Nuclear magnetic resonance studies of fluorine-19 containing hydrazides as probes for membrane glycoprotein.

Nadarajah, Karuppiah

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RÉCU
NUCLEAR MAGNETIC RESONANCE STUDIES OF FLUORINE-19 CONTAINING HYDRAZIDES AS PROBES FOR MEMBRANE GLYCOPROTEINS

by

Nadarajañ Karuppiah

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, Canada 1983
ABSTRACT

Two fluorine-containing acyl hydrazides were evaluated for glycoprotein labelling and subsequent monitoring by $^{19}$F NMR. The sialoglycoproteins studied were glycophorin and fetuin. The first reagent, a thiol hydrazide (2-acetamido-4-mercaptobutyric acid hydrazide), was incorporated following periodate oxidation and its sulfhydryl group alkylated with 3-bromo-l,l,l-trifluoropropanone (BrTFA). Though incorporation of this hydrazide with erythrocyte glycoprotein has been reported and sulfhydryl groups alkylation with BrTFA is known, no detectable signal was observed in the present system. The second reagent was based on N-trifluoroacetyl glycine hydrazide incorporation, and was found to be covalent as judged by gel filtration and fluorine NMR. In the fluorine NMR spectrum a signal of lower intensity was always observed occurring about 14 Hz upfield from the major signal. The possibility that this signal could be due to reversibility of the hydrazone bond was ruled out by performing sodium borohydride reduction to stabilize the hydrazone bond. It was concluded that the weaker signal could be a fluorine-containing hydrazone or reduced hydrazone moiety on a sugar residue other than sialic acid. Similarly derivatized erythrocyte ghosts showed an appreciable broadening of about 18 Hz; however, this assessment was complicated by the presence of two or
more-components. Interaction of the lectin, wheat germ agglutinin with the derivatized soluble glycoprotein revealed apparent linewidth narrowing of the signals. Lectin-treated derivatized ghosts also showed an apparently narrowed spectrum. Though measurement of correlation times was not attempted, apparent narrowing of linewidths was interpreted as an indication of increased mobility of oligosaccharide moieties and of the absence of direct interaction of the lectin with the sialyl residues. It was concluded that trifluoroacetyl glycine hydrazide could be used to probe and manipulate membrane glycoproteins in an effort to determine their dynamic properties.
DEDICATION

To my family
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere appreciation and gratitude to Dr. Keith E. Taylor, my research adviser, for his continuous guidance which involved a tremendous amount of time and labour in directing the present study to its understanding.

I would also like to extend my heartfelt thanks to my fellow colleagues, in particular, A. Sharma and D. Sbissa for their moral support throughout this endeavour.

Many thanks also go out to the technical staff of this department, especially Mike Fuerth for assisting me in certain aspects of this research. I would also like to thank the typist for the excellent typing of this thesis.

Last, but not least, I would like to thank this department for offering me the opportunity to expand my knowledge and practical experience in the field of Biological Chemistry.
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ABBREVIATIONS

cm = centimeters
δ = chemical salt
CP₃COONa = sodium salt of trifluoroacetyl glycine hydrazide
D₂O = deuterium oxide
¹⁹F NMR = fluorine-19 nuclear magnetic resonance
g max = gravitational maximum
h = hour
Hz = hertz
max = maximum
µg = microgram
mg = milligram
min = minute
ml = milliliter
mM = millimolar
M = molar
NS = number of scans
PAS = periodic acid Schiff
PBS = phosphate buffered saline
r.p.m. = revolutions per minute
SDS = sodium dodecyl sulfate
TFG = trifluoroacetyl glycine hydrazide
WGA = wheat germ agglutinin
MBTH = 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate
CHAPTER I

INTRODUCTION

An overwhelming amount of data has implicated the cell surface as the primary site for control of cell growth, division, development, communication, differentiation and death. Cells must react to their environment and to extracellular stimuli through their surfaces, particularly their plasma membranes. Consequently, this organelle has evoked the interest of a large number of investigators in many fields over the last 70 years (1). Histochemical staining experiments provided the first clear indication that carbohydrate-containing macromolecules formed part of the surface membrane of animal cells (2). These studies were initially carried out by light microscopy and have largely been confirmed by recent studies combining electron microscopy with specific staining methods.

Under appropriate conditions the outer surface of a variety of cell types can be stained by periodic acid silver methenamine and the reaction is inhibited by specific glycosidases (3). Some cells have an easily visualized surface coat which binds the stain, but in most cases (the red blood cell is one example), a distinct structure is not visible.
Other evidence that glycoproteins are present on cell surfaces has been obtained by studying the biological effects of treating cells with specific hydrolytic enzymes. For example, it has been shown that removal of sialic acid residues from the glycoprotein of tumor cell surfaces by neuraminidase alters the capacity of the cells to metastasize (4). In a similar kind of experiment, Sanford (5) found that neuraminidase treatment of transplantable tumor cells has altered their immunological reactivity. Studies by Gesner and co-workers (6,7) have provided further evidence for the idea that membrane glycoproteins are involved in cell recognition phenomena. The role that carbohydrates play in determining the chemical specificity of cell surfaces is best documented in the case of ABO and Lewis blood group determinants (8). On the basis of these and other studies, it has been suggested that glycoproteins (and glycolipids) mediate the social behaviour of mammalian cells. This hypothesis (8) implies that contacts between cells are regulated in some way by macromolecules on their exposed surface and the specificity of the reaction is determined by unique patterns of carbohydrate configuration and orientation.

The human erythrocyte has been an essential workhorse for membrane data and the development of new techniques and theories. Owing to the ease of isolation and the purity and quantity of materials available, the erythrocyte has
been utilized as a plasma membrane source by almost every membrane researcher at one time or another (9-20). Compared to most plasma membranes, the erythrocyte ghost membrane is relatively simple. Of the protein components in the membrane, two integral glycoproteins are of special interest. These are (a) a glycoprotein of 90,000-100,000 apparent molecular weight, Band 3 (21,22) and (b) the major sialoglycoprotein of 30,000-50,000 apparent molecular weight (23) which is also called the MN-glycoprotein (24) or glycophorin (25). Band 3 and glycophorin are the major proteins exposed to the external environment (26-29) and are in addition the receptors for a variety of viruses, lectins, and specific antibodies, hormones and they mediate particular functions such as mitogenesis, morphogenic movement or cell adhesion. Glycophorin has been well characterized and is composed of a single polypeptide chain of approximately 131 amino acids (25-30). It contains about 60% carbohydrate by weight, mainly in the form of two principal types of oligosaccharides (31,32) which bear a variety of antigens (ABO,MN) and receptors for several lectins (wheat germ agglutinin, Phaseolus vulgaris and Lens culinaris phytohemagglutinin) and viruses (influenza, NDV) (24,30,33-35). Glycophorin has been proposed to be a trimodal amphipathic molecule with a hydrophilic-hydrophobic-hydrophilic structure (25). It spans the membrane exposing its N-terminal glycopeptide
region to the extracellular environment, an internal hydrophobic region to the lipid hydrophobic zone of the membrane and its C-terminal hydrophilic region to the cell interior (25,30). The oligosaccharide moieties are covalently attached to the N-terminal region of the polypeptide. These oligosaccharide moieties have frequently been implicated in surface recognition events (33-35). Though the chemical composition of surface oligosaccharides has been studied in some detail (35,36) no detailed investigation of their physical properties has been determined. It is likely that an understanding of their dynamic and conformational properties will be of considerable importance in understanding their functional role.

A number of techniques have been developed in recent years to provide an approach to studying the dynamic properties of these complex biological structures. Fluorescence spectroscopy has been used for many years to obtain molecular information of specific carbohydrate residue in highly heterogenous biological systems. Once the location of the reporter molecule is defined, changes in the fluorescence lifetime or quantum yield of a membrane bound probe can signal a conformation change (37-42). Another major technique involves electron paramagnetic resonance studies of spin-labelled systems. The technique has been used extensively by Grant and co-workers to study
dynamic properties of the oligosaccharide moieties of the erythrocyte major transmembrane glycoprotein (glycophorin) (43,44), oligosaccharide moieties of gangliosides (45), and glycolipids (46,47). Similar spin labelling studies of glycoproteins and glycolipids have also been carried out by Feix et al. (48) who reached a different conclusion with respect to the dynamic properties of these oligosaccharide moieties.

Nuclear magnetic resonance (NMR) techniques have become powerful tools for investigating the physical properties of many biological systems. NMR spectroscopy has been shown to be a valuable method for studying the contents of intact cells (49,50), where most of these studies involved nuclei such as $^1\text{H}$, $^{13}\text{C}$, $^{15}\text{N}$, and $^{31}\text{P}$ (51-54). The fluorine-19 nucleus has also become a useful probe for such studies because of its high sensitivity, wide chemical shift range and the absence of endogenous signals. Fluorine-19 NMR spectroscopy has been used to investigate the structure function relationships in protein molecules in cases where an appropriate $^{19}\text{F}$ label could be incorporated biosynthetically or could be attached (covalently or non-covalently) to specific groups in a protein molecule (55-58). When resonances in a protein NMR spectrum can be resolved and assigned to individual nuclei in the protein, these resonances then serve as probes of the structure, chemical state, and dynamic properties (in terms
of reaction and molecular motion) of the protein in solution. Sykes et al. (55) have demonstrated that fluorine-19 nuclear magnetic resonance spectroscopy of specifically $^{19}\text{F}$-labelled enzymes as large as alkaline phosphatase (M.Wt. 86,000) provides a very valuable probe for conformational change in proteins. Fluorine-19 magnetic resonance studies have also been useful in describing the structure and dynamics of the phospholipids of model membrane systems (59,60). For example, fluorinated fatty acids have been incorporated biosynthetically as unsaturated fatty acid analogues into the phospholipids of Escherichia coli and subsequently monitored by fluorine-19 nuclear magnetic resonance spectroscopy to determine membrane protein-lipid interaction. Such studies suggested the technique to be more sensitive than spin labels or fluorescent probes. Miura et al. (61) have provided valuable information on the flavin-protein interaction in flavoproteins in general as well as in riboflavin employing $^{19}\text{F}$ NMR spectroscopy. Maugras et al. (62) had great interest in the development of new techniques for identifying the nature of the chemical bonds between the cell surface macromolecules and hapten groups. They used a fluorine-containing molecule, CF$_3$-TNBS(4-trifluoromethyl-2,6-dinitrobenzene sulphonate), an analog of 2,4,6-trinitrobenzene sulphonate (TNBS), to probe membrane events following membrane protein-hapten interactions by the use of $^{19}\text{F}$ NMR spectroscopy.
Though $^{19}$F-NMR spectroscopy has been used in the investigation of these biological systems, its application has never been extended to studying the dynamic properties of the oligosaccharide moieties of cell surfaces. Fluorine-labelled saccharides have been used to study interactions with lysozyme (63) and concanavalin (64). Lectin sugar interactions were first investigated by Midoux et al. (65) and Jordan et al. (66). The latter group's report constituted the first application of $^{19}$F NMR studies to wheat germ agglutinin (WGA) binding sites and demonstrated the advantages of this probe.

Since the dynamic properties of the erythrocyte oligosaccharide moieties have been determined using spin labels and fluorescence probes and have never been attempted with $^{19}$F NMR spectroscopy, the ideas of Midoux et al. (65) and Jordan et al. (66) evoked our interest in synthesizing fluorine-containing molecules for covalent incorporation into specific sugars of the erythrocyte oligosaccharide moieties where they might act as reporter groups.

Fluorine-$^{19}$F is superior to most nuclei, since it has 83% of the sensitivity of the proton, or higher if advantage is taken of nuclear Overhauser effect, yet there is no significant endogenous "background" in biological systems as there is for proton NMR.

Previous work in this laboratory has focused on hydrazides as reagents for derivatization of cell surface
sugar aldehydes as the corresponding hydrazones as shown.

\[
\begin{align*}
0 & \quad 0 \\
R-C-H & + \quad R-C-N-NH_2 & R-C=N-N-C-R' & + \quad H_2O
\end{align*}
\]

Many variations of the hydrazide-hydrazone incorporation system have been reported by others. For example, biotin hydrazide (67) sugar-containing hydrazides (68), 2,4-dinitrophenyl hydrazide (69), methionine sulfone hydrazides (70), fluorescent hydrazide probes (71), acetyhydrazide (71), a thiol-containing hydrazide (72), and N-(nitroazidophenyl)-\(\beta\)-alanine hydrazide (73) have been covalently attached to the cell surface sugar aldehydes generated by mild periodate oxidation and/or by treatment with galactose oxidase.

Two such fluorine-containing hydrazides were available in this laboratory and it was the goal of the present project to study, (a) the incorporation of these reagents into soluble and membrane-bound glycoproteins, and (b) to evaluate any incorporated groups as potential reporters for ligand association events. The study was restricted to the soluble sialoglycoprotein fetuin, solubilized glycophorin and erythrocyte ghosts, all of which were oxidized by periodate treatment. The latter method has been reported to be selective for sialyl residues (74).

Two acyl hydrazides were evaluated in this laboratory. In the first case, a thiol hydrazide was to be incorporated following Y. C. Wu of this lab (72), Scheme 1, and its
sulfhydryl group-alkylated, as shown in Scheme 2. For alkylation 3-bromo,1,1,1-trifluoropropanone (BrTFA) was used in the present study. BrTFA has been used as an alkylation reagent for many sulfhydryl-containing proteins and peptides (75-80), and the trifluoroacetonyl group has been shown to be a sensitive monitor of environmental changes. The second case, a one-step reagent, was based on N-trifluoroacetyl glycine hydrazide incorporation, as shown in Scheme 3.

WGA has been used in determining the dynamic properties of the oligosaccharide moieties of glycophorin (43-45). WGA sugar interactions have been reported by Jordan et al. (66) employing $^{19}\text{F}$ NMR. Therefore, in the present study, WGA was chosen as the saccharide binding ligand.
\[ R' = \begin{array}{c}
\text{CH}_3 \text{C-N-CH}\text{-} \\
\text{H} \text{-CH}_2\text{-CH}_2\text{-SH}
\end{array} \]

in N-acetyl-DL-homocysteine hydrazide

SCHEME 1: Incorporation of R' [thiol hydrazide]
SCHEME 2: Alkylation of Sulfhydryl Group with BrTFA
SCHEME 3: Incorporation of $R'$ Trifluoroacetylglucose [glycine hydrazide]

$R' = \text{CF}_3\text{C}-\text{N}-\text{CH}_2^-$

$R'$ in Trifluoroacetylglucose hydrazide
CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Chemicals

   (i) Basic fuchsins, Sephadex G-25, Coomassie Brilliant
       Blue-R, sodium dodecyl sulphate and Trizma Base were
       commercially available from Sigma Chemical Company, St.
       Louis. Sodium phosphate (monobasic and dibasic), ethyl-
       enediamine tetracetate (EDTA), sodium potassium tartrate,
       copper sulphate, ferric chloride, sodium borate, boric
       acid, formaldehyde, sodium hydroxide, hydrochloric acid,
       charcoal, sodium arsenite, sodium metabisulphite, sodium
       acetate, acetone and sodium chloride were purchased from
       Fisher Scientific Company, Fair Lawn, New Jersey. From BDH
       Chemicals Canada, sodium metaperiodate, periodic acid,
       isopropyl alcohol, phenol, n-butanol, Triton X-100 and
       phenol reagent were purchased. Trifluorethanol, 3-methyl-2-
       benzothiazolinone hydrazone hydrochloride monohydrate,
       acrylamide and bisacrylamide were obtained from Aldrich
       Chemicals, Milwaukee, Wisconsin. Biobeads SM2, ammonium
       persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED)
       were commercially available from Bio-Rad, Richmond, Cali-
       fornia; Pyronin Y stain was from Eastman Kodak, Rochester,
New York: fetoin was from GIBCO (Grand Island Biological Company) Grand Island, New York; 3-bromo-1,1,1-trifluoropropanone was from PCR Research Chemicals, Gainesville, Florida; and wheat germ agglutinin from BRL (Bethesda Research Laboratories), Gaithersburg, MD.

(ii) Outdated blood was obtained from the Canadian Red Cross Society, Windsor Branch, and London Regional Centre.

(iii) Fluorine-containing molecules.

(a) thiol hydrazide (2-acetamido-4-mercaptopbutyric acid hydrazide) was prepared and characterized by Y. C. Wu of this lab (72).

(b) Trifluoroacetylglucose hydrazide was prepared and characterized as the trifluoroacetate salt by Dr. K. E. Taylor of this lab.

(iv) Equipment. All aqueous solutions were prepared using distilled deionized water. pH measurements were obtained on a Radiometer model 26 pH meter with semi-micro combination electrode, GLK2301-C. Centrifugation was performed on a Sorvall centrifuge RC-2-B with SS-34 rotor. Spectrophotometric measurements were performed on a Beckman model 35 spectrophotometer using appropriate cuvettes of 1cm path length. Sephadex G-25 medium was packed in a 1.5x90cm column KL5 obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The fraction collector used was model
2070 from LKB Bromma equipped with a UV-cord S absorbance monitor operating at 280nm. Stained gels were scanned with a ORTEC model 4310 densitometer.

(v) Fluorine NMR instrumentation and conditions.

Fluorine NMR spectra were recorded at 84.6MHz with a Bruker model CXP 100 pulse NMR spectrophotometer operating in the Fourier transform mode. The field was locked to the D₂O resonance. A typical spectrum was recorded using 2000Hz sweep width and 4K points although some earlier spectra were recorded using 6024Hz sweep width and 4K points. An acquisition time of 1.024s, delay time of 1.1s pulse time of 1 µs (ca. 90° pulse width) were used. Chemical shifts were assigned with respect to external 1M aqueous CF₃COONa. Linewidths were estimated in Hz by measuring the height of the peak to the centre of the noise background and then taking the width of the line at half its height. This value in cm was multiplied by the frequency Hz per cm obtained from the printout minus line broadening to give the effective value of the linewidth.

B. Methods

1. Analytical Methods

   (i) Protein Estimation (81). A standard solution of BSA (Bovine Serum Albumin) was made in distilled water. Samples of different concentrations were made and the absorbance determined by the method of Peterson (81). The
actual concentration of the various BSA solutions was determined by taking the absorbance at 280nm wavelength to be divided by the molar extinction coefficient \((E_{1%}_{1cm} = 6.7)\) of BSA. A standard curve of the absorbance versus the actual concentration was thus plotted.

(ii) Sialic Acid Estimation. A MBTH assay. A stock solution of sialic acid at 20mg/100ml was made in water and oxidized with sodium periodate (final concentration being 1mM) for 10min at 0°C. Formaldehyde released on oxidation was diluted accordingly to give various concentrations of formaldehyde. Fifty μl of each dilution was taken for assay following the standard procedure. The actual concentration of sialic acid was calculated assuming 98% purity of the commercial product.

2. Electrophoretic Procedures

SDS polyacrylamide gel electrophoresis was executed according to the method of Fairbanks et al. (15) with the modifications of Yu et al. (83). Samples of ghosts, pellet (Triton X-100), extract (Triton X-100), and purified glycophorin were gel electrophoresed.

(i) Triton X-100 glycophorin. Each gel was loaded with 40 and 80 micrograms of protein for Coomassie Blue and periodic acid Schiff staining, respectively.

(ii) Purified glycophorin. Each gel was loaded with 20μg of protein for both staining procedures.
(iii) Polyacrylamide Gel Electrophoresis in SDS.
Concentrated stock solutions were mixed in the order and
proportions given in Table 1 and added to 15cm glass tubes
of 6mm inner diameter. The tubes, which had been cleansed
with chromic acid, washed and rinsed with distilled water
were coated with 1% Prosil-28 for half an hour and dried
at 100°C for half an hour. Each column was overlaid gently
with distilled water (~200μl) and left aside for polymeriza-
tion. When polymerization was complete (left to stand
for at least 12hrs) the overlaid distilled water was re-
moved and protein samples at concentrations stated above
were added containing 0.2% S.D.S., 5% sucrose, 10mM DTT
Tris-HCl(pH8), 1mM EDTA(pH8), 40mM DTT and 10μg/ml of
Pyronin Y (tracking dye). The columns were then filled
to the top with the electrophoresis buffer.

Electrophoresis was carried out with the voltage
gradient at about 7-8v/cm and the current at 8mA/tube.
The running time under these conditions was about 2h.
Position of the tracking dye was marked in each gel by
pricking the centre of the dye mark with a needle dipped
in drafting ink.

(iv) Coomassie Blue Staining. Gels were placed
in 70ml glass tubes and were treated with the fixing,
staining and destaining solutions given below. Solutions
were stirred vigorously at room temperature: (1) 25%
isopropyl alcohol, 10% acetic acid, 0.025% Coomassie Blue,
Table 1: Formulas for Stock Solutions, Buffers, and Gels

A. Stock Solutions

**Con Ac Bis**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Acrylamide (40g)</td>
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</tr>
<tr>
<td>Bis (1.5g)</td>
<td></td>
</tr>
<tr>
<td>H₂O to 100ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10xBuffer (pH7.4)</td>
<td></td>
</tr>
<tr>
<td>1.0M Tris (40mL)</td>
<td></td>
</tr>
<tr>
<td>2.0M sodium acetate (10mL)</td>
<td></td>
</tr>
<tr>
<td>0.2M EDTA (10mL)</td>
<td></td>
</tr>
<tr>
<td>Acetate acid to pH7.4</td>
<td></td>
</tr>
<tr>
<td>H₂O to 100mL</td>
<td></td>
</tr>
</tbody>
</table>

20% (w/v) SDS
1.5% (w/v) ammonium persulfate
0.5% (v/v) TEMED

B. Electrophoresis buffer (per liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>100mL</td>
</tr>
<tr>
<td>20% SDS</td>
<td>10mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>890mL</td>
</tr>
</tbody>
</table>

C. Gels (per 10mL of solution 5.0% in acrylamide)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con AcBis</td>
<td>1.25mL</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>1.0mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>6.25mL</td>
</tr>
</tbody>
</table>

1.5% ammonium persulfate (1.0mL)
0.5% TEMED (0.5mL)
overnight; (2) 10% isopropyl alcohol, 10% acetic acid, 0.0025% Coomassie Blue, 9h; (3) 10% acetic acid with 0.0025% Coomassie Blue, overnight; (4) 10% acetic acid, several hours, until the background was clear.

(v) PAS staining procedure. Steps (1)-(4) as above (omitting the protein stain) were carried out; (5) 0.5% periodic acid, 2h; (6) 0.5% sodium arsenite, 5% acetic acid, 30-60min.; (7) 0.1% sodium arsenite, 5% acetic acid, 20min—repeated twice; (8) acetic acid 10-20min. Following these steps the gels were transferred to tubes containing 20ml of Schiff reagent and left overnight; then (9) returned to the large tubes for incubation in 0.1% sodium metabisulfite (\(\text{Na}_2\text{S}_2\text{O}_5\)), 0.01N HCl, for several hours; (10) left in 10% acetic acid.

(vi) Preparation of the Schiff reagent (15). 2.5g of basic fuchsin was dissolved in 500ml of water, then 5g of sodium metabisulfite and 50ml of 1N HCl were added. The solution was stirred for several hours, and then decolourized with about 2g of activated charcoal.

3. Characterization of fetuin

Fetuin stock was made in 5mM sodium phosphate buffer pH7.4 and aliquots were taken for analysis of protein (81) and sialic acid (82) content according to the standard curves, Appendix I and Appendix II, respectively.
4. Preparation of Triton X-100 enriched glycophorin extract (83)

Outdated blood was mixed with 1ml of 0.2M EDTA for every 25ml of blood, then diluted with an equal volume of cold 5mM sodium phosphate (pH7.4)–0.15M NaCl. The suspension was centrifuged for $10^4 g_{\text{max}}$ min at 4°C.

Supernatant and "buffy coat" were aspirated. The packed cells were washed several times until most of the "buffy coat" was removed. The cells were lysed with cold 5mM sodium phosphate buffer pH7.4 and the suspensions were centrifuged for $3 \times 10^5 g_{\text{max}}$ min. The resulting deep red supernatants were aspirated, leaving red, translucent pellets of packed ghosts over minute, opaque, cream-coloured "buttons". Each tube was tilted and rotated to allow the loose ghost pellet to slide off the tightly packed material, which was then aspirated with little loss of ghosts. After several washes with 5mM sodium phosphate–15mM NaCl pH7.4 under the same centrifugal conditions, the ghosts were obtained almost white. The hemoglobin-free erythrocyte ghosts obtained were equilibrated with 56mM sodium borate, pH8, centrifuged and incubated with five volumes 0.05% (v/v), Triton X-100 in 56mM sodium borate pf pH8 for 20min at 0°C. Centrifugation was then done for 1h at 15,000 r.p.m. (16.2 $\times 10^5 g_{\text{max}}$ min) to separate the extract from the residue. The separated residue and extract were then characterized for protein (81) and sialic acid (82) content and by SDS polyacrylamide gel electrophoresis (15,83).
5. Removal of Triton X-100 from glycoporphin samples with Bio-Beads SM-2 (84)

(i) Washing of Bio-Beads. Methanol (100ml) was added to 15g Bio-Beads SM-2. The mixture was stirred for 15 min and the copolymer beads were collected on a sintered glass funnel and washed with a further 250ml of methanol. The beads were not allowed to dry but were immediately washed with 500ml of water. The moist beads were washed several times over a sintered glass funnel. The moist beads were stored under water at 4°C until required.

(ii) Removal of Triton X-100. Moist copolymer beads (0.6g for every 2ml) were added to glycoporphin extract containing 1% Triton X-100. The mixture was gently stirred at 4°C for at least 5hrs to assure complete removal of Triton X-100.

6. Preparation of purified glycoporphin (85)

Purified glycoporphin was prepared by the method of Taraschi et al. (85). To 1 volume of hemoglobin-free erythrocyte ghosts prepared above was added 0.2 volumes of cold 1M Tris-HCl, pH8, and 1.2 volumes of cold n-butanol with stirring for 30 min at 0°C. The resulting suspension was centrifuged at 8000xg for 10 min and the lower aqueous phase was collected with a syringe. The aqueous phase which contained a small amount of butanol was dialyzed against 10mM Tris-HCl, pH8, at room temperature
for 2 hr. The aqueous phase was extracted with an equal volume of 50% phenol, centrifuged at 8000xg for 10 min and the phenol-poor upper aqueous phase was dialyzed for 3 days at 4°C against 1 mM Tris-HCl pH 8. To 1 volume of the dialyzed phase was added 0.2 volume of 1.0 M Tris-HCl, pH 8. This volume was mixed with an equal volume of cold (-20°C) acetone. After 30 min, the glycophorin precipitate was collected by centrifugation at 8000xg for 15 min. The clear supernatant (1 volume) which was 60% ethanol was mixed with an equal volume of cold (-20°C) acetone. After 30 min, the glycophorin precipitate was collected by centrifugation at 5000xg for 10 min, and redissolved in 1 mM Tris-HCl, pH 7.4 and was dialyzed overnight at 4°C against distilled water. The dialyzed glycophorin was freeze-dried and stored at -20°C. The glycophorin thus prepared was characterized for the protein (81) sialic acid (82) contents and by SDS polyacrylamide gel electrophoresis (15,83).

7. Derivatization of ghosts with thiol hydrazide

Erythrocyte ghosts prepared as above were oxidized selectively at sialic acid residues with sodium metaperiodate (final concentration being 1 mM) for 10 min at 0°C. The ghosts were then washed and centrifuged twice to remove iodate and excess periodate. Packed ghosts were then resuspended in wash buffer (5 mM sodium phosphate buffer-15 mM NaCl), pH 7.4, at 50% suspension followed by incubation
with thiol hydrazide (final concentration being 10mM) for 2h at room temperature. The suspension was then spun down to remove the supernatant containing most of the unbound reagent. The system (packed ghosts) was then treated with 3-bromo-1,1,1-trifluoropropanone (final concentration being 10mM) for 20min at room temperature. The ghost suspension was centrifuged and washed several times, equilibrated with 56mM sodium borate pH8, and extracted with Triton X-100 as described above. The extract obtained after centrifugation at 15,000 r.p.m. for 1h was lyophilized and reconstituted with distilled water. (In this case made to volume of 5mL.)

8. Derivatization of Triton X-100 glycophorin extract and purified glycophorin

Triton X-100 extract reconstituted in distilled water (800nmoles per ml of sialic acid) or purified glycophorin reconstituted in 5mM phosphate buffer pH7.4 (1400nmoles per ml of sialic acid) were oxidized with 2 equivalents of periodate per sialyl residue for 10min at 0°C, followed by incubation with trifluoroacetyl glycine hydrazide (final concentration 10mM) for 2h at room temperature. The sample was then eluted through the Sephadex column with 5mM sodium phosphate pH7.4 at 4°C. The void volume fractions were pooled together, freeze-dried and stored at -20°C. For fluorine-19 NMR spectroscopy of these samples, they were reconstituted in 1ml D₂O.
9. Derivatization of fetuin

A 1ml aliquot (containing 3000nmoles per ml of sialic acid) of fetuin in 5mM sodium phosphate buffer was derivatized and gel filtered as for the Triton X-100 extract and purified glycophorin samples above.

10. Borohydride reduction of TFG derivatized samples

The washed derivatized sample was treated dropwise with sodium borohydride solution in 5mM phosphate buffer pH7.4 to give a final concentration of 50mM and was left for 30min at room temperature. The reduced sample was then eluted through the Sephadex column and the void volume fraction was freeze-dried, stored at -20°C and reconstituted for fluorine-19 NMR spectroscopy as above.

11. Derivatization of erythrocyte ghosts with the trifluoroacetyl glycine hydrazide salt

Hemoglobin-free erythrocyte ghosts were resuspended in wash buffer pH7.4 (50% suspension) and oxidized with two equivalents of periodate per sialyl residue at 0°C for 10min. The ghost suspension was washed twice and centrifuged at $3 \times 10^5 g_{\text{max}} \text{ min}$. The ghosts were resuspended in wash buffer (50% suspension) and incubated with trifluoroacetyl glycine hydrazide for 2hrs at room temperature (final concentration 5mM). Ghosts were then washed several times by centrifugation to remove excess reagent before recording the fluorine-19 NMR spectrum.
12. Wheat germ agglutinin titre studies

(i) Wheat germ agglutinin titre with fetuin serial
dilutions of WGA were made from stock WGA at a concentration
of 8mg/ml in a disposable polystyrene microtitre plate with
PBS, pH7.4, as represented in Scheme 4. Fetuin at a
concentration corresponding to 500nmoles/ml of sialyl
residues was added to the above serial dilutions such that
the final concentrations of WGA and fetuin were obtained,
as shown in Scheme 4.

(ii) Wheat germ agglutinin titre with red blood cells.
Serial dilutions of WGA were made as above with PBS pH7.4
to give the final concentrations (as represented in Scheme
5) on addition of a 4% suspension of red blood cells.

(iii) Wheat germ agglutinin titre with TFG derivatized
red blood cells. Five ml of washed red blood cells at 50% 
suspension in PBS was oxidized with sodium metaperiodate
for 10min at 0°C (final concentration 1mM). Excess periodate
was washed away by centrifugation. The packed red blood
cells were resuspended in PBS. To 4ml of this suspension
was added TFG reagent (final concentration 5mM) and left
for 2hrs at room temperature. Excess of reagent was washed
several times before performing hemagglutination titration
described above.
WGA —— 1mg/ml
Fetuin — 250nmoles/ml
A = 8mg/ml WGA
B = PBS buffer pH 7.4
C = 500nmoles of sialic acid containing
2 mg of protein per ml.

\[ \text{\textsuperscript{a}} = 100\mu l \text{ of the dilution mixture was removed to the adjacent well before addition of 100\mu lC.} \]

**Scheme 4.** Serial dilutions made for wheat-germ agglutinin-fetuin titre with their final concentration of WGA and fetuin.
GA. 200μg/ml 100μg/ml 50μg/ml 25μg/ml 12.5μg/ml 6.75μg/ml
Blood suspension

A = 800μg/ml of WGA.

B = PBS pH 7.4

C = 4% washed red blood cell suspension

a = 100μl of the dilution mixture was removed to the adjacent well before addition of 100μl C.

Scheme: 5 Serial dilutions made for wheat-germ agglutinin-red blood cell titre with their final concentrations of WGA and red blood cell suspension.
CHAPTER III
RESULTS AND DISCUSSION

Two approaches were taken to obtain defined, soluble glycoporphin preparations. One (83) involved selective extraction with a non-ionic detergent to yield an enriched sample, while the second (85) involved a procedure reputedly yielding a homogenous preparation.

Hemoglobin-free human erythrocyte membranes (ghosts) prepared according to Fairbanks et al. (15) from outdated blood in 5mM sodium phosphate pH7.4, were extracted with 0.5% Triton X-100 in 56mM sodium borate pH8, as described by Yu et al. (83), with similar results. Electrophoresis in 5.0% polyacrylamide gels containing 0.2% sodium dodecyl sulphate executed as previously detailed (15,83) showed that 0.05% Triton X-100 released most of each glycoprotein peak made visible by periodic-Schiff reagent (Fig. 1). Coomassie blue staining revealed bands 3, 5, 6 and 7 whilst bands 1, 2, 4.1 as well as undesignated minor components were retained in the pellet (Fig. 2). Protein and sialic acid content of the Triton X-100 extract, presented in Table 2, confirmed the extract to be enriched with glycoporphin based on a concentration of 268nmoles of sialic acid (81) per mg of protein (82) relative to the ghosts with 112nmoles of sialic acid per mg of protein.
Figure 1

Gel scans showing the release of membrane glycoproteins by Triton X-100.

Legend

Gels containing 80µg of protein were stained with periodic acid-Schiff reagent and scanned. TD represents the tracking dye marked with drawing ink.

a) PAS staining of Triton X-100 extract.
b) PAS staining of Triton X-100 pellet.
Figure 2

Gel scans showing the release of membrane proteins by Triton X-100.

Legend

Gel containing 40μg of protein were stained with Coomassie Blue and scanned. TD represent the tracking dye marked with drawing ink.

a) Triton X-100 pellet.
b) Triton X-100 extract.
Table 2: (i) Chemical Composition of the Triton X-100 Extract and Residue<sup>a</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>Ghosts</th>
<th>Extract&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg)</td>
<td>3.68</td>
<td>0.88</td>
<td>2.60</td>
</tr>
<tr>
<td>Sialic Acid (nmoles)</td>
<td>412</td>
<td>235</td>
<td>158</td>
</tr>
<tr>
<td>nmoles sialic acid/mg protein</td>
<td>112</td>
<td>268</td>
<td>60</td>
</tr>
</tbody>
</table>

(ii) Chemical Composition of the Purified Glycophorin

| Protein (mg)                  | 3      |
| Sialic Acid (nmoles)          | 5700   |
| nmoles sialic acid/mg protein | 1900   |

<sup>a</sup>Data are expressed per ml of packed ghosts input.

<sup>b</sup>Packed ghosts were incubated in five volumes of 0.05% Triton X-100 in 56nM Na Borate pH8 for 20min on ice before centrifugation (15,000 r.p.m. per lhr) and analysis.
Similarly, the purified soluble glycophorin extract obtained by the method of Taraschi et al. (85) was also characterized by protein and sialic acid contents and SDS-PAGE (Fig. 3). Data obtained, Table 2, showed the sialic acid content to be about 1900 nmoles of sialic acid (82) per mg of protein (81) which was similar to that obtained by Taraschi et al. (85), 2000 nmoles of sialic acid per mg protein, where sialic acid was determined by the method of Warren (86) and protein was determined by the method of Lowry (87). Both experimental values obtained seem to be twice that estimated by calculations based on the known (89) polypeptide and oligosaccharide composition of human erythrocyte glycophorin A. This could be due to bovine serum albumin being used as a standard protein to determine the concentration of protein in the purified glycophorin sample. Disc gel electrophoresis of the purified glycophorin showed two bands when stained with Coomassie blue and with periodic Schiff reagent (PAS2 and PAS3) (Fig. 3). The band closer to the origin of the gel stained more than the other.

Fetuín, the predominant glycoprotein of fetal calf serum, which was commercially available, was characterized only for its sialic acid (83) and protein content (82) which showed it to have about 250-260 nmoles of sialic acid per mg of protein.
Figure 3
Gel scans of purified membrane glycoprotein (glycophorin).

Legend
a) Soluble membrane glycoprotein (glycophorin) prepared by the method of Taraschi et al. were electrophoresed and the gels stained with Coomassie Blue. Gels contained 20μg of protein.

b) Densitometer scan of the electrophoresed gel stained with periodic acid-Schiff reagent. Gel contained 20μg of protein.
Figure 3
Nuclear Magnetic Resonance Studies

Periodate-treated erythrocyte ghosts were earlier derivatized by Wu et al. (72) of this lab with the radioactive form of a thiol hydrazide (Fig. 1a) and shown to incorporate about 50nmols of the reagent per mg of membrane protein. In the present study, erythrocyte ghosts and fetoin were similarly derivatized with the non-radioactive form of the reagent and later alkylated with 3-bromo-1,1,1-trifluoropropanone (75-79). Neither showed any signals after 30,000 scans when monitored for fluorine-19 NMR. (The derivatized erythrocyte ghosts had been subjected to selective Triton X-100 extraction procedure described above (83) and the extract had been dialyzed before monitoring for fluorine-19 NMR.) It was thought at this juncture that perhaps oxidation of the sulphhydryl group by the dissolved oxygen in the buffer was taking place to form disulphide linkages hence making it impossible for alkylation by 3-bromo-1,1,1-trifluoro- propanone. Hence, to rectify this problem all buffer systems used were N$_2$-saturated to remove the dissolved oxygen. Washing of the ghosts with N$_2$-saturated buffer before and after derivatization did not lead to samples with any detectable $^{19}$F NMR signals. No further investigations were attempted with this reagent but, rather, all additional experimental investigations were carried out
with the other hydrazide compound, N-trifluoroacetic acid glycine hydrazide (TFG).

The hydrazide compound (TFG) provided as the corresponding trifluoroacetate salt was synthesized in this laboratory by a two-step synthesis based on the synthesis by Rando et al. (71) of acethyldrazide. The first step involved the treatment of trifluoroacetyl glycine with t-butyl carbazate and in the second step the filtrate was treated with trifluoroacetic acid to obtain the transparent oil-like trifluoroacetyl glycine hydrazide salt as shown in Scheme 6. Fluorine-19 NMR spectra relative to the sodium salt of trifluoroacetic acid (Fig. 4) shows two peaks of comparable intensities, about 12 Hz apart (Fig. 4a). The two peaks indicate that the hydrazide compound contains two types of fluorine-containing molecules, one peak being of the hydrazide's covalent trifluoroacetyl group and one of the trifluoroacetate (TFA⁻) counter ion. The covalent trifluoroacetyl group is the portion required for covalent labelling of glycoproteins after periodate oxidation.

Hence, in order to determine the respective components, authentic sodium trifluoroacetate (CF₃COONa) was added to the TFG sample and the fluorine-19 NMR spectrum examined again. Figure 4b, representing the spectrum of the mixture, shows the signal on the right side (upfield) to be decreased in relative intensity, thus signifying that the upfield
SCHEME 6: Synthesis of Trifluoroacetylglycine hydrazide
Figure 4

$^{19}$F Fourier transform nuclear magnetic resonance spectrum of trifluoroacetyl glycine hydrazide (TFG) 20mM.

Legend

Chemical shifts relative to CF$_3$COONa.

(a) Reagent Standard Number of scans NS = 4

(δ) A = -0.15 ppm (-12.6Hz)
    B = -0.3 ppm (-25.4Hz)

(b) Reagent Standard with authentic CF$_3$COONa added NS = 2

(δ) C = -0.13 ppm (-10.7Hz)
    D = -0.3 ppm (-24.4Hz)
signal, occurring at a chemical shift of -24 Hz, is of the covalent trifluoroacetyl group.

Stability of the hydrazide with respect to hydrolysis was investigated by $^{19}$F NMR. No hydrolysis of the reagent was observed when it was kept at room temperature for 2-3 days in solution or in phosphate buffer pH=8. Also, no hydrolysis at 37°C occurred when it was observed for 3-8h. Hydrolysis of the compound was, however, observed within two hours when the temperature was raised to 80°C (Fig. 5). The NMR spectrum showed results similar to those when authentic CF$_3$COONa was added suggesting that the right-hand peak (upfield signal) was that of the hydrazide trifluoroacetyl group. These results indicate that if an appreciable number of scans had to be collected, which might extend for 3-8h, no hydrolysis of the reagent would be expected if spectral accumulation was at or below 37°C.

The first protein chosen for derivatization was fetuin, the predominant glycoprotein of fetal calf serum, which can be isolated in a high degree of purity and good yield (88-90). The carbohydrate of fetuin makes up approximately 23% of its molecular weight and consists of a total of 52 sugar residues in the form of sialic acids, D-galactose, D-mannose, N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D glucose) and N-acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactose). The commercially available material was derivatized with TFG following oxidation of its sialyl
Figure 5

$^{19}\text{F}$ Fourier transform nuclear magnetic resonance spectrum ($^{19}\text{F}$ NMR) of trifluoroacetyl glycine hydrazide (20mM).

Legend

Reagent hydrolysed for 2h at 80°C.

Chemical shifts relative to CF$_3$COONa.

(a) \( \delta \)  
\[ A = -0.023 \text{ ppm} \ (\pm 1.95\text{Hz}) \]
\[ B = -0.184 \text{ ppm} \ (\pm 15.6\text{Hz}) \]
groups with sodium periodate (mild periodate oxidation is reported to be selective for only these carbohydrate residues (91-93). After passing through a Sephadex G-25 column to remove excess reagents, the eluate was lyophilized and reconstituted in D₂O for ¹⁹F NMR. Figure 6b represents the spectrum obtained from such an experiment. A sharp signal was observed at a chemical shift of -1.95Hz (width at half height = 6.8Hz). This signal seemed to correspond to the chemical shift of trifluoroacetate anion in aqueous solution. However, since we do not have chemical shifts of model hydrazine or a macromolecular aldehyde coupled with the hydrazide compound for comparison, the signal could be that of the covalently bound reagent and not of the trifluoroacetate ion since the latter ions ought to have been removed when passed through the Sephadex G-25 column. Nevertheless, the same derivatized sample was eluted through the Sephadex column again to ensure removal of any trifluoroacetate. A similar spectrum of the NMR was observed when the void volume fraction was lyophilized and reconstituted again.

Similar experiments were carried out with Triton X-100 glycophorin enriched extract (83) and pure glycophorin (85). These glycophorin preparations were derivatized with TFG and passed through the Sephadex G-25 column once before recording the ¹⁹F NMR spectrum. Though a signal was observed after 4000-5000 scans at a chemical shift of -7.8Hz,
Figure 6

$^{19}\text{F}$ Fourier transform nuclear magnetic resonance spectrum of derivatized fetuin.

Legend

Derivatized sample (3000nmoles of sialic acid containing 12mg of protein) eluted once through the Sephadex column, eluate pooled together, lyophilized and reconstituted to 1ml with $\text{D}_2\text{O}$ before recording $^{19}\text{F}$ NMR spectrum.

Chemical shifts relative to $\text{CF}_3\text{COONa}(1\text{M})$

(a) Reagent Standard (20mM) NS = 44

(δ) A = 0.13 ppm (10.7Hz)
B = 0.02 ppm (1.95Hz)

(b) TFG derivatized fetuin NS = 6800

(δ) A = -0.02 ppm (-1.95Hz)
another weaker signal (relative height 68%, relative area 41%) at a chemical shift of -19.5Hz was also observed (Fig. 7) for the Triton X-100 glycophorin. Similarly, resonances of chemical shifts of 1.5Hz (strong) and -13.2Hz (weak) (relative height 35%, relative area 16.6%) were observed for the purified glycophorin sample (Fig. 8). In all cases, the two peaks were about 14Hz apart which conformed to that of the standard Figure 4, 7a. However, the relative height of the small peak was different in all cases and even larger variations will be seen in samples below. Such an observation was also made with a freshly derivatized fetuin sample (Fig. 9) with chemical shifts at -3.9Hz (strong) and -18.6Hz (weak) (relative height 40%, relative area 15%). Since the $^{19}$F NMR spectra only sometimes showed the upfield signal and, if present, at widely different relative intensities, it was considered at this stage that the upfield signal might either be due to hydrolysis of the bound reagent via Schiff base breakdown or via loss of covalent trifluoroacetyl group as illustrated in Scheme 7. The latter doubt could be ruled out since most of the experimental techniques were carried out at room temperature or below and no hydrolysis of reagent takes place under the above conditions. Furthermore, a Triton X-100 glycophorin sample and a fetuin sample were separately passed through the Sephadex column for the third time to ensure that the upfield
Figure 7.

$^{19}$F Fourier transform nuclear magnetic resonance spectrum of TFG derivatized Triton X-100 glycophorin extract after removal of Triton X-100.

Legend

Triton X-100 removed from glycophorin extract with Bio-Beads SM2 before derivatization. Sample containing 1500nmoles of sialic acid and 6mg of protein was derivatized and eluted three times through the Sephadex column. Each time the eluate (void volume) was pooled and lyophilized. For $^{19}$F NMR observation the sample was reconstituted to 1ml with D$_2$O.

Chemical shifts relative to CF$_3$COONa(1M).

(a) Reagent Standard (20mM) NS = 39

(δ) A = -0.046 ppm (-3.9Hz)

B = -0.195 ppm (-16.6Hz)

(b) TFG derivatized glycophorin (Triton X-100 extract) NS = 15000

(δ) A = -0.09 ppm (-7.813Hz)

B = -0.23 ppm (-19.531Hz)
Figure 8

$^{19}$F Fourier transform nuclear magnetic resonance spectrum of TFG derivatized purified glycoporphin.

Legend

Derivatized sample (1900nmoles of sialic acid containing 1mg of protein) eluted two times through the Sephadex column.

Chemical shifts relative to CF$_3$COONa.

(a) Reagent Standard (20mM) $\text{NS} = 8$

\begin{align*}
(\delta) \quad A &= -0.06 \text{ ppm (-5.8Hz)} \\
B &= -0.21 \text{ ppm (-17.6Hz)}
\end{align*}

(b) TFG derivatized purified glycoporphin

$\text{NS} = 10470$

\begin{align*}
(\delta) \quad A &= 0.02 \text{ ppm (1.5Hz)} \\
B &= -0.16 \text{ ppm (-13.2Hz)}
\end{align*}
Figure 9

$^{19}$F Fourier transform nuclear magnetic resonance spectrum of TFG derivatized fetuin.

Legend

Derivatized sample eluted two times through the Sephadex column.

Chemical shifts relative to CF$_3$COONa.

(a) Reagent Standard (20mM) $NS = 5$

($\delta$) A = -0.118 ppm (-10Hz)

B = -0.26 ppm (-22Hz)

(b) TFG derivatized fetuin $NS = 5500$

($\delta$) A = -0.05 ppm (-3.9Hz)

B = -0.218 ppm (-18.6Hz)
SCHEME 7: Various Possibilities of Hydrolysis of the Reagent
peak was not CF$_3$COO$^-$. The resultant $^{19}$F NMR spectrum was unchanged.

Rando et al. (71) have measured the stability of hydrazones formed with radioactively labelled acethyldrazide incorporated into bovine erythrocyte membranes. They reported that periodate-treated cells yielded some hydrazone bonds of limited stability so that after 6h at 30°C 40-50% of the radioactivity was lost while the remaining 50% of the hydrazone moieties were bound in a stable fashion without further loss over the next few hours. Such a situation might exist in the present system with TFG. The hydrazone moieties bound after periodate oxidation followed by treatment with the hydrazide could be reversible or unstable (71) giving rise to the signal. If complete reversibility occurred in the present samples, then passing them three times through the Sephadex G-25 column should eliminate the major peak or cause it to appear with lower intensity, hence many more scans would be required for adequate signal to noise ratio. This did not occur in the present cases. On the other hand, if 50% of the hydrazone moieties were stable to hydrolysis (71) for more than 6h, three passes through the gel filtration column would be expected to wash out the labile hydrazones in a stepwise manner. Thus a sharp signal at the chemical shift of free hydrazide might be expected until loss of the
labile hydrazones was complete. In any case, the series of gel filtrations ruled out the possibilities that the downfield signal was either trifluoroacetate or the free hydrazide. Though it was not possible to make a quantitative measurement of the bound hydrazones, the fact that a well-defined signal was obtained within 10,000 scans after successive gel filtrations indicated more than 50% of the original trifluoroacetyl hydrazone moieties were still bound. To further address the possibility of free hydrazide's being formed to account for the loss of "labile" (71) hydrazones, a different approach was taken. Sodium borohydride, which is known to stabilize hydrazone bonds (94), was added to a newly derivatized fetuin sample. Whether the sample treated was eluted through G-25 once, twice or three times (Fig. 10) the results were similar in that only a small upfield signal (relative height 30%, relative area 6%) was observed. This small peak could possibly be due to free hydrazide formed from hydrazones that escaped sodium borohydride reduction although it is unlikely that it would survive three gel filtrations. Alternatively, it could perhaps be due to a fluorine-containing hydrazone or reduced hydrazone moiety on some other sugar residue. Incorporation into other sugar residues would be a distinct possibility if the periodate oxidation was not strictly confined to sialyl residues (91–93). Incorporation of TFG could have been further
Figure 10

$^{19}\text{F}$ Fourier transform nuclear magnetic resonance spectrum of TFG derivatized fetuin sodium borohydride reduced.

Legend

Derivatized sample (3000nmoles of sialic acid containing 12mg of protein) eluted three times through the Sephadex column.

Chemical shift relative to CF$_3$COONa NS = 6000

\[
\begin{align*}
\delta & = -0.09 \text{ ppm} (-7.8\text{Hz}) \\
B & = -0.025 \text{ ppm} (-21.5\text{Hz})
\end{align*}
\]
investigated by neuraminidase treatment (before and after derivatization) to strengthen the specific labelling interpretation of the reagent's behaviour. However, control experiments with fetuin and Triton X-100 glyco- phorin in which periodate oxidation was omitted prior to incubation with TFG reagent followed by one gel filtration indicated no incorporation of the reagent even after accumulation of approximately 30,000 spectra. This suggests that incorporation of the reagent is specific for aldehyde groups.

Small chemical shift differences in sugar-bound trifluoroacetyl groups have been reported by Midoux et al. (65). They found the two trifluoroacetyl groups of 1-O-methyl-di-N-trifluoroacetyl β-chitobioside to have chemical shifts differing by 6Hz. Similar small differences, about 30Hz, were reported by Millett and Raftery (95) for N-trifluoroacetyl groups on acylated glucosamine oligomers and about 20Hz by Alter and Magnuson (96) for α and β anomers of N-trifluoroacetyl-D-glucosamine. In view of these examples, the present observations of chemical shifts differing by about 14Hz could indeed be due to different types of sugar-bound trifluoroacetyl groups.

$^{19}F$ chemical shifts occur over a much larger range than $^1H$ NMR chemical shifts reflecting the much greater sensitivity of $^{19}F$ chemical shifts to the chemical environ-
ment of the nucleus (99) and therefore it should be a 
good nucleus to use as a reporter group. However, the 
sensitivity of individual fluorine nuclei to inductive 
and field effects is largely negated for the trifluoro-
methyl group. Examples of small molecules are given 
above. In the case of macromolecules, for example, 
studies on wheat germ agglutinin–sugar interaction (66), 
WGA–sugar glycoside interaction (65), fluorotyrosine 
alkaline phosphatase (55) and lysozyme (98) report small 
changes in chemical shifts upon association with or 
incorporation into the protein. In the present investi-
gation, the trifluoroacetyl chemical shift appears to move 
about 14 Hz downfield for the bound reagent relative to 
the free. The respective changes in chemical shifts were 
similar to chemical shift values determined by the fore-
going workers. However, this change in chemical shift 
could (a) be due to the difference between hydrazide and 
hydrazone bound trifluoroacetyl groups, or (b) could 
reflect genuine differences in environment due to the 
macromolecules. At present, this has not been resolved.

One noteworthy aspect of the TFG-derivatized proteins 
presented here is that linewidths, approximately 6 to 11 Hz 
are not greatly different from that of the free reagent. 
This is in contrast to results of protein sulphydryl 
alkylation by 3-bromo-1,1,1-trifluoropropanone where line-
widths of 20–40 Hz were reported (75–78). This lack of
broadening in the present samples may be indicative of segmental motion of the glycoprotein oligosaccharides which allows them to appear more mobile than the macromolecule as a whole. Other studies have pointed to the same phenomenon (41,98-100).

It was clear at this stage that the major peak or downfield peak at about -9±3Hz was due to the covalently bound hydrazone or its reduction product and therefore the investigation was extended to wheat germ agglutinin interaction studies. Wheat germ agglutinin is a protein with a molecular weight of 36,000 composed of two identical polypeptide chains each possessing two binding sites for sugars (101-103). The sugar-binding specificity of this lectin (107) has been generally accepted to be based on 2-acetamido-2-deoxy-D-glucose(GlcNAc), its β-1,4 linked oligomers (103,105-106) and sialyl residues of glyconjugates (113,114). Bhavanandan et al. (107) investigated hemagglutination with WGA employing sialyl and asialoglycoproteins as hemagglutination inhibitors. Some of their results, shown in Table 3, indicated that very low concentrations of α-1-acid of the corresponding asialoglycoproteins were required for 50% inhibition of the agglutination activity of wheat germ agglutinin. Ovine submaxillary mucin and fetuin WGA interactions have also been extensively investigated by Peters et al. (108). Both of these groups clearly indicated the direct role of
Table 3: Inhibitory Effect of Glycoconjugates on the Agglutinating Activity of Wheat Germ Agglutinin

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration needed for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$-Acid glycoprotein</td>
<td>1.4\mu M</td>
</tr>
<tr>
<td>Asialo $\alpha_1$-acid glycoprotein</td>
<td>&gt;20.8\mu M</td>
</tr>
<tr>
<td>Fetuin</td>
<td>20.8\mu M</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>&gt;52.7\mu M</td>
</tr>
<tr>
<td>Glycophorin MM, NN</td>
<td>0.16, 0.32\mu M</td>
</tr>
<tr>
<td>Asialoglycophorin</td>
<td>&gt;32.5\mu M</td>
</tr>
</tbody>
</table>

Data taken from (107).
N-acetylneuraminic acid residues of sialoglycoproteins in the specific interaction with WGA. The lectin has also been extensively used as a tool for the isolation and characterization of a variety of glycoconjugates including cell surface components (109-112). Dynamic properties of the oligosaccharide moieties of membrane glycoproteins (43,44) and gangliosides (45) have been investigated employing WGA.

A fetuin sample derivatized, borohydride reduced and gel filtered three times, was observed by $^{19}$F NMR spectroscopy. Figure 11 represents the spectra taken in the absence and presence of WGA. No apparent chemical shift difference was observed but measurement of the linewidth at half height of the peak, 11Hz, did show some broadening relative to that of authentic hydrazide or TFA$^-$, 3Hz. Upon treatment with WGA narrowing of the linewidth to about half, 5.3Hz, (Fig. 11) was observed. Another derivatized fetuin sample that had undergone sodium borohydride reduction but had been eluted only once through the Sephadex G-25 column showed linewidths of 8Hz both in the absence and presence of WGA (Fig. 12a and b). The same linewidths were observed with a third fetuin sample derivatized under similar conditions and at a concentration of 500nmoles sialyl groups per ml, treated with lectin at a concentration of 6mg per ml (Fig. 12c and d). Likewise, a purified glycophorin sample
Figure 11

$I^F$ Fourier transform nuclear magnetic resonance spectrum of fetuin (before and after treatment with WGA).

Legend

Derivatized sample gel filtered three times.

Chemical shifts relative to CF$_3$COONa.

(a) Fetuin (3000nmol of sialic acid containing 12mg of protein) derivatized with TFG followed by sodium borohydride reduction.

$\text{NS} = 11200$

(δ) $A = -0.08 \text{ ppm} (-6.8\text{Hz})$

$B = -0.25 \text{ ppm} (-21.5\text{Hz})$

(b) Fetuin (as above) after incubation with WGA (4mg/ml) for 2h. $\text{NS} = 20000$

(δ) $A = -0.09 \text{ ppm} (-7.8\text{Hz})$
Figure 12

$^{19}F$ Fourier transform nuclear magnetic resonance spectrum of fetuin, TFG derivatized and sodium borohydride reduced. (Before and after treatment with WGA.)

Legend

Derivatized sample eluted once through the Sephadex column.

Chemical shifts relative to CF$_3$COONa (1M).

(a) Fetuin derivatized with TFG (1500nmoles of sialic acid per 6mg of protein) NS = 2600

(δ) A = -0.07 ppm (-5.9Hz)

B = -0.24 ppm (-21.5Hz)

(b) Same as (a) with WGA (4mg/ml) NS = 2600

(δ) A = -0.07 ppm (-5.9Hz)

B = -0.24 ppm (-20.5Hz)

(c) Fetuin derivatized with TFG (500nmoles of sialic acid/ml with 2mg of protein) NS = 5100

(δ) A = -0.07 ppm (-5.9Hz)

(d) Fetuin (500nmoles of sialic acid/ml with 6mg/ml of WGA) NS = 12000

(δ) A = -0.07 ppm (-5.9Hz)

B = -0.24 ppm (-20.5Hz)

No apparent change in linewidth data (both cases about 8Hz).
that had been derivatized without sodium borohydride reduction and passed once through the Sephadex G-25 column revealed an apparent linewidth narrowing from 6 Hz to 3 Hz (Fig. 13) in the presence of WGA. In two of the cases above and one later to be seen with erythrocyte ghosts, the presence of WGA seems to cause linewidth narrowing and no chemical shift in the $^{19}$F resonance of the probe. Spin-labelling investigations of glycophorin (free and incorporated into model lipid bi-layers) (43,44) and $^{19}$F NMR of glycoside sugars (98) revealed noticeable broadening effects when bound to WGA and concanavalin A (plant lectin), respectively. With the present results, it was thought that the lectin binding site on sialyl residue was perturbed by TFG derivatization, preventing interaction with WGA. This possibility was eliminated by performing a WGA titre with TFG derivatized red blood cells. The titre values (Table 4) were similar to those of the TFG and non-TFG derivatized. Therefore, the narrowing of the linewidth could be an indirect phenomenon of WGA interaction and that there is no direct interaction of WGA with the sialyl residue. This suggestions is in agreement with the work of Capaldi and Taylor (73) in this laboratory who observed no cross-linking of wheat germ agglutinin with sialyl residues after incorporation of a photolabile hydrazide and subsequent monitoring by gel electrophoresis. However, the apparent narrowing of
Figure 13

\(^{19}\text{F}\) Fourier transform nuclear magnetic resonance spectrum of purified glycophorin (before and after treatment with WGA).

Legend

Derivatized sample eluted two times through the Sephadex column.

Chemical shifts relative to CF\(_3\)COONa.

(a) Reagent Standard (20mM) \(\text{NS} = 8\)

\(\delta\)  
\(A = -0.08\ \text{ppm} \ (5.8\text{Hz})\)
\(B = -0.21\ \text{ppm} \ (17.6\text{Hz})\)

(b) Glycophorin (1900nmoles of sialic containing 1mg of protein) derivatized with TFG. 
\(\text{NS} = 11200\)
\(\delta\)  
\(A = 0.02\ \text{ppm} \ (1.5\text{Hz})\)
\(B = -0.16\ \text{ppm} \ (13.2\text{Hz})\).

(c) As (b) treated with WGA (4mg/ml) \(\text{NS} = 20000\)
\(\delta\)  
\(A = 0.02\ \text{ppm} \ (1.5\text{Hz})\)
\(B = -0.13\ \text{ppm} \ (10.3\text{Hz})\)
Table 4: Wheat Germ Agglutinin Titre Studies of Fetrin and Red Blood Cells.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Component</th>
<th>Before derivatization</th>
<th>After derivatization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetrin</td>
<td>1mg/ml</td>
<td>--</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>12.5µg/ml</td>
<td>12.5µg/ml</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Concentrations given are the lowest WGA concentration at which agglutination of 2mg of fetrin or 2\% (v/v) human erythrocytes were observed in a set of 2 fold serial dilutions of lectin.
the linewidth may also indicate the possibility of increased motion of the sialic acid residues. Strict conclusions of this apparent property needs further investigation in particular with respect to linewidth measurements (Table 5). The trifluoroacetyl group was chosen as the reporter in the present study because it would provide a strong singlet spectrum and therefore alleviate potential sensitivity problems. However, one drawback of this choice is that the group is not as responsive to environmental changes as might be wished (95).

The WGA interaction study was extended to erythrocyte ghosts. The TFG-derivatized ghosts exhaustively washed free of excessive TFG reagent were packed into the NMR tube without solubilization or extraction. Linewidth comparison with respect to the standard revealed an appreciable broadening of up to 18Hz although this assessment was complicated by the presence of two or more components. This may indicate that the erythrocyte oligosaccharide moieties are restricted in the native form. This indication appears to concur with fluorescence studies carried out by Low et al. (113) who compared the mobility of fluorescent probes incorporated at the sialic acid residues on the intact membrane and the trypsin-liberated glycopeptides. A four-to-nine-fold decrease in the rotational correlation time accompanied proteolytic removal of the labelled glycopeptides from the membrane (113).
Table 5: Linewidth Data of the Various Derivatized Sample

<table>
<thead>
<tr>
<th>Components</th>
<th>G-25</th>
<th>NaBH₄</th>
<th>Linewidth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin (Fig. 6)</td>
<td>1X</td>
<td>NONE</td>
<td>6.8Hz</td>
</tr>
<tr>
<td>Fetuin (Fig. 10)</td>
<td>3X</td>
<td>NONE</td>
<td>8Hz</td>
</tr>
<tr>
<td>Fetuin (Fig. 9)</td>
<td>2X</td>
<td>NONE</td>
<td>6Hz</td>
</tr>
<tr>
<td>Glycophorin (Fig. 8)</td>
<td>2X</td>
<td>NONE</td>
<td>6Hz</td>
</tr>
<tr>
<td>Glycophorin⁺ + WGA (Fig. 13)</td>
<td>2X</td>
<td>NONE</td>
<td>3Hz</td>
</tr>
<tr>
<td>Triton X-100 glycophorin (Fig. 7)</td>
<td>3X</td>
<td>NONE</td>
<td>6Hz</td>
</tr>
<tr>
<td>Fetuin (Fig. 12)</td>
<td>1X</td>
<td>YES</td>
<td>8Hz</td>
</tr>
<tr>
<td>Fetuin + WGA (Fig. 12)</td>
<td>1X</td>
<td>YES</td>
<td>8Hz</td>
</tr>
<tr>
<td>Fetuin (Fig. 11)</td>
<td>3X</td>
<td>YES</td>
<td>11Hz</td>
</tr>
<tr>
<td>Fetuin + WGA (Fig. 11)</td>
<td>3X</td>
<td>YES</td>
<td>5.5Hz</td>
</tr>
<tr>
<td>Ghosts (Fig. 14)</td>
<td>--</td>
<td>NONE</td>
<td>Broadened spectra with multiple components</td>
</tr>
<tr>
<td>Ghosts + WGA (Fig. 14)</td>
<td>--</td>
<td>NONE</td>
<td>Narrowed and simplified</td>
</tr>
</tbody>
</table>

ᵃGlycophorin sample as Fig. 8.
Figure 14

$^{19}F$ Fourier transform nuclear magnetic resonance spectrum of TFG derivatized erythrocyte ghost samples.

Legend

Chemical shifts relative to CF$_3$COONa (1M).

(a) Ghost (3.6mg of protein and 400nmoles of sialic acid) TFG derivatized NS = 20000

(δ) $A = -0.02$ ppm (-1.95Hz)

(b) As (a) incubated with WGA (1mg/ml) NS = 11000

(δ) $A = 0.01$ ppm (1.0Hz);

$B = -0.1$ ppm (-8.8Hz)

(c) Ghost (newly derivatized under similar conditions) NS = 9600

(δ) $A = -0.06$ ppm (-5.6Hz)

$B = 0.08$ ppm (7Hz)

$C = 0.125$ ppm (10.6Hz)

(d)$^a$ Ghosts (100nmoles of sialic acid/ml with 4mg/ml of WGA) NS = 10000

(δ) $A = -0.06$ ppm (-5.6Hz)

(e) Ghosts (400nmoles of sialic acid/ml with 1mg/ml WGA) NS = 9500

Spectra not shown. Similar chemical shifts as (c) were obtained.

---

$^a$Apparent change in linewidth (6Hz in the absence and 4.8Hz in the presence of lectin).
Although no attempt was made to measure the correlation time, the TFG-derivatized erythrocyte ghosts treated with WGA in the present work showed an apparently narrowed spectrum again with at least two components. Although exact estimation of linewidths was not possible, comparison of Figure 14a and b, the signal to noise ratio and the number of scans accumulated for each, it is clear that the spectrum taken in the presence of WGA was markedly sharpened.

On the other hand, a newly washed erythrocyte ghost preparation derivatized under similar conditions showed two or more components but these were well resolved. The linewidth with lectin at a concentration of 1mg per ml in the presence of 400nmoles of membrane sialyl groups per ml was 4.8Hz (Fig. 14, legend e). The same linewidth was observed with lectin at 4mg per ml in the presence of 100nmoles of membrane sialyl groups per ml (Fig. 14d). These linewidths may be compared to the ones which were 6.0Hz for the corresponding sample without lectin, (Fig. 14c). The apparent narrowing of linewidth in the former case (Fig. 14a and b) contradicts the spin-labelling investigations carried out on glycophorin (free and incorporated into lipid bilayers) by Grant and co-workers (43,44) who observed a large increase in the correlation time on interaction with WGA at concentrations over a narrow range (0.04mM-0.08mM) (Fig. 15). This was
Figure 15

Oligosaccharide immobilization as a function of WGA concentration (43).

Legend

Spin-labelled glycophorin was incorporated into a lipid bilayer system and correlation time was calculated by measuring linewidths as a function of WGA concentration. The ESR spectra were run at 21°C in buffered saline containing 2mM Ca$^{2+}$ and Mg$^{2+}$. 
Figure 15 (43)
interpreted to arise from decreased mobility of the spin probe due to the binding of WGA in close proximity to the nitroxide. The sharp concentration dependence (Fig. 15) of the observed effect was interpreted (43) as evidence for positive co-operativity for the binding of the lectin to its receptor. Feix and Butterfield (48,114) found correlation times for the spin labels on membrane-bound glycophorin to be similar to that determined by Grant and co-workers (43,44) with purified glycophorin incorporated into liposomes. In contrast, in the presence of WGA, Feix and Butterfield found an increased correlation time. They attributed the differences to the different systems used, liposomes versus whole ghosts. In addition, Feix and Butterfield used hypotonic buffers compared to the physiological ionic strength used by Grant and co-workers for glycophorin-WGA interaction studies. In the present study, however, low ratios of lectin to sialyl groups concentrations (Figs. 11, 12a and b, 13, 14a and b) and higher ratios of lectin to sialyl group concentrations (Figs. 12c and d, 14b and d) did not lead to any observation of significant linewidth differences although an apparent linewidth narrowing was observed in some of the lectin-sugar interaction studies (Figs. 11, 13, 14a and b). However, these apparent linewidth narrowing effects observed with lectin-glycoprotein mixtures, should be reversed in the
presence of the inhibitory sugar, N-acetylglucosamine. This was not observed upon addition of N-acetylglucosamine (final concentration 100 mM) although agglutination clumps were solubilized. Therefore, to make concrete conclusions, further investigations have to be carried out, such as obtaining the linewidths of the $^{19}$F NMR peak over a range of WGA concentrations as investigated by Grant and co-workers (43,44) and subsequent effects in the presence of the inhibitory sugar.

In conclusion, the major findings of the present project indicate that TFG hydrazone covalently binds to the selectively oxidized sialic acid (91-93) of glycophorin by hydrazone formation. Good spectra were obtained within 500 scans at 1500 nmol of sialyl groups per ml and within 10,000 scans for lower concentrations of sialyl groups, such as 200 nmol per ml, reasonable spectra were obtained. Another small signal of varying intensity was observed at about 13±3 Hz upfield relative to the major peak. This could possibly be interpreted as a TFG hydrazone moiety on some other sugar residue (due to non-selective periodate oxidation). The trifluoroacetyl group chosen as the reporter group appears to be less sensitive to changes in chemical constitution or environmental change. Preliminary investigations of interactions of TFG-derivatized glycophorin (soluble) and erythrocyte ghosts with WGA appear to agree with the work
of Feix et al. (114), but further analysis are required to make strong conclusions.

Future $^{19}$F NMR investigations of this apparent property with the present TFG reagent could be examined after oxidation with galactose oxidase, before and after neuraminidase treatment of soluble and membrane-bound glycophorin followed by treatment with the appropriate lectins including WGA. This would help to resolve the question of narrowing or broadening effect (commonly observed for WGA-sialyl residue interaction) of sugar lectin interaction. Such spectroscopic probes would be useful for characterizing cell surface-ligand interactions and for monitoring changes in cell surface components as, for example, those accompanying malignant transformation.
APPENDIX I

Protein Standard Curve (82)

Legend

Protein standard curve by Peterson's method using Bovine Serum Albumin as the standard. Absorbance measured at 750nm.
ABSORBANCE at 750 nanometers

B.S.A. CONCENTRATION (mg/ml)
APPENDIX II

Sialic Acid Standard Curve (82)

Legend

Sialic acid standard curve (MBTH Assay).

N-Acetylneuraminic acid from *Escherichia coli* was used as the standard. Absorbance measured at 670nm.
ABSORBANCE at 670 nanometers

SIALIC ACID CONCENTRATION (nanomoles/ml)
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