous aldehydes and Schiff's bases (49). Non-specific label with $^3\text{H}$-borohydride alone is quite high, although use of unlabelled borohydride prior to oxidation could reduce this background. Borohydride can also promote $\beta$-elimination of seryl or threonyl linked oligosaccharides (50) and thereby change the nature of the glycoprotein being studied.
With the $^{14}\text{C}$-thiol hydrazide some of these problems would not be encountered and satisfactory labelling of the cell surface at physiological pH could be achieved. Incorporation of the thiol hydrazide into the cell surface also serves to introduce a SH group "handle" into glycoproteins thus providing a means for effecting further reactions at the cell surface.

The labelled reagent reported here contained carbon-14 although the synthesis described could, without modification, be used for incorporation of $^{3}\text{H}$ or $^{13}\text{C}$-acetyl groups and for a synthesis starting from the commercially available $^{35}\text{S}$-homocysteine thiolactone hydrochloride. The application studied here has been the derivatization of membrane sugar aldehydes but it is suggested that the reagent could also be useful in labelling, cross-linking, and/or immobilizing-soluble glycoproteins and other supramolecular aggregates in which glycoproteins are involved. In the reverse sense, the reagent might equally serve to derivatize non-glycosylated proteins for subsequent attachment to synthetic aldehyde-containing matrices.
Appendix 1. $^{14}$C Quench Correction Curve (from Least Square Analysis) Using Sample Channels Ratio Method

\[ y = mx + b \]

Correlation Coeff. \( r^2 = 0.9994 \)

Intercept \( b = 98.13 \)

Slope \( m = -18.419 \)
Appendix 2. Standard Curve for BSA from Least Square Analysis

\[ y = mx + b \]

Correlation Coeff. \( r^2 = 0.9992 \)
Intercept \( b = -1.795 \)
Slope \( m = 0.8647 \)

Log \( \mu g \) BSA

Log Absorbance (750nm)
Appendix 3. Chromatogram of Thiol Hydrazide Synthesized from Intermediate Unpurified N-Acetylhomocysteine Thiolactone

<table>
<thead>
<tr>
<th>Solvent Front</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
</tr>
</tbody>
</table>

- \( \square \)
- \( \bigotimes \)
- \( \bigodot \)
- \( \mathcal{Y} \)
- \( \mathcal{T} \)
- \( \mathcal{M} \)
- \( \mathcal{E} \)
- \( \mathcal{H} \)
- \( \mathcal{R} \)
- \( \mathcal{D} \)
- \( \mathcal{F} \)
- \( \mathcal{G} \)
- \( \mathcal{L} \)
- \( \mathcal{O} \)

Notes:

a) Sample A - from authentic N-acetylhomocysteine thiolactone.
Sample B - from standard thiol hydrazide.
Sample C - from ring-opening reaction (hydrazinolysis) in CHCl_3.
Sample D - from ring-opening reaction in ethyl acetate.
Sample E - from ring-opening reaction in methanol.

b) ○ - detected by uv lamp.

γ, γ1 - shown yellow with thiol spray reagent.
γ2, γ2 - shown with iodine vapor.

c) The figure is a composite from three tlc plates.
Appendix 4. Chromatogram of Synthetic $^{14}$C-Thiol Hydrazide with Parallel Samples Developed in Solvent System A

<table>
<thead>
<tr>
<th>Solvent Front</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
</tr>
<tr>
<td>O</td>
</tr>
</tbody>
</table>

Notes:

a) Sample A - authentic N-acetylhomocysteine thiolactone.
Sample B - standard thiol hydrazide.
Sample C - synthetic $^{14}$C-thiol hydrazide.
Sample D - acethyldrazide.
Sample E - hydrazine.

b)  
   - detected by uv lamp.
   $\gamma, \gamma', \gamma''$ - shown with thiol spray reagent.
   $\delta$ - shown with iodine vapor.
Appendix 5. Chromatogram of the Samples Developed in Solvent System B

Solvent Front

<table>
<thead>
<tr>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage from Scintillation Counting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 0.04 %</td>
<td>★</td>
<td></td>
</tr>
<tr>
<td>3. 0.55 %</td>
<td>△</td>
<td>□</td>
</tr>
<tr>
<td>2. 90.8 %</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>1. 8.57 %</td>
<td>★</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

a) Spots shown uv lamp.

b) Y means "yellow" with thiol spray reagent.

c) Sample A - synthetic $[^{14}\text{C}]$-hydrazide.

Sample B - acetyldrazide.

Sample C - standard thiol hydrazide.
Appendix 6. Calculation of Specific Activity Incorporated into Erythrocyte Membranes (from the Data of Liao et al. (28)).

The specific activity of tritiated sodium borohydride was 12.5 mCi/mmol. With four hydride equivalents per borohydride the specific activity of one $^3\text{H}$-hydride equivalent would be 3.125 mCi/mmol.

$$3.125 \text{ mCi/mmol} = 3.125 \text{ nCi/nmol}$$

$$3.125 \text{ nCi/nmol} \times 2.22 \times 10^3 \text{ dpm/nmol}$$

$$= 6937.5 \text{ dpm/nmol}$$

From their Fig. 2, we chose the point at which oxidation of sialyl residues were complete. The incorporation was 160,000 cpm/mg. Assume 35% efficiency for tritium, then

$$160,000 \text{ cpm/mg} \div 35\% = 457,140 \text{ dpm/mg}$$

$$457,140 \text{ dpm/mg} \div 6937.5 \text{ dpm/nmol}$$

$$= 65.6 \text{ nmol/mg}$$
REFERENCES


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