SPIN LABELING AND ANALYSIS OF ERYTHROCYTE SURFACES

by

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(Department of Chemistry)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February, 1985

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Date Apr. 25 1985
Spin labeling the oligosaccharides of the red cell membrane was achieved via selective oxidation of gal/galNAc (with galactose oxidase) or sialic acid residues (with mild periodic acid) followed by reductive amination of the oxidized sugars with NaBH$_3$CN and TEMPAMINE.

Spin labeling the galactose residues resulted in low yields and specificity, hindering analysis of the spin labeled cells (SL-RBC). Higher specificity and yields were obtained by labeling sialic acids. A protocol was devised which gave maximum yields with no Heisenberg exchange or membrane alterations (as detected by gel electrophoresis). Detailed analysis of the product showed the majority of the spins to be on the PAS positive membrane proteins (glycophorin A, B and C), only 8% being associated with the lipids. Isolation of glycophorin A, the major sialoglycoprotein of the red cell membrane, revealed two modified sialic acids per molecule.

Successful ESR interpretations could only be done by lysing the SL-RBC (producing SL-ghosts), eliminating spins which had become internalized (rather than covalently attached to the surface) during the reductive amination step. Assuming a random distribution of biradicals (since there were two spins per glycophorin), an average separation of 16 ± 2 angstroms was calculated between the nitroxides.

The spin labeled sialic acids exhibited relatively mobile spectra with $\tau_c = 9 \times 10^{-10}$ s. Upon addition of wheat germ agglutinin (WGA), a
A lectin known to bind to glycophorin, the mobility of the spin label decreased. Even though WGA binding to SL-ghosts showed complex behaviour as detected by Scatchard plots (which required compensation for WGA impurities and non-specific binding), the ESR was only sensitive to the specific binding, the spin mobility decreasing with increasing WGA.

The fact that the spin probe was monitoring sialic acids interactions was confirmed by addition of other lectins. Only lectins which interact with glycophorin altered the ESR signal.
# TABLE OF CONTENTS

## INTRODUCTION

1

## CHAPTER 1  BACKGROUND

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 The Red Blood Cell</td>
<td>5</td>
</tr>
<tr>
<td>1.1(a) The Red Cell in vivo</td>
<td>5</td>
</tr>
<tr>
<td>1.1(b) History of the Cell Membrane Studies</td>
<td>8</td>
</tr>
<tr>
<td>1.1(c) The Red Blood Cell Membrane</td>
<td>11</td>
</tr>
<tr>
<td>1.1(c)(i) Lipids</td>
<td>12</td>
</tr>
<tr>
<td>1.1(c)(ii) Proteins</td>
<td>14</td>
</tr>
<tr>
<td>- The cytoskeleton</td>
<td>17</td>
</tr>
<tr>
<td>- Catalytic functions, Band 3</td>
<td>21</td>
</tr>
<tr>
<td>- Band 4.5</td>
<td>23</td>
</tr>
<tr>
<td>- Contact or receptor proteins</td>
<td>24</td>
</tr>
<tr>
<td>- Minor components of the membrane</td>
<td>27</td>
</tr>
<tr>
<td>1.2 Lectins</td>
<td>34</td>
</tr>
<tr>
<td>1.3 Chemical Modification</td>
<td>36</td>
</tr>
<tr>
<td>1.3(a) NANA Oxidation</td>
<td>36</td>
</tr>
<tr>
<td>1.3(b) Galactose Oxidation</td>
<td>37</td>
</tr>
<tr>
<td>1.3(c) Reductive Amination</td>
<td>38</td>
</tr>
<tr>
<td>1.4 Electron Spin Resonance</td>
<td>40</td>
</tr>
<tr>
<td>1.4(a) Nitroxides</td>
<td>40</td>
</tr>
<tr>
<td>1.4(b) Electron Spin Resonance:General</td>
<td>41</td>
</tr>
<tr>
<td>1.4(c) Nitroxide ESR</td>
<td>42</td>
</tr>
<tr>
<td>1.4(d) Artifacts</td>
<td>51</td>
</tr>
<tr>
<td>1.4(e) Advantages</td>
<td>53</td>
</tr>
</tbody>
</table>

## CHAPTER 2  SPIN LABELING THE GALACTOSE RESIDUES OF THE RED CELL SURFACE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Introduction</td>
<td>54</td>
</tr>
<tr>
<td>2.2 Materials and Methods</td>
<td>55</td>
</tr>
<tr>
<td>2.2(a) Collection of Red Blood Cells</td>
<td>55</td>
</tr>
<tr>
<td>2.2(b) Galactose Oxidase Oxidation of Red Cells</td>
<td>55</td>
</tr>
<tr>
<td>2.2(c) Spin Labeling</td>
<td>56</td>
</tr>
<tr>
<td>2.2(d) Ghost Preparation</td>
<td>56</td>
</tr>
<tr>
<td>2.2(e) Microelectrophoresis</td>
<td>57</td>
</tr>
<tr>
<td>2.2(f) Viscometry</td>
<td>57</td>
</tr>
<tr>
<td>2.2(g) Electron Spin Resonance</td>
<td>58</td>
</tr>
<tr>
<td>2.2(h) Quantitation</td>
<td>58</td>
</tr>
<tr>
<td>- (i) Red Cell or Ghost Count</td>
<td>58</td>
</tr>
<tr>
<td>- (ii) ESR</td>
<td>59</td>
</tr>
<tr>
<td>- (iii) Protein</td>
<td>59</td>
</tr>
<tr>
<td>2.2(i) Miscellaneous</td>
<td>60</td>
</tr>
</tbody>
</table>
CHAPTER 3 SPIN LABELING THE SIALIC ACID RESIDUES OF THE RED CELL SURFACE

3.1 Introduction ............................................. 86
3.2 Materials and Methods
   3.2(a) Periodate Oxidation of Red Cells
         and Ghosts ........................................... 89
   3.2(b) Periodate Oxidation of Glycophorin
         and Fetuin .......................................... 89
   3.2(c) Spin Labeling of Red Cells .................. 90
   3.2(d) Spin Labeling Ghosts by the Method of
         Felix and Butterfield (1980) .................. 91
   3.2(e) Formaldehyde Assay for Red Cells ........ 91
   3.2(f) Sialic Acid Assays ............................... 92
   3.2(g) Rouleaux .......................................... 92
   3.2(h) ESR .................................................. 92
   3.2(i) Measurements of the Distance Between
          Nitroxide Labels ................................... 93
   3.2(j) Reversibility ..................................... 93
   3.2(k) Hydrolysis of the Spin Labeled Ghosts ... 94
   3.2(l) Isolations ......................................... 94
   3.2(m) Tritium Analysis ................................ 95
3.3 Results
   3.3(a) Rouleaux vs Periodate Modification ......... 96
   3.3(b) Formaldehyde Formation ....................... 99
   3.3(c) Tritiated Glycophorin ......................... 102
   3.3(d) SDS PAGE Analysis ............................... 104
   3.3(e) Spin Labeling Red Cells ..................... 104
   3.3(f) Background Signal ................................ 110
   3.3(g) Visual Inspection of Oxidized and
          Spin Labeled Cells ................................. 110
   3.3(h) Spin Labeled Ghosts .............................. 112
   3.3(i) F-B SL-ghosts ..................................... 112
   3.3(j) Distance Measurements ......................... 115
   3.3(k) Reversibility ..................................... 115
3.3(1) Neuraminidase or Acid Hydrolysis of SL-ghosts .................................. 116
3.3(m) Lipid Extraction ......................................................... 118
3.3(n) Selective Solubilization ............................................... 119
3.3(o) Isolation of Glycophorin ............................................. 121
3.3(p) Tritium Distribution of SDS PAGE gels.......................... 122
3.4 Discussion ................................................................. 125
3.4(a) Periodate Oxidation .................................................... 125
3.4(b) Spin Labeling ........................................................... 128
3.4(c) Quantitation ............................................................. 129
3.4(d) Location ................................................................. 130
3.4(e) ESR Interpretation ..................................................... 133
3.4(f) Distance between Nitrooxides ...................................... 135
3.4(g) F-B SL-ghosts .......................................................... 137
3.5 Conclusion ........................................................................ 139

CHAPTER 4 WHEAT GERM AGGLUTININ PURIFICATION AND BINDING

4.1 Introduction ................................................................. 141
4.2 Materials and Methods
  4.2(a) Wheat Germ Agglutinin ............................................... 145
  4.2(b) Protein Assays ......................................................... 145
  4.2(c) Agglutination Assay .................................................... 145
  4.2(d) Iodination ............................................................... 146
  4.2(e) SDS PAGE ............................................................. 148
  4.2(f) Spin Labeled Ghosts and Extractions ......................... 148
  4.2(g) Binding Assay .......................................................... 149
  4.2(h) Other Lectins ......................................................... 150
4.3 Results
  4.3(a) WGA Addition to Extractions ...................................... 151
  4.3(b) WGA Plus Various SL-Ghosts ..................................... 153
  4.3(c) Binding Assay .......................................................... 156
  4.3(d) Other Lectins .......................................................... 164
4.4 Discussion
  4.4(a) WGA broadening of the ESR signal .............................. 166
  4.4(b) Mechanism of Broadening ......................................... 169
  4.4(c) WGA Binding Isotherm ............................................... 170
  4.4(d) WGA Receptor on Red Cells ...................................... 173
  4.4(e) Correlation between SP and WGA Binding .................... 176
  4.4(f) Binding of Other Lectins .......................................... 178
4.5 Conclusion ........................................................................ 179
4.6 Summarizing Discussion .................................................. 180

APPENDIX A SDS PAGE ....................................................... 181
APPENDIX B ESR Integration .................................................. 184
# LIST OF TABLES

## TABLE

### CHAPTER 1

1.1 Composition of the red cell membrane ........................................... 11  
1.2 Lipid composition of the red cell membrane ................................. 12  
1.3 Properties, associations and function of the red cell membrane fractions ...................................................... 20  
1.4 Enzymes found in the red cell membrane ....................................... 28  

### CHAPTER 2

2.1 Ghost counting method and grams/ghost obtained .............................. 63  
2.2 Cell electrophoresis of GO or NANase treated and spin labeled red cells .................................................. 66  
2.3 SP for NAGO SL-RBC with the addition of gal binding lectins ......................... 67  
2.4 SP at different hematocrits of RBC plus 1 mM TEMPAMINE ..................... 69  
2.5 Sample manipulation of SL-RBC and resultant SP ................................ 70  
2.6 Spin labeled samples made into ghosts and their SP ........................... 70  

### CHAPTER 3

3.1 Formaldehyde formation as a function of periodate concentration and exposure time to red cells ......................... 99  
3.2 Formaldehyde formation as a function of periodate concentration and exposure to ghosts ........................................... 100  
3.3 Formaldehyde formation as a function of periodate concentration and exposure to glycoporphin and fetuin .......... 102  
3.4 Initial TEMPAMINE concentration and the resultant spins per SL-ghost .................................................. 106  
3.5 Other attempts to improve spins/SL-ghost ...................................... 107  
3.6 Periodate concentration along with spins/SL-ghost and SP in comparison to optimal protocol ................................. 108  
3.7 Spins/SL-ghost and resultant $w_0$ ............................................. 112  
3.8 $d_1/d$ of SL-ghosts and 1:4 and 1:8 SLAN ghosts .............................. 115  
3.9 Percentage release of tritium and spins from SL-ghosts with days incubated at 4°C ................................................ 116  
3.10 Percentage release of tritium, spins and sialic acid compared to neuraminidase treatment or acid hydrolysis of SL-ghosts or F-B SL-ghosts ........................................... 117  
3.11 Percentage extraction of spins and tritium by chloroform:methanol of SL-ghosts and F-B SL-ghosts .............................. 118  
3.12 Percentage extraction of spins and tritium along with their SP of SL-ghosts by NaOH, SDS and Triton X-100 .............................. 119  
3.14 Distribution of tritium on SDS PAGE of various samples ................... 124
CHAPTER 4

4.1 SP of various selective extractions of SL-ghosts after the addition of WGA ........................................ 141
4.2 SP after lectin added to SL-ghosts ........................ 164

APPENDIX D

D.1 Percentage binding of iodinated impure Sigma WGA to chitin and ovomucoid columns ............................ 199
D.2 CM-Sepharose chromatography of commercial WGA .......... 202
D.3 FPLC chromatography of Vector and Sigma WGA .......... 202
D.4 Amino acid composition of WGA from various literature sources ......................................................... 206
LIST OF FIGURES

CHAPTER 1

1.1 Chronological order of proposed membrane models .......... 10
1.2 SDS PAGE of red cell membranes separated by Fairbanks et al 1971 and Laemmli, 1970 ................................. 16
1.3 Nomenclature for the PAS staining bands of red cell membranes ......................................................... 16
1.4 Schematic drawing of the red cell membrane skeleton unit cell ................................................................. 19
1.5 Proposed organization of band 3 in the cell membrane ... 22
1.6 Amino acid sequence of glycophorin A and its arrangement in the red cell membrane .............................. 26
1.7 Diagrammatic representation of the PAS proteins in the red cell membrane ........................................... 26
1.8 Structures of the oligosaccharides found on band 3 and glycophorin A ...................................................... 32
1.9 Cartoon of the outer surface of the red cell membrane ... 33
1.10 Oxidation of sialic acid by periodate .............................. 37
1.11 Oxidation of galactose termini by galactose oxidase ...... 38
1.12 The structure of TEMPAMINE .................................. 39
1.13 Reductive amination of an aldehyde with TEMPAMINE 39
1.14 Effect of the magnetic field upon the unpaired electron and the absorption of microwaves and the resultant ESR spectrum ................................................................. 42
1.15 The effect of the nuclear spin state of N on the unpaired electron ............................................................. 43
1.16 (A) The principal axis for the nitroxide (B) Direction dependence of the Zeeman and the hyperfine interactions of the nitroxide .................................................. 45
1.17 Effect of viscosity on the ESR spectra of nitroxides ... 47
1.18 Powder spectrum showing the heights $d_1$ and $d$ used in calculating average distances between nitroxides ............................... 51

CHAPTER 2

2.1 Percentage of the red cell or ghost population and the trigger levels of the particle counter ...................... 62
2.2 ESR spectra of control RBC and NAGO SL-RBC plus SBA 68
2.3 ESR spectrum of GO SL-ghosts .................................. 72
2.4 SDS PAGE gels of normal and GO SL-ghosts .................. 73
2.5 ESR spectra of NAGO SL-ghosts before and after addition of PNA and SBA .................................................. 74
2.6 Cell viscometry of normal and NAGO SL-RBC with the addition of PNA ...................................................... 76
CHAPTER 3

3.1 Pictures of red cells or periodate treated red cells in 80% plasma .......... 97
3.2 Pictures of red cells or periodate treated red cells in 3% dextran .......... 98
3.3 SDS PAGE gels of periodate oxidized ghosts .................. 101
3.4 SDS PAGE gels of glycophorin and tritium labeled glycophorin .............. 103
3.5 SDS PAGE gels of various red cell samples, oxidized and spin labeled. Stained with PAS or Basic Fuchsin ... 105
3.6 SDS PAGE gels of normal, SL-ghosts and double the periodate spin labeled ghosts ................. 109
3.7 ESR spectra of SL-RBC and controls before and after lysis .................. 111
3.8 ESR spectrum of SL-ghosts showing parameters measured to determine SP .................. 113
3.9 SDS PAGE gels of SL-ghosts and F-B SL-ghosts .................. 114
3.10 SDS PAGE gels of the NaOH pellet and the Triton X-100 pellet and extract of SL-ghosts .................. 120
3.11 SDS PAGE gels of isolated SL-glycophorin and SL-ghost ............. 123

CHAPTER 4

4.1 Schematic illustration of the disposition of the primary and secondary binding locations on the WGA dimer ...... 142
4.2 ESR spectra of the Triton X-100 extract of SL-ghosts before and after the addition of WGA .................. 152
4.3 ESR spectra of SL-glycophorin isolated from SL-ghosts before and after the addition of WGA .................. 154
4.4 Graph of SP of SL-ghosts after addition of WGA (from various commercial sources) compared with the initial WGA added and SP for the binding experiment with impure Sigma WGA of Figs. 4.12 and 4.15a .................. 155
4.5 ESR spectra of SL-ghosts and WGA and the resultant supernatant and pellet after centrifugation .................. 157
4.6 ESR spectra of F-B SL-ghosts before and after addition of WGA .................. 158
4.7 Binding isotherm of $^{125}$I WGA to SL-ghosts for the total and non-specific binding of WGA .................. 159
4.8 Binding isotherm of $^{125}$I WGA to SL-ghosts for specific binding .................. 160
4.9 Scatchard plot of specific binding of Fig. 4.13 ............. 161
4.10 SP and binding of $^{125}$I WGA/SL-ghost for impure Sigma and pure Vector $^{125}$I WGA .................. 162
4.11 ESR spectra of SL-ghosts (A) plus WGA (C) and 2 [D] SL-ghosts (B) plus WGA (D) along with their calculated SP's .................. 163
4.12 ESR spectra of SL-ghosts (A) plus WGA (C). (A) relsysised (B) and WGA (D) along with their calculated SP's ....... 165

APPENDIX B

B.1 ESR spectra before and after being digitized along with the resultant first integration (also rezeroed) and the second integration of the rezeroed first integration .. 185
B.2 Placement of ESR spectrum on the plotter bed and the location of $P_1$ and $P_2$ ................................. 187

APPENDIX D

D.1 SDS PAGE gels of reduced impure $^{125}$I Sigma WGA and pure $^{125}$I Vector WGA ................................. 197
D.2 SDS PAGE gels of impure $^{125}$I Sigma WGA before and after exposure to red cells .................................. 198
D.3 SDS PAGE gels of reduced $^{125}$I Sigma WGA fractions run on ovomucoid and chitin columns .......................... 200
D.4 SDS PAGE gels of reduced Vector WGA run on FPLC and CM-sepharose columns ................................. 203
ABBREVIATIONS

- ab₀:
  isotropic hyperfine constant

- AChe:
  acetylcholinesterase

- ATP:
  adenosine triphosphate

- ATPase:
  adenosine triphosphatase

- B:
  Bohr magneton

- BSA:
  bovine serum albumin

- CHO cells:
  Chinese Hamster Ovary cells

- CM-sepharose:
  carboxymethyl-sepharose

- Con A:
  Concanavalin A

- cpm:
  counts per minute

- d, d₁:
  peak-to-peak intensities in powder spectra as defined in Figure 1.18

- DTT:
  dithiothreitol

- dpm:
  distigrations per minute

- E.C. (followed by a number):
  Enzyme Commission, followed by numbers indicating classification of the enzyme

- EDTA:
  ethylenediamine tetraacetate

- EPR:
  electron paramagnetic resonance

- ESR:
  electron spin resonance

- F-B SL-ghosts:
  SL-ghosts made by the method of Felix & Butterfield

- FPLC:
  fast protein liquid chromatography

- G:
  gauss
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
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<td>$g_{xx}, g_{yy}, g_{zz}$</td>
<td>principal values of the g-tensor</td>
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<td>$g_{//}, g_{\perp}$</td>
<td>parallel and perpendicular components of an axially symmetric g-tensor</td>
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<tr>
<td>gal</td>
<td>galactose</td>
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<td>galNAc</td>
<td>N-acetylgalactosamine</td>
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<td>G-3-PD</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
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</tr>
<tr>
<td>GO SL-RBC</td>
<td>galactose oxidized and spin labeled red cells</td>
</tr>
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<td>GO SL-RBC made into ghosts</td>
</tr>
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<td>externally applied magnetic field</td>
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<td>Peak-to-peak height of a first derivative ESR signal</td>
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<td>integrator sensitivity</td>
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<td>mono sulfate</td>
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<td>M</td>
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<td>$m_I$</td>
<td>magnetic quantum number associated with the component of the nuclear spin</td>
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<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>$m_s$</td>
<td>magnetic quantum number associated with the component of the electron spin</td>
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<td>$m_W$</td>
<td>milliwatts</td>
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<tr>
<td>NAGO SL-RBC</td>
<td>neuraminidase, then galactose oxidase treated and spin labeled red cells</td>
</tr>
<tr>
<td>NAGO SL-ghosts</td>
<td>NAGO SL-RBC made into ghosts</td>
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<td>NANA</td>
<td>N-acetyl neuraminic acid</td>
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</tr>
<tr>
<td>OSM</td>
<td>ovum submaxillary mucin</td>
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<tr>
<td>PAGE</td>
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<tr>
<td>PAS</td>
<td>periodic acid Schiff base</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>Abbr.</td>
<td>Definition</td>
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<tr>
<td>PBS/azide</td>
<td>phosphate buffered saline containing azide</td>
</tr>
<tr>
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<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>pI</td>
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<tr>
<td>SL-ghosts</td>
<td>periodate oxidized and spin labeled red cells made into ghosts</td>
</tr>
<tr>
<td>SL-RBC</td>
<td>periodate oxidized and spin labeled red cells</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>$T_{xx}, T_{yy}, T_{zz}$</td>
<td>principal values of the hyperfine tensor</td>
</tr>
<tr>
<td>$\tau_c$</td>
<td>correlation time</td>
</tr>
<tr>
<td>$\tau_{\parallel}, \tau_{\perp}$</td>
<td>motion about the principal and molecular axis of a nitroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TD</td>
<td>tracking dye</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>microwave frequency</td>
</tr>
<tr>
<td>VC</td>
<td>neuraminidase <em>Vibrio cholerae</em></td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w₀</td>
<td>linewidth of the center line of an nitrooxide ESR signal</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight per weight</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENT

This project was greatly aided by the assistance of numerous people, among these are Foon Yip, who aided in the ESR and lipid work, Mandy Hoskins for doing the cell viscometry work and John Cavanagh, in the isolation of glycophorin. I would also like to thank John Cavanagh and Charlie Ramey for their general help. Stimulating discussions are acknowledged with Dr. Geoffrey Herring, who also provided access to his ESR machinery and commented liberally on the manuscript as did Dr. Manssur Yalpani. My thanks are also due to Drs. Phil Reid, Evan Evans, John Waterton and Mike Bernstein for advice and assistance. A great debt is owed to my wife Sandra Sturgeon, for emotional support and critical reading of this thesis among other things. Thanks are also due to people such as Kim Sharp, Johan Janzen, Tim Webber, Jim Van Alstine and Basil Chui for their discussions and comments.

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INTRODUCTION

Chemistry is a science which deals with isolated systems in a controlled, well defined manner. Molecules can be assigned three-dimensional structures, reactions are known in great detail. Analysis at the atomic level can be easily achieved, giving the chemist an extremely detailed picture of the system of interest.

Biology, on the other hand, is much less clearly defined at the molecular and atomic level. Many interactions of varying complexity occur at the same time. Some of these interactions are unknown and it is hard to isolate these to study individual components. The biologist usually can't determine the cause of an event, but can only infer.

The question asked in this thesis is can techniques, which have been used to analyse complex, isolated systems such as carbohydrates, peptides, oligomers etc., be extended and applied to less well defined biological systems such as cell membrane lectin interactions and still be useful? Can chemical analysis be applied and give answers which are meaningful to biologists?

Of particular interest is the chemical and biochemical function of the plasma membrane. Cell surfaces play a critical role in the response of cells to the external environment, which may contain drugs, hormones, antigens, toxins or infectious agents and may result in agglutination, adhesion or motility (Hughes, 1976; Monsigny, 1979; Rauvala, 1983). The carbohydrate-containing substances, glycolipids and glycoproteins, are good candidates for participating in intercellular recognition and in the binding of regulatory molecules (Hughes, 1975).

Studies concerning membrane carbohydrates have shown all the
carbohydrate to be anchored to the external face of the cell (Winzler, 1972; Gahmberg, 1976). This carbohydrate-rich forest-like structure on the outer surface is known as the glycocalyx (Geyen & Makovitzky, 1980) and typically contains four to eight kinds of sugars (glucose, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, fucose and sialic acid (Hughes, 1975; Schrevel et al, 1982)). It is the glycocalyx that is in contact with the external environment, be it plasma, another cell surface or some in vitro solution or surface. The possible form of linkages for the hexoses is enormous. Three amino acids can produce only six different tripeptides while 1056 trimers are possible with three different hexoses. Biosynthesis of these oligosaccharides is less exact than for proteins and nucleic acids (being under enzymatic control rather than that of a direct genetic template) and leads to variability of structure known as microheterogeneity. This biosynthesis is sensitive to extracellular stimuli and the microheterogeneity may reflect some pathophysiological state of the organism (Hatton et al, 1983). A considerable amount of information, environmentally sensitive, can be "carried" by the glycocalyx, therefore.

The red blood cell is well suited for use in membrane studies. It is easy to obtain in large numbers virtually free of contaminants, a criterion not easily met in biology. Model recognition systems which react with specific elements of the glycocalyx are also available in the form of lectins, proteins which are easily obtained in large quantities that bind certain sugar sequences, offering a means of manipulating the system and mimicking recognition behavior in biology.

In the present work therefore, a lectin/red cell system was selected for
study by quantitative chemical means, to discover the limitations and advantages of such an approach.

When modifying a biological entity such as a red cell, one is constrained by the media compatible with these systems. Conditions near physiological (pH 7, 280 milliosmole, aqueous) must be employed throughout the procedure if information is to be obtained. The next major constraint is selectivity and detectability of the modification. Biological systems for all their complexity are all made up of relatively few building blocks combined in a variety of ways.

Selectivity in modification would render interpretation easier but it is made difficult by the chemical similarity of completely different compounds. Also, the more selective one becomes, the lower the degree of incorporation. For example, to selectively modify the most abundant protein on the red cell membrane (Band 3 at $1 \times 10^6$ copies per cell) at a one to one basis, results in a concentration of $1.8 \times 10^{-5}$ M for packed cells. The reporter group introduced therefore has to be easily detectable as well as report useful information about its environment.

Spin probes (nitroxides are used in this study) are the best suited for analysis of membranes. They offer several very important features which overcome the above restrictions. They can be detected at low concentrations (down to $10^{-6}$ M). Natural occurrence of paramagnetism in biological systems is relatively low, thus there is no ambiguity regarding the source of the signal. They are sensitive to molecular motion and environment, making them a good reporter group. The only significant price paid in using spin probes is the possible perturbation of the system by introduction of the spin label.
Galactose (and N-Acetyl galactosamine) and sialic acid (sugars commonly contained in the glycocalyx) can be selectively oxidized and a spin probe attached to these oxidized sugars via reductive amination, a technique successfully used previously for a variety of macromolecules (Aplin, 1979; Yalpani, 1980; Bernstein, 1983). Thus, in principle, selectivity and sensitivity are obtainable using the spin label method. A careful study of spin labeling of the red cell glycocalyx was thus undertaken to examine the limitations and usefulness of this technique as an in situ membrane probe.

In what follows, to provide the background necessary for interpretation, Chapter one reviews each of the above aspects of this study (chemical modification, ESR, the red cell and lectins). Chapter two deals with quantitation and the effect of spin labeling the galactose residues. Chapter three discusses the effects of periodate modification of red cells and compares it with fetuin (a serum sialoglycoprotein) and glycophorin (the major sialoglycoprotein of the red cell membrane) as well as discussing the results of spin labeling the oxidized sialic acids on red cells. Chapter three also describes attempts at isolation of the spin labeled components of the red cell membrane. To study a model recognition event, the lectin wheat germ agglutinin (WGA) is used. Chapter four deals with the binding of WGA to spin labeled ghosts.
CHAPTER ONE

This chapter deals with the approach used to modify the glycocalyx of the red cell and gives the background material necessary to understand the experimental results in the following chapters. Section 1.1 outlines what is known about red cells with emphasis on the membrane structure and Section 1.2 explains the use of lectins in this study. Section 1.3 deals with the chemical modification necessary to insert spin probes covalently into the glycocalyx and Section 1.4 the theory of electron spin resonance needed to understand and interpret spectra.

1.1 THE RED BLOOD CELL

An enormous amount of information is available on the red blood cell (RBC) especially since the advent of blood transfusions. A better understanding of the red cell results from knowledge of its function and production. The first part of this review briefly covers what is known about red cell production and function followed by a more extensive overview of its membrane structure.

1.1(a) The red cell in vivo

Anton van Leeuwenhoek was the first to see red blood cells, in 1674, describing them as "small globules driven through a cristaline humidity of water" (Grimes, 1980). In 1685, Bidloo described the red cell as "a ballon-like-body in which fluid solutions of hemoglobin were enclosed
by an external envelope" (Lee et al, 1974). This description is surprisingly accurate. Many reviews have been written about the red cell (Berlin & Berk, 1975; Brewer, 1975; Tanner, 1978; Hillman & Finch, 1974; Grimes, 1980; Williams et al, 1983) so only a brief description is offered here.

The red blood cells (or erythrocytes) are produced from stem cells located in the bone marrow of adults. Each stem cell undergoes a series of mitotic divisions producing sixteen smaller cells called orthochromatic normoblasts. Hemoglobin production has already started and the nucleus is compact. Incapable of mitosis, these normoblasts produce more hemoglobin, eliminate the nucleus, organelles and enzymatic pathways not needed for red cell survival yielding a cell called a reticulocyte. The reticulocyte enters the circulation and with the loss of residual RNA becomes a red blood cell. This whole process of specialization takes about seven days.

As the cell differentiates and becomes more specialized, organelles and metabolic pathways are eliminated. Protein and lipid synthesis stops and what remains is a discoid shaped cell filled with hemoglobin surrounded by the cell membrane. Inside this cell are four enzymatic pathways. The major pathway (utilizing 90% of the glucose) is the Embden-Meyerhof pathway, producing ATP and NADH (which are used in the maintenance and flexibility of the cell) and lactate. The other three pathways are used to maintain the function of hemoglobin and, due to the high levels of O₂, to maintain oxidative, reductive homeostasis. Hemoglobin is regulated by a variety of organic phosphates, pH and CO₂ levels. Carbonic anhydrase, the second most abundant protein, facilitates CO₂ transport. In the membrane are found transport proteins for glucose, lactate and HCO₃⁻ with Na⁺/K⁺
and Ca\(^{2+}\)/Mg\(^{2+}\) ATPase pumps to maintain concentration gradients. These metabolic pathways ensure red cell survival and efficient O\(_2\) transfer. There are other enzymes in the cell but at much lower concentrations and some with no known function.

The red cell travels about 175 miles during its lifetime of 120 days. It passes through blood vessels of varying sizes and is exposed to a wide range of shear stresses, spending most of its life in the capillary channels of the microcirculation, sometimes squeezing through openings 1/20\(^{th}\) its diameter. As the cell ages its pliability decreases, membrane is lost, the mean hemoglobin concentration increases and certain glycolytic enzyme activities decrease. The reticuloendothelial tissue (liver, spleen and bone marrow) are phagocytic towards these old cells. The spleen is the most sensitive of these organs. Its network slows down the blood flow, forcing red cells to flow through 3-5 \(\mu\) diameter orifices where cell membranes and abnormal particles are removed.

The red cell in an adult male has an average volume (mean corpuscular volume (MCV)) of 90.1 \(\mu\)m\(^3\). The mean corpuscular hemoglobin concentration is 33.9 g/dl RBC. The average surface area is 145 \(\mu\)m\(^2\) with a diameter from 7.5 to 8.3 \(\mu\)m and a thickness of 2 \(\mu\)m. Hemoglobin constitutes 97.5% of the protein in red cells with 0.8% being enzymes and 1.7% being membrane proteins.

When studying red blood cells, one must be aware that no sample contains only normal human erythrocytes. Three major sources of heterogeneity are genetic variability, especially of many enzymes and hemoglobin (there are at least 500 genetically distinct hemoglobins catalogued in the International Hemoglobin Information Centre), a population variability due to the fact
that red cells age and all stages are contained in a red cell sample, and
variations between donors of different age, especially the very young and

It might be asked if the red cell is too specialized to be useful as a
representative model but all mammalian cells are highly differentiated. The
technical advantages of being able to obtain large, relatively homogeneous
quantities makes it useful. Due to its specialized function, it has a
static membrane, so one can study it without the additional confusion of
membrane turnover. The red cell is also fairly stable to a variety of in
vitro manipulations, making it suitable for membrane studies.

1.1(b) History of cell membrane studies

Early observations by Overton (1895) indicated the cells were surrounded
by a semipermeable membrane which was more permeable to nonpolar than polar
solutes. Gorter & Grendel (1925), measured the surface area of a monolayer
of lipid extracted from red cells and found that there was enough lipid in
an erythrocyte membrane to cover twice the surface area of the cell. They
thus proposed a bilayer structure for the lipid. Luckily, their
underestimation of the surface area compensated for incomplete extraction of
the lipids in their experiments.

Danielli & Davson (1935), suggested that the lipid bilayer was covered
by layer(s) of protein to account for the low surface tension observed.
Electron micrographs of fixed membranes resulted in the
Davson-Danielli-Robertson model (Robertson, 1959). Criticism of this model
arose because one would expect to see beta-sheets of protein instead of the
alpha helix found. Extracted lipid still gave the same electron micrographs as intact membranes and certain ions which in theory shouldn't be permeable in fact were. A huge variety of models was postulated (Fig. 1.1, taken from Finean & Mitchell, 1981). The best accepted one, proposed by Singer & Nicholson in 1972 is the "fluid mosaic" model. This model agrees best with available data. It includes alpha helices in membrane proteins, fluid lipids, lipids not directly interacting with membrane proteins and allows for apparent mobility of macromolecules in the plane of the membrane (Juliano, 1973). The membrane consists of a two dimensional fluid matrix where proteins are intercalated into the fluid lipid bilayer, some all the way through, some only partially, while others rest on the lipid surface.

Although refinements to the fluid mosaic model are still being published (e.g. Israelachvili, 1977) most models currently being presented are detailed studies of individual membranes rather than general structures (e.g. the last model proposed in Fig. 1.1).
Fig. 1.1 Diagram illustrating the chronological order in which the most influential models have been proposed. Each of the structures indicated are described in more detail in the source publication (Finean & Mitchell, 1981).
1.1(c) The red blood cell membrane

"The red cell membrane is giving up some of its secrets, but only slowly and, seemingly, grudgingly" (Brewer et al, 1982). One of the major breakthroughs in red cell membrane analysis came when Dodge et al (1963) were able to lyse red cells free of cytoplasmic contaminants. These membrane preparations are called ghosts because they are difficult to see unless looked at with a phase contrast microscope. Unlike previous methods, that of Dodge et al (1963) gave reproducible ghosts with the major contaminant, hemoglobin, reduced to about 0.04% of the total weight. These ghosts were extensively analysed; Table 1.1 (taken from Juliano, 1973) lists a typical analysis of Dodge ghosts.

**TABLE 1.1** (taken from Juliano, 1973)

<table>
<thead>
<tr>
<th>COMPOSITION OF THE RED CELL MEMBRANE OF DODGE TYPE GHOSTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>Phospholipids</td>
</tr>
<tr>
<td>Hexose</td>
</tr>
<tr>
<td>Hexosamine</td>
</tr>
<tr>
<td>Sialic Acid</td>
</tr>
<tr>
<td>Glycolipid</td>
</tr>
</tbody>
</table>

Analysis of membranes can be broken down into the studies of lipids and proteins and each will be dealt with individually.

1.1(c)i Lipids

There are approximately 5 mg of lipid/10^{10} cells (Rendi et al, 1976) and all are located in the membrane (see Zwaal et al, 1973 and van Deenen & de Grier, 1974 for reviews). Table 1.2 (taken from Grimes, 1980) shows typical values for red cells. Variation in lipid content is found and is due mainly to different isolation techniques (Nelson, 1972).

**TABLE 1.2**

TYPICAL VALUES FOR NORMAL HUMAN RED CELL LIPIDS, EXPRESSED AS % DRY WEIGHT (taken from Grimes, 1980)
The lipid is largely in a bilayer configuration and serves as a barrier or boundary between the outside and the inside of the red cell. The phospholipids are asymmetric in their distribution in the bilayer: 80% of the sphingomyelin, 75% of the phosphatidylcholine (PC) and all the glycolipids are in the outer layer while 100% of the phosphatidylserine (PS) (the only major phospholipid to be charged (negatively) at physiological pH), 80% of the phosphatidylethanolamine (PE) and the majority of the phosphatidylinositol are at the inner or cytoplasmic side of the membrane (Zwaal, 1978; van Deenen, 1981; Eton, 1981). Asymmetry in the head groups is accompanied by asymmetry in the hydrocarbon tail composition and thus in the fluidity of the bilayer (Cogan & Schachters, 1981; Seigneuret et al, 1984).

The glycolipids of red cells contain A, B, H, II blood group determinants (Hakomori, 1981a) as well as the P antigens (Marcus et al, 1981). Hakomori (1981b) raises the possibility that there may be glycolipid-associated proteins. These antigens could regulate, or be regulated by, glycolipids.

The red cell lipids are known to exchange with serum lipids. Cholesterol is in rapid equilibrium with unesterified plasma cholesterol (produced by the plasma enzyme lecithin-cholesterol acyltransferase). PC and sphingomyelin also undergo exchange while it appears that PS and PE do not (Weed & Reed, 1966).

Wallach & Verma (1975) found membrane associated carotenoids (at 7 x 10^{-4} % of lipid) which Lippert et al (1975) also found in variable amounts from donor to donor, possibly washed from ghosts in some experiments. Lewis
antigens are lipids which are transferred from the serum into the red cell membrane (Marcus & Cass, 1969).

It has been shown that shape changes can be induced in the red cell by amphipathic molecules affecting the lipid bilayer (Lange et al., 1982; Landman, 1984). Lipids are also involved in cell fusion processes.

1.1(c)ii Proteins

The membrane proteins give the red cell its shape, stability and supply the majority of surface charge. Analysis of red cell membrane proteins was greatly impeded by their insolubility in ordinary buffers and until the advent of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), they were not very well understood.

SDS disrupts noncovalent interactions and binds to polypeptide chains. The amount of SDS is usually proportional to the mass of the polypeptide and thus proteins migrate on PAGE as a function of molecular size (Reynolds & Tanford, 1970). Kale et al. (1978) and Makino (1979) have shown that SDS does bind to membrane proteins, but certain proteins such as glycoproteins appear to migrate in an anomalous fashion. Other problems are the choice of standards used to determine molecular weights (log(molecular size) vs mobility is not always linear), and that several protein species may migrate at the same place on the gel since biological systems typically contain a huge variety of proteins.

Hjelmeland & Chrambach (1981) review SDS PAGE in general and Thompson & Maddy (1982) discuss technical aspects for red cells. Fairbanks et al. (1971) used a one dimensional SDS PAGE system which clearly showed at least
7 major protein bands. The numerical labeling of these bands (1 for the highest molecular weight, 2 for the next highest and so on) provided a reference point for discussion of particular red cell membrane proteins. It was later found that the system of Laemmli (1970) gave better resolution and more bands (Thompson & Maddy, 1982). Fig. 1.2 (adapted from Thompson & Maddy, 1982) shows the protein gel profile for the Fairbanks and the Laemmli systems along with the numbering system.

As the sialoglycoproteins stain poorly with the common protein stain coomassie blue, they are visualized via periodic acid-schiff stain (PAS). These glycoproteins, being heavily glycosylated, didn't run "ideally" on SDS PAGE (having an apparent molecular weight greater than actual (Grefrath & Reynolds, 1974)) and were discovered to combine not only with themselves but with other PAS proteins. Due to the fact that under different gel and buffer conditions they run differently relative to the coomassie blue stained proteins, the PAS proteins are treated separately. There are now believed to be four individual PAS stainable components (and possible minor, uncharacterized bands) which interchange with each other. Fig. 1.3 shows the gel profiles of the PAS proteins (adapted from Anstee, 1981) along with the most common nomenclatures and their sources.

The red cell membrane proteins can be distinguished on the basis of their ease of solubilization and/or function. Singer & Nicolson (1972) separated proteins into two categories by their ease of solubilization. Extrinsic (or peripheral) membrane proteins were easily extracted by manipulation of the ionic strength or pH. Bands 1, 2, 4, 5, and 6 are included in this category and comprise from 30 to 35% of the membrane protein. The remaining proteins were extremely difficult to remove,
Fig. 1.2 The total polypeptide content by coomassie blue staining (A and C) and glycoproteins by PAS staining (B and D) separated by Fairbanks system (A and B) or separated by the Laemmli system (C and D). Polypeptides are numbered according to Fairbanks et al (1971) and the glycoproteins by the nomenclature of Anstee (1981). Hb = hemoglobin, TD = tracking dye. (Adapted from Thompson & Maddy, 1982)

Fig. 1.3 Nomenclature for the PAS staining bands. SDS PAGE of membranes from normal human erythrocytes separated by the Laemmli system. The gel was stained with PAS. (Taken from Anstee, 1981)
requiring detergents, and were believed to be intimately connected with the membrane. They were called integral (or intrinsic) proteins. These include bands 3, and 7, PAS 1, 2, 3, and 4 as well as minor proteins like the ATPases, acetylcholinesterase (AChe) and uncharacterized glycoproteins.

The extrinsic proteins are believed to be on the inner layer of the bilayer while the integral proteins are intercalated into the bilayer and in some cases span it (e.g. band 3 and PAS 1) although band 7 is believed to be on the inside.

The other categorization, proposed by Marchesi (1979a), separates the membrane proteins into three functional groups. The first group has a contact or receptor function. These are proteins exposed to the outer surface which interact with the environment. The PAS proteins are examples of these. The second functional group contains the catalytic units. The red cell membrane undergoes vigorous transactions with the surrounding blood plasma, exhibits HCO$_3^-$ and Cl$^-$ exchange, glucose uptake and maintains Na$^+$/K$^+$ and Ca$^{2+}$ gradients. Transporter proteins such as band 3 would be in this catalytic category. The third group contain the supporting elements, the proteins which combine to produce a supporting skeletal structure.

The cytoskeleton

Several recent reviews have been written about spectrin and the cytoskeleton (Marchesi, 1979b, 1983; Palek, 1983) so only a brief synopsis will be presented here.

The cell owes its shape, integrity and elasticity to the cytoskeleton (Gratzer, 1981). Band 1 (240k) and band 2 (220k), collectively known as spectrin, comprise 25% of the membrane protein and form a tetramer composed of two heterodimers (band 1+2) joined head to head.
Near the tail end, actin (band 5) binds as does band 4.1; 4.1 is actually two distinct proteins called 4.1a (78k daltons) and 4.1b (80k daltons) (Lelo & Marchesi, 1984). Ankyrin (band 2.1) binds to spectrin at a site 200 angstroms distal to the end of spectrin and also to the cytoplasmic end of band 3 (Weaver & Marchesi, 1984; Weaver et al, 1984). It appears that bands 4.2, 4.9 and 7 are also involved but little is known about them (Lux, 1979).

Mueller & Morrison (1981) found that PAS 2, which they call "glycoconnectin", interacts with the cytoskeletal structure, most likely through band 4.1a,b. The result is a meshwork of spectrin underlying the membrane connected to band 3 via ankyrin and to PAS 2 via band 4.1a,b. If one assumes the spectrin tetramer is the basic unit, there are equal numbers of 4.1 and 2.1 per tetramer ($1 \times 10^5$/cell). There are about 5 times as many actins (Pinder & Gratzer, 1983) but only $3.5 \times 10^4$ PAS 2/cell (Furthmayr, 1978) or 1/3 as many. Fig. 1.4 (taken from Cohen, 1983) shows a proposed structure for the cytoskeleton. It has been estimated that the skeleton covers from 50 to 70% of the inner surface (Haest, 1982).

Nakajima (1979) and Anderson & Lovrien (1981) postulate an association between glycophorin A (PAS 1) and spectrin although no other evidence has supported this hypothesis. The spectrin complex may also bind to the inner bilayer of the phospholipids and aid in maintaining the phospholipid asymmetry (Kuypers et al, 1984; Haest et al, 1980). Haest (1982) has written an excellent review concerning the interactions between the skeleton and the red cell membrane which is summarized in Table 1.3.

Recently Fowler & Bennett (1984) discovered a membrane protein (a dimer consisting of a 29k and a 27k dalton monomer and about 1% of the total
membrane protein) they identify as tropomyosin. This molecule is known to bind to muscle F-actin and they point out that since there is 1 tropomyosin dimer to 7-8 actin molecules, the possibility of actomyosin contractile mechanisms should be reconsidered.

Fig. 1.4 (A) Schematic drawing illustrating some of the molecular associations present in a hypothetical red cell membrane skeleton "unit cell." The number of actin molecules shown in the short filament and the number of attached spectrin molecules may vary from unit cell to unit cell. (B) Two-dimensional membrane skeletal network which results from the head-to-head association of the spectrin dimers in the unit cells shown in A. (Taken from Cohen, 1983)
TABLE 1.3
PROPERTIES, ASSOCIATIONS AND FUNCTION OF MEMBRANE PROTEIN FRACTIONS
(Taken from HAEST, 1982)

<table>
<thead>
<tr>
<th>Peptide fraction</th>
<th>$M_r$ (kDa)</th>
<th>Copies per cell ($\times 10^{-5}$)</th>
<th>Associated state</th>
<th>Function</th>
<th>Association with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1 (spectrin)</td>
<td>240000</td>
<td>2.2</td>
<td>Dimers of band 1 and 2 form tetramers and oligomers</td>
<td>Membrane skeleton</td>
<td>Band 3 (to band 1)</td>
</tr>
<tr>
<td>Band 2 (spectrin)</td>
<td>220000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 2.1 (ankyrin)</td>
<td>210000</td>
<td>1.1</td>
<td>Monomer</td>
<td>Connects membrane skeleton with intrinsic domain</td>
<td>Band 2, band 3</td>
</tr>
<tr>
<td>Band 2.2 (ankyrin)</td>
<td>183000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 2.3 (ankyrin)</td>
<td>165000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 3</td>
<td>95000</td>
<td>12</td>
<td>Tetramer in equilibrium with dimers</td>
<td>Inorganic anion transport system</td>
<td>Band 1, ankyrin</td>
</tr>
<tr>
<td>Band 4.1 a</td>
<td>80000</td>
<td>2.3</td>
<td>Dimers?</td>
<td>Connects membrane skeleton with intrinsic domain, Spectrin-actin interaction</td>
<td>Spectrin, glycophorin C</td>
</tr>
<tr>
<td>Band 4.1 b</td>
<td>78000</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 4.2</td>
<td>72000</td>
<td>2.3</td>
<td>Tetramer</td>
<td>Unknown</td>
<td>Band 3</td>
</tr>
<tr>
<td>Band 4.5/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>59000</td>
<td>1.3</td>
<td>Possibly monosaccharide, l-lactate and nucleoside transfer system (Refs. 209, 211 and 212 resp.)</td>
<td>Membrane skeleton (degradation product?)</td>
<td>Band 2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 4.9</td>
<td>48000</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 5 (actin)</td>
<td>43000</td>
<td>5.1</td>
<td>Oligomers of 10 monomers</td>
<td>Membrane skeleton</td>
<td>Spectrin, band 4.1</td>
</tr>
<tr>
<td>Band 6</td>
<td>35000</td>
<td>4.1</td>
<td>Tetramer</td>
<td>Glyceraldehyde-3-P-dehydrogenase</td>
<td>Band 3, spectrin-actin</td>
</tr>
<tr>
<td>Band 7</td>
<td>29000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycophorin A</td>
<td>29000</td>
<td>2</td>
<td>Dimer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycophorin B</td>
<td></td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycophorin C</td>
<td>$-25000^b$</td>
<td>0.35</td>
<td>Skeleton attachment site</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: $^b$ refers to negative values.
Catalytic functions

Band 3

The most abundant intrinsic protein of the red cell membrane is band 3. There are $1.2 \times 10^6$ copies/cell (Fairbanks et al, 1971) and it comprises 24% of the total membrane protein (Jones & Nickson, 1980). Several review articles on the subject have been written (Jenkins & Tanner, 1977; Steck, 1978; Macara & Cantley, 1983).

On SDS PAGE band 3 always appears as a broad band centered at about 100,000 daltons implying a heterogenous population (Matsumoto & Osawa, 1980). This is partially due to the heterogeneity in the glycosylated portion of band 3 (Mueller & Morrison, 1979; Fukuda et al, 1979; Tsuji et al, 1981) and to other minor proteins comigrating with band 3. These minor proteins include acetylcholinesterase (Ott et al, 1983), $\text{Na}^+/\text{K}^+$ ATPase (Erdmann, 1982), the $\text{Ca}^{2+}-\text{Mg}^{2+}$ ATPase (Gietzen & Koland, 1982; Zurini et al, 1984), $\text{Mg}^{2+}$ ATPase (White & Ralston, 1980) and the insulin receptor (Grigorescu et al, 1983).

Blood groups A, B (Hakomori, 1981a) and I (Childs et al, 1978) are located on band 3 in its carbohydrate structure. This has been named erythroglycan, a large branched oligosaccharide of repeating gal and glucNAc units (Jarnefelt et al, 1978; Suji et al, 1980). The senescent cell antigen has been postulated to reside on band 3 (Kay et al, 1983).

Band 3 is known to be the anion transporter ($\text{H}_2\text{PO}_4^-$, $\text{Cl}^-$, $\text{HCO}_3^-$ etc.) (Cabantchik et al, 1978) and can also transport a small fraction of the neutral amino acids (Saleh & Wheeler, 1982; Young et al, 1981), phosphoenol pyruvate (Hamasaki et al, 1978), water (Brown et al, 1975) and possibly $\text{Ca}^{2+}$ (Waisman et al, 1982).
The cytoplasmic part of Band 3 is the binding site for ankyrin and cytoplasmic components such as hemoglobin (Cassoly & Salhany, 1983) aldolase, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase (G3PD) (Gilles, 1982) and possibly others.

Fig. 1.5 (taken from Macara & Cantely, 1983) is the proposed model for band 3. Although the exact structure isn't known, the molecule must traverse the membrane several times to explain all the proteolysis and chemical modification data accumulated about it. Band 3 may be a dimer or tetramer in the membrane (Klingenberg, 1981) and may interact with glycophorin A (Nigg et al, 1980) but doubts were raised by Schweizer et al (1982) who were unable to crosslink band 3 to itself or other components using instantaneous crosslinking conditions.

Fig. 1.5 Organization of the Band 3 polypeptide in the red cell membrane, showing its two-domain structure. (Taken from Macara & Cantely, 1983)
Band A.5

One function that band 3 was thought to have had was that of glucose transport. Mullins & Langdon (1980), using a potent glucose inhibitor affinity label, labeled exclusively band 3 getting $3 \times 10^5$ transporters/cell. Band 4.5 labeled if band 3 was enzymatically degraded by endogenous proteases in Triton X-100. However, others, using cytochalasin B binding, labeled band 4.5 (Shanahan, 1983). The amount varied from $3.3 \times 10^4$ (Jones & Nickson, 1980) to $2.5 \times 10^5$ sites/cell (Lin & Snyder, 1977). Carruthers & Melchior (1984) claim that band 4.5 is the transporter but band 3 also appears to mediate cytochalasin B and transport activity.

Whitfield et al (1983) state that bands 3 and 4.5 are immunologically and structurally related. Most investigators now believe band 4.5 to be the glucose transporter (Wagner et al, 1984; Wu et al, 1983b; Klip et al, 1984). This glucose transporter comprises 3.5% of the membrane protein and is a glycoprotein, 15% carbohydrate by weight (Lienhard et al, 1983).

Band 4.5 is actually a region of overlapping polypeptides and 6 bands can be seen on Laemmli gels (Jones & Nickson, 1980). They range in size from 45000 to 55000 daltons and comprise a total of 10% of the membrane protein (each band numbering a little over $1 \times 10^5$ copies/cell). Like band 3 they are also heterogenously glycosylated (Gorga et al, 1979) and carry blood groups A, B, H, and Ii (Hakomori, 1981a).

Catalase, a cytosol enzyme of 60000 daltons also appears in this area (Deas et al, 1978).

Band 4.5 is also known to be the nucleoside carrier (Wu et al, 1983a), although band 7 has been implicated along with band 4.5 (Wu et al, 1983b).
It appears to be a multimer in the membrane, having a radiation target size of 122000 daltons (Jarvis et al, 1984). The nucleoside transporter comprises 0.1% of the membrane protein, numbering about $1.38 \times 10^4$ copies/cell (Jarvis & Young, 1982).

**Contact or receptor proteins**

The only contact proteins considered in this section are the PAS proteins, and as can be seen from Fig. 1.3, there are at least four. Reviews on these molecules have been written by Furthmayr (1981) and Anstee (1981).

The most predominant and best known is glycophorin A (PAS 1 or alpha) comprising 70% of all PAS protein. It numbers about $5 \times 10^5$/cell, although values as high as $2 \times 10^6$ have been quoted (Anstee, 1981). It comprises about 1.6% of the membrane protein and carries 60% of the sialic acid. The amino acid sequence has been worked out, it has 131 amino acid residues with residues 71 to 90 being hydrophobic in nature and just large enough to span the lipid bilayer. The carboxyl end of the molecule resides on the cytoplasmic side and the amino terminus on the outer surface is heavily glycosylated. Sixty percent of the molecule is carbohydrate, consisting of 15 O-glycosidically linked and 1 N-glycosidically linked oligosaccharides. Fig. 1.6 depicts the structure of the peptide chain and the location of the oligosaccharides. From the amino acid and carbohydrate composition, a molecular weight of 31000 daltons was calculated for glycophorin A. This molecule possesses M, N, and Pr blood groups as well as malarial parasite and virus receptors.

Glycophorin B (PAS 3 or delta) comprises about 15% of the PAS protein and numbers about $7 \times 10^4$/cell (Furthmayr, 1978). Its molecular weight is
27000 daltons (Dohnal et al, 1980). The amino end of this molecule is identical to that of glycophorin A, blood group N, up to residue 26, which is found for glycophorin B not to be N-glycosylated. It is believed that it doesn't carry a N-glycosidically linked complex oligosaccharide. Thus, glycophorin B carries the N blood determinant as well as Ss and U (all three being determined by amino acids) and the Pr group.

Glycophorin C (3.5 x 10^4/cell) according to Anstee is actually two PAS proteins, gamma and beta. Beta is also known as PAS 2 or glycoconnectin. Together they comprise about 10% of the PAS proteins, the remainder presumably being minor uncharacterized fractions (even band 3 stains slightly with PAS due to its glycosylation). Absence of peptides 3000 to 4000 daltons in size following tryptic digest of these PAS molecules (except for glycophorin A) suggested no significant cytoplasmic segment for gamma or beta although glycoconnectin has been shown to bind to band 4.1 on the cytoplasmic side.

Fig. 1.7 represents a proposed model for the structure of these glycophorins (except gamma for which there is insufficient data) (Anstee, 1981). All are believed to extend through the membrane with the amino terminus and the glycosylated part on the outside, and the carboxyl end at the cytoplasmic side.

The conformation of these oligosaccharides adjacent to the membrane is not known. Are they jutting straight out, or do they extend along the surface of the lipid layer? Due to their behavior on SDS PAGE, it has been postulated that these molecules could be dimers or oligomers in the membrane although direct evidence is lacking.
Fig. 1.6 The amino acids of glycophorin A, arranged to simulate, in a very general way, the positions they might have if the molecule was perpendicular to the lipid bilayer of the membrane. The solid vertical line approximates the location of the inner half of the phospholipid bilayer. The outer edge of the bilayer can only be approximated and is defined by the dashed vertical lines. The 15 diamond shaped structures are the O-glycosidically linked and the other is the N-glycosidically linked oligosaccharide. (Taken from Marchesi, 1979)

Fig. 1.7 Diagrammatic representation of the location of antigens on normal erythrocyte sialoglycoproteins. The small circles are the O-glycosidically linked oligosaccharides and the big shaded circles the N-glycosidically linked oligosaccharides. (Taken from Anstee, 1981)
The function of these PAS proteins is unknown. Healthy individuals exist who don't contain glycophorin A (Tanner & Anstee, 1976) and two people are known who lack gamma and delta (although there is altered glycosylation of the other membrane proteins (Anstee, 1981)). The PAS proteins do supply the major source of sialic acid and thus carry the majority of the surface charge. The age related antigen has been proposed to reside on glycophorin (Alderman et al, 1981).

The glycophorins interact with the cytoskeleton directly (via glycoconnectin) and possibly indirectly (Lovrien & Anderson, 1980; Nakajima, 1979). Bowles & Hanke (1977) have even postulated that glycophorin has lectin activity, binding specifically to galNAC residues which may be involved in some recognition process. Even though they contain receptors for virus and malarial parasites, it is probably better to think of these proteins as contact proteins, their function being one of keeping the red cells apart through electrostatic repulsion and steric stabilization.

Minor components of the membrane

There are a number of minor proteins associated with or in the membrane which can't be assigned roles or structures. Gahmberg (1976) labelled the red cell surface with galactose oxidase and [³H] and found over 20 stainable glycoproteins. Using two dimensional gel electrophoresis (separating proteins on the basis of molecular weight and iso-electric point) 100-200 reproducibly detectable proteins can be seen in red cell membrane preparations (Rubin & Milikowski, 1978; Rosenblum, 1981). The majority of these proteins appear to be enzymes. Schrier (1978) reviews the membrane enzymes and lists over 40 he believes are definitely in the membrane (Table 1.4),
TABLE 1.4

ENZYMES WHOSE ACTIVITY IS FOUND IN THE MEMBRANE OF RED BLOOD CELLS
(Taken from Schrier, 1978)

I. Enzymes of nucleotide metabolism (ATPases considered separately)

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>2',3' cAMP cyclic nucleotide 3'-phosphohydrolase</td>
</tr>
<tr>
<td>Adenyl cyclase</td>
</tr>
<tr>
<td>UTPase</td>
</tr>
<tr>
<td>5' AMP phosphatase</td>
</tr>
</tbody>
</table>

II. Enzymes of carbohydrate metabolism

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP-N-acetylneuraminic acid: glycoprotein sialyltransferase</td>
</tr>
<tr>
<td>N-acetyl-D-glucosaminidase</td>
</tr>
<tr>
<td>α-D-glucosidase</td>
</tr>
<tr>
<td>β-D-glucosidase</td>
</tr>
<tr>
<td>α-D-galactosidase</td>
</tr>
<tr>
<td>α-D-galactosidase</td>
</tr>
<tr>
<td>α-L-fucosidase</td>
</tr>
<tr>
<td>α-L-fucosidase</td>
</tr>
<tr>
<td>β-D-sialidase</td>
</tr>
<tr>
<td>β-D-sialidase</td>
</tr>
<tr>
<td>β-D-xylosidase</td>
</tr>
<tr>
<td>β-D-mannosidase</td>
</tr>
<tr>
<td>N-acetyl-β-D-galactosaminidase</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
</tr>
<tr>
<td>N-acetyl-galactosaminyl transferase</td>
</tr>
</tbody>
</table>

III. Phosphatases

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₆ phosphate phosphatase</td>
</tr>
<tr>
<td>p-nitrophenyl phosphatase (not K⁺ dependent, for K⁺ dependent see ATPases)</td>
</tr>
<tr>
<td>Mg²⁺ dependent phosphoprotein phosphatase</td>
</tr>
</tbody>
</table>

IV. Proteinases

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 proteinases pH optima 7.4, 7.4, 3.2</td>
</tr>
<tr>
<td>2 proteinases pH optima 3.4, 7.4</td>
</tr>
</tbody>
</table>

V. ATPases

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPases not linked to fibrous membrane proteins</td>
</tr>
<tr>
<td>Mg²⁺ ATPase</td>
</tr>
<tr>
<td>Na⁺, K⁺ ATPase, ouabain inhibited</td>
</tr>
<tr>
<td>(K⁺ dependent p-nitrophenyl phosphatase, ouabain inhibited)</td>
</tr>
<tr>
<td>Co²⁺, Mg²⁺ ATPase</td>
</tr>
<tr>
<td>Possibly two affinities for Ca²⁺, the low affinity enzyme may be the Ca²⁺ transporting enzyme</td>
</tr>
<tr>
<td>Monovalent cation stimulated, not related to active Co²⁺ extrusion</td>
</tr>
<tr>
<td>ATPases associated with fibrilar membrane proteins (spectrin)</td>
</tr>
<tr>
<td>Co²⁺ ATPase inhibited by Mg²⁺ (approximately 5% as active as the Co²⁺, Mg²⁺ ATPase)</td>
</tr>
<tr>
<td>Mg²⁺ ATPase, low activity, stimulated by actin, thus resembling actomyosin</td>
</tr>
</tbody>
</table>

VI. Protein kinases

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP independent, stimulated by mono and divalent cations to phosphorylate band 2, spectrin</td>
</tr>
<tr>
<td>cAMP stimulated, Co²⁺ inhibited, monovalent cation inhibited</td>
</tr>
</tbody>
</table>

VII. Miscellaneous

<table>
<thead>
<tr>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺ glycohydrolase (DPNase)</td>
</tr>
<tr>
<td>NADP⁺ glycohydrolase (TPNase)</td>
</tr>
<tr>
<td>NADH: acceptor oxidoreductase</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
</tr>
</tbody>
</table>
Juliano (1973) puts the enzymes into three classes. The first contain cytoplasmic enzymes, which may also be associated with the membrane and whose release usually parallels that of hemoglobin. The next class contains those which require more stringent conditions for extraction, similar to those of extrinsic proteins like spectrin. The last class (shown in Table 1.4) consists of the intrinsic enzymes such as NADH oxidoreductase, protein kinase, AChE, Na\(^+\)K\(^+\) ATPase, etc. The proteases found in the membrane are known to cleave band 3, the PAS proteins and spectrin (Nickson & Jones, 1980; Siegel et al, 1980; Pontremoli et al, 1980). Yatzio et al (1978) and Kahane et al (1980) stress the fact that when analysing red cells for membrane enzymes, one must be sure no other blood cells (such as platelets and white cells) contaminate the preparation.

Sample preparation may also cause artifacts. Hahanan (1973) states osmotic stress may lead to artifactual association of enzymes with the inner surface. Aldolase, pyruvate kinase, fructose-bisphosphatase (Tillmann et al, 1975) as well as hemoglobin and 2% of the calmodulin bind to the membrane (Klinger et al, 1984). The emerging view is that there is no abrupt interface between the cytosol and the inner layer of the membrane (Salhany, 1983). Methemoglobin reductase is 35% membrane bound in a 45000 dalton form and is cleaved to the 29000 dalton form which elutes to the cytosol (Choury et al, 1983). This association of cytosol enzymes with the membrane may be part of a complex regulatory system (Haest, 1982).

Red cell metabolites also react with and alter membrane components. Protein kinases are known to phosphorylate proteins 2, 2.1, 2.3, 3, 4.1, 4.5, 4.8, and 5 (Rsienbenlist & Taketa, 1983). Methylases alter the charge of membrane proteins by methylating the acidic amino acids reversibly on
bands 2.1, 3, 4.1, 4.5, and 6 (Ro et al, 1984; Green et al, 1983). Glutathione is known to react with the membrane protein sulfhydryl groups of spectrin bands 3, 4.1, 4.2, 4.5, and 5 (Haest et al, 1979).

The outer surface of the membrane is also exposed to a variety of proteins as well as hormones, drugs, etc. For instance, N-acetyl-D-galactosaminytransferase is found in serum and on red cells (Kim et al, 1971). Antibodies are known to bind to red cells (Muller & Lutz, 1983; Alderman et al, 1981; Bartosz & Bryszewska, 1983). Davis & Weiss (1980) extracted by gentle agitation (glc)3-cys-glu-gly-(gly-ser)-ala, the removal of which seemed to decrease glucose transport.

Serum glucose has been shown to react with membrane proteins nonenzymatically (Miller et al, 1980) providing an average of $7 \times 10^5$ glucoses/cell (Schleicher et al, 1982).

The red cell interacts with many biological substances in the nanomolar range, such as insulin, growth hormone, acetylcholine, adrenergic agents and prostaglandins, some at only a few molecules/cell (Gratzer, 1981).

Other minor components on the membrane are antigens of which there are over 300 types known (Rosse, 1984) and transport proteins for compounds (such as lactate (Dubinsky & Racker, 1978), glutathione (Kondo et al, 1981) and amino acids (Saleh & Wheeler, 1982)) for which no protein has yet been isolated.

No mention has been made of the changes which occur to a red cell as it ages in vivo, about alterations which can occur in a disease state, or to the fact that there is considerable genetic variability. Each of these could be chapters in themselves. Suffice to say they add to the problem and complexity of red cell membrane analysis.
A major problem at present concerns the conformation of the membrane proteins in situ. How they interact with each other and with the rest of their environment is not known.

Of particular interest in this study are the carbohydrates located on the PAS proteins and bands 3 and 4.5 which are depicted in Fig. 1.8. At 15 O-glycosidic oligosaccharides per glycophorin A, there must be approximately $8 \times 10^6$ of these per red cell and about $5 \times 10^5$ N-glycosidically linked oligosaccharides per red cell. From band 3, about $1 \times 10^6$ oligosaccharides must reside on the surface. They appear to constitute the majority of the glycocalyx. Fig. 1.8 shows the proposed structures for these oligosaccharides. Fig. 1.8a is the proposed structure for the O-glycosidically linked oligosaccharide of glycophorin A (Furthmayr, 1981; Thomas & Winzler, 1969; Sadler et al, 1979; Gahmberg & Andersson, 1981; Prohaska et al, 1981). Fig. 1.8b is the N-glycosidically linked oligosaccharide of glycophorin A (Irimura et al, 1981; Yoshima et al, 1980) and Fig. 1.8c the structure of the band 3 oligosaccharide (N-glycosidically linked) as proposed by Fukuda et al (1984). Fig. 1.9 depicts this outer surface of the red cell (taken from Sharp, 1985).
Fig. 1.8 The proposed structures for the oligosaccharides of glycophorin and band 3. (A) the O-glycosidically linked oligosaccharides of glycophorin, taken from Furthmayr, 1981; (B) the N-glycosidically linked oligosaccharide of glycophorin taken from Irimura et al, 1981 ($R_1 = H$ or NANA 2-6 (Yoshima et al, 1980)); and (C) the oligosaccharide attached to band 3. $R = H$, Fuc 1-2, NANA 2-3, or NANA 2-6; $R_2 = R$-Gal 1-4 Glc 1-6, n = 4-5. (Taken from Fukuda et al, 1984)
Fig. 1.9 A scale drawing attempting to show the outer surface of the red cell membrane. Major intrinsic proteins: band 3 (B), glycophorin A (G). Major extrinsic proteins: spectrin (S), actin (Ac), band 2.1 (ankyrin) (A). Lipids: phospholipid bilayer (PL), glycolipids (GL). Carbohydrate is shown in solid black. No attempt has been made to show the true conformations, shapes and distribution of these components. However the relative amounts, average volumes and separation between band 3, glycophorin A, the glycolipids and the phospholipids are shown to scale, based on current estimates of their molecular weights, % carbohydrate and amounts per cell. Modification of Peters & Grant (1983). Calculated and drawn by K.A. Sharp (1985).
A useful tool in the study of the glycocalyx would be one that interacted with the glycocalyx, mimicking recognition behavior. Lectins, known to bind to carbohydrate sequences, appear well suited for use in model studies therefore.

1.2 LECTINS

"A macromolecule contains information in its sequence of subunits that determines the three-dimensional contours of its surface. These contours in turn govern the recognition between one molecule and another, or between different parts of the same molecule, by means of weak noncovalent bonds." (Alberts et al., 1983). This statement describes a wide variety of biological interactions including enzyme-substrate, antibody-antigen and cell-cell recognition. Lectins are proteins (or glycoproteins) which bind noncovalently and reversibly to carbohydrate groups without modifying them. Excluded from this group are proteins with known functions such as antibodies, transport proteins, and enzymes.

Lectins were discovered by Stillmark in 1888 and were originally called phytohemagglutinins because they could agglutinate red cells and were isolated mainly from plants. In 1952, Watkins & Morgan found that specific monosaccharides could inhibit such hemagglutination and were thus the first to show the presence of sugars on the outer surface of membranes. Indeed developments in lectin studies parallel advances in our knowledge of surface carbohydrates. In 1954, Boyd & Shapleigh chose the more general term, lectin, from the latin Lego, to pick or choose, because lectins could be isolated from animal sources also.
Lectins are in many ways analogous to antibodies, both recognizing certain molecular "contours." Kabat (1978) has even found similarities in the binding sites of lectins and antibodies, both showing large differences in site sizes and specificities. Although antibodies are more specific and bind to proteins as well as sugars, they are hard to obtain pure in large amounts. Lectins can be isolated relatively cheaply, from a variety of sources. Several excellent reviews on lectins are Brown & Hunt (1978), Goldstein & Haynes (1978), Lis & Sharon (1973) and Nicolson (1974).

Due to their ability to bind saccharides, lectins have become useful for isolation and purification of glycoproteins and cells. They are used as surface probes (e.g. blood group typing, monitoring variations in malignant cells) as well as for structural studies and for model studies of carbohydrate binding. Lectin binding to cells may elicit a number of responses, depending on the lectin and the cell receptor. Agglutination, mitogenesis, toxicity, altered transport and mimicking of an active agent in vivo (such as insulin) have been seen. Lectins thus enable a researcher to manipulate cells in vitro to study these phenomena.

Lectin binding is affected by the nature of the lectin (number of binding sites, binding constants, net charge and size) and the experimental conditions such as temperature, time, pH, ionic strength, nonspecific interactions, cofactors, tertiary structure of the oligosaccharides, number of receptors per cell and properties of the cell surface. Binding can occur without agglutination (Sharon & Lis, 1975). The fact that lectins bind strongly to cells, mimicking a variety of natural agents, makes them well suited for studying the complex phenomenon of protein binding.
1.3 CHEMICAL MODIFICATION

Of all the carbohydrates on the cell surface, sialic acid (also known as N-acetyl neuraminic acid or NANA) and galactose are the easiest to modify specifically, sialic acid because of its exocyclic triol (not known to exist on other sugars) and galactose because of the selectivity of enzymes. As reviewed earlier, the red cell surface is known to contain both these residues. In this work they were selectively modified by a procedure similar to that of Aplin et al (1979), (see also Aplin, 1979 and Bernstein, 1983). This involves selective oxidation of the NANA or galactose residues followed by reductive amination via NaBH$_3$CN and a compound with a reactive amine group. The first step is the oxidation of the terminal NANA or galactose, generating a reactive aldehyde.

1.3(a) NANA oxidation

It has been known that mild oxidation by periodate results in selective oxidation of the exocyclic triol of NANA (Van Lenten & Ashwell, 1971) (Fig. 1.10). Periodate oxidation is commonly used in Smith degradation to determine intersugar linkages in oligosaccharides, with its reactivity towards vicinal diols in the order extracyclic greater than intracyclic cis greater than intracyclic trans (Wagh & Bahl, 1981). If the periodate concentration is kept low enough and the times short enough, only the most reactive diols will be oxidized, those on the exocyclic triol of NANA, leaving the integrity of the carbohydrate chain intact and ensuring specificity in the reaction.
1.3(b) Galactose oxidation

The oxidation of galactose is best performed with the enzyme galactose oxidase (G.O.) (E.C. No 1.1.3.9). This enzyme oxidizes the 6-CH$_2$OH of galactose or N-acetyl galactosamine (galNAc) producing an aldehyde and H$_2$O$_2$ (see Fig. 1.11). The H$_2$O$_2$ formed inhibits G.O. by a feed-back control mechanism and is usually removed by addition of the enzyme catalase. The addition of catalase to red cells being oxidized by G.O. is not necessary, however, due to the natural presence of catalase in these cells (Liao et al, 1973; Deas et al, 1978).

Galactose is usually found with NANA linked to it so to label these subterminal residues also one must first remove the NANA. This can be done by either mild acid hydrolysis (usually 0.1 N H$_2$SO$_4$ at 80°C for one hour) or with the enzyme neuraminidase (E.C. No. 3.2.1.18).
Fig. 1.11 Oxidation of galactose termini by galactose oxidase. R = red cell.

1.3(c) Reductive amination

The aldehydes generated on NANA or galactose can be modified with the spin label 2,2,6,6-tetramethyl-4-amino-piperidine-1-oxyl (often called TEMPAMINE and indicated in chemical pathways as SL-NH$_2$) (Fig. 1.12). The amine group is reductively aminated onto the aldehyde using NaBH$_3$CN as depicted in Fig. 1.13. As can be seen from this scheme, addition of NaB[$^3$H]$_3$CN results in a tritium being placed on the covalently attached probe, resulting in a second tag which can be followed during the reactions. NaBH$_3$CN is used to reduce the imine ion instead of NaBH$_4$ due to its resistance to aqueous hydrolysis and its specificity for the intermediate Schiff base over the aldehyde, NaBH$_4$ reducing the aldehyde as well (Borch et al, 1971; Lane, 1975). One also has the option of using other compounds or probes, the only criteria being that they contain a reactive amine, are soluble in aqueous media and normally don't react with the biological system unless previously oxidized.
Fig. 1.12 The structure of TEMPAMINE, the spin label used in this study.

\[
\begin{align*}
\text{Fig. 1.13 Reductive amination of an aldehyde with TEMPAMINE, using NaBH}_3\text{CN with or without NaB}[^3\text{H}]_3\text{CN.}
\end{align*}
\]
1.4 ELECTRON SPIN RESONANCE

Electron spin resonance (ESR), also known as electron paramagnetic resonance (EPR), is an important spectroscopic tool for studying biological systems. This is usually achieved by incorporation of a spin label into the system of interest. Information regarding membranes (especially the lipid portion), enzymes and other biological entities has been expanded by the use of ESR. A number of excellent reviews and monographs covering many aspects of theory, application and practical problems are cited below (Hudson & Luckhurst, 1969; Smith, 1972; Keith et al, 1973; Likhtenstein, 1976; Berliner, 1976, 1978, 1979; Schreier et al, 1978; Jost & Griffith, 1978; Marsh, 1981; Poole Jr., 1983).

1.4(a) Nitroxides

ESR is performed on paramagnetic substances which in this study are introduced into the system by means of spin labeling (see Section 1.3). The term "spin label" was first coined by McConnell and coworkers (Stone et al, 1965) and refers to stable free radicals, usually a nitroxide-containing compound which contains an unpaired electron localized on the nitrogen and oxygen atoms (Jost & Griffiths, 1978).

Nitroxides are amongst the most stable of free radicals and were first synthesized about 1960 (Rozantsev, 1970). The nitroxide itself is usually not involved in chemical reactions, the reactive group being located away from the nitroxide (as is seen for TEMPAMINE in Fig. 1.13). Fig. 1.12 shows the structure of TEMPAMINE, the spin probe used in this study. The methyl
substituents at the 2 alpha carbons prevent the nitroxide from undergoing disproportionation. At pH 6-8 the reduction potential for the nitroxide is about -150 mv, which means it can be reduced by such agents as ascorbate, dithiothreitol, and mercaptoethanol but not NaBH₄, LiAlH₄ or NaCNBH₃. The oxidation by the nitroxide of a number of reducing agents has been used to investigate the accessibility of this group.

1.4(b) Electron Spin Resonance: General

Electron spin resonance occurs with a paramagnetic molecule. This molecule contains at least one unpaired electron which has a spin of + or - 1/2. With the application of a magnetic field, H, energy levels between the two spin states occur as depicted in Fig. 1.14a. With the application of a microwave frequency, ν, perpendicular to the magnetic field, transitions occur between these two levels (known as the Zeeman levels) at

\[ hν = gμH \]  

where \( h \) = Planck's constant, \( B \) = the Bohr magneton (\( \mu B/2m \)) where m is the mass and e the charge of the electron and g is a dimensionless parameter related to the effective magnetic moment of the electron, equaling 2.00232 for a free electron.

The frequency of resonance is dependent upon the applied field and most experiments, including the ones here, are conducted at the X-band (about 9.5 GHz, equivalent to a 3 cm wavelength) which corresponds to an external field of about 3.4 kG.
Fig. 1.14 (A) Effect of external magnetic field strength on the energy level of the orbitals of unpaired electrons having spin states of $m_s = +$ and $-1/2$. (B) Absorption of microwaves of frequency $v$ as a function of field strength. (C) The resultant ESR spectrum, the first-derivative spectrum taken from (B). (Taken from Benga, 1983)

Net absorption of microwave energy from $H_1$ occurs at resonance due to more spins being at the lower energy state (Fig. 1.14b). These spins remain close to the equilibrium energy distribution due to rapid relaxation and a weak $H_1$. Absorption is monitored with the aid of phase-sensitive detection using field modulation at $10^5$ Hz and recorded as the first derivative of the absorption signal (Fig. 1.14c).

1.4(c) Nitroxide ESR

In the nitroxide spin label, the electron also interacts with the nuclear spin of the nitrogen nucleus, a phenomenon known as hyperfine
interaction. The spin Hamiltonian for a nitroxide electron is given by

\[ \mathcal{H} = \mathcal{H}(\text{Zeeman}) + \mathcal{H}(\text{hyperfine}) + \mathcal{H}(\text{dipolar}) + \mathcal{H}(\text{exchange}) \]  

[2]

The nuclear Zeeman term, being negligible, has been omitted.

The hyperfine interaction occurring between the electron and the nitrogen nucleus (I = 1), results in the characteristic 3-line spectrum (m = 1, 0, -1, Fig. 1.15). Small hyperfine interactions occur with the beta-hydrogens but are not resolved. Hyperfine interactions also occur with the alpha \(^{13}\)C nuclei (1.1% natural abundance) and \(^{15}\)N (0.4% natural abundance) and are seen as satellite peaks at high signal-to-noise.

Fig. 1.15 The effect of the spin state of nitrogen nuclei (I = +1, 0, -1) on the energy level of the unpaired electron. The magnitude of the magnetic field felt by the electron will now depend on the magnetic field from the nearby nucleus as well as the applied magnetic field. Also shown are some parameters used to analyse the spectrum, \(h_1\), \(h_0\) and \(h_{-1}\) are the height of the low-field, mid-field and the high-field lines respectively and \(w_0\) is the linewidth of the mid-field line (see equations 9 and 10). (Adapted from Benga, 1983)
Fig. 1.16a shows the principal axes for the nitroxide. The direction-dependence of the Zeeman and hyperfine interactions is seen in Fig. 1.16b. Here a spin label is in a diamagnetic host crystal and the spectrum run at various orientations to the magnetic field. The $g$- and $T$-tensors are then measured at the different orientations. For most nitroxides, $g_{xx} = 2.009$, $g_{yy} = 2.006$ and $g_{zz} = 2.002$ with $T_{xx} = T_{yy} = 6G$ and $T_{zz} = 32G$ ($T_{zz} = 37G$ for TEMPAMINE). These values differ from compound to compound and the polarity of the environment (Berliner, 1978). Both terms can be approximated as axially symmetric with

$$g_{xx} = g_{yy} = g_{\perp}$$ \hfill [3]

and

$$T_{xx} = T_{yy} = T_{\perp}$$ \hfill [4]

with

$$g_{zz} = g_{\parallel}$$ \hfill [5]

and

$$T_{zz} = T_{\parallel}$$ \hfill [6]
Fig. 1.16 (A) Schematic representation of the nitroxide group showing the unpaired electron in the nitrogen $p_z$ orbital (taken from Smith, 1972).

(B) T- and g- anisotropies in di-t-butyl nitroxide oriented in a host crystal at room temperature. The crystal was rotated in the molecular $yz$ plane. At $0^\circ$ and $90^\circ$ the external field lay along the $z$ and $y$ axes respectively. (Taken from Jost & Griffiths, 1978).
The electron distribution in the nitroxide is influenced by the polarity of the environment. This is easily seen when one pictures the nitroxide bond in its resonance form

$$N\cdot O' \leftrightarrow N^+\cdot O^-$$

Polar solvents stabilise the ionic form resulting in increased spin density at the nitrogen and hence increased magnitude of the isotropic splitting constant $a_o$, defined below. The value of $a_o$ of TEMPAMINE varies from 16.99 G in water to 15.22 G in n-hexane (Knauer & Napier, 1976).

In dilute solutions at room temperature with fast isotropic motion occurring, the nitroxide spectrum is usually three sharp lines (Fig. 1.16). The $g$ and $T$ anisotropies are averaged out and are characterized by the isotropic splitting constant $a_o$.

$$a_o = \frac{1}{3}(T_{xx} + T_{yy} + T_{zz})$$  \[7\]

The $g$ values also average out

$$g = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$$  \[8\]

Fig. 1.17 shows the effect of altering the spin probe motion via temperature. Decreasing the temperature increases solvent viscosity and thus decreases the motion of the probe. At low temperatures, a polycrystalline array of nitroxides is obtained and the limiting lineshape spectrum is obtained (often called the "powder," "rigid" or "glass" spectrum). Very little molecular motion occurs. All possible orientations
Fig. 1.17 The effect of viscosity on ESR spectra. The structures of the spin labels used are given at the head of the corresponding column of spectra. The molecular motion was controlled by altering the solvent viscosity. Most mobile, top spectrum, least mobile (characterized by the splitting 2T), bottom spectrum. (Taken from Jost & Griffiths, 1978)
of the nitroxide contribute to the spectrum, which is simply the sum of resonances due to the orientations in Fig. 1.16 together with all others. The outer extremes are due to the nitroxides orientated about the z-axis, parallel to the external field, with splitting = 2T_{zz}.

The correlation time for rotational reorientation is denoted \( \tau_c \). Between the non-viscous solution for which \( \tau_c = 10^{-11} \) s and the rigid state, characterized by \( \tau_c = 10^{-7} \) s, partial averaging of the anisotropic quantities occurs. The spectrum is sensitive to motional effects between these two extremes, a time scale relevant to the motions of a variety of biological systems. As the motion of the probe slows down, asymmetric broadening of the spectrum occurs (see Fig. 1.17). The direction of the g anisotropy is such that the high-field transition, which corresponds to \( m_I = -1 \), begins to broaden before the low-field line, which in turn broadens before the centre line.

From ESR spectra one can calculate an apparent correlation time by measuring linewidths and peak-to-peak heights (h). Two often used formulae are:

\[
\tau_c = 6 \times 10^{-10} w_0 [(h_{0}/h_{-1})^{1/2} + (h_{0}/h_{1})^{1/2} - 2]s \tag{9}
\]

or

\[
\tau_c = 6 \times 10^{-10} w_0 [(h_{0}/h_{-1})^{1/2} - 1]s \tag{10}
\]

(see Nordio, 1976, Felix & Butterfield, 1980 and Keith et al., 1970) where \( h_1 \) are the peak heights of the 1, 0 or -1 lines and \( w_0 \) is the linewidth of the centre line (see Fig. 1.15).
This treatment assumes that the molecular motion is isotropic, that tumbling motion is sufficiently slow to influence the linewidth and that the three lines do not overlap. At the X-band, the equations are applicable from $5 \times 10^{-11}$ to $5 \times 10^{-9}$ s. Very slow tumbling may be characterized by the splitting ($2T$, Fig. 1.17) between the outermost extrema of the spectra. For accurate calculations, spectral simulation is necessary to minimize effects of unresolved hyperfine splitting and conformational interconversions of the probe, which also contribute to spectral broadening (Schreier et al., 1978).

Small probes in isotropic solutions can still undergo anisotropic motion (Schreier et al., 1978) and if attached to other compounds may also exhibit anisotropy. If anisotropic motion is present, at least two $T$ values must be calculated, $T_{||}$ about the principal axis (the $z$ axis in Fig. 1.16) and $T_{\perp}$ about the molecular axis the $x$ axis in Fig. 1.16) (see Luoma et al., 1982 and Schreier et al., 1978).

When the rotational anisotropy is small and motion rapid, $T_C$ is often calculated using equations [9] and [10]. Sometimes $T_C$ is dispensed with completely and the $h_0/h_{-1}$ or $h_0/h_{-1} + h_0/h_{-1}$ quoted as empirical indices of mobility as was done by Sharom & Grant (1977) and Davoust et al. (1981). In the present work the exact nature of the tumbling motion is considered less important than changes in tumbling rates due to external perturbations or the physical state of the system.

Two aspects of the Hamiltonian yet to be discussed are the electron-electron exchange and the electron-electron dipolar interaction.

In electron-electron, or Heisenberg exchange, the wavefunctions of two different unpaired electrons overlap and electrostatic interactions tend to
couple the spins. The exchange energy is isotropic; in solution exchange occurs when the radicals diffuse together and collide. The electrons exchange spin states rapidly, shortening the lifetime of the states without affecting the total energy of the system. In dilute solutions such as those used in this thesis, such events occur sufficiently rarely that they don't contribute significantly to the linewidth (Chapter 3, Table 3.6).

Exchange broadening by the paramagnetic metal ion complex Fe(CN)$_6^{3-}$ occurs, resulting in a broadened nitroxide signal without frequency shift or exchange narrowing (Morse, 1977; Aplin, 1979). All nitroxides accessible to this agent broaden completely at high enough Fe concentration, leaving only protected nitroxides to give a signal. This is one way of assaying the location of nitroxides in complex systems.

The dipolar interaction is direction-dependent and is averaged to zero by rapid tumbling, so that it has an opposite viscosity/T dependence to that of strong exchange (which depends on collision frequency), increasing as motional averaging becomes less complete at high viscosity. It reaches a limiting value which depends on \( (1-3\cos^2\theta)/r^3 \) (where \( \theta \) is the angle between the vector \( \mathbf{r} \) and the external field \( \mathbf{H} \) and \( r \) is the interelectronic distance between the two nitroxides).

From empirical calibration methods (originally done by Kokorin et al, 1972, and specifically for TEMPAMINE by Waterton & Hall, 1979; Aplin, 1979 and Yalpani, 1980), one is able to estimate the mean nearest-neighbour distance in a polycrystalline array between nitroxides if kept below 2 mM (Heisenberg exchange also affects lineshape at high radical concentrations). At these low concentrations, partial saturation is difficult to avoid, even at the lowest microwave power of \( 1.6 \times 10^{-4} \) W.
Fig. 1.18 shows the spectral parameters used to calculate the distances. From studies at 77°K, equation [11] was phenomenologically derived (see Waterton & Hall, 1979; Aplin, 1979 and Yalpani, 1980)

\[
d_1/d = (d_1/d)_{\text{dil}} + 0.58r^{-3}
\]

[11]

where \((d_1/d)_{\text{dil}}\) are the spectral parameters defined in Fig. 1.18 at infinite dilution of the spin probe (where no dipolar interactions occur).

The above equation is valid in the range 1.0 to 2.4 nm.

1.4(d) Artifacts

Assuming the instrumentation is acceptable (see Jost & Griffiths, 1978
and Marsh, 1981) and the sample contains no other paramagnetic compounds to broaden spectra, the only real problem in deriving information from ESR experiments is spectral misinterpretation. One must have an experienced eye when analysing spectra to avoid such misinterpretation (Berliner, 1978). For instance, localized increased concentrations of the spin label may result and/or the spin probe may be insoluble and precipitate. This results in spectra ranging from a single broad line (due to Heisenberg exchange) to a powder-type spectrum. Both would be superimposed over the typical three lines giving the resultant spectrum a broadened appearance.

Conversely, the probe may hydrolyse off or be attached to a proteolytic fragment, resulting in a more mobile spectrum. More mobile signals are easier to detect (it takes only 1/40 as much free signal to give the same peak height as an immobilized signal (Jost & Griffith, 1978)) and thus even very minor hydrolysis will yield a resultant spectrum which looks more mobile than that which characterizes the bulk of the sample.

One must remember also that a reporter group is being introduced which could significantly perturb the system of interest.

There is also ambiguity as to the type of motion the probe reflects. Is it attached to a segment which is more mobile or more rigid than the rest of the system? Is a change induced in the spectrum just a change in the immediate vicinity of the nitroxide or is the rest of the system also changing in a similar manner? It is difficult to approximate the anisotropic tumbling model (Berliner, 1978), so computer simulations used to fit experimental results often provide a number of values which equally well describe the data (Schreier et al, 1978).
1.4(e) Advantages

One advantage of the spin probe method over other spectroscopic ones is the sensitivity of ESR: very little sample is required, a necessary condition for working on many biological systems. Distances between spin labels and other paramagnetic materials can be determined. The polarity of the nitrooxide environment is easily obtainable as is its molecular tumbling rate. The time scale of $10^{-7}$s to $10^{-11}$s is useful and (although not used in this thesis) with the advent of saturation transfer ESR (see Hyde & Dalton, 1972 and Devaux et al, 1981), correlation times from $10^{-7}$s to $10^{-3}$s are accessible. Modern spectrometers are equipped with computers which can digitize spectra and utilize sophisticated programs to manipulate the data, resulting in more accurate interpretations. One can quantitate the spin label yields by double integration of spectra, and, with the chemistry now available, label almost any material.
CHAPTER TWO

2.1 INTRODUCTION

The introduction of spin labels into red cells is not a new technique. Numerous studies have dealt with spin labeled lipids being inserted into the bilayer (e.g. Shiga et al, 1977; Suda et al, 1980; Butterfield, 1982). The peptide parts of membrane proteins have been modified with spin labels (e.g. Fung & Simpson, 1979; Yamaguchi et al, 1982) and in some cases the spin probe of interest wasn't attached to any component but was allowed to permeate into the red cell (e.g. Ross & McConnell, 1975; Schnell et al, 1983). Information on properties such as membrane fluidity (Shiga & Maeda, 1980), protein conformation changes (Lammel & Maier, 1980; Schneider & Smith, 1970), protein-lipid interaction (Bieri et al, 1975; Wallach et al, 1974), transport mechanisms (Ross & McConnell, 1975; Zimmer et al, 1981) and viscosity and elasticity of the cell membrane (Noji et al, 1981) have been elucidated by the above techniques.

Only one other laboratory (that of Butterfield) aside from those associated with the present work has attached the spin probe to the glycocalyx of the red cell, an important part of the membrane (see Introduction). The findings of this laboratory will be discussed in the following chapters.

This chapter deals with quantitation of the spin labeled red cell system and covers the modification of galactose in the glycocalyx. These studies show the necessity for eliminating the background spin probe (by lysing the cell) and the need for high yields and specificity in the modification of the red cell membrane for spectral interpretation.
2.2 MATERIALS AND METHODS

2.2(a) Collection of red blood cells

Blood was drawn from healthy human volunteers by venipuncture into the anticoagulant sodium citrate (0.38%) or EDTA (10.5 mg/7 ml blood). Occasionally, blood was obtained from the Red Cross (in citrate-phosphate-dextrose (Masouredis, 1972)). Blood was used the same day it was collected unless otherwise indicated. Plastic ware was used in all handling of blood. Outdated blood was rejuvenated to restore depleted ATP levels by the method of Valeri & Zaroulis (1972).

Red cells were separated from the plasma by table top centrifugation (1000 x g for five min) and then washed three times in phosphate buffered saline (16.7 mM Na$_2$PO$_4$, 3.3 mM NaH$_2$PO$_4$, 130.4 mM NaCl pH 7.4 (PBS) in 0.025% NaN$_3$ (PBS/azide)) removing the buffy coat (containing white cells and platelets) each time. A wash ratio of one volume red cells to 25 volumes PBS/azide was used unless otherwise indicated.

All blood samples were kept at 4°C or on ice unless otherwise indicated.

2.2(b) Galactose oxidase oxidation of red cells

Red cells were first treated with the enzyme neuraminidase from Vibrio cholerae (Calbiochem-Behring Corp., La Jolla, CA) at one volume of red cells to five volumes of neuraminidase (0.04 U per 10 ml of 37 mM tris HCl pH 6.9, 114 mM NaCl, 4 mM CaCl$_2$) and incubated for one hour at 37°C. These
cells were then washed three times in PBS and incubated with one volume of galactose oxidase (*Dactylium dendroides* from ICN Pharmaceuticals Inc., Cleveland, Ohio) at 10 units/ml PBS for one hour at room temperature. In some cases the neuraminidase step was left out. In one instance the neuraminidase and galactose oxidase were added together in PBS and incubated for an hour at 37°C and then washed three times in PBS/azide. In all procedures controls were also run and treated identically except no oxidizing agent was present.

2.2(c) Spin labeling

To one volume of washed oxidized cells or controls was added one volume of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (TEMPAMINE) from Aldrich Chemical Co., Milwaukee, Wisconsin. (Originally TEMPAMINE from Molecular probes Inc. (Plano, Texas) was used but was found to be impure (M. Yalpani, personal communication)). The concentration of TEMPAMINE was varied from 0.6 to 4 mg/ml PBS/azide. One volume of NaBH$_3$CN (Aldrich Chemical Co.) at five times the molar concentration of TEMPAMINE in PBS/azide was added at the same time and the cells incubated for two hours at room temperature. The reaction was stopped by diluting the solution by a factor of three with PBS/azide and washing three times in PBS/azide.

2.2(d) Ghost preparation

Lysis of red cells, oxidized cells, labeled cells and controls was done by the method of Dodge et al (1963) in 20 ideal milliosmoles phosphate
buffered solution at pH 8.0 in 0.025% azide. One volume of cells was lysed with 40 volumes of the lysis buffer. Ghosts were centrifuged at 20,000 x g's at 4°C for 20 minutes. Care was taken to remove any dense small white pellet at the bottom of the tubes which could contain proteolytic enzymes (Fairbanks et al, 1971). The procedure was repeated three more times producing (usually) pearly white membrane preparations (ghosts).

2.2(e) Microelectrophoresis

The electrophoretic mobilities of red cells or modified red cells were measured in a cylindrical chamber essentially as described by Seaman & Heard (1961) (see also Seaman, 1975). The chamber was immersed in a water bath at 25°C and measurements were made at an electric field strength of 4.0 volts/cm. Electrodes were of silver/silver chloride.

2.2(f) Viscometry

A viscometric assay was performed in a Couette viscometer (Contraves LS 20) as described by Greig & Brooks (1979). This involves measurement of the shear stress resulting from the application of a constant shear rate to a red cell suspension. If upon exposure to a lectin an increase in the shear stress occurs, agglutination has taken place. The fractional increase provides a quantitative index of the degree of aggregation present.
2.2(g) Electron Spin Resonance

Spectra were recorded at the X-band in the derivative absorption mode on a Varian E-3. Spectrometer settings (modulation amplitude, filter time constant and scan rate) were chosen to avoid spectral distortion and power levels were non-saturating and the field always increased from left to right. The settings chosen were 5.7 mW power and a 1 gauss (G) modulation. All aqueous samples were placed in a flat cell of 200 µl capacity and recorded at ambient temperature. Samples were also run at constant temperature on a homodyne spectrometer with a Varian 12 inch magnet and digitized. This was kindly provided by Dr. F.G. Herring.

2.2(h) Quantitation

(i) Red cell or ghost count

Red cell counts were determined by their hematocrit (volume fraction) in capillary tubes spun down for five min on an International micro-capillary centrifuge (International Equipment Corp., Needham Hts., MA), assuming 1.1 x 10^10 packed cells/ml. At low concentrations, red cells were counted in a hemocytometer (a chamber which holds a known volume of red cells over a grid pattern; cells are then counted visually with a microscope) or a Model 112 CL TH/RWP particle counter (Particle Data Inc., Elmhurst, Ill). Ghosts were originally counted microscopically in an improved Neubauer hemocytometer, but due to their poor visibility and low density were later determined on the particle counter also. For ghosts, the counter trigger levels were altered from the red cell settings (10 lower to 50 upper) to 5 lower and 50 upper, (other settings were current 1/4, gain 68, function switch at delta,
linear control and a 76 μ orifice). Dilutions (from 200-400) for counting were done in PBS/azide by weight to ensure accuracy of dilution.

(ii) ESR

Double integrations were done using free spin label of known concentration in PBS/azide as standards. These were originally performed on the Varian E-3 with a Pacific Precision Co. MP-1012A integrator. The second integration was obtained by cutting out and weighing the peaks. Integrator sensitivity (IS) was defined as (mass/gain) x # spins/ml of standard. The spins/ml of the unknown was determined by its (mass/gain) x IS.

The double integrations could also be performed on the spectra with a Hewlett Packard 9815A calculator interfaced with a 9872A plotter using a program written by K.A. Sharp (see Appendix B). Double integrations were also performed via computer on digitized spectra run at constant temperature on the homodyne spectrometer kindly provided by Dr. F.G. Herring. Sample size in this case was limited to 24 μl.

(iii) Protein

Protein concentrations of ghost suspensions were determined by the Lowry procedure (Lowry et al, 1951) as modified by Markwell et al (1978) using human serum albumin (Sigma Chemical Co., St. Louis, Mo) as the standard.

Absorbance at 280 nm was determined by the method of Victoria et al (1981) except in 0.5% SDS instead of 0.2% SDS.
2.2(i) Miscellaneous

$K_3\text{Fe(CN)}_6$ (80mM) (BDH Chemicals Canada Ltd, Vancouver) was added to spin labeled red cells and red cells plus spin label according to Morse (1977).

Lectins from *Glycine max* (soybean agglutinin (SBA)) type VI, *Bandeiraea simplicifolia*, *Dolichis biflorus* (horse gram), *Abrus communis* (jequirity bean agglutinin, and *Ricinus communis* (Castor bean type 11 (RCA 11)) all bind galactose derivatives and were purchased from Sigma. Concanavalin A (Con A), a mannose binding lectin, was from Calbiochem-Behring.

Bound sialic acid was assayed as in Reid et al (1977).
2.3 RESULTS

2.3(a) Quantitation

(i) Ghost counts

It was found using the hemocytometer that only 8 of the 80 squares could be counted sufficiently accurately. This was due to the fact that red cell ghosts didn't settle onto the grid pattern so one had to constantly raise and lower the microscope's field of vision to ensure that one had counted all the ghosts. Thus 8 random squares were counted and the number multiplied by 10 to give the total in that grid.

Using the particle counter it was found that the average apparent volume of the ghosts was lower than that of red cells (Fig. 2.1) so the window settings had to be lowered to ensure all the ghosts were counted. It was observed that the % of the total population that occupied the lowest volume interval (5-10 setting), although consistent for each ghost preparation, varied considerably from sample to sample. The only correlation of sample treatment to the % in this interval was that the ghosts incubated at 37°C were larger in size and thus occupied a smaller % of this window (Fig. 2.1).

The internal consistency was much better for the particle counter than for the hemocytometer. For the particle counter, each sample was consistent (within 9 ± 7%, n = 30) regardless of dilution. When samples of known ghost counts were accurately diluted and counted again the results were within 7 ± 6% of that calculated. The % in the 5-10 window was also very consistent (+ 5%) for a given sample, but varied tremendously from sample to sample, giving rise to inconsistencies in the 10-50 window results.
Fig. 2.1 Graph of the distribution of red cell populations and the window settings of the particle counter. Abscissa, the % distribution/window ordinate, the window setting. •••• red cells; ○○○○ ghosts ——— ghosts after incubation at 37°C for 40 hours.
The advantages of the particle counter are that it is faster, more consistent, and more cells can be counted per sample (thousands compared to about 100 for the hemocytometer). Using the particle counter one can count ghost populations to within 10% accuracy.

(ii) Protein quantitation of ghosts

The modified Lowry gave a good standard curve (coefficient of determination = 0.992). Table 2.1 tabulates the results and also lists values quoted in the literature.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>GRAMS PROTEIN/GHOST X10^13 (+ SD)</th>
<th>RANGE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemocytometer</td>
<td>3.67 ± 0.38</td>
<td>3.4-4.0 (6)</td>
</tr>
<tr>
<td>Cell counter</td>
<td>6.15 ± 0.64</td>
<td>5.7-6.6 (6)</td>
</tr>
<tr>
<td>Fairbanks et al (1971)</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Dodge et al (1963)</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Khodadad &amp; Weinstein (1982)</td>
<td>5.5</td>
<td></td>
</tr>
</tbody>
</table>

The OD at 280 nm of ghosts (1 mg/ml) in 0.5% SDS (used to solubilize the protein and prevent light scatter) was 1.2 ± 0.1 (range 1.07-1.32, n = 6), identical to that of Victoria et al (1981) in 0.2% SDS.

The results of the last two sections support the use of the particle counter as the method of choice for determining ghosts/ml.
(iii) ESR integration

Originally the integrations were performed on the Varian E-3 using a Pacific Precision integrator. This was found to be tedious and inaccurate. The trace had to be cut out and weighed to obtain the second integration and there was always baseline drift on the integrator at the gains used (5-10 x 10^5).

Errors came in the placement of the sample, the reproducibility of power settings and the microwave coupling (see Goldberg, 1978). When a sample was taken out, put back in, the machine retuned and another integration run, the reliability turned out to be low (a 24% error). Calculating spins/ml would have approximately doubled this error due to error in IS (calculated from the spin standard) plus the error in the integration of the unknown.

A program written by K.A. Sharp for a Hewlett Packard calculator and plotter utilized manual digitization of the ESR spectrum (150 points, see Appendix B). Over an extended period of time (3 years), the IS using Sharp's program was 2.56 ± 0.82 x 10^{-20} (n = 55, range 1.36-5.79), an uncertainty of 33%. The advantage of this program is ease. One can integrate the spectrum when time is available and can reintegrate if needs be (reintegrating the same spectrum has a 4 ± 1% accuracy, n = 6, range 1.7-5.5). Integrating the same sample but a different spectrum was fraught with the problems cited above (gain accuracy and sample reproducibility) and was found to produce differences from 3 to 23%, averaging 15 ± 10%. The problem with this program is that the spectrum can't be too broad or have too much baseline drift. Considering how the first integration is zeroed (see Appendix B), one would expect the SL-ghost integrations to be slightly underestimated, due to slight broadening, compared to the free spin label.
Quantitation via the temperature controlled, computer digitized spectrometer was different. Sample size was smaller due to the temperature controlled cavity (24 µl compared with 200 µl for the Varian E-3). One put in a standard sample containing a known number of spins (as opposed to a known concentration); unknown samples had accurately measured volumes (measured by weight). The spectra were digitized, stored on tape and integrated via a computer program devised by P.S. Phillips and F.G. Herring (Herring & Phillips, 1985). The baseline of this integration was then zeroed and the second integration performed. The answers obtained for spins/ml were very close to those calculated via the Hewlett-Packard (H-P) program on a identical sample run on the Varian E-3. The H-P program yielded $2.5 \times 10^{16}$ spins/ml and the computer digitized spectrum yielded $2.35 \times 10^{16}$ (another sample gave $7.3 \times 10^{16}$ for the H-P and $9.9 \times 10^{16}$ for the computer digitized).

All values quoted as spins/ml or spins/ghost are derived from the H-P program.

The end result of this quantitation is that when quoting spins/ghost an uncertainty of about 20-40% is inherent in the calculation.

2.3(b) Galactose spin labeling of red cells

It was found from cell microelectrophoresis data (Table 2.2) that the simultaneous combination of neuraminidase and galactose oxidase (GO) wasn't as efficient in removing sialic acid as was the sequence of neuraminidase digest followed by GO. This is shown by the fact that the electrophoretic mobility of the cells treated simultaneously didn't decrease as much as in
the sequential experiment (Table 2.2). In these experiments (collaborating with M.A. Bernstein and R. Greig) a total of $5 \times 10^4$ spins/cell was found for the sequential digest and spin labeling of the red cell.

**TABLE 2.2**

**CELL ELECTROPHORESIS OF ENZYME TREATED AND SPIN LABELED RED CELLS**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mobility ($\mu m \ sec^{-1} \ V^{-1} \ cm$)</th>
<th>After spin labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$-1.08 \pm 0.03$</td>
<td>$-1.08 \pm 0.01$</td>
</tr>
<tr>
<td>NANase treated red cells</td>
<td>$-0.65 \pm 0.03$</td>
<td>$-0.66 \pm 0.01$</td>
</tr>
<tr>
<td>NANase then GO treated red cells</td>
<td>$-0.66 \pm 0.05$</td>
<td>$-0.65 \pm 0.05$</td>
</tr>
<tr>
<td>NANase with GO treated red cells</td>
<td>$-0.90 \pm 0.02$</td>
<td>$-0.90 \pm 0.07$</td>
</tr>
</tbody>
</table>

Further experiments were conducted with either neuraminidase followed by GO (called NAGO labeled cells) or with GO by itself (called GO labeled cells), followed by reductive amination.

Analysis of these spin labeled cells (SL-cells), labeled at 0.6 mg/ml TEMPAMINE, showed a freely mobile spin probe population whose $\tau_c$ (correlation time, Chapter 1, section 4) didn't change upon addition of a variety of galactose binding lectins, even though in all cases the cells were obviously agglutinated (Fig. 2.2 and Table 2.3). All ESR spectral data are expressed in terms of their spectral parameter, SP, defined below (the spectral parameter is a reflection of the spins' mobility, the higher the parameter the slower the mobility):
\[ SP = (h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2 \]

TABLE 2.3
SP FOR NAGO SL-RBC PLUS LECTINS

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>RELATIVE GAIN(^1)</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAGO SL-RBC</td>
<td>1</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Control(^2)</td>
<td>40</td>
<td>0.11</td>
</tr>
<tr>
<td>NAGO SL-RBC + 250 ug lectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBA</td>
<td>1.24</td>
<td>0.07</td>
</tr>
<tr>
<td>RCA 11</td>
<td>1.24</td>
<td>0.11</td>
</tr>
<tr>
<td>PNA</td>
<td>2.5</td>
<td>0.09</td>
</tr>
<tr>
<td>Bandeirae simplicifolia</td>
<td>2.5</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\(^1\)Gain of samples on the Varian E-3 spectrometer relative to that set for SL-RBC
\(^2\)Red cells treated as in section 2.2 b and c but without enzymes NANase and GO

It became apparent that the signal observed was that of free spin label trapped inside the red cell (first observed by Morse, 1977).

Washing red cells exposed to spin label (at 4 mg/ml) until no signal was detected in the supernatants still resulted in an easily detectable signal from the red cell pellets (SP = 0.19). Lysing these cells at a 1:1 ratio with ghost buffer produced a spin label population in the supernatant (SP = 0.03). In another experiment, 80mM K\(_3\)Fe(CN)\(_6\) (isosmomolar to red cells) was added to these cells and a signal was still detected in the red cell pellets (with an increased SP = 0.19). Addition of 80 mM K\(_3\)Fe(CN)\(_6\) to
Fig. 2.2 ESR spectra of (A) control cells (treated as in (B) but without neuraminidase or GO added) and (B) NAGO SL-RBCs made as described in protocol, section 2.2(b) and (c). (C) is sample (B) with the addition of 250 μg of SBA lectin. See Table 2.3 for details.
one mM TEMPAMINE in PBS resulted in a completely broadened spectrum barely detectable at a gain 48 times higher. Table 2.4 lists the spectral parameter vs the hematocrit of red cells exposed to TEMPAMINE with or without 80mM K$_3$Fe(CN)$_6$.

**TABLE 2.4**

<table>
<thead>
<tr>
<th>Hematocrit of red cells plus 1 mM TEMPAMINE</th>
<th>SP</th>
<th>SP in the presence of 80mMK$_3$Fe(CN)$_6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>41%</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>76%</td>
<td>0.13</td>
<td>0.23</td>
</tr>
<tr>
<td>84%</td>
<td>0.16</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Another confirmation of spin label noncovalently associating with the red cell came from the observation that the signal decreased with increased washing. Perhaps the most sensitive indicator was the ESR signal itself. Samples containing free spin label always exhibited a free population spectrum which overrode that of the covalently bound species. Table 2.5 lists the SP observed following different sample manipulations.

In some cases extensive washing resulted in spectral changes. A NAGO SL-red cell after 10 washings produced a spectrum (SP = 0.33) which was noticeably changed from that of the controls (SP = 0.15), but addition of the jequirity bean lectin (250 μg) still resulted in no further spectral changes (SP = 0.34) even though obvious agglutination had occurred.
TABLE 2.5
SAMPLE MANIPULATION OF SL-RBC AND RESULTANT SP

<table>
<thead>
<tr>
<th>SAMPLE MANIPULATION</th>
<th>SPINS/CELL</th>
<th>SP</th>
<th>SPINS/CELL</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed five times</td>
<td>2.5 x 10^6</td>
<td>0.24</td>
<td>1.7 x 10^6</td>
<td>0.16</td>
</tr>
<tr>
<td>Washed seven times</td>
<td>1.9 x 10^5</td>
<td>0.30</td>
<td>1.1 x 10^5</td>
<td>0.22</td>
</tr>
<tr>
<td>Made into ghosts</td>
<td>1.9 x 10^5</td>
<td>0.62</td>
<td>1.1 x 10^5</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Better analysis could be done utilizing ghosts made from spin labeled red cells. NAGO and GO SL-ghosts had SP varying from 0.41 to 0.62 averaging 0.54 ± 0.09 (n = 4) (Table 2.6) with the GO labeled ghosts being more consistent.

TABLE 2.6
SPIN LABELED SAMPLES MADE INTO GHOSTS AND THEIR SP

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SPINS/GHOST</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAGO SL-ghosts</td>
<td>2.0 x 10^5</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>0.8 x 10^5</td>
<td>0.42</td>
</tr>
<tr>
<td>GO SL-ghosts</td>
<td>5 x 10^5</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>1 x 10^5</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Fluctuations are to be expected due to low yields (the maximum yield was $5 \times 10^5$ spins/ghost with the resultant gain being $10 \times 10^5$). Fig. 2.3 shows a typical spectrum of GO SL-ghosts. The resultant SDS PAGE (Fig. 2.4) shows that these labeled ghosts are comparable to the controls (red cells treated as stated in the methods for spin labeling red cells but no galactose oxidase present).

Addition of 250 µg (50 µl) of PNA or SBA to 1 ml of NAGO SL-ghosts resulted in visible agglutination and a slight increase in the spectral parameters (Fig. 2.5).
Fig. 2.3 An ESR spectrum of GO SL-ghosts made as described in the text. It was run on a Varian E-3 with the gain set to $10 \times 10^5$, modulation 1 G, and power at 5.7 mW. SP = 0.58
Fig. 2.4 SDS PAGE of (A) GO SL-ghosts made as in protocol and (B) untreated ghosts. The top scans are densitometric tracings of coomassie blue stained gels loaded with 45 μg of membrane protein. The bottom scans are of PAS stained gels loaded with 180 μg of membrane protein. The numbering system is that of Fairbanks et al, 1971.
Fig. 2.5 ESR spectrum of (A) NAGO SL-ghosts made as described in the text along with the spectra of these ghosts with the addition of 250 μl (50 μl) lectins, (B) PNA and (C) SBA. The spectral parameters, SP, are also indicated.
To further study the effects of PNA upon red cells, neuraminidase treated cells and NAGO SL-red cells were exposed to PNA in a viscometer to assay the agglutination induced by PNA with these treated cells.

Unmodified red cells sheared in the presence of PNA showed no noticeable agglutination in the viscometer. The neuraminidase treated and the NAGO SL-cells, however, exhibited behaviour characteristic of strong agglutination. Fifteen micrograms of PNA added to a 47% solution of NAGO SL-cells showed the typical shear-enhanced agglutination response when plotted as shear stress against time (Fig. 2.6) (Greig & Brooks (1979) analysed the system with respect to Con A agglutination). This system, however, was not inhibited or reversed by galactose, the monosaccharide inhibitor of PNA. Because this measurement works only on red cells, the free spin label couldn't be eliminated and the resultant spectrum from the highly agglutinated cells was no different from the cells not exposed to PNA or to the viscometer, all giving the typical free spin label spectra.
Fig. 2.6 The shear stress recorded as a function of time for aggregating erythrocytes. Red cells were suspended at a final hematocrit of 47% and 0.9 ml placed in the cup of the viscometer. Temperature was maintained at 37.0 ± 0.1°C. At a constant shear rate of approximately 49s⁻¹ (37 rpm) a steady baseline shear stress, due to the viscosity of the unaggregated cell suspension is obtained. PNA was added as indicated in a volume of 40 ul. (A) is the neuraminidase treated red cells with 20 µg of PNA added and (B) is the NAGO SL-RBC with 10 µg PNA added.
2.4 DISCUSSION OF SPIN LABELING RESULTS

(i) Red cells

(a) Microelectrophoresis

The neuraminidase and GO treatment together was not as efficient as the sequential addition of these two enzymes as seen by their electrophoretic mobility measurements. Mobility is a measure of the surface charge distributed through the glycocalyx (Levine et al, 1983) to which sialic acid is the major contributor (Eylar et al, 1962; Seaman et al, 1977). Vassar et al (1972) found a 60% decrease in electrophoretic mobility of red cells after neuraminidase treatment (however, Luner et al (1975) found that removal of 83% of the sialic acid with neuraminidase resulted in an 80% drop in the electrophoretic mobility). Table 2.2 shows that mobility decreased 40% for the neuraminidase and NAGO treated cells, indicating that not all the sialic acid had been removed. The red cells that had been treated simultaneously with neuraminidase and GO showed only a 16% decrease in mobility indicating that less sialic acid had been removed. This decrease in sialic acid removal is probably due to the incubation buffer being optimal for GO and not neuraminidase (requiring lower pH and Ca$^{2+}$), thus the neuraminidase was not as efficient.

The important result from this data is that the mobilities of these cells were not altered by the reductive amination step. This indicates that this type of modification results in no further change in the surface charge of the red cell as detected by microelectrophoresis. The SDS PAGE gels of GO SL-ghosts don't appear different from those of normal red cells (Fig. 2.4), showing that the GO labeling method is mild.
(b) Spin labeling

Due to low yields ($5 \times 10^5$ spins/cell maximum) the spectra are noisy (gains set to $10 \times 10^5$) and drift can occur (times for running a spectrum are from 8-30 min). The resultant signal-to-noise ratio is thus lower than desired. For these reasons spectral parameters (SP) have been quoted instead of $T_C$ calculations, since $w_0$ (the center line peak width) is hard to determine accurately (Lee et al., 1980). These weak signals make spectral interpretation more difficult and uncertainties high (20% accuracy at the gains quoted). This could mask slight systematic alterations in the samples, since changes of 10% would go unnoticed and 20% changes would be viewed as questionable.

The major problem in spin labeling biological systems is the removal of non-reacted spin probe. This population is usually free unless partitioned into a different environment or precipitated out. Spin probes measure the micro-environment, as opposed to the macro-environment, of a solution. The solution may appear very viscous and still give ESR signals of freely spinning nitroxides, if the micro-environment of that nitroxide has a low viscosity (see Aplin, 1979 and Morse et al., 1979). This freely spinning population is readily detected because its spectrum exhibits the typical three sharp lines which can be seen down to about $10^{-6}$ M. This spectrum is superimposed over the covalently attached spectrum and can override the bound signal.

This free spin probe is inside the red cell (first observed by Morse, 1977, see also Morse et al., 1979, and Bartosz & Leyko, 1980). Extensive washing of the red cells never eliminated the signal from the controls (cells + TEMPAMINE) even if washed, let sit overnight and washed again. No
signal was detected in the supernatants of these cells, but upon lysing (1:1 with ghost buffer) a signal was observed in the supernatant (SP = 0.03).

For red cell controls (red cells plus TEMPAMINE), SP was 0.135 ± 0.015, indicating a slight increase in $\tau_C$ ($\tau_C$ for free TEMPAMINE in aqueous solutions has been reported to be $4.3 \times 10^{-11}$ s (Bartosz & Leyko, 1980) or $4.6 \times 10^{-11}$ s (Morse et al., 1979), which corresponds to a spectral parameter of about 0.04 assuming $\omega_0 = 1.5$ G and $K = 6.5 \times 10^{-10}$ and using equation [9] in Chapter 1 (p. 48) which agrees well with the SP obtained for TEMPAMINE in PBS/azide in this work: 0.03 ± 0.01 (n = 10)). As the hematocrit of controls was increased, so did SP (Table 2.4). The fact that SP increased with hematocrit indicates that the spin probe inside was spinning at a slower rate, the more mobile signal in solution extra-cellularly originating from slow leakage out of the cell. If one extrapolates the data in Table 2.4 to 100% hematocrit, an SP of 0.198 is obtained (coefficient of determination = 0.997) which results in a calculated $\tau_C$ of $1.9 \times 10^{-10}$ s. Interestingly, using the data in Table 2.4, it was calculated that at hematocrits of 36% or less, the signal obtained from control suspensions would be equivalent to that of TEMPAMINE in PBS, the signal from inside the red cells being totally undetectable.

Additional proof of the location came using the method of Morse, 1977 (addition of 80 mM Fe(CN)$_6^{3-}$, a paramagnetic ion which broadens the nitroxide signal of TEMPAMINE (Morse, 1977; Aplin, 1979)). At 80 mM K$_3$Fe(CN)$_6$, the signal is completely broadened out for the spin label standard in PBS (as was found by Morse, 1977) but its addition to red cells spin labeled or with spin label added to them still resulted in a signal from the red cell pellets (Table 2.4) (Morse, 1977). K$_3$Fe(CN)$_6$ doesn't
enter red cells (Morse, 1977; Mishra & Passow, 1969; Kaplan et al, 1973), thus the observed signal must be from spins protected by the red cell, presumably inside it. A $\tau_c$ of $1.9 \times 10^{-10}$ s was calculated for the TEMPAMINE inside red cells, in good agreement with Morse (1977) ($1.84 \times 10^{-10}$ s) and Morse et al (1979) ($2.0 \times 10^{-10}$ s).

Spin labeling the red cells via NAGO or GO resulted in spectra such as that shown in Fig. 2.2. As seen in Table 2.3, addition of a variety of gal/galNAc binding lectins (and Con A, a mannose binding lectin) at concentrations which produce visible agglutination resulted in no change in the spectra. The non-covalently bound spectrum predominated. Extensive washing resulted in a decreased signal but the free signal couldn't be eliminated unless the cells were lysed and made into ghosts (Table 2.5, Fig. 2.3). For the washed NAGO SL-red cells (SP = 0.33), the spectra of which began to resemble those of SL-ghosts (SP = 0.54), the addition of jequirity bean lectin still resulted in no detectable spectral change upon agglutination (SP = 0.34). This could be due to the free spin label masking any minor change induced by the lectin, or the lectin agglutination may actually produce no change.

Only when the samples had been lysed were the signals visually different and SP increased by about 50% (Table 2.5 and 2.6). It appears that very few, if any, spins were removed upon lysing the cells, yet the SP were dramatically different. It might be argued that the nitrooxide environment was altered upon the formation of ghosts, but the spectra of NAGO SL-cells also start to show this type of increase if enough free spin label can be removed (SP = 0.33).

Only when these cells were made into ghosts (all the free spin label
removed) was there any detectable change upon the addition of PNA and SBA, and even then it was only about 20% in the spectral parameter (Fig. 2.5).

The spin labeled ghosts produced are still agglutinable by lectins. Since PNA binds only to desialylated red cells (Goldstein & Haynes, 1978) the NAGO SL-method was used to see if the spectrum would be altered upon addition of PNA or SBA lectins. PNA agglutinates NAGO SL-red cells as seen from the viscometry of these cells, PNA producing a typical lectin profile (Greig & Brooks, 1979, Fig. 2.6). Cells not treated with neuraminidase don't agglutinate while neuraminidase digested and NAGO SL-cells do. This shows that even though the cells are modified with the spin label, they still bind PNA. Shear induced agglutination was not reversed by galactose, possibly due to other PNA binding sites being exposed during shearing.

PNA binding to the NAGO SL-cells was also indicated by the fact that visible agglutination occurred with these cells and the resultant SL-ghosts upon addition of the PNA lectin.

Interpretation of the effects of PNA on NAGO SL-ghosts is complex due to the multiple membrane components being labeled, low yields, the possibility of unbound label contributions and lack of knowledge of the PNA binding sites. Carter & Sharon (1977) isolated, via a PNA affinity column, desialylated glycophorin and a 27 K component from red cells. Jaffe et al (1979) tentatively identified desialylated glycophorin A and a second glycoprotein (of 58-61 K) as the PNA receptors.

GO has a specificity for terminal C\textsubscript{4} and C\textsubscript{6} OH groups of galactose and galNAc (Hamilton et al, 1973; Schlegel et al, 1968). Yields as high as 2-4 \times 10^7 \textsuperscript{3}H/cell have been quoted for just the GO/NaB\textsubscript{3}H\textsubscript{4} method (Gattegno et al, 1981; Aminoff et al, 1981) and are improved employing NAGO
vs GO, with two additional proteins being labeled (Gahmberg, 1976), indicating that a large number of exposed terminal gal and galNAc residues are present on the red cell. The GO/NaB[^3H]_4 labeling method shows at least 18 proteins labeled (Gahmberg, 1976) (PAS proteins, band 3, band 4.5 Mueller et al, 1979 and high molecular weight components, Jokinen, 1981; Abraham & Low, 1980) with about 40% of the label on the lipids (Gahmberg & Hakomori, 1973; Steck & Dawson, 1974). Since PNA appears to bind to only two of the 18 proteins and since 40% of the label is on the lipids, one wouldn't expect a large change in the ESR spectrum with PNA binding, as only a small fraction of the total spin population would be perturbed.

As can be seen from Fig. 2.5, there is a slight increase in SP with the addition of PNA and SBA. This increase could be due to the spin label slowing down slightly but the changes are very close to experimental uncertainties. The fact that both PNA and SBA increase SP supports the assumption that these lectins are binding to the spin labeled gal/galNAc residues, however.

One problem resulting from removal of sialic acid to label the galactose residues is that a sugar unit which also carries a negative charge has been lost. Desialylating red cells renders them agglutinable to PNA. The question arises, is this due to gal residues being exposed or is it, wholly or in part, a result of removal of steric or electrostatic hindrance by the sialic acid. Removal of sialic acid decreases the surface charge (Table 2.2) and this fact alone appears to render them more agglutinable (Luner et al, 1975; Greenwalt & Steene, 1974). The system has been perturbed and it is hard to determine if the effect one sees is due to this perturbation alone or to secondary effects.
(ii) $\tau_C$ of labeled ghosts

GO or NAGO SL-red cells made into ghosts had a calculated $\tau_C$ of $7.7 \times 10^{-10}$ s (assuming the SP = 0.54 $\pm$ 0.09, $w_0 = 2.2$ (Chapter 3, Table 3.7) and $K = 6.5 \times 10^{-10}$). This value is close to that found for the NAGO-treated fetuin ($5.2 \times 10^{-10}$ s) and NAGO treated BSM ($3.5 \times 10^{-10}$ s) of Aplin et al (1979), but is much closer to the values obtained for spin labeled sialic acid residues on ghosts by Felix & Butterfield ($8.4 \times 10^{-10}$ s) (1980), for fetuin by Aplin et al ($7.9 \times 10^{-10}$ s) (1979) and for glycophorin by Lee & Grant ($9.6 \times 10^{-10}$ s) (1979). All of the above have faster molecular motion than anticipated based on their molecular size, implying that these sugar units are more free than the bulk of the molecule. The fact that SL-sialic acid residues gave similar results to the labeled gal residues implies a general freedom of motion for the oligosaccharides. This implies that the glycocalyx of the red cell surface (or more specifically, the terminal gal/galNAc residues) are fairly mobile. The spectrum of the GO SL-ghosts may be a composite of several different populations (lipids, PAS proteins, bands 3 and 4.5), although the yields were too low for this type of analysis (labeling with NaB[$^3$H]$_3$CN still resulted in low tritium labeling). The fact that PNA and SBA didn't alter the signal very much implies a heterogeneous population (or possibly only minor interactions with the SL-gal/galNAc residues). If the population is heterogeneous, then this result is very surprising, because about 40% of this population are glycolipids and the appearance of fairly homogeneous spectra indicates that the majority of the gal/galNAc residues are experiencing the same type of environment. The actual values for $\tau_C$...
quoted and in the literature are only estimates of the true $\tau_c$, however, which requires computer simulation to calculate properly (and a knowledge of how the probe is actually rotating).

The advantage of using SL-sugar residues in this context, on the other hand, is that one now has, in principle, an independent confirmation that the molecule of interest is actually binding to the sugar unit, something which is usually taken for granted but not shown.
2.5 CONCLUSION

A spin label was successfully attached to the surface of red cells via the GO or the NAGO method followed by reductive amination with TEMPAMINE. No detectable surface charge alterations occurred due to the reductive amination step. The red cell samples had to be lysed to eliminate unbound spin label, to allow for proper spectral interpretation and to observe any possible spectral alterations upon the addition of lectins.

From the $\tau_c$ calculations, it appears that the terminal gal/galNAc residues on the glycocalyx are all fairly mobile, having a correlation time of $7.7 \pm 1 \times 10^{-10}$ seconds. They appear to be heterogeneous in their distribution, being only partially sensitive to PNA and SBA agglutination.

Converting NAGO SL-red cells into ghosts does not appear to alter the mobility of these terminal sugars and thus one can use this system for studying the glycocalyx.
3.1 INTRODUCTION

Due to low yields and the lack of specificity found in the galactose oxidase reactions, the sialic acids on the red cell surface were chosen to be modified. On the red cell membrane, the main sialic acid containing proteins are the PAS proteins (glycophorins A, B and C and minor components). There is no enzymatic way of oxidizing these sugars, so mild periodate treatment was used, which under proper conditions is specific for sialic acid.

Periodate modification of red cell surfaces was first carried out in 1948 by Hirst on fowl red cells. This was one of the first indications that the cell surface contained sugars. The cells' ability to absorb influenza virus was altered. In 1949, St. Groth made the same observation for human red cells and found that this effect was sensitive to the extent of periodate modification. Stewart (1949) found these periodate treated cells were able to form antibodies in rabbits and that these cells could become agglutinable by the rabbit's own serum. Springer (1963), reviewing the literature, states that blood type activity was decreased upon periodate modification, with the M and N being the most susceptible followed by the Rh(D), indicating that these two blood types are most likely carbohydrate in nature.

In 1964, Spiro showed that sialic acid was selectively oxidized in the periodate modification of fetuin, and was completely destroyed after thirty
min exposure to periodate at a 7:1 ratio of periodate to sialic acid. Van Lenten & Ashwell (1971) confirmed this with the periodate oxidation of orosomucoid.

Blumenfeld et al (1972) were the first to periodate oxidize red cells and reduce the product with NaB[\(^3\)H]\(_4\), thus labeling the newly generated aldehydes of the cell surface. They found a correlation between the \(^3\)H profile and the PAS stainable proteins on SDS PAGE. Neuraminidase digest of these treated ghosts (Liaos et al, 1973) showed the major released product to be NANA\(_7\) (sialic acid with the C\(_8\) and C\(_9\) removed) with a concomitant decrease in the PAS and \(^3\)H staining. Mueller et al (1976), using the Laemmli gel system, labeled the ghost via the periodate/NaB[\(^3\)H]\(_4\) method and found all the PAS proteins (8 stainable components visible) labeled.

The periodate oxidation method has become the method of choice for labeling the sialic acids of cell membranes. Lymphocytes (Presant & Parker, 1976; Spiegel & Wilchek, 1983), synaptic plasma membranes (Cruz & Gurd, 1980) and platelets (Rotman et al, 1980; Steiner et al, 1983) have been labeled this way. Thiol mannosyl hydrazide (Rando & Bangerton, 1979), biotin hydrazide (Heitzmann & Richards, 1974; Skutelsky et al, 1977), \(^3\)H (via NaB[\(^3\)H]\(_4\)) (Steiner et al, 1983; Steck & Dawson, 1974; Kahane et al, 1976), arylalkylamines (Schweizer et al, 1982), eosin derivatives (Cherry et al, 1980), thiolhydrazide (Taylor & Wo, 1980), fluoresceine amine (Abraham & Low, 1980) and spin labels (Aplin et al, 1979, Felix & Butterfield, 1980) have been attached to the cell surface using this method.
This chapter deals with the selective modification of sialic acid via mild periodate oxidation followed by spin labeling with TEMPAMINE and NaBH₄CN (reductive amination). Effects of periodate oxidation on red cells and ghosts was analysed and compared to sialoglycoproteins glycophorin and fetuin. Attempts at improving yield of spins/cell with minimal perturbation of the membrane followed. Higher yields (compared to GO SL-ghosts) resulted in the opportunity to analyse SL-ghosts in more detail. Results indicate selective labeling had occurred and information about the SL-sialic acids could be obtained.
3.2 MATERIALS AND METHODS

3.2(a) Periodate oxidation of red cells and ghosts

To one volume of packed red cells (washed as in Chapter 2, section 2.2(a)) or ghosts (prepared as in Chapter 2, section 2.2(d)) was added one volume of NaIO₄ of varying concentrations (0-10 mM) in PBS/azide (defined in Methods, Chapter 2, section 2.2(a)). These cells were incubated for 10 min unless otherwise indicated and the reaction stopped by diluting the solution by a factor of three with PBS/azide and then washed three times at a wash volume ratio of 25:1 (PBS to red cells) spun at 1,000 x g for five min or 40:1 (Dodge buffer (20 ideal milliosmole phosphate buffer, pH 8.0 in 0.025% azide) to ghost) spun down at 20,000 x g at 4°C for 20 min.

3.2(b) Periodate oxidation of glycophorin or fetuin

Glycophorin was isolated from red cells by the method of Marchesi & Andrews (1971). Neuraminidase digest was performed on glycophorin as follows: to 10 mg of glycophorin in 10 ml Krebs-Ringer solution (0.121 M NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1 mM MgSO₄, 17 mM Na₂HPo₄) was added 0.2 U of *Vibrio cholerae* neuraminidase (Calbiochem). This was incubated at 37°C for 24.5 hours. The sample was dialysed against Kreb-Ringers solution until no free sialic acid (determined by the method of Reid *et al.*, 1977) could be detected in the sample. Lowry assays were performed as in Lowry (1951) as modified by Peterson (1977).

Fetuin (type IV) was purchased from Sigma.
To a known concentration of sialoglycoprotein (glycophorin or fetuin) was added NaIO\textsubscript{4} in PBS. After certain time intervals the solution was monitored for formaldehyde (by the method of Nash, 1953, as modified by Reid et al, 1977).

\textsuperscript{3}H labeling of glycophorin

Five milligrams of glycophorin in 5 ml of 0.1 M sodium acetate pH 5.5 in 0.025% azide was incubated with a 2 molar excess of NaIO\textsubscript{4} for 2 hours at 4\textdegree{}C in the dark. This reaction was stopped by the addition of 0.3 ml of 0.1 M glucose and the sample dialysed against water and 0.1 M phosphate buffer, pH 7.1. Next, 25 mCi of Na\textsubscript{3}H\textsubscript{4} (Amersham) was added and incubated for 1 hour at room temperature followed by 2.8 mg of cold NaBH\textsubscript{4} and incubation for another 35 minutes. The product was dialysed against 0.1 M sodium acetate pH 5.0 and then against water/0.025% azide until no more \textsuperscript{3}H was released. The product was then freeze dried.

3.2(c) Spin labeling of red cells

Experimental conditions were varied for the periodate treated cells to optimize labeling and are tabulated in the results. Occasionally, Na\textsubscript{3}H\textsubscript{3}CN (Amersham) at about 3 \times 10\textsuperscript{8} cpm/ml in the NaBH\textsubscript{3}CN solution was used. Other reagents were borane dimethyl amine (Aldrich), 2,2,6,6-tetramethyl-4-amino-piperidine-1-oxyl (Sigma), the spin label analogue (SLAN). Optimal conditions for labeling the red cells are quoted in the Results (section 3.3(e)) for ease of comparison.
3.2(d) Spin labeling ghosts by the method of Felix & Butterfield (1980)

To 50 ml of ghosts (at 5.4 x 10^9 ghosts/ml) was added 50 ml of 2 mM NaIO\textsubscript{4} in 0.1 M sodium acetate pH 5. This was incubated for 10 minutes at 4\textdegree{}C with gentle mixing. The oxidation was stopped by addition of 450 ml of 5 mM arsenite in 50 mM phosphate buffer pH 9.2. The samples were spun down at 20,000 x g for 20 minutes at 4\textdegree{}C. To the pellet was added 100 ml of 1 mM TEMPAMINE and 100 ml of 1 mM NaBH\textsubscript{3}CN with an activity of 8 x 10\textsuperscript{7} cpm/ml (both in 5 mM phosphate buffer, pH 8) and incubated overnight at 4\textdegree{}C (a total of 19.5 hours). The suspension was spun down as before and then washed in Dodge buffer five times at a volume ratio of 1:25 ghosts to buffer. Controls were treated identically except no periodate was added.

3.2(e) Formaldehyde assay for red cells

The formaldehyde was assayed via a modification of the method of Nash (1953). Due to slight lysis of red cells by the periodate oxidation step, an additional step was included. After the addition of the periodate and its cessation by the addition of KI/Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, the protein in solution was precipitated via the method of Somogyi (1945). In this procedure 50 \( \mu \)l of ZnSO\textsubscript{4} (5% by weight) was added to the 300 \( \mu \)l sample, mixed, then 50 \( \mu \)l of Ba(OH)\textsubscript{2} (0.3 M) added and the sample mixed and centrifuged on an Eppendorf table top centrifuge. This method was found to successfully precipitate the interfering protein (more effectively than 10% trichloroacetic acid or (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}). The supernatant was then assayed according to Nash (1953). Standard samples were treated identically.
3.2(f) Sialic acid assays

Sialic acid was assayed by the thiobarbituric acid method of Aminoff (1961) as modified by Culling et al (1977), or by the resorcinol assay of Jourdain et al (1971). Bound sialic acid was assayed as in Reid et al (1977).

3.2(g) Rouleaux

Red cells attach side-by-side to form what is called rouleaux in the presence of certain macromolecules (e.g. fibrinogen and globulins found in the plasma or dextran solutions). An assay of cell-cell surface interactions are their ability to form rouleaux. A 5% suspension of washed periodate oxidized red cells was exposed to an 80% plasma solution (in PBS) or a 3% solution of dextran 70 (Pharmacia) in 1% albumin. The dextran concentration was determined in a polarimeter.

Red cells that had been oxidized with 3.5 mM NaIO₄ and washed, were exposed to plasma or dextran and a varying concentration of N-Acetyl glucosamine (GlcNAc), glucosamine (glcNH₂) or glucose.

All data was obtained via microscopic inspection of the mixtures.

3.2(h) ESR

ESR samples were run as described in Chapter 2. Temperature controlled experiments were done on a temperature controlled homodyne spectrometer employing a Varian 12 inch magnet. The samples were put in 20 µl capillary
tubes and flame sealed at one end making sure no heat damage occurred to the sample.

3.2(i) Measurements of the distance between nitroxide label

Various samples of red cells were labeled according to the optimal protocol with the inclusion of a varying ratio of spin label analogue to TEMPAMINE, from 9:1, 4:1 to no spin label analogue. This ensured that the reacting amine group concentration stayed the same and only the nitroxide concentration decreased by the dilutions quoted. These labeled cells were then made into ghosts as in the protocol. Spectra were run at 77°K on all the samples using a Dewar insert on the Varian E-3 containing liquid nitrogen. Spectra were run at 0.16 mW microwave power, the lowest available to prevent saturation. Silica tubing (3 mm o.d.) sealed at one end was used and solutions were introduced using a teflon syringe.

3.2(j) Reversibility

Spin labeled H labeled ghosts, made via the optimal protocol were left sitting at 4°C in PBS/azide. At selected times aliquots were removed and spun down and the supernatant and pellet analysed for spin label and tritium content.
3.2(k) Hydrolysis of the spin labeled ghosts

Neuraminidase digest was performed on the spin labeled ghosts (SL-ghosts) with enzyme from *Clostridium perfringens* (Sigma type VI) or *Vibrio cholerae* (Calbiochem-Behring Corp., La Jolla, CA) at 1.3 IU per 6 x 10⁹ ghosts at 37°C for 39 hours at pH 5.1. Native red blood cells were incubated only for 1.5 hours under identical conditions.

Acid hydrolysis was performed with 0.1 N H₂SO₄ at 80°C for one hour at one ml H₂SO₄ to one ml of SL-ghosts (6 x 10⁹ ghosts/ml). The samples were then spun down (20,000 x g for 20 min at 4°C), the supernatant and the pellet separated and neutralized with NaOH.

In all cases, appropriate controls (SL-ghosts or normal ghosts were treated identically, except no neuraminidase or acid was added) were included.

3.2(l) Isolations

SL-ghosts were selectively solubilized with 0.1 M NaOH or 0.5% Triton X-100 in 56 mM borate buffer, pH 8 as described by Steck & Yu (1973) and Yu et al (1973).

Lipids were isolated with chloroform:methanol as described by Saito & Hakomori (1971) or via the method of Folch et al (1957).

Glycophorin was isolated from ghosts or SL-ghosts by the method of Marchesi & Andrews (1971). Briefly, to every gram of lyophilized ghosts are added 43 ml of 0.3 M LIS (lithium diiodosalicylate) in 0.05 M tris pH 7.4. After incubating for 20 min, 80 ml of ice cold water was added and allowed
to incubate for one hour at 4°C. The supernatant was collected by centrifugation at 18,000 x g for one hour at 4°C and 50% phenol in water added at equal volume to the supernatant. The phases were allowed to separate and the upper phase collected and dialysed against water. This was freeze-dried and then the protein washed twice in ice cold absolute ethanol and resuspended in water, dialysed and freeze dried.

3.2(m) Tritium analysis

SDS PAGE was performed as described in Appendix A. The gels were sliced at 1 mm intervals and counted for $^3$H by the method of Aloyo (1979). In some cases the gels were frozen at -70°C immediately after being run and sliced, and in other cases the gels were fixed and stained before being sliced and counted. No differences in the $^3$H profiles were noticed but a decrease of 50% in the cpm/slice was obtained for the stained gels.

Tritiated samples (5-10 μl) were solubilized in 10 ml Atomlite (NEN, Boston, MA) or in 10 ml toluene (scintanalyzed (Fisher)) to which had been added 6 g of PPO (Kodak-Eastman), 10 ml NCS tissue solubilizer (Amersham) and 10 ml hyamine hydroxide (Packard) per liter of toluene. Samples were allowed to incubate at room temperature for two days, with occasional shaking, before being counted. Absolute disintegration rates (disintegration per minute (dpm)) were determined by adding various amounts of erythrocytes mixed with a constant known level of tritium. With the use of the experimental quench curve, the number of counts per minute (cpm) was converted into dpm. The moles of NaBH$_3$CN which reacted was calculated from the specific activity (2 to 308 x 10$^{-12}$ mmole/dpm).
3.3 RESULTS

3.3(a) Effects of periodate modification on rouleaux formation

Red cells were modified as described in the Methods section 3.2(a) with concentrations varying from 0 to 10 mM periodate. Up to 3.5 mM periodate, no obvious alterations were visible but 5 mM exposure resulted in noticeable swelling and 10 mM periodate caused lysis. These modified cells were exposed to blood plasma. Control cells in 80% plasma formed rouleaux (Fig. 3.1). As the periodate concentration increased, less rouleaux were formed. At 0.5 mM, the rouleaux formation had decreased to about 50% that of controls and by 3.5 mM periodate, the cells were swollen and clumps had formed; at 5 mM and higher, cell lysis resulted, and large clumps formed.

This pattern was also observed for red cells suspended in dextran solutions (Fig. 3.2), with 2 mM periodate modification, significant decreases were observed in rouleaux formation. In place of rouleaux clumps were formed which got bigger the higher the periodate concentration used to modify these cells. At 2 mM periodate modification, the clumps were already being observed.

A concentration of 3.5 mM periodate was chosen to modify cells due to noticeable clumps being formed but no lysis occurring. To these systems of oxidized red cells and plasma or dextran were added the sugars glcNAc, glcNH₂ and glucose at 4, 10 and 40 mg/ml. For plasma, glcNAc at 40 mg/ml completely inhibited the formation of clumps and this inhibition was noticeable even at 4 mg/ml. The controls at 40 mg/ml glcNAc were crenated however. Forty mg/ml glcNH₂ partially inhibited clumping and had no effect
Fig. 3.1 Oxidation of red cells vs their ability to form rouleaux. (A) is a picture of normal rouleaux in 80% serum. (B), (C), and (D) are periodate treated red cells exposed to 80% serum, (B) 1 mM, (C) 2 mM, (D) 5 mM periodate. Magnification 1600 X.
Fig. 3.2  The effect of periodate oxidation of red cells upon their ability to form rouleaux in 3% dextran. (A) normal rouleaux formation with unmodified red cells and (B), (C), and (D) periodate treated cells. (B) 0.5 mM, (C) 1 mM, (D) 2 mM periodate. Magnification 1600 X.
on the controls (they still formed rouleaux). No effect was produced by
glucose. For the dextran system, whether the cells were oxidized or not,
there was only a slight decrease in the agglutination with all the sugars.

3.3(b) Formaldehyde formation

Table 3.1 lists the amount of formaldehyde formed due to periodate
oxidation of red cells as performed in the protocol with varying times and
periodate concentrations. The amount of formaldehyde is expressed as the %
of sialic acid modified assuming 3 x 10^7 sialic acids/cell.

TABLE 3.1
FORMALDEHYDE FORMATION\(^1\) AS A FUNCTION OF PERIODATE CONCENTRATION TO RED CELLS

<table>
<thead>
<tr>
<th>Exposure time of periodate (min)</th>
<th>Molar excess of periodate to sialic acid(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Four</td>
</tr>
<tr>
<td></td>
<td>Eight</td>
</tr>
<tr>
<td>10</td>
<td>7%</td>
</tr>
<tr>
<td>20</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td>12%</td>
</tr>
<tr>
<td></td>
<td>15%</td>
</tr>
</tbody>
</table>

\(^1\)Expressed as the % of total sialic acid on a red cell
\(^2\)Assuming the total sialic acid on a red cell is 3 x 10^7

Repeated experiments at four fold molar excess (2 mM) of periodate to
sialic acid proved to be variable, with the average being 15 ± 9% of the
sialic acid being modified (n = 5, range 7-22).

Table 3.2 lists the amounts of formaldehyde formed upon the oxidation of
ghosts and neuraminidase treated ghosts for various times and oxidation
conditions in PBS.
TABLE 3.2

FORMALDEHYDE FORMATION\(^1\) WITH PERIODATE OXIDATION OF GHOSTS

<table>
<thead>
<tr>
<th>Periodate exposure</th>
<th>Molar excess of periodate to total sialic acid(^2)</th>
<th>Ghosts</th>
<th>NANase treated Ghosts(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td>42.5±.5</td>
<td>74±8</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>55±6</td>
<td>66±2</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>72±6</td>
<td>76±1</td>
</tr>
</tbody>
</table>

\(^1\)Expressed as the % of total sialic acid on a red cell, results an average of four experiments  
\(^2\)Assuming the total sialic acid on a red cell is 3 x 10\(^7\)  
\(^3\)80 ± 8% if the sialic acid removed  

Fig. 3.3 shows the SDS PAGE gels of the oxidized ghosts. Table 3.3 lists the amounts of formaldehyde formed upon the oxidation of glycophorin, neuraminidase treated glycophorin and fetuin for various times and oxidation conditions in PBS.

Glycophorin contained 37.6% (w/w) lowry protein and 18.2 ± 0.7% (w/w) sialic acid. Fetuin contained 6.45% (w/w) sialic acid.
Fig. 3.3. Densitometric scans of SDS PAGE gels (5% pH 6.1) scans of (A) coomassie blue (45 μg membrane protein 595 nm) or (B) PAS (180 μg membrane protein 525nm) of (1) untreated ghosts, (2) seven molar excess of periodate to sialic acid or (3) 15 molar excess periodate oxidation of ghosts. Numbering according to Fairbanks et al, 1971. TD = tracking dye.
TABLE 3.3

FORMALDEHYDE FORMATION WITH PERIODATE OXIDATION OF GLYCOPHORIN AND FETUIN

<table>
<thead>
<tr>
<th>Periodate exposure (min)</th>
<th>Glycophorin</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Fetuin</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>NANase treated</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9+1</td>
<td>9+5</td>
<td>9+1</td>
<td>16+6</td>
<td>5+1</td>
<td>21+10</td>
<td>21+10</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>58+2</td>
<td>54+6</td>
<td>100+3</td>
<td>82+8</td>
<td>9</td>
<td>80+5</td>
<td>98+3</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>93+3</td>
<td>111+5</td>
<td>121+5</td>
<td>122+5</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1expressed as a % of the total sialic acid (average of 4 experiments)
2average of two experiments
35 ± 5% of the bound sialic acid remaining

3.3(c) Tritiated glycophorin

The sample of glycophorin used was found to contain 31.4% protein and 18.9 ± 1.0% sialic acid. In 0.1 M sodium acetate 1 mg/ml glycophorin has an absorbance of 0.428 at 280 nm.

After tritiation, it was found that over 99% of the unreacted tritium was released during the first two hours of dialysis against acetate buffer, pH 5. The resultant activity was $1.43 \times 10^7$ cpm/mg glycophorin. Fig. 3.4 shows the gel profiles of the tritium labeled and unmodified glycophorin. These gels were sliced immediately after being run.
Fig. 3.4  SDS PAGE of 50 µg glycophorin (reduced) run on a 10% laemmli gel. (A) PAS stain of isolated glycophorin. (B) Periodate and NaB[3H]4 treated glycophorin. The gel was sliced in 1 mm intervals and counted for tritium. TD = tracking dye.
3.3(d) SDS PAGE analysis

(i) PAS staining

PAS staining as in Appendix A, yielded the typical PAS profile for red cell membranes (Figs. 3.5, 3.11, 3.12). If periodate oxidation of the gels was omitted in the staining of the gels via PAS but everything else kept constant, only the lipids stained. This staining procedure has been called Basic Fuchsin staining. Gels of periodate treated cells with or without spin labeling were analysed by this method and a new band corresponding to PAS 1 was found also to stain (at a lower intensity). Fig. 3.5 shows the results of staining variously treated red cells.

This additional staining technique (i.e. omitting oxidation on the gels) was applied to various samples throughout this work and is noted where used.

3.3(e) Spin labeling red cells

The optimal conditions used to spin label red cells were as follows: to one volume of freshly washed packed red cells was added one volume of 2 mM NaIO₄ (a four molar excess to sialic acid) in PBS/azide. After incubation at room temperature for 10 minutes, the oxidized cells ([O] RBC) were diluted three fold with ice cold PBS/azide and washed three times in ice cold PBS/azide. One volume of 23 mM TEMPAMINE (4 mg/ml PBS/azide) and one volume of 48 mM NaBH₃CN (3 mg/ml PBS/azide) with or without 1-4 x 10⁸ cpm/ml (specific activity 2.9 to 308 x 10⁻¹² mmole/dpm) NaB[³H]₃CN were added. After two hours incubation at room temperature, the solution was diluted three fold with ice cold PBS/azide and washed three times.
Fig. 3.5 SDS PAGE of 5% gels, pH 6.1 of (A) PAS staining and (B) Basic fuschin staining of 180 μg of membrane protein of SL-ghosts made as in protocol, section 3-3(e), with varying amounts of spins/SL-ghost. (1) $7.3 \times 10^6$, (2) $1.7 \times 10^6$, (3) $0.18 \times 10^6$ and (4) cells periodate oxidized but not exposed to TEMPAMINE. The insert scans in (B) are samples at full gain on the densiotometer. TD = tracking dye.
The resultant spin labeled red cells (SL-RBCs) were then lysed by the method of Dodge et al. (1963) and denoted spin labeled ghosts (SL-ghosts).

The following data shows how this protocol was arrived at. Various attempts were made to optimize the yield. Tables 3.4-3.6, show the results of alterations from the above protocol. All conditions were as quoted above unless stated in the table (i.e. the tables only quote the variations from the above protocol).

Table 3.4 shows the spins/ghost vs the initial TEMPAMINE concentration. Table 3.5 lists other manipulations done to try to improve labeling yields along with the initial TEMPAMINE concentrations used.

### Table 3.4

TEMPAMINE CONCENTRATION AND SPINS/GHOST

<table>
<thead>
<tr>
<th>TEMPAMINE conc (mM)</th>
<th>Spins/ghost x 10^-6</th>
<th>% of total sialic acid modified</th>
<th>% of oxidized sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.4 ± 0.3</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>1.6 ± 1.3</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>2.0 ± 1.7</td>
<td>6</td>
<td>6.5</td>
</tr>
<tr>
<td>17</td>
<td>3.5 ± 2.0</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>23</td>
<td>4.0 ± 1.7</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>29</td>
<td>3.1 ± 1.8</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>35</td>
<td>3.9 ± 1.3</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>45</td>
<td>3.1</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

1 assuming the total sialic acid content in a red cell is 3 x 10^7
2 assuming 15% of the sialic acids are periodate oxidized
### Table 3.5

**Other Attempts to Improve Spin Labeling of Red Cells**

<table>
<thead>
<tr>
<th>Manipulation</th>
<th>TEMPAMINE conc (mM)</th>
<th>Resultant spins/ghost X 10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejuvenated old blood</td>
<td>23</td>
<td>1.13</td>
</tr>
<tr>
<td>0.3 M Sorbitol (diluted 8:1 with PBS) instead of PBS</td>
<td>35</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.6</td>
</tr>
<tr>
<td>O₂ bubbled in the PBS buffer</td>
<td>23</td>
<td>2.8</td>
</tr>
<tr>
<td>Incubation overnight at 4°C</td>
<td>23</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.2</td>
</tr>
<tr>
<td>Recrystallized NaBH₃CN</td>
<td>23</td>
<td>1.2</td>
</tr>
<tr>
<td>Boranedimethyl complex instead of NaBH₃CN</td>
<td>23</td>
<td>0.95</td>
</tr>
<tr>
<td>No reducing agent</td>
<td>23</td>
<td>0.34</td>
</tr>
<tr>
<td>No oxidation</td>
<td>23</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 3.6 compares results of variation in the initial oxidation conditions with that of the final spins/ghost and also when SL-RBCs are reoxidized (when still red cells) and the whole labeling procedure repeated. The shift in the spectral parameter compared to the standard method is also shown. Fig. 3.6 shows SDS PAGE gel scans of various products quoted in Table 3.6.
TABLE 3.6
INCREASING PERIODATE CONCENTRATION ALONG WITH SPINS/GHOST AND SP

<table>
<thead>
<tr>
<th>% increase in periodate initially used(^1)</th>
<th>% increase in resultant spins/ghost(^1)</th>
<th>% increase in resultant SP(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>79 ± 52 (n = 3)</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

\(^1\)In comparison to the protocol used in section 3.3(e)

SL-RBC reoxidized and labeled as in optimum protocol resulted in a 19% increase in the resultant spins/ghost
Fig. 3.6 5% SDS PAGE (pH 6.1) gels of (1) 45 μg membrane protein coomassie blue stained and scanned, (595 nm), and (2) 180 μg membrane protein PAS stained and scanned, (525 nm), of (A) untreated red cells, (B) SL-ghosts made as described in optimum protocol section 3.3(e) and (C) SL-ghosts made as in (B) except at twice the periodate concentration. The numbering system according to Fairbanks et al (1971). TD = tracking dye, Hb = hemoglobin.
3.3(f) Background signal

As in Chapter 2, it was found that a large population of spin label had entered the cell which could be removed only by lysing the cells. Fig. 3.7 shows the difference in the spectra before and after lysing of spin labeled cells and controls (red cells plus TEMPAMINE).

Care must be taken in lysing red cells. It was found that the samples could sit overnight at 4°C but could not be frozen or else the membranes appeared to fragment.

The control (red cells plus TEMPAMINE then washed and lysed) spectrum varied from nondetectable to an average of $6 \pm 2 \times 10^5$ spins/ghost ($n = 25$). Fig. 3.7b shows a typical spectrum obtained for control ghosts.

3.3(g) Visual inspection of oxidized and spin labeled cells

Another reason for lysing the red cells came from the observation that a gradual change overcame the periodate oxidized red blood cells whether or not they were spin labeled. A solution of 23 mM TEMPAMINE/NaBH$_3$CN caused slight hemolysis as determined by the visual appearance of hemoglobin in the solution. As the TEMPAMINE concentration increased, lysis was more noticeable. In all cases, the hemolysis was minor. Over an extended period of time (6 hours or more), these oxidized (spin labeled or not) red cells became darker red in color and more viscous compared to the controls. If left overnight at 4°C, the alterations were even more noticeable.

Rouleaux formation by SL-RBCs was also inhibited (they formed clumps like the periodate treated cells), while the controls formed rouleaux.
Fig. 3.7 ESR spectra of the control (red cells plus TEMPAMINE then washed) before (A) and after (C) lysing and of SL-RBCs before (B) and after (D) lysing (cells prepared as described in the optimal protocol section 3.3(e)). $A = 7 \times 10^5$, $C = 4 \times 10^5$, $B = 1.8 \times 10^6$ and $D = 1.4 \times 10^6$ spins/cell.
3.3(h) Spin labeled ghosts

Fig. 3.8 shows a typical spectrum for spin labeled ghosts along with the parameters measured. The spectral parameter, $SP$, was defined as on page 67

$$SP = \left( \frac{h_0}{h_{-1}} \right)^{1/2} + \left( \frac{h_0}{h_+} \right)^{1/2} - 2$$

and for SL-ghosts, $SP = 0.64 \pm 0.097$ ($n = 50$).

Table 3.7 shows the peak-to-peak linewidth of the center line ($w_0$) vs the spins/ghost and shows that over the range quoted the change in $w_0$ is negligible ($\pm 2\%$).

<table>
<thead>
<tr>
<th>SPINS/SL-ghost</th>
<th>$w_0$ (guass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$7.3 \times 10^6$</td>
<td>2.26</td>
</tr>
<tr>
<td>$1.7 \times 10^6$</td>
<td>2.16</td>
</tr>
<tr>
<td>$0.18 \times 10^6$</td>
<td>2.21</td>
</tr>
</tbody>
</table>

The SP of SL-ghosts decreased 21% as the temperature increased from $20^\circ C$ to $29^\circ C$ and increased 21% as the temperature decreased from $20^\circ C$ to $12^\circ C$.

3.3(i) F-B SL-ghosts

Ghosts spin labeled by the method of Felix & Butterfield (1980) as described in section 3.2(d). The resultant spins/ghost was $0.14 \times 10^6$ (0.5% of the total sialic acid modified). The SP of the F-B SL-ghosts was 0.52. Fig. 3.9 show the SDS PAGE gels of SL-ghosts and F-B SL-ghosts.
Fig. 3.8 A typical spectrum of SL-ghosts made as described in the text section 3.3(e). \( w_0 \) is the linewidth of the mid-field line, \( h_+ \), \( h_0 \), and \( h_- \) are the height of the low-field, mid-field and the high-field lines respectively, and are used to measure the spectral parameter \( SP \) as defined in section 3.3(h).
Fig. 3.9 Densitometric scans of SDS PAGE gels (12% Laemmli). On each gel was loaded 180 μg of reduced membrane protein of (A) SL-ghosts or (B) F-B SL-ghosts. PAS stain of (1) control ghosts (no oxidation) and (2) ghosts made as in optimal protocol section 3.3(e) for SL-ghosts and section 3.2(d) for F-B SL-ghosts. Gel (3) is (2), sliced in 1 mm intervals and counted for tritium.
3.3(j) Distance measurements

Table 3.8 lists the parameters measured (as defined in Chapter 1, p. 51) for samples run at 77°K prepared as stated in Methods. It was found that baseline drift was substantial and had to be accounted for before meaningful measurements could be taken (due to the gain being high). The SLAN:SL of 8:1 and 4:1 represent the infinite dilution of the spin label. Given

\[ d_1/d \text{ (for SL-ghosts)} = (d_1/d) \text{ at infinite dilution} + 0.58r^{-3}. \]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spins/SL-ghost</th>
<th>(d_1/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-ghosts</td>
<td>5.2 x 10^6</td>
<td>0.539 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>3.0 x 10^6</td>
<td>0.541</td>
</tr>
<tr>
<td>1:4 SLAN ghosts</td>
<td>9.0 x 10^5</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3.4 x 10^5</td>
<td>0.49</td>
</tr>
<tr>
<td>1:8 SLAN ghosts</td>
<td>2.0 x 10^5</td>
<td>0.49</td>
</tr>
</tbody>
</table>

An average distance of 23 ± 2 angstroms (n = 5) between nitroxides of the spin labeled ghosts was calculated.

3.3(k) Reversibility

SL-ghosts made as described by optimal conditions (section 3.3(e)) with the inclusion of 1.5 x 10^8 cpm/ml of NaB[3H]_3CN produced SL-ghosts with 3.0 x 10^6 spins/ghost and 4.7 x 10^{-5} dpm/ghost. Table 3.9 lists
the time and the quantity of spins and tritium found in the supernatant as well as the supernatant spectral parameters (SP).

**TABLE 3.9**

% RELEASE OF SPINS AND TRITIUM WITH TIME OF INCUBATION OF SL-GHOSTS

<table>
<thead>
<tr>
<th>Incubation (days)</th>
<th>% of total found in the supernatant</th>
<th>Tritium</th>
<th>Spins</th>
<th>SP of spins in the supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5.8</td>
<td>11</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>14</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>26</td>
<td>13</td>
<td>10</td>
<td></td>
<td>0.03</td>
</tr>
</tbody>
</table>

The signal on the SL-ghosts was stable; it could still be detected months later. Some samples exhibited a decrease in their spectral parameter with time (weeks) while others increased. Re-lysing the spin labeled ghosts again the next day resulted in only a 2% increase in the parameter.

3.3(1) Neuraminidase or acid hydrolysis of SL-ghosts

Attempts were made to hydrolyse the spin labeled sialic acid off the red cell membranes of SL-ghosts or F-B SL-ghosts. The majority of effort was via neuraminidase digest because it is milder than acid hydrolysis. Table 3.10 lists the results of the various attempts, quoting the % of the sialic acid, spin label and tritium recovered in the supernatant as well as the relevant spectral parameters, SP.
TABLE 3.10
HYDROLYSIS OF SL-GHOSTS OR F-B SL-GHOSTS

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Sample</th>
<th>Method1</th>
<th>% recovered  in the supernatant</th>
<th>SP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>SL-ghosts</td>
<td>CP</td>
<td>82</td>
<td>5</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0.46</td>
</tr>
<tr>
<td>27.5</td>
<td>SL-ghosts</td>
<td>VC</td>
<td>76+11</td>
<td>20</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0</td>
<td>20</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>control ghost</td>
<td>VC</td>
<td>100</td>
<td>10</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0</td>
<td>8</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>F-B SL-ghost</td>
<td>VC</td>
<td>47</td>
<td>0</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0</td>
<td>30</td>
<td>0.78</td>
</tr>
<tr>
<td>39.5</td>
<td>SL-ghost</td>
<td>CP</td>
<td>38</td>
<td>4</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0</td>
<td>5</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>SL-ghost</td>
<td>H⁺</td>
<td>17</td>
<td>73</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>F-B SL-ghost</td>
<td>H⁺</td>
<td>30</td>
<td>96</td>
<td>0.11</td>
</tr>
</tbody>
</table>

1 Neuraminidase from: CP - Clostridium perfringens, VC - Vibrio Cholerae, none - no enzyme present in incubation buffer, H⁺ - acid hydrolysis
2 Ghosts prepared identically to SL-ghosts but not exposed to periodate

The absorption spectra of the sialic acids assay products for the various fractions showed no differences from the standards for the thiobarbituric acid or the resorcinol assay.
3.3(m) Lipid extraction

Tritiated samples of SL-ghosts or F-B SL-ghosts were made as in the protocol. Analysis was carried out on the spins and the tritium counts for SL-ghosts but due to the low spins/ghost of F-B SL-ghosts only the tritium could be analysed. In both cases SDS PAGE samples were run and the amount of tritium in the sample associated with the lipid (the peak that runs with the tracking dye) (Fig. 3.9) was determined (Table 3.13). Table 3.11 lists the results found for each type of analysis.

\[
\begin{array}{ccc}
\text{Sample} & \text{Spins} & \text{Tritium} & \text{% tritium associated with lipid on SDS PAGE} \\
\hline
\text{SL-ghosts} & 10 \pm 3 & 20 \pm 4 & 10 \pm 2.5 \\
\text{Control}^1 & 13 & 53 & 63 \\
\text{F-B SL-ghosts} & 13 & 23 & 26 \\
\text{F-B control}^1 & 13 & 54 & \\
\end{array}
\]

\(^1\)cells treated identically except not periodate oxidized

Only 30% of the chloroform:methanol extracted tritium associated with the Folch upper layer extraction, the layer associated with the glycolipids.
3.3(n) Selective solubilization

Extractions of SL-ghosts and F-B SL-ghosts were done as in Methods (section 3.3(e)) and the quantity of spins or tritium solubilized by each of these methods listed in Table 3.12. Included in this table are the SP of these fractions as well as for 1% SDS extraction of SL-ghosts (F-B SL-ghosts having too weak a spin label signal to be detected in any of these fractions).

**TABLE 3.12**

EXTRACTION OF SPINS AND TRITIUM FROM SL-GHOSTS

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>% extracted</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spins</td>
<td>Tritium</td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>82 ±4</td>
<td>60</td>
</tr>
<tr>
<td>extracted</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>pellet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>11 ±6</td>
<td>13</td>
</tr>
<tr>
<td>extracted</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>pellet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% SDS</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 3.10 shows the accompanying SDS PAGE gel analysis of these fractions.
Fig. 3.10 PAS stain of SDS PAGE of extractions of SL-ghosts. (1) 0.1 M NaOH pellet, (2) Triton X-100 pellet and (3) Triton supernatant. Samples made as described in the text (section 3.2(n)) and diluted 1:1 with sample buffer, reduced and 100 μl of each run of 5% gels (pH 6.1). TD = tracking dye.
3.3(o) Isolation of glycophorin

SL-ghosts made as in the protocol resulted in $3.0 \times 10^6$ spins/ghost and $4.7 \times 10^{-5}$ dpm/ghost. Non-labeled ghosts were run in parallel during the isolation procedure. From 25 ml of SL-ghosts 5.8 mg of SL-glycophorin was recovered. Calculating that 25 ml SL-ghosts equaled $4.29 \times 10^{17}$ spins and $7.09 \times 10^6$ dpm total, 54% of the spins co-isolated with the glycophorin fraction and $15 \pm 2\%$ of the tritium did (over 25% of the tritium dialysed away).

At 8.3 mg/ml in PBS, SL-glycophorin had an SP = $0.73 \pm 0.04$ and at 0.6 mg/ml, an SP = 0.64.

For SL-glycophorin, $106 \pm 4 \mu g$ of bound sialic acid was found per mg of SL-glycophorin and $131 \pm 4 \mu g$ sialic acid/mg of normal glycophorin (19% decrease for SL-glycophorin, equivalent to 2.5 sialic acid). The resorcinol assay was used and all samples were found to have identical absorption spectra to standards. SL-glycophorin had a calculated 2.1 spins/glycophorin ($4.1 \times 10^{16}$ spins/mg or 16% of the total sialic acid) and $2 \times 10^5$ dpm of $^3$H/mg glycophorin (at a specific activity of $2.18 \times 10^{-10}$ mmole/dpm, this is equivalent to 1.35 moles of NaBH$_3$CN to mole glycophorin). The interpretation of the values is in doubt since Burness & Pardo (1981) showed that glycophorin isolated by the method used here resulted in co-isolation of PAS 3, 4 and minor proteins (14% of the total PAS stain). The isotope effect for NaB[$^3$H]$_3$CN is unknown (for NaB[$^3$H]$_4$, it appears to be 0.5 eq per NANA$_7$ aldehyde, not the expected 1 eq, according to Liao et al, 1973). If one assumes that the only site for $^3$H in these preparations is the reduced NANA-SL bond, comparing the ratio of $^3$H to spins in
SL-glycophorin implies an isotope effect of \( \frac{1.35}{2.1} = 0.64 \) (Borch & Durst, 1969, observed an isotope effect for LiBD\(_3\)CN). Fig. 3.11 shows some SDS PAGE gels run of isolated SL-glycophorin in parallel with SL-ghosts and normal ghosts for comparison.

The SP of SL-glycophorin (at 8.3 mg/ml) decreased 22% as the temperature increased 9.5°C (from 20.5°C to 30°C) and increased 13% when the temperature dropped from 20.5°C to 11°C.

3.3(p) Tritium distribution on SDS PAGE gels

Table 3.13 lists the distribution of the tritium on SDS PAGE gels of variety of samples, comparing them to isolated SL-glycophorin and \(^3\text{H}\)-glycophorin made by the IO\(_4^-/\text{NaB}[^3\text{H}]_4\) method.
Fig. 3.11 Densitometric scans of 12% Laemmli SDS PAGE gels of reduced samples of (A) 278 ug isolated SL-glycophorin and (B) 180 ug membrane protein of SL-ghosts. (1) PAS stain, (2) basic fuschin stain, (3) gel (1), sliced into 1 mm thicknesses and counted for tritium. TD = tracking dye.
TABLE 3.13

DISTRIBUTION OF TRITIUM IN VARIOUS SDS PAGE SAMPLES

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>above PAS 1</th>
<th>PAS 1</th>
<th>between PAS 1 and Lipid</th>
<th>Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) SL-ghosts:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. gel run and sliced</td>
<td>23±2%</td>
<td>52±1%</td>
<td>22±1%</td>
<td>5.5±0.7%</td>
</tr>
<tr>
<td>2. gel run, stained and sliced</td>
<td>19±8%</td>
<td>52±7%</td>
<td>20±2</td>
<td>6.6±2.5%</td>
</tr>
<tr>
<td>3. 1.6x10^6 spins/ghost</td>
<td>12%</td>
<td>60%</td>
<td>20%</td>
<td>7.8%</td>
</tr>
<tr>
<td>4. 4x10^5 spins/ghost</td>
<td>11%</td>
<td>56%</td>
<td>19%</td>
<td>8.7%</td>
</tr>
<tr>
<td>5. control</td>
<td></td>
<td></td>
<td></td>
<td>63%</td>
</tr>
<tr>
<td>(B) F-B SL-ghost:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. gel run, stained and sliced</td>
<td>15%</td>
<td>33%</td>
<td>13%</td>
<td>26%</td>
</tr>
<tr>
<td>2. control</td>
<td></td>
<td></td>
<td></td>
<td>54%</td>
</tr>
<tr>
<td>(C) SL-glycophorin:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. isolated from SL-ghosts</td>
<td>28±2%</td>
<td>58%</td>
<td>13±2%</td>
<td>1.5±0.7%</td>
</tr>
<tr>
<td>2. IO_4/Na[3H]_4 labeled</td>
<td>18%</td>
<td>61%</td>
<td>16%</td>
<td>4.8%</td>
</tr>
</tbody>
</table>

1 cells treated identically but not periodate oxidized
3.4 DISCUSSION

The effects of spin labeling upon red cells were studied in two parts: periodate oxidation and reductive amination (addition of the spin label TEMPAMINE and NaBH$_2$CN). Analysis and optimization of these two steps was done with the aim of producing maximum yields with the least perturbation to the red cells. Once this was accomplished, analysis of the product took place to determine where the modification occurred.

3.4(a) Periodate oxidation

Exposure of red cells to high periodate concentrations (10 molar excess to sialic acid (equivalent to 5 mM periodate) or higher) results in immediate swelling and hemolysis. For instance, it was reported in 1949 (St. Groth), that decreased osmotic resistance occurred in red cells exposed to 16 molar excess periodate. Stewart (1949) found that even a four molar excess will result in hemolysis and cell instability if the incubation times are long (30 min compared with the 10 min used in this work).

Short incubation times and low periodate concentrations therefore are important not only for selective sialic acid modification (Chapter 1, p. 36) but also to maintain red cell integrity.

(i) Altered surface properties

These periodate modified cells behave differently from normal cells in other ways as well in that surface properties have been altered. Periodate
treatment inhibits the formation of rouleaux (Figs. 3.1, 3.2). Large, more symmetrical aggregates are produced instead, more typical of antibody-induced agglutination than rouleaux. The theory of rouleaux formation is not completely known, but macromolecular bridging of polymers in solution is believed to be the cause (Brooks, 1976; Skalak et al, 1981; Fritz Jr, 1984).

Dextran and plasma appear to induce agglutination by different mechanisms as seen by the effects of the monosaccharides glcNAc and glcNH$_2$. GlcNAc (at 181 mM) inhibited and glcNH$_2$ partially inhibited agglutination of periodate (3.5 mM) oxidized red cells by plasma (also shown by Novagrodsky (1973) for glcNAc and galNAc), but had no effect on dextran agglutinated oxidized cells. Periodate oxidized cells have been found to be agglutinable by all adult sera (Stewart, 1949; Moskowitz & Treffers, 1950) due to the presence of a natural antibody (Yachmin & Gardner, 1961). This antibody is probably preferentially bound to the monosaccharides while dextrans are likely non-specifically adsorbed onto a variety of structures on the red cell surface.

(ii) Formaldehyde formation

Oxidation of red cells by periodate results in the formation of formaldehyde (Table 3.1) due to the cleavage of the C$_9$-C$_8$ diol of sialic acid, resulting in an aldehyde on C$_8$ (reduction of this aldehyde to the alcohol results in a product called NANA$_8$). Another molecule of periodate can then oxidize the C$_8$-aldehyde C$_7$-OH, producing CHO$_2$OH and an aldehyde on C$_7$ (which, when reduced, produces NANA$_7$). Mild oxidation of sialic
acid with periodate has the potential for producing two products, the C₈ or the C₇ aldehyde. In either case, for each formaldehyde produced, one sialic acid has been oxidized. Removal of sialic acid by the enzyme neuraminidase resulted in no formaldehyde formation (Tables 3.2, 3.3).

Increased periodate concentration or exposure times results in increased formaldehyde formation (Table 3.1) and lysis of red cells. The reactivity of red cells to periodate appeared to be lower than ghosts (also seen by Blumenfeld et al, 1972 and Taylor & Wu, 1980), isolated glycophorin or fetuin (Tables 3.2 and 3.3).

Periodate modification didn't appear to alter isolated glycophorin (Fig. 3.4) or red cells (Fig. 3.5) as analysed by SDS PAGE, but oxidized ghosts showed major changes in the gel profiles compared to untreated ghosts (Fig. 3.2). High molecular weight components were seen in the coomassie blue stain (protein) profiles of periodate treated ghosts and the protein bands were broader compared to untreated ghosts. The PAS staining profile also showed material at the top of the gel which was unable to penetrate the gel due to extensive denaturation and cross-linking. This sensitivity of ghosts to periodate has also been seen by Gahmberg et al (1978), who found concentrations as low as 0.1 mM periodate altered SDS PAGE profiles of periodate treated ghosts while concentrations of 2 mM had no effect on red cells. Hence, although ghosts are more reactive to periodate than red cells, one can't increase the degree of oxidation by using ghosts due to this sensitivity to periodate, which apparently induces membrane protein crosslinking.
The value of 2 mM periodate (a four to one ratio of periodate to sialic acid; exposure time of 10 min at room temperature) appears to be the upper limit for red cells (altered PAS profile of 4 mM treated cells also Fig. 3.6). Under these conditions, 15 ± 9% of the sialic acids are oxidized as determined by formaldehyde formation (section 3.3(b)). Massamiri et al (1979) found at a molar ratio of 10 periodates to one sialic acid, 28% of the sialic acids were oxidized. Assuming linearity, 14% would have been oxidized at a five to one ratio, in close agreement to that found here.

3.4(b) Spin labeling

Spin labeling the oxidized red cells (by reducing the aldehyde via reductive amination, Chapter 1, section 1.3) didn't appear to reverse any effects induced by periodate oxidation. These spin labeled cells also showed inhibited rouleaux formation (in the presence of plasma) and underwent a slow change with time (six hours or more), as did the oxidized cells. The oxidized or spin labeled oxidized cells became more viscous and darker red compared to normal cells. No changes were noticed in the SDS PAGE coomassie blue profile (Fig. 3.6). These changes could have been due to some metabolic pump or internal metabolite being altered as postulated by Brossmer & Bohm (1974) who found that the red cell glucose utilization decreased and K⁺ efflux increased after exposure to periodate, even at periodate levels lower than needed to cause red cell lysis.

Spin labeling the red cells also resulted in a large population of spins going inside the cell rather than being attached to the oxidized sugars (as found in Chapter 2, section 2.3(b)). The presence of TEMPAMINE inside the
red cells and the slow irreversible alterations induced by the oxidation step necessitated the additional step of lysing the cells after they had been spin labeled (Fig. 3.7), removing the majority of non-covalently attached spin label and stopping the slow alterations in the red cells.

3.4(c) Quantitation

The optimal concentration of TEMPAMINE required to maximize the spins/ghost was 23 mM (Table 3.4), producing $4 \pm 1.7 \times 10^6$ spins/ghost. This value is similar to that found for periodate treated red cells by Cherry et al (1980) of $10^6$ eosin molecules/cell, Taylor & Wo (1980) $1.5 \times 10^6$ thiol hydrazides and Schweizer et al (1982) $7.2 \times 10^6$ arylalkyldiamines per cell. Table 3.5 shows that no manipulation of the reaction media other than altering the periodate concentration (Table 3.6) improved labeling. Much higher yields were not expected since only 15% of the sialic acids were oxidized at 2 mM periodate.

At 23 mM TEMPAMINE, $89 \pm 38\%$ of these oxidized sialic acids were reductively aminated (Table 3.4). Incomplete reduction of the aldehydes was anticipated due to short incubation times (two hours as opposed to the usual 12 hours or longer, Lane (1975)). Incomplete reduction of the oxidized sialic acids was also detected by the basic fuschin stain, staining only the membrane components already oxidized. Figs. 3.11 and 3.12 show a faint band corresponding to PAS 1 for SL-ghosts indicating that some oxidized sialic acid still remained. Untreated ghosts had no basic fuschin stain except in the lipid region (believed to be due to auto-oxidation of lipids and labile
acetal phospholipids whose products react with the Schiff's reagent (Pearse, 1968)).

The labeling procedure labels approximately 15% of the sialic acids of red cell membranes (13% as detected by spins/SL-ghost, Table 3.4, 16% as detected by the decrease in detectable sialic acid for isolated SL-glycophorin and between 12 to 24% as determined by the hydrolysis attempts, Table 3.10). The values from the hydrolysis experiments are based on the assumption that the modified sialic acids (i.e. those spin labeled) are insensitive to neuraminidase. The hydrolysed sialic acids showed no maximum shift in the resorcinol assay as was seen by Van Lenten & Ashwell (1971) and Liao et al (1973) for NANA<sub>7</sub>, implying only unmodified sialic acid was released. Decreased sensitivity of neuraminidase to NANA<sub>7</sub> has been seen by Suttajit & Winzler (1971), Blumenfeld et al (1972) and Liao et al (1973) (the exocyclic hydroxyls are needed for optimal activity (Ashwell & Morell, 1974)).

3.4(d) Location

The spin labeled components appear to be associated mainly with the PAS proteins, predominately glycophorin A. Red cells oxidized but not spin labeled showed a very heavily stained PAS 1 with basic fuschin (Fig. 3.4). As the degree of spin labeling increased (by increasing the initial TEMPAMINE concentration up to 23 mM) the amount of basic fuschin stain at PAS 1 decreased, until only a very faint band was seen for SL-ghosts made as described in the optimal protocol.
The PAS stain showed the same trend, decreasing in intensity the higher the degree of spin labeling occurring (Fig. 3.4). In this case only the non-reductively aminated sialic acids were stained. Dahr (1974, 1976), Tanner (1978) and Furthmayr (1981) believe that the PAS stain depends almost entirely on the sialic acid in the membrane glycoproteins (lipids stain oxidized or not and are therefore insensitive to PAS staining for sialic acid, Fig. 3.4). Glycophorin A contains 70% and glycophorin B 10% of the total sialic acid (Singer & Morrison, 1974; Anstee, 1981) and 70% and 15% of the PAS stain respectively (Burness & Pardue, 1981; Furthmayr, 1981). The PAS proteins (glycophorins A, B and C) comprise at least 80% of the sialic acid in the red cell membrane. Band 3 (Furthmayr et al, 1976; Fukuda et al, 1979; Koziarz et al, 1978), the glucose transporter (band 4.5) (Sogin & Hinkle, 1978) and acetylcholinesterase (Bjerrum & Bog-Hansen, 1976; Ravazzolo et al, 1983) also contain sialic acid, as do the lipids (about 2% of the total sialic acid appear on the lipids, Wherrat1, 1973, Marcus et al, 1981, Beeley et al, 1977).

Using NaB[3H]3CN in the reductive amination step resulted in the SDS PAGE gel 3H profiles of SL-ghosts corresponding to the PAS profiles (Figs. 3.9, 3.11, 3.12). Over 50% of the tritium was associated with PAS 1 (Table 3.13). Published data obtained via the periodate/NaB[3H]4 method shows similar results (Blumenfeld et al, 1972; Steck & Dawson, 1974; Mueller et al, 1976; Gahmberg & Anderson, 1977; Gattegno et al, 1983).

Selective extractions of SL-ghosts, made in the presence of NaB[3H]3CN, with NaOH, Triton X-100 and chloroform:methanol showed that the tritium co-isolated with the spin population which co-isolated with the PAS proteins (Table 3.12). It was found that 89% of the spins and 87% of
the tritium was not extracted by 0.1 M NaOH and 82% of the spins and 60% of the tritium was extracted with the Triton. These two fractions are known to contain the majority of PAS proteins (Fig. 3.10) (Yu et al., 1973; Steck & Yu, 1973; Mueller & Morrison, 1981). Chloroform:methanol, used to extract lipids, extracted only 7% of the spins and 6.5% of the tritium. This was comparable to the amount of tritium found in the lipid area on SDS PAGE gels of the SL-ghosts (8.4%) (Fig. 3.9, Table 3.13). Only about 30% of the chloroform:methanol extracted tritium and spins co-isolated with the glycolipid fraction in a Folch extraction (the upper layer) (section 3.3 (1)), which represents about 2% of the total. This low degree of lipid labeling of periodate treated red cells was also found by Cherry et al. (1980), Abraham & Low (1980), Steck & Dawson (1974), Mueller et al. (1976) and Gahmberg & Anderson (1977).

Isolation of glycophorin from SL-ghosts labeled with NaB[\(^3\)H]CN showed that 54% of the spins co-isolated with the glycophorin fraction. The tritium profile of SDS PAGE gels of the isolated SL-glycophorin was identical to that of the PAS profile (Fig. 3.11) with 58% of the tritium associated with PAS 1. Coomassie blue staining was identical to the PAS stain suggesting no other proteins were isolated (data not shown).

The amount of detectable bound sialic acid in the glycophorin isolated from SL-ghosts was decreased by a factor of 2.5 (19%) compared to glycophorin isolated from identical cells not treated. This decrease was probably due to the non-detectable sialic acids being modified (spin labeled) and no longer sensitive to the bound sialic acid assay (due to the exocyclic triol being destroyed). There is good agreement between the decrease in the bound sialic acid and the number of spins/glycophorin (2.1
spins/SL-glycophorin, which equals 84% of the oxidized sialic acids or 16% of the total sialic acid on SL-glycophorin), verifying that the two spin labels per glycophorin isolated from SL-ghosts were located on the sialic acids.

A problem with the spin labeling procedure of red cells was the appearance of a small population of spins which appeared to be non-covalently associated with the red cell membranes. This population seemed to slowly leach out into solution in suspensions of SL-ghosts and control ghosts (red cells exposed to TEMPAMINE and then washed and lysed). Reversibility experiments (section 3.3(i)) show a spin population which migrates into solution with time (approximately 10% of the total spins in 10 days, Table 3.8). This spin population was also found in the supernatants of hydrolysis experiments; spins could be recovered in the supernatants of SL-ghosts not exposed to neuraminidase (Table 3.10). The population was characterized by a spectral parameter, SP, (0.06-0.14) smaller than that of SL-ghosts (SP = 0.64) and was seen also in the NaOH and chloroform:methanol extracts of SL-ghosts. These fractions (the neuraminidase, NaOH, chloroform:methanol, and PBS extractions) represented only a minor component of the total spins per SL-ghost, however.

3.4(e) ESR interpretation

SL-ghosts have an SP of 0.64 ± 0.097 and exhibited negligible Heisenberg exchange (Table 3.6). This SP can be converted to a correlation time \( \tau_c \) using the formula

\[
\tau_c = 6.5 \times 10^{-10} \omega_0 SP \text{ (seconds)}
\]
Although accurate $\tau_C$ calculations need rigorous computer simulations (Chapter 1, p. 49), the formula is valid if one compares values within a known system, assuming the same type of motion is always occurring to the probe.

For SL-ghosts, $\tau_C$ is $9.2 \pm 1.4 \times 10^{-10}\text{s}$ and $7.4 \times 10^{-10}\text{s}$ for F-B SL-ghosts. Literature values for sialic acid spin labeled red cells are $9.6 \times 10^{-10}\text{s}$ (Felix & Butterfield, 1980) and $10 \times 10^{-10}\text{s}$ (Aplin et al., 1979). Ross et al (1983) obtained $12-13 \times 10^{-10}\text{s}$ spin labeling the sialic acids of lymphocyte membranes.

Cherry et al (1980) found two molecular motions for an eosin probe attached to the sialic acids of red cells, a fast (nanosecond range) motion believed to be the probe itself and a slower ($10^{-5}$ to $10^{-7}\text{s}$) motion believed to be cooperative motion of the oligosaccharide chains. Unfortunately, the slower time scale was not examined in the present ESR experiments.

For isolated SL-glycophorin at $8.3 \text{ mg/ml}$, $SP = 0.73$ ($\tau_C = 10 \times 10^{-10}\text{s}$) and at $0.64 \text{ mg/ml}$, $SP = 0.64$ ($\tau_C = 9.2 \times 10^{-10}\text{s}$). Higher concentrations of SL-glycophorin appear to cause the spin label to slow down. Lee & Grant (1979) also observed a decrease (18%) in the mobility of sialic acid spin labeled glycophorin as the concentration of glycophorin increased (from $0.6 \text{ mg/ml}$ to $69.1 \text{ mg/ml}$) and attributed it to an increase in the local head group density. This increase in $SP$ with increased glycophorin may also reflect solution behavior of glycophorin, since isolated glycophorin is known to aggregate in aqueous solutions (Grefrath & Reynolds, 1974). From 10 to 20 monomers per aggregate have been reported (Edgmond et al, 1979; Springer et al, 1966), possibly in the form of a loose
aggregate of dimers (Silverberg et al, 1976). The change in SP may reflect a change in the size of the glycophorin aggregate with increased glycophorin concentration.

Lee & Grant (1979) calculated a $\tau_c$ of $9.6 \times 10^{-10}$s for their SL-glycophorin and Aplin et al (1979) $7.9 \times 10^{-10}$s for sialic acid spin labeled fetuin. Low et al (1982) found isolated glycophorin from fluorescein amine labeled red cells had an increased rotational freedom compared to labeled red cells and concluded that on the membrane, oligosaccharide density may immobilize the sialic acids via hydrogen bonding.

All the $\tau_c$ calculations for spin labeled sialic acid either on the red cell membrane or on isolated proteins give values ($7.4 - 10 \times 10^{-10}$s) fairly similar regardless of whether on the membrane or not. No mobility increase was found upon isolation of SL-glycophorin or in the Triton X-100 and SDS extractions (Table 3.12). The difference between the observations of Low et al (1982) and the spin label studies may reflect the type of probes used (spin label as opposed to fluorescence).

3.4(f) Distance between nitroxides

At a spin concentration of $5 \times 10^{-5}$M ($5 \times 10^6$ spins/SL-ghost, $6 \times 10^9$ ghosts/ml), the average separation of nitroxides would be 174 angstroms if all were randomly distributed in three dimensions in solution (Aplin, 1979; Yalpani, 1980). If one assumes that the spins are evenly distributed over the cell surface, a distance of 53 angstroms would separate the nitroxides (the red cell has a surface area of $145 \, \mu m^2$). Clearly both assumptions are wrong because the sensitivity of the $d_1/d$ measurement is
from 10 to 24 angstroms (Aplin, 1979; Yalpani, 1980). Changes in $d_1/d$
were noticed above $10^6$ spins/SL-ghosts, however, indicating that the spins
were closer than predicted if their distributions were random (Table 3.8).
Using equation [11] in Chapter 1, an average nitroxide separation of 23
angstroms is implied from the experimental $d_1/d$ values assuming random
3-dimensional distribution. Assuming the majority of the spins are on
glycophorin (at two spins/glycophorin, this equals 1 to 4 x $10^6$ spins/cell
or 25 to 100% of the total spins/SL-ghost), there are a potential 31 NANA
molecules to label on glycophorin A and a similar number on glycophorin B.
Lovrien & Anderson (1980) state glycophorin has an average radius of 25
angstroms and an average distance of 220 angstroms between glycophorin
molecules in the membrane. If there are two spins/glycophorin, the above
averages suggest the distance measured is intramolecular. In this case, a
more appropriate model would be to assume a random distribution of
biradicals. This results in a calculated 16 angstrom average distance
between nitroxides (the distance altered by a factor of 0.7, Kokorin et al,
1972). Due to low signal-to-noise and baseline drift, a more detailed
analysis was not attempted.

There is little information available regarding how glycophorin is
situated on the cell surface. Stibenz & Geyer (1980), based on model
calculations, believe that glycophorin is hydrogen-bonded to the headgroups
of the lipid bilayer, looping back on itself. Skutelsky et al (1977),
through electron microscopy studies, and Levine et al (1983), through
electrophoretic mobility studies, found the sialic acid to be 50-70
angstroms away from the membrane. If fully extended, glycophorin could
reach 168 angstroms beyond the lipid bilayer (Stibenz & Geyer, 1980).
Ruppel et al (1982) found that the conformation of glycophorin in vesicles was concentration dependent, small amounts of glycophorin spreading out onto the lipid-water interface and high concentrations protruding into the aqueous phase. The lack of a good model for glycophorin oligosaccharides makes the randomly distributed biradical the best approximation.

The sialic acids are the terminal residues of the 16 oligosaccharide chains and certainly could be more than 16 angstroms apart. The 16 angstrom distance separating the spins, which are attached to two of the possible 31 sialic acids, suggests that only selective sialic acids are being labeled, perhaps the most exposed and accessible residues.

3.4(g) F-B SL-ghosts

In the work discussed throughout this chapter, spin labeled ghosts called F-B SL-ghosts, made by the method of Felix & Butterfield (1980) have also been analysed. They were shown to contain $0.14 \times 10^6$ spins/F-B SL-ghost and had 25% of the tritium label associated with lipid (Fig. 3.9, Table 3.13) compared to $4 \times 10^6$ spins/SL-ghost and 8% associated with the lipid for SL-ghosts made via the present protocol (section 3.3(e)). The increase in the amount of tritium associated with the lipid for F-B SL-ghosts could be due to the higher reactivity of ghosts to periodate, compared with red cells. Subtle rearrangement of the lipids seems to occur when red cells are made into ghosts (Juliano, 1973; Zwaal et al, 1973; Marchesi et al, 1976) which may make them more reactive. Increased labeling of the lipid in ghosts by $\text{IO}_4^-/\text{NaB}[^3\text{H}]_4$ was also seen by Liao et al (1972) and Gahmberg & Anderson (1977) (but not Steck & Dawson, 1974). Felix
& Butterfield (1980) also found that improved yields (by increasing the initial TEMPAMINE concentration) resulted in Heisenberg exchange, thus limiting them to low yields of spins/ghost. They calculated that 40% of the sialic acids had been labeled by their technique. If this were true, they would have obtained $1.2 \times 10^7$ spins/cell, 2.7 times higher than obtained here for SL-ghosts. The figure of 40% came from determining the decrease of tritium incorporation into periodate oxidized ghosts before and after spin labeling, however. They are thus really stating that 40% of the oxidized sialic acids had been labeled.

The reason for the careful analysis carried out above will become clear in Chapter 4, when the effects of the wheat germ agglutinin lectin binding to these SL-ghosts will be analysed to examine the possible use of this spin labeling technique for monitoring changes in sialic acid.
3.5 CONCLUSION

Although oxidation and labeling of red cells has become routine, new information is still obtainable. In this work, careful analysis has been done on the spin labeling of red cells. Extensive quantitation was carried out at a variety of levels and correlations have been found between the amount of formaldehyde formed, the amount of spins/SL-ghost and the isolation of SL-glycophorin and tritium from SL-ghosts. It is quite clear that the protocol devised results in two spins/glycophorin, showing that in spite of a complex substrate, specificity and quantitation are both possible.

Surface alterations were produced by periodate oxidation and lysis occurred at high periodate levels. Conditions were found where minimal perturbation of the resultant SL-ghosts occurred as detected by SDS PAGE and lack of Heisenberg exchange. The labeling procedure was limited by the periodate oxidation step. The only other published procedure for spin labeling red cell sialic acids (Felix & Butterfield, 1980) was limited by the TEMPAMINE concentration (increased concentration resulted in Heisenberg exchange). The protocol in this work differs from Felix & Butterfield in that the red cell not the ghost is periodate oxidized, producing SL-ghosts with more spins/ghost and without Heisenberg exchange.

Detailed analysis of the SL-ghosts showed that the majority of the label was on the PAS-stainable proteins (as detected by tritium labeling, selective solubilization and by a new staining procedure called Basic Fuchsin) with only about 8% being associated with the lipids. Occasionally, a small fraction of the spin label appeared to be associated with, but not
covalently attached to, the SL-ghosts. It appeared in the supernatants of NaOH extractions and in various buffer incubations. This fraction appeared very mobile in comparison to the SL-ghosts and slowly leached out into the solution.

Since there are two spins/glycophorin molecule, it appears that certain sialic acids were selectively spin labeled. Assuming a random biradical distribution, the average distance between the spins was calculated to be 16 \( \pm 2 \) angstroms.
4.1 INTRODUCTION

The lectin wheat germ agglutinin (WGA), was used as a model to study the specific binding of a protein to spin labeled ghosts.

WGA was first discovered in 1963 as an impurity in wheat germ lipase (Aub et al, 1963). It is now believed to be a dimer of 36000 daltons, high in disulfide bonds (no free sulfhydryl groups are detectable (Nagata & Burger, 1974; Rice & Etzler, 1975)). This makes WGA very stable in 0.1 N HCl, 0.05 M NaOH or at 60°C (Nagata & Burger, 1974). From centrifugation studies, it was found that the WGA dissociates into its monomer form at low pH with a $pK_a = 4$ for this reaction (Monsigny et al, 1979).

The binding sites on wheat germ agglutinin (WGA) were first postulated to be specific for glcNAc by Burger & Goldberg (1967) when that monosaccharide was found to inhibit WGA binding to cells. Sialic acid appeared important also since neuraminidase treated cells showed decreased binding. Inhibition of WGA binding (by mono-, di-, tri- and oligosaccharides) to red cells, glycoproteins etc. became a standard method for determining the specificity of WGA. Allen et al (1973) were the first to propose that WGA contained subsites, due to the increased affinity for (glcNAc)$_n$ up to $n = 3$, postulating that WGA could accept three glcNAc molecules in the beta 1-4 configuration. This subsite theory has been the framework on which all other models have been based. It is accepted that subsites exist, but how they interact is still in question (see Grivet et al, 1983; Kronis & Carver, 1984c).
Sialic acid also appears to interact with the WGA binding site, as shown by fetuin and OSM binding to WGA affinity columns (Peters et al, 1979; Bhavanadan & Katlic, 1979; Monsigny et al, 1980).

All reported X-ray crystallography work on WGA has been done by Wright (1974, 1977, 1979, 1980, 1981 and 1984). The crystal consists of two monomers (or protomers). The binding sites are located in the contact region between the protomers and binding requires participation of both protomers.

Fig. 4.1 Schematic illustration of the disposition of the primary and secondary binding locations on the WGA dimer. The domains of protomer 1 are labelled A₁, B₁, C₁, D₁, and those of protomer II A₁₁, B₁₁, C₁₁, D₁₁. Each unique binding location is subdivided into subsites (small circles). GlCNAC oligomers bind at subsites 1, 2, and 3 in both binding locations, whereas NANA oligosaccharides utilize subsites in the primary binding location only. Subsite 1 is shaded to indicate strongest binding interactions. (Taken from Wright, 1980)
There are two primary sites which are accessible to glcNAc, NANA and sialyllactose, and two secondary sites which are poorly accessible to \((\text{glcNAc})_2\) and not at all to NANA (although there is no obvious reason for the inaccessibility, Wright, 1980). The primary site can be divided into subsites 1, 2, and 3 and, although the secondary site is less well defined, can also be divided into three subsites (Fig. 4.1).

An unsubstituted acetoamido group at C-2, and equatorial OH at C-3 and C-4 are required for binding. Substitutions at C-1, C-4 and C-6 are allowed, and WGA can also accommodate internal sequences of glcNAc in oligosaccharides.

Analysis of binding data (for a ligand binding to a macromolecule or cell) usually involves binding isotherms (a plot of the amount bound \((B)\) vs the equilibrium concentration of the ligand \((F)\)) or Scatchard plots (Scatchard, 1949) which is a plot of \(B/F\ vs F\) derived from the equation \(B/F = K(B_0 - B)\), where \(B_0\) is the number of potential binding sites and \(K\) is the association constant. Analysis of this type assumes that the system is in equilibrium.

Scatchard plots are useful because the number of binding sites and association constants can easily be extrapolated if these plots are linear. Deviations from linearity (a single straight line implying only one type of binding site) can be sensitive indicators of the type of binding. Positive cooperativity results in a concave line with a well pronounced maximum. Negative cooperativity results in a concave line as does heterogeneity of the binding sites. Systems which don't show a single affinity can be treated by curve fitting the data to predictions of mathematical models (see Dahlquist, 1978).
WGA binding has been analysed via Scatchard plots. Solution studies employing equilibrium dialysis (Nagata & Burger, 1974), circular dichroism (Thomas et al, 1977), proton nmr (Jordan et al, 1977; Kronis & Carver, 1982), deuterium nmr (Neurohr et al, 1981), fluorine nmr (Midouz et al, 1980), intrinsic fluorescence (Kronis & Carver, 1984c), and fluorescently labeled saccharides (Clegg et al, 1983) in which saccharides were added to WGA in solution and some parameter measured, all showed linear Scatchard plots at all WGA concentrations. (glcNAc)$_n$, NANA and sialylactose all bound to WGA. Unlike the crystallographic studies of WGA, these studies all showed four equivalent sites/WGA dimer (except Jordan et al (1981) who found two sites/dimer). In solution, therefore, no secondary sites are observed.

The binding of WGA to cells appears to be more complex and of higher affinity than to simple sugars. WGA binding to Chinese hamster ovary (CHO) cells (Stanley & Carver, 1977), fat cells (Cuatrecasas, 1973), kidney 21 CB cells (Monsigny et al, 1980), mouse thymus cells (Monsigny et al, 1979) and red cells (Adair & Kornfeld, 1974) all gave nonlinear Scatchard plots of a type that implied multiple and heterogeneous sites.

This chapter deals with the binding of WGA to spin labeled ghosts and to various fractions isolated from these ghosts. Emphasis is placed on the effect of WGA on the spin probe. Other lectins (of varying specificity) were also used to determine the sensitivity of the spin probe.
4.2 MATERIALS AND METHODS

4.2(a) Wheat germ agglutinin

Wheat germ agglutinin was obtained from Sigma Chemical Co, St. Louis Mo, EY laboratories Inc, San Mateo, CA, Bethesda Research Laboratories (BRL) Inc, Gaithersburg, MD, and from Vector Laboratories Inc, Burlingame, CA.

Purification of the commercial WGA samples was done by cation exchange chromatography (Appendix D). All commercial preparations contained some impurity (a yellow pigment) varying from 7% for Vector WGA to 14% for EY WGA with Sigma WGA varying (depending on the lot) in its impurity. Detailed analysis is shown in Appendix D. The WGA SL-ghost experiments state which WGA was used.

4.2(b) Protein assays

Protein assays were originally done by the method of Lowry et al (1951) as modified by Peterson (1977) with human serum albumin (Sigma) as the standard. O.D.\textsubscript{280} was found to be more reliable and consistent and was used even though it was less sensitive. O.D.\textsubscript{280} was determined on a Beckman Model 25 spectrophotometer (Beckman Instruments Inc., Irvine, CA).

4.2(c) Agglutination assay (microtitres)

The assay is a modification of that of Sever (1962). A 50 µl suspension of red blood cells (4% v/v) in phosphate buffered saline (PBS) was mixed
with 50 µl of a WGA solution of varying concentrations (in PBS). The suspensions were left undisturbed in the microtitre plate for 60 minutes. The non-agglutinated cells settle and flow down the sides of the conical well, forming a small knob at the bottom. The agglutinated cells do not settle but coat the sides of the cone, appearing more diffuse by eye. The microtitre quoted is the lowest final concentration of WGA which caused agglutination. Unless stated the final concentration of the red cell suspension was 2%. Originally fresh red cells (washed) were used for these microtitres but glutaraldehyde fixed red cells (stable for several months at 4°C) were later used for ease and because consistent red cell samples could be used for all studies (see Turner & Liener, 1975).

The cells were fixed in 0.1% glutaraldehyde (Ladd Research Industries) and then extensively washed in PBS and stored at 4°C. Because fixed cells, being rigid, don't deform and pack down as much as fresh cells (Vassar et al, 1972), a final concentration of 2.5% (v/v) was used in the microtitre assay.

4.2(d) Iodination

Iodination of proteins involves the initial oxidation (with lactoperoxidase or iodo-beads, N-chloro-benzenesulfonamide derivatized polystyrene beads) of the radioiodide which then reacts with the tyrosines of proteins (electrophilic substitution of the ortho hydrogens of the phenolic ring, Regoecki, 1984). Iodination was originally done with lactoperoxidase (Sigma) in a solution of 22 mg WGA/ml PBS containing 50 mM glcNAc (to prevent iodination of the binding sites). A four ml aliquot of
this WGA solution was combined with 0.1 mg lactoperoxidase and one mCi of carrier free Na\textsuperscript{125}I (Amersham, Arlington Heights, Ill) at room temperature. The reaction was initiated by the addition of 20 \mu l of 0.06% \text{H}_2\text{O}_2 and 15 minutes later an additional 10 \mu l of \text{H}_2\text{O}_2 was added. The reaction was stopped with the addition of 0.1% (w/v) of KI/\text{NaN}_3 after 30 minutes. The solution was then dialysed (using 8000 MW cut off dialysis bags) against four l of PBS at 4^\circ\text{C} repeatedly until \textsuperscript{125}I in the solution was negligible.

This procedure was modified in some experiments by substituting 0.54 ml Biorad Enzymobeads (lactoperoxidase immobilized on Sepharose) for the lactoperoxidase.

With the advent of Pierce Chemical Co (Rockford, Ill) Iodo-beads, the procedure was modified again. To two ml of a 20 mg/ml of WGA in PBS/azide or 0.05 M sodium acetate, 0.05 M NaCl, pH 4.3/azide, both containing 50 mM glcNAc, were added five iodo-beads and 0.1 mCi Na\textsuperscript{125}I. After occasional mixing, the solution (minus the iodo-beads) was loaded onto a CM-Sepharose CL-6B column and eluted with 0.05 M NaCl acetate buffer, pH 4.3 in 0.025% sodium azide. This column was washed until all free \textsuperscript{125}I was eluted. The bound \textsuperscript{125}I WGA was then eluted with the same buffer at 0.45 M NaCl.

In all cases the iodination step was monitored via trichloroacetic acid (TCA) precipitation. A very small volume (1 \mu l) of the iodination mixture was mixed with 1 ml of 1 mg/ml bovine serum albumin, 1 ml of 20% TCA added, mixed, spun down and 1 ml of the supernatant taken off and both fractions counted. The free \textsuperscript{125}I distributed evenly amongst the two fractions but the bound \textsuperscript{125}I pelleted with the albumin and the % incorporation was calculated.
The free $^{125}\text{I}$ was separated from the bound via dialysis or column chromatography either on a sephadex G-10 or a CM-cellulose CL-6B column. Analysis of the $^{125}\text{I}$ WGA and various fractions was also performed via instant thin layer chromatography (TLC). A drop of solution was added near the base of a 10 x 2 cm strip of polysilicic acid gel (Gelman Instruments Co, Ann Arbor, Mi.) This was put into a PBS chamber and the PBS solvent allowed to run almost to the top. The strip was air dried, cut into 1 cm strips and counted for $^{125}\text{I}$. Free $^{125}\text{I}$ ran with the solvent and the $^{125}\text{I}$ protein stayed at the base.

4.2(e) SDS PAGE

SDS PAGE was performed as described in Appendix A.

4.2(f) Spin labeled ghosts and extractions

Spin labeled ghosts were made as in the optimum protocol of Chapter 3 (Section 3.3(e)). The extracted fractions of Chapter 3 were tested for their ability to react with various preparations of WGA (from different sources).

The lipid extract was concentrated, dried, resuspended in chloroform, degassed, dried again and resuspended in PBS. This was kindly done by Foon Yip.
4.2(g) Binding assay

To varying concentrations of iodinated WGA (± 50 mM glcNAc, Calbiochem-Behring) were added SL-ghosts or normal ghosts. After one hour of incubation at room temperature (with mild shaking) these samples were spun down at 20000 x g at 4°C for 20 minutes. The supernatant was removed and its $^{125}\text{I}$ activity determined on a LKB Compugamma. The activities of the pellets were determined in a similar manner.

ESR spectra were run on the pellets from the binding assay as described in Chapter 2.

Binding isotherms were obtained by plotting the amount bound to ghosts or SL-ghosts vs the concentration of iodinated WGA in solution at equilibrium.

The total amount of WGA bound/SL-ghost is referred to as total binding. Non-specific binding is defined as the amount of WGA bound/SL-ghost in the presence of 50 mM glcNAc. Specific binding was defined as the total binding minus non-specific binding.

To analyse the binding data correctly, several things had to be accounted for:

(i) Free $^{125}\text{I}$. This was monitored via the quick TLC plate method as described in the iodination section (4.2(d)). Duplicate samples were run before and after the binding experiment and the average result taken as the amount of free $^{125}\text{I}$. This varied from 3-7% of the total activity.

(ii) Impurity in the WGA. Initial experiments were done on nonpurified commercial preparations of WGA (58% pure, see Appendix D Fig. D.1)). This impurity was compensated for by assuming only the pure WGA bound to the
pellets (SDS PAGE analysis of the pellets showed that the binding WGA was between 80 and 100% pure). Later experiments were run with WGA purified on the CM CL-6B cation exchange column. The iodinated WGA preparations all had microtitres of 5-9 μg/ml showing that they were still active.

(iii) Trapped volume in the pellet. Ghosts are leaky and less dense than red cells. It was found (by taking samples of the pellets and spinning them down hard in capillaries) that 55 ± 8% (40-71%) of the pellet volume was trapped supernatant. This was confirmed by washout experiments in which the WGA-ghost pellet was repeatedly washed in PBS, eluting all the non- or weakly bound WGA.

The amount of WGA binding to the sample tubes was found to be negligible (a maximum of 0.9% to the gamma counting tubes).

All data shown are the average of duplicate points.

4.2(h) Other lectins

*Ricin communis* l (a galactose binding lectin), peanut agglutinin (a galactose binding lectin; (Chapter 2) and soybean agglutinin (a galNAc/galactose binding lectin) were all purchased from EY laboratories. Concanavalin A (Con A) (a mannose binding lectin) and slug lectin (*Limax flavus*) (a sialic acid binding lectin) were purchased from Calbiochem-Behring Corp, La Jolla, CA.

Lectin solutions (0.5 ml at 10 mg/ml in PBS except Con A at 50 mg/ml and slug at 2 mg/ml) were added to 0.25 ml of SL-ghosts and incubated for 30 minutes. The mixture was then spun down and the pellet ESR run.
4.3 RESULTS

4.3(a) ESR analysis of WGA addition to extractions of SL-ghosts

Table 4.1 lists the spectral parameters for the mixtures of WGA and the SL-ghost-derived fractions described in Chapter 3.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>volume (µl)</th>
<th>volume WGA added (µl) (mg)</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pellet</td>
<td>200</td>
<td>100 (2.5 mg)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 (7.5 mg)</td>
<td>0.63</td>
</tr>
<tr>
<td>supernatant</td>
<td>200</td>
<td>75 (1.9 mg)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>225 (5.7 mg)</td>
<td>0.19</td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pellet</td>
<td>200</td>
<td>100 (2.5 mg)</td>
<td>0.55</td>
</tr>
<tr>
<td>supernatant</td>
<td>200</td>
<td>7.5 mg/ml final</td>
<td>0.11 (resultant supernatant)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5 mg/ml final</td>
<td>1.36 (pellet)</td>
</tr>
<tr>
<td>Isolated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL-glycophorin</td>
<td>500</td>
<td>500 (0.5 mg)</td>
<td>0.02 (resultant supernatant)</td>
</tr>
<tr>
<td>(1 mg/ml)</td>
<td></td>
<td></td>
<td>1.23 (pellet)</td>
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</table>

For Triton X-100 extractions of SL-ghosts, it was found that a precipitate formed upon addition of Sigma WGA if the final WGA concentration was 4 mg/ml or greater. The ESR spectra are shown in Fig. 4.2.

Addition of 0.5 ml iodinated purified Vector WGA in PBS (1 mg/ml at 4.94 \times 10^6 \text{cpm/mg}) to an equal volume of a SL-glycophorin solution (1 mg/ml)
Fig. 4.2 ESR spectra of the Triton X-100 extract of SL-ghosts before and after the addition of Sigma WGA (4.5 mg/ml final concentration).
resulted in the formation of a pellet containing 96% of the spins with a ratio of 1.5 moles glycophorin to 1 mole WGA. The supernatant contained 3.6% of the spins and an equilibrium concentration of 142 µg/ml of WGA. Fig. 4.3 shows the ESR spectrum and the resultant spectral parameters. The SP decreased by 20% with an increase of 9°C (from 20°C to 29°C) and increased by 26% with a decrease in temperature from 20°C to 10°C.

The lipid extract (isolated from SL-^3H-ghosts with chloroform: methanol) was concentrated and dried down. This lipid was extracted from the equivalent of about 1.25 ml of SL-ghosts. Only 6.6% of the tritium in the lipids resuspended into the PBS solution. The resultant ESR signal was very weak. Addition of an equal volume of WGA (4 mg/ml PBS) resulted in no change in this signal (SP = 0.02) (detailed analysis was very difficult, due to the extremely weak signal).

Addition of WGA to the supernatants of the neuraminidase treated SL-ghosts of Chapter 3 resulted in no change in SP, remaining that of free spin label (SP = 0.03).

4.3(d) WGA plus various SL-ghosts

Fig. 4.4a is a plot of the resultant SP of pellets from various commercial WGAIs added 1:1 (v/v) to SL-ghosts (of various preparations). The total WGA concentrations are listed because no binding assays were performed on these samples. Fig. 4.4a is an accumulation of a variety of experiments and shows the variability that arises. For comparison, Fig. 4.4b is a plot of SP vs total WGA (the initial amount added 1:1 with SL-ghosts) from a binding assay done with impure Sigma WGA. The binding data are shown in
Fig. 4.3 ESR spectra of SL-glycophorin (1 mg/ml) before and after the addition of an equal volume of purified Vector WGA (1 mg/ml).
Fig. 4.4 Graph of SP after addition of WGA (1:1) (v/v) with SL-ghosts. Total WGA concentration added on the abscissa and the resultant SP of the SL-ghosts/WGA pellet on the ordinate. (A) the result of a variety of SL-ghost and WGA samples. (B) initial conditions used in the binding of impure Sigma WGA of Fig. 4.12 to 4.15a. WGA source, * Sigma, + Vector, ▲ EY
Figs. 4.7 to 4.9 and the SP vs WGA bound/SL-ghost shown in Fig. 4.10a.

Fig. 4.5 shows the ESR spectra of WGA binding to SL-ghosts and the resultant supernatant signal after the pellet was incubated at 4°C for two days.

Spin labeled ghosts were also made according to the protocol of Felix & Butterfield (1980), Chapter 3. Fig. 4.6 shows the effect of adding an equal volume of vector WGA (10 mg/ml PBS) to these ghosts along with their spectral parameters.

4.3(e) Binding assay

WGA binding to SL-ghosts was the same as that to unlabeled ghosts.

Fig. 4.7 shows the resultant binding isotherms for a variety of binding experiments. Fig. 4.8 shows the resultant specific binding of these experiments. Fig. 4.9 is the Scatchard plot for the specific binding of Fig. 4.8 (B is defined as the bound WGA x 10⁻⁵ and F is defined as the free WGA, which is the equilibrium WGA concentration left in the binding supernatant after the WGA has bound to the SL-ghosts).

Fig. 4.10 shows the graphs of the spectral parameter, SP, vs the amount of WGA bound for the total, non-specific and the specific binding.

Fig. 4.11 compares the results of two binding experiments which vary in the way the SL-ghosts were prepared. One was prepared by the optimal protocol and the other identically except with twice the periodate concentration. The figure illustrates the ESR spectra before and after the addition of WGA to these samples.
Fig. 4.5 ESR spectra of SL-ghosts (200 μl) plus 6.6 mg EY WGA (100 μl) incubated at 4°C for two days followed by separation of the supernatant and pellet (the supernatant contained 10% of the total spins). Also quoted are their calculated SP's.
Fig. 4.6 ESR spectra of F-B SL-ghosts before and after addition of an equal volume of purified Vector WGA (10 mg/ml). Also quoted are the calculated SP.
Fig. 4.7  The graph of WGA bound per SL-ghost vs the amount of WGA left in solution (binding isotherm). The solid lines are the total binding of $^{125}$I WGA to SL-ghosts and the dotted lines the nonspecific binding (WGA binding to SL-ghosts in the presence of 50 mM GlcNAc).  ○  Sigma WGA (58% pure)  *  pure Sigma WGA  +  pure Vector WGA (using SL-ghosts in Fig 4.13b, i.e. relysed again overnight). The bottom graph is an expanded region of the upper graph.
Fig. 4.8 The binding isotherm of $^{125}\text{I}$ WGA to SL-ghosts as described in Fig. 4.6. Specific binding is defined as the total binding minus the non-specific binding (binding in the presence of 50 mM GlcNAc). Symbols as defined in Fig. 4.7.
Fig. 4.9 Scatchard plot (B/F vs F) of specific binding of Fig. 4.8. B is defined as the amount of specific WGA bound per SL-ghost ($\times 10^5$) and F is defined as the amount of WGA left in the solution (equilibrium binding in ug/ml) after WGA binding to SL-ghosts. Extrapolation of the graph to the X-axis (the amount of specific WGA bound) yields the number of binding sites for the specific WGA binding per SL-ghost. The lower graph is an expansion of the upper graph. Symbols as in Fig. 4.7.
Fig. 4.10 Graph of SP vs the number of molecules of $^{125}\text{I}$ WGA bound/SL-ghost from the binding isotherms of Figs. 4.7 and 4.8. (A) (spectra run on the Varian E-3 at ambient temperature) for 58% pure Sigma WGA. (B) (spectra run at a constant temperature of 20°C) pure Vector WGA binding to SL-ghosts of Fig. 4.17b. □ total WGA/SL-ghost, * non-specific (binding of WGA in the presence of 50 mM GlcNAc) and ○ specific binding (defined as the total minus the non-specific binding).
Fig. 4.11 ESR spectra of SL-ghosts (A) prepared as in the optimum labeling protocol in Chapter 3 section 3-3(h) and (C) prepared the same way except at twice the normal periodate concentration (2[0]) SL-ghosts). (B) is SL-ghosts plus Sigma WGA binding at 6.9 X 10⁵ total and 4.2 x 10³ specific WGA/SL-ghost. (D) is 2[0] SL-ghosts plus Sigma WGA at 6.3 x 10⁶ total and 4.2 x 10⁶ specific WGA/ghost. The calculated SP for each spectrum is also quoted.
Fig. 4.12 shows the same SL-ghosts prepared as in the protocol, WGA added, reysed to eliminate what appeared to be an unbound signal (incubated overnight in ghost buffer and washed at 40:1 volume) and WGA added again. This was done as a test to determine the reliability of the SL-ghost preparation. These SL-ghosts were then used in a binding assay (with Vector WGA) with the results shown in Figs. 4.7, 4.8 and 4.10.

4.3(f) Other lectins

Table 4.2 lists the spectral parameters of the lectin pellets formed as described in Methods.

<table>
<thead>
<tr>
<th>Lectin added</th>
<th>% increase in SP</th>
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<tbody>
<tr>
<td>RCA</td>
<td>29</td>
</tr>
<tr>
<td>PNA</td>
<td>0</td>
</tr>
<tr>
<td>ConA</td>
<td>6</td>
</tr>
<tr>
<td>Soybean</td>
<td>5</td>
</tr>
<tr>
<td>Slug</td>
<td>30</td>
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10.5 ml lectin at 10 mg/ml in PBS (except Con A at 50 mg/ml and Slug at 2 mg/ml) added to 0.25 ml SL-ghosts.
Fig. 4.12 ESR spectra of SL-ghosts (A) before and after addition, (C) of equal volumes of purified BRL WGA (37.5 mg/ml). Spectrum (B) is the SL-ghosts of (A) relysed in Dodge buffer and (D) is the result of adding equal volumes of purified Sigma WGA (21.5 mg/ml) to (B). Both (A) and (B) had $2.9 \times 10^6$ spins/SL-ghost. Also quoted are the SP for each spectrum. SL-ghosts (B) were used in the binding isotherm of Fig. 4.7 with pure Vector WGA, but the spectra on which the data in Fig. 4.10 were based were run 10 hours later than those shown here.)
4.4 DISCUSSION

4.4(a) WGA broadening of the ESR signal

Addition of WGA results in an increase in the spectral parameter of SL-ghosts (Figs. 4.4, 4.10 and 4.12) and extracted preparations (Table 4.1, Figs. 4.2 and 4.3). The only cases in which WGA didn't increase the SP were those involving the NaOH and the neuraminidase supernatants. When WGA was added to fractions which resulted in precipitates (SL-ghosts, SL-glycophorin and the Triton X-100 extraction) the remaining signal (about 5%) had the characteristics of a free spin label, with a low SP (Figs. 4.2 4.3, and 4.5). This implies that there is a faint unbound signal present in SL-ghosts. It either slowly dissociates with time, or incomplete reduction could allow slow reversal of labeling. These spins didn't interact with the WGA.

Addition of all the various WGA preparations to SL-ghosts (Fig. 4.4) caused the spectral parameters to increase. The biggest changes in SP didn't come at the highest WGA concentrations because the SL-ghost preparations differ in the amount of the unbound (background) ESR signal which is insensitive to WGA. This background signal caused the variations in SP changes with WGA concentration.

This insensitive fraction, although low in concentration, interferes with spectral interpretation of WGA binding to SL-ghosts. Addition of WGA to SL-ghosts treated with double the amount of periodate yielded a signal which was a superposition of two separate spin populations, one broadened by WGA and the other WGA insensitive (Fig. 4.11). This insensitive population was
believed to be a second spin label site which was produced by the higher amount of periodate oxidation but it could also represent non-interacting, noncovalently attached spins since no differences were found in the SDS PAGE tritium profiles of these ghosts compared to SL-ghosts. This type of interference was also noticed in other binding experiments where an addition of over 10 mg/ml Vector WGA resulted in only minor broadening of the ESR signal (Fig. 4.10b). Plotting the spectral parameter vs the amount of WGA bound/SL-ghost in this case showed little correlation between the two (Fig. 4.10b). Letting the samples sit for 4 days at 4°C resulted in a 20% decrease in the spectral parameters, suggesting either proteolysis or a leaching out of the non-bound spins.

No experimental method was found which would reliably eliminate this signal. The SL-ghosts in which the signal appeared had been made as described in the protocol in Chapter 3 (Section 3-3(e)) and tested with the addition of WGA to see if there was homogeneous broadening (Fig. 4.12a and c). A faint free signal was observed so the ghosts were incubated at a 40:1 ratio with lysis buffer overnight at 4°C, lysed again and analysed by the addition of WGA (Fig. 4.12b and d). The spectrum appeared to be satisfactory so the binding experiment was performed and spectra recorded with the results illustrated in Fig. 4.10b. No obvious decreases or alterations in these spectra were visible in the SL-ghosts which were relysed. Visual inspection of the spectra obtained for Fig. 4.10b, taken 10 hr after that shown in Fig. 4.12d, again revealed a weak but noticeable WGA insensitive population. Seigneuret et al (1984) also noted this problem with spin labeled lipids inserted into the red cell bilayer where a small but noticeable percentage became free.
The only experiments to give an increase in SP with increasing WGA in which detailed binding measurements were made were the initial experiments done with the impure Sigma WGA. However, in most cases, SP increased as a function of increasing WGA concentration (Fig. 4.4). The broadening of the spectra by WGA has been reported by Lee & Grant (1979, 1980) for SL-glycophorin, as was also found here for isolated SL-glycophorin (Fig. 4.3), for spin labeled NANA of fetuin (Kwok & Landsberg, 1982) and for spin labeled NANA of lymphocytes (Ross et al 1983, although they don't mention which lectins they used). Binding of antibodies to nitroxides also show spectral broadening (Rey & McConnell, 1976).

An unusual result was reported by Felix et al (1982) in that they found a decrease in $\tau_C$ with the addition of WGA to their SL-ghosts (F-B SL-ghosts). As shown in Chapter 3 (Discussion), F-B SL-ghosts are comparable to those made here except have a lower number of spins/ghost and a greater proportion associated with the lipids. The signal from this type of ghost is extremely low (at $2 \times 10^6$ gain on a Varian E-3 with a 200 $\mu$l sample size) and not detectable from the small volumes needed for temperature controlled experiments (24 $\mu$l). In the present work addition of an equal volume of 10 mg WGA/ml to these ghosts resulted in a 55% increase in the spectral parameters (Fig. 4.6), not a decrease as Felix et al (1982) observed. As discussed above, the discrepancy could be associated with a large population of non-interacting spins, or, since their lectin concentration was low (1.5 mg/ml) possibly not enough binding occurred and the alteration was due to experimental fluctuations. They had a considerable attachment of the spin probe to glycolipids (30%). At low WGA concentrations spin labeled gangliosides appear to first decrease in their $\tau_C$ and as the WGA increases $\tau_C$ also does (Lee et al, 1980). Felix et al
(1982) attribute their decrease in $\tau_C$ (33%) to the hypotonic buffers they used, as opposed to the higher ionic strength PBS used in the other experiments, and to complex glycophorin-membrane protein interactions which, following WGA binding, were felt to leave the probe in a less restrictive environment.

In this work the yields of spins/ghost were low for F-B SL-ghosts so no detailed analysis could be done. Errors were high due to the low signal-to-noise ratio. The literature supports the finding that WGA does broaden the ESR signal of SL-sialic acid moieties.

The above results, and the shape of the broadened spectra (Figs. 4.4 and 4.11) cast considerable uncertainty on the use of SP to describe the reduction in spin label motion associated with the experimental manipulations used in this study. Clearly, the broadened signal is complex and most likely contains contributions from several types of spectra. This is particularly true when the background signal is appreciable (Figs. 4.5 and 4.10b). In the absence of a detailed spectral analysis, however, SP is used in this thesis as an index of changes in spin label motion. It should not be interpreted as representing a uniform change in correlation time for the entire spin population.

4.4(b) Mechanism of broadening

Peters et al. (1979) found WGA bound better to ovine submaxillary mucin and fetuin if the NANA was converted to NANA$_7$, suggesting that the exocyclic chain is a potential source of steric hindrance. NANA$_7$ glycophorin glycopeptides are retained on WGA affinity columns (Bahavanadan & Katlic, 1979) and Kahan et al. (1976) used I$_4$/NaB[3H]$_4$ treated
red cells to monitor the isolation of glycophorin on a WGA affinity column. Hence, oxidation per se does not seem to reduce WGA affinity for NANA. However, Lee and Grant (1980) found that SL-glycophorin (labeled on the sialic acids) was a weaker inhibitor of WGA agglutination than unmodified glycophorin, again suggesting that side chain constituents can interfere with WGA binding to some degree. In this work, since only about 13% of the sialic acids were modified on the SL-ghost, specific WGA binding to SL-ghosts didn't differ from that of normal ghosts.

The fact that the spin label signal broadens upon the addition of WGA implies it is attaching to, or very near, sialic acid. The exocyclic triol is the furthest removed from the binding site in crystal studies (Wright 1980), however, and since WGA can also bind internal sugar units, direct binding might not be expected. Using the Stokes-Einstein relationship $t_T = \frac{4\pi\eta r^3}{3kT}$ (where $r$ = the radius of the protein, equal to 25 angstroms for WGA (Wright, 1980), and $\eta$ = the viscosity of the solution) a value of $1.7 \times 10^{-8}$s at 22°C is calculated for WGA in solution. For the largest SP of a WGA/SL-ghost pellet (1.7) a $T_C$ of about $3 \times 10^{-9}$s is implied using equation [9] in Chapter 1. For the WGA/SL-glycophorin pellet, $T_C = 1.7 \times 10^{-9}$s (SP = 1.23, Table 4.1). If WGA was binding to the nitroxide itself, therefore, much higher SPs would have been produced. Hence it seems likely that the signal broadening is due to steric hindrance as opposed to actual binding to the spin probe.

4.4(c) WGA binding isotherm

WGA binding to red cells is complex. WGA appears to bind specifically to red cells in a positively cooperative fashion (i.e. initially bound
material enhances subsequent binding) at low WGA concentrations and there appears to be two types of specific binding sites as detected by Scatchard plots (Anderson & Lovrien, 1981; Adair & Kornfeld, 1974), as well as considerable non-specific adsorption.

The cooperativity and two binding sites have also been found for CHO cells by Stanley & Carver (1977). Sigmodial curves (an indication of positive cooperativity) have been seen in the isotherms of WGA binding to glycoporin-containing liposomes (Ketis et al, 1980; Redwood et al, 1975) and to ganglioside liposomes (Redwood & Polefka, 1976).

Addition of 50 mM glcNAc usually results in instantaneous release of WGA from cells (Anderson & Lovrien, 1981; Evans & Leung, 1984). This reversibility by glcNAc has usually been taken to infer that WGA is binding to glcNAc residues. However, glcNAc is found to reverse and inhibit binding of WGA to glycolipid vesicles not containing any glcNAc (Boldt et al, 1977). Wright (1980) and Cuatrecasas (1973) postulate that glcNAc binds to an unoccupied site on WGA causing either conformational changes in the lectin resulting in dissociation of the WGA from the cell (Cuatrecasas, 1973) or destabilizing WGA:WGA aggregates on the cell surface (Wright, 1980) (native WGA crystals disintegrate in the presence of glcNAc but are stable in the presence of NANA, Wright, 1979). Whether NANA on the cell binds to all four WGA sites is unknown.

In all cases, 50 mM glcNAc appears not to be an effective inhibitor of total WGA binding to ghosts or SL-ghosts. Non-specific binding appears to be very large (Fig. 4.7). Perhaps lysing the red cells into ghosts exposes other sites not normally available to WGA, as suggested by Horisberg & Rosset (1977). These investigators found twice the number of receptors on isolated membranes as was found via colloidal gold-WGA labeling of red
cells. The non-specific binding was highest for SL-ghosts that had been re-lysed overnight (Fig. 4.7). These SL-ghosts also had a WGA insensitive signal (as shown by the lack of correlation of SP vs WGA bound per SL-ghost, Fig. 4.10b), showing that the details of sample preparation were important. Perhaps excessive lysing produced inside-out ghosts (Steck, 1974) which rendered the SL-sialic acid inaccessible to WGA.

If impurities and non-specific binding are compensated for, all the specific binding data, taken over an extended period of time, are reproducible (Fig. 4.8) even though there was scatter for the total WGA/SL-ghost in the binding isotherm (Fig. 4.7). Points from both purified WGA and impure WGA preparations for which the amount of binding was calculated from the WGA band on SDS PAGE (Appendix D) fall on the same line. This indicates that non-specific binding fluctuates and causes the scatter in the data, due apparently to variations in ghost preparations. Cooperativity plus at least two different classes of binding sites for WGA result in a Scatchard plot far removed from the straight line obtained for simple saccharide binding to WGA in solution. There are also indications that WGA binding to cells may not be in equilibrium. Scatchard plots of WGA binding to CHO cells or fat cells are effected by the number of cells in the assay (Stanley & Carver, 1977; Cuatrasas, 1973). Data sent to E. Evans by Lovrien shows the same phenomenon for red cells (Evans, personal communication). One possible explanation for this is the fact that WGA binding to cells is very fast (Schneble & Bachi, 1975) and the rate of release slow (Cutrecasas, 1973) possibly resulting in the cells initially exposed to lectin binding most of the WGA, leaving less in solution for the remaining cells.
For WGA binding to SL-ghosts, therefore, it is not surprising that the Scatchard plots are complex. Detailed analysis of all the specific binding data via Scatchard analysis indicated that binding did not fit a simple model (Fig. 4.9). Inconsistencies in the Scatchard plots for the various experiments are also due to the sensitivity of this plot compared to binding isotherms (compare Fig. 4.8 to 4.9). At low levels of binding, there is positive cooperativity (as depicted by a positive slope for the Scatchard plot, Fig. 4.9), where the WGA broadening is strongest. The best high WGA concentration data gives saturation at about $9 \times 10^6$ WGA/SL-ghosts, similar to that cited in the literature ($8 \times 10^6$, Adair & Kornfeld, 1974; $1.2 \times 10^7$, Anderson & Lovrien, 1981 and from $1.0 \times 10^7$ for old to $1.7 \times 10^7$ for young red cells, Choy et al, 1979).

4.4(d) WGA receptor on red cells

Although WGA binding to red cells has been studied for some time, the exact location of the receptor(s) has not been identified with certainty, as is seen from the following. WGA affinity columns will bind glycophorin (Kahane et al 1976; Adair & Kornfeld, 1974), acetylcholinesterase (Ravozzolo et al, 1983), band 3 (Bjerrum et al, 1981, although Futhmayr et al, 1976 isolate band 3 by collecting the material that fails to bind to a WGA affinity column), band 4.5 (the glucose transporter) (Froman et al, 1981) and glycolipids (Bowles & Hanke, 1977). WGA will bind to glycophorin in solution (Fig. 4.3 and Lee & Grant, 1979) and to glycophorin-containing vesicles (Ketis & Grant, 1982; Redwood et al, 1975).

WGA will also bind to vesicles made of human red cell lipids if they are high in sialoglycolipids (Van der Steen et al, 1983), to ghosts so
extensively protease treated the no protein can be detected (Gordon et al, 1977) and to vesicles containing globoside and ceramide trihexose isolated from red cells (Boldt et al, 1977), although Rendi et al (1976) reported that WGA would not bind to vesicles made from red cell lipids.

Although WGA binds to desialylated red cells (Adair & Kornfeld, 1974; Schnebli & Bache, 1975 and Anderson & Lovrien, 1980), it is with a weaker association constant (Adair & Kornfeld, 1974). WGA columns bind desialylated glycophorin (Kahne et al, 1976), implying sialic acid is not the only binding site. However, Bhavanandan & Katlic (1979) found asialoglycophorin to be a poor inhibitor of WGA hemagglutination.

Burness & Pardoe (1983) found as the sialic acid content of glycophorin decreased, so did its ability to inhibit WGA hemagglutination (a 28% decrease in the sialic acid content resulted in a 42% decrease in inhibition). However, Fukuda & Osawa (1973) could release 50% of the sialic acid with no effect on its ability to inhibit WGA hemagglutination. This suggests that certain sialic acids are more important than others for WGA binding. A similar conclusion was drawn by Kronis & Carver (1982) based on studies of sialylactose binding to WGA, the alpha 3 structure binding better than the NANA alpha 6-lactose (the alpha 3 gal and the alpha 6 galNAC both are found on glycophorin).

Adair & Kornfeld (1974) found the RBC components released from a WGA affinity column inhibited better than glycophorin isolated via the LIS/phenol method. Kahane et al (1976) didn't find any PAS 3 in the material released from a WGA column by glcNAC, implying that not all the PAS positive components react equally with WGA (although Bjerrum et al, 1980 found that PAS 3 did react with WGA).
Bhavanandan & Katlic (1979) state that WGA specificity is relative rather than absolute, depending on the density of receptors. High densities of sialic acid and GlcNAc lead to strong WGA binding, thus the topography of cell surfaces is important in the formation of stable bonds.

The results presented in this thesis show that WGA added to various fractions isolated from ghosts reacts to the fractions rich in PAS proteins (the NaOH pellet, the Triton X-100 extract and to isolated SL-glycophorin) (Table 4.1 and Figs. 4.2 and 4.3). SDS PAGE gels of these fractions show that unless the WGA is reduced a large fraction of PAS stainable components won't run into the gel, implying that the WGA is complexing with the PAS proteins (data not shown). The isolated lipid, however, didn't appear to interact with the WGA when resuspended in PBS.

The experimental evidence obtained in this work strongly supports the idea that initial binding of WGA to red cells occurs at one site per glycophorin. Cooperativity is seen in Scatchard plots up to 2 to $10^5$ WGA/cell (Fig. 4.9) (Anderson & Lovrien, 1980 reported $5 \times 10^5$), approximately equal to the number of glycophorin copies in the red cell membrane (Anstee, 1981; Furthmayr, 1981). SL-glycophorin reacted strongly with WGA, completely precipitating out upon the addition of WGA (except for 3.6%, believed to be non-covalently associated spins) at a ratio of 1.5 moles glycophorin to 1 mole of WGA (Fig. 4.7). For a precipitate to form, 2 moles of glycophorin to 1 mole WGA is expected, but due to the oligomeric nature of glycophorin in aqueous solutions (existing in aggregates of 10 to 20 monomers, Chapter 3, Discussion), this lower molar ratio of glycophorin to WGA is expected. The resultant ESR signal in this precipitated pellet was broad ($SP = 1.23$), like that of SL-ghost with WGA. Visual inspection of
the pellet spectrum reveals two superimposed spectra, one broad, which may possibly be a combination of unbound and WGA bound SL-glycophorin. SDS PAGE gels of this pellet again wouldn't run into the gel unless reduced first, even though the individual components ran into the gels, reduced or not (data not shown). No non-covalently-associated spins were detected in the pellet even when the sample was run at $10^0C$ (as determined by visual inspection of the spectrum for sharp lines).

Since most of the spins are associated with the PAS proteins (Chapter 3) and as shown here, this spin population is sensitive to the addition of WGA, WGA is almost certainly binding to glycophorin on ghosts. Even though there is a variety of sites on the red cell capable of binding WGA, the initial binding is to only glycophorin as shown by the altered ESR spectrum of SL-ghosts after the addition of the lectin.

4-4(e) Correlation between SP and WGA binding

The fact that the SL-ghost spectral parameters do increase with the addition of WGA suggests that the spin probe is sensitive to WGA binding. This increase is linear in the amount specifically bound up to $5 \times 10^5$ WGA/ghost (for impure Sigma WGA, Fig. 4.10a). For nonspecific binding there appears to be no effect upon spin motion until about $3.6 \times 10^5$ WGA/ghost, where again SP starts to increase. These breaks in the plot at 3.6 and 5.4 $\times 10^5$ WGA/SL-ghost are close to the number of glycophorins/red cell. This suggests that 1:1 binding of WGA to SL-glycophorin on the cell results in spin label broadening. After this 1:1 binding with glycophorin, the non-specific binding broadens also.
The selective broadening at low WGA levels indicates the WGA does indeed bind to glycophorin. The correlation is only seen if one calculates the specific binding, implying that the non-specific binding occurs to something other than the PAS proteins. In this low WGA concentration region, the binding is cooperative (Fig. 4.9) yet one still sees the correlation indicating labeling doesn't affect the cooperative mechanism. Scatchard analysis (Fig. 4.9) shows complex binding yet the SP vs WGA bound is linear in this region. One can conclude that ESR provides a more specific picture of WGA binding than adsorption measurements in this instance since the effects of non-specific binding or of the impurities in impure radiolabeled preparations are not seen.

It has been postulated that there is a population of sialic acids on red cells (40% of the total sialic acids) which are more "external" than the others, as indicated by their accessibility to neuraminidase and periodate treatment in intact cells in comparison to ghosts (Singer & Morrison, 1974; Blumenfeld et al, 1972). The lower accessibility of a fraction of the red cell sialic acids may be due to the size of neuraminidase and periodate since it is known that tetramethyl ammonium ions are excluded relative to K⁺ and Na⁺ (Brooks, 1973) or possibly due to subtle rearrangements in the glycocalyx after lysis. Since only 15% of the sialic acids get oxidized (roughly two per glycophorin for isolated SL-glycophorin at 131 ug sialic acid/mg, Chapter 3), one would assume the most accessible would react with periodate and thus be spin labeled. One would also expect these external sialic acids to be the first seen by WGA. Since it appears that the spin labels are on the average 16 angstroms apart, (Chapter 3) WGA could easily accomodate both SL-sialic acids located on glycophorin (the distances
between binding sites on WGA are from 22 angstroms, from a primary to a secondary site to 42 angstroms for the two secondary sites with the primary sites being 31 angstroms apart (Wright, 1980). As the WGA concentration increases, the binding would be to a variety of sites and the spin label broadening would become less (in proportion). This would explain why the specific broadening starts to level off and the non-specific to increase.

4.4(f) Binding of Other Lectins

The selectivity of this spin label for sialic acid-binding proteins is also seen in Table 4.2. Lectins such as PNA, ConA and SBA don't bind to glycophorin or sialic acid residues and have no effect on the SP after addition to SL-ghosts. Slug lectin (specific for sialic acid) binding to SL-ghosts does cause an increase in SP (30%). Of interest is the RCA lectin which also increased SP upon exposure to SL-ghosts. It has been postulated that RCA doesn't bind to glycophorin (Adair & Kornfeld, 1974; Jakobovits et al, 1981; Triche et al, 1975) yet glycophorin can inhibit RCA hemagglutination (Adair & Kornfeld, 1974), RCA binds all $^3$H/G0 labeled glycoproteins of red cells (Tsao et al, 1981) of which glycophorin is one, (Chapter 2) and it binds to band 3 and PAS 1 on SDS PAGE gels (Tanner & Anstee, 1976). The data in Table 4.2 implies that RCA does indeed bind to glycophorin.
4.5 CONCLUSION

Addition of WGA to SL-ghosts, selective extractions of SL-ghosts or isolated SL-glycophorin results in decreased spin label mobility as detected by an increase in the spectral parameter. WGA appears to bind to glycophorin as determined by its interaction with selective extractions of ghosts and its precipitation of isolated glycophorin. WGA binding to SL-ghosts was indistinguishable from that to normal ghosts, showing that spin labeling the cells didn't alter them detectably with respect to this reaction. Binding analysis showed high and variable non-specific binding. If noncovalent (background) ESR signal was removed a linear increase of spectral parameter vs the amount of specifically bound WGA/SL-ghost was observed. The binding of impure Sigma WGA was more efficient at broadening the ESR signal than Vector WGA which had been purified. For specific binding, the change in SP started to level off at about $5 \times 10^5$ WGA/ghost and was interpreted to indicate specific binding of one mole WGA per mole of glycophorin. More general binding occurs at higher WGA levels as determined by an increase in plots of SP vs non-specific binding and by a reduced correlation between specific binding and spectral alterations.

WGA also broadened the ESR signal of F-B SL-ghosts, contrary to the results reported by Felix & Butterfield (1980). The proposed mechanism of broadening by WGA is that of steric hindrance by the WGA molecule of the spin probe.

Only sialic acid-binding proteins affected the ESR signal of SL-ghosts indicating that the spin probe sensitivity was limited to this moiety.
4.6 SUMMARIZING DISCUSSION

The original question asked in this thesis was can techniques used in chemistry on complex isolated systems be applied successfully to less well defined biological systems in situ? A positive answer is implied by the results presented above.

ESR proved to be sensitive enough to analyse a specific component of the glycocalyx of the cell membrane. Conditions could be found where maximum labeling and minimal perturbations occurred with retention of specificity for glycophorin. Elimination of interfering spins was a prerequisite for understanding and interpreting spin labeling data, however.

Spin labeling sialic acid proved to be more informative than spin labeling the gal/galNAc residues, perhaps due to their accessibility (the sialic acids are the terminal residues of oligosaccharides on membrane sialoglycoproteins). This accessibility also appeared to be responsible for the sensitivity of these spin labeled sialic acids to WGA binding. Spin labeling reported selectively on the specific binding of WGA (detected as an increased SP) without necessitating compensation for non-specific binding or impurities in WGA. In fact, impure WGA was found to be more effective in ESR studies than purified commercial WGA.

Out of the complex binding of WGA to red cell membranes, the spin label monitored specific binding to glycophorin sialic acids. This selectivity was also found with Slug and RCA lectin binding. Hence, in situ spin labeling would seem to be a promising way to study any receptor activity in which sialic acid is thought to be involved.
SDS PAGE was run on 5 or 10% cylindrical gels as follows, following general instructions provided by Bio-rad Laboratories. All reagents were of electrophoresis purity from Bio-Rad Laboratories, Richmond CA.

The gel was first cast into 7 x 12.5 mm glass tubes. The gel solution consisted of 5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide (or 10% acrylamide and 0.26% bisacrylamide), 0.1% (w/v) SDS, 0.03% (v/v) tetramethylethylenediamine (TEMED) and 0.05% (w/v) ammonium persulfate for the 5% acrylamide and 0.01% ammonium persulfate for the 10% acrylamide, in a buffer of 0.205 M tris acetate pH 6.1. The gel was layered with isobutanol to ensure a flat surface and allowed to polymerize at room temperature for one hr. The isobutanol was then rinsed off with distilled water.

The sample buffer consisted of 12% sucrose, 1% (w/v) SDS in 0.041 M tris acetate pH 6.1. If the sample was to be reduced 0.04 M dithiothreitol (DTT) or 10% mercaptoethanol was used. Samples were incubated at 100°C for three minutes and then the tracking dye, Pyronin Y was added. If the sample was in solution, the sample buffer was added at a volume to ensure that the sample buffer was never diluted more than 50% (ghost samples were usually mixed 50:50 with the sample buffer). If the sample was freeze-dried protein, final protein concentrations of 1 mg/ml sample buffer were usually made.
The electrophoresis buffer was 0.205 M tris acetate pH 6.1 with 0.1% (w/v) SDS. A Hoefer Scientific Instrument (San Francisco CA) gel electrophoresis chamber model DE 102 was used. Electrophoresis was run at slightly less than 1 watt/tube at 4°C.

A discontinuous system as described by Laemmli (1970) was also used with a 5% stacking gel and either a 10, 12 or 15% separating gel.

Staining.

Gels were first fixed (after being run) in methanol: acetic acid: water (5:1:5) overnight, and if staining for protein were also incubated with 0.02% coomassie blue. The coomassie blue stained gels were then destained in 10% (v/v) acetic acid until the background was clear. If staining for carbohydrate, the PAS (periodic acid Schiff base) stain was used. The fixed gels were incubated in 1% periodate in 5% acetic acid for one hour. The periodate was then eliminated by first washing in 0.5% arsenite in 5% acetic acid and then in 0.1% arsenite in 5% acetic acid. The arsenite was then washed out with 5% acetic acid and the Schiff reagent was added and the gels incubated overnight. The gels were then destained with 0.1% sodium metabisulfite in 0.01 M HCl repeatedly until no pink color could be seen with the addition of formaldehyde. The Schiff reagent consisted of one g of Basic Fuchsin with one g of sodium metabisulfite in 200 ml of 0.1 N HCl. The mixture was filtered with activated charcoal. For Basic Fuchsin stain, the same procedure was followed except the periodate oxidation step was eliminated.
Scanning gels

Gels were scanned on a Helena Auto Scanner model R4-077 (Helena Laboratories, Beaumont, Texas) which sets the largest peak to 100% absorbance or on a Beckman model 25 spectrophotometer attached to a 24-25 ACC recorder. Coomassie blue stained gels were recorded at 595 nm and the PAS and Basic Fuchsine stained gels at 525 nm.

Radioactive gels were sliced in 1 mm intervals with a Bio Rad gel slicer model 195. Iodinated gel slices were counted with an LKB Compugamma counter and tritiated gel slices were counted by the method of Aloyo (1979).

Molecular weight estimations

Molecular weight standards hemoglobin (16000 daltons), human serum albumin (66000 daltons), cytochrome c (12400 daltons) and ovalbumin (45000 daltons) were used. Their dimers and trimers also appeared on the gels. A plot of log(molecular weight) vs their $R_f$ (distance protein traveled/distance tracking dye traveled) was made and from the $R_f$ of the unknowns a molecular weight was calculated.
ESR INTEGRATION

ESR spectra were run on the Varian E-3 at ambient temperature using a flat cell of 200 µl capacity. Samples were run several times, scan rates and response times such that the maximum peak-heights were ensured for each run. The spectrum, at a later time, was put onto a Hewlett Packard 9872A plotter in conjunction with the 9815A Hewlett Packard calculator, digitized (150 points) and plotted (program written by K.A. Sharp, listed below). This digitized spectrum was integrated and the result also plotted. Four points were selected as zero points as in fig. B.1 and a second integration was performed (on this rezeroed first integration) and its numerical value stated. Fig. B.1 shows the initial ESR spectrum, the digitized spectrum and the resultant integrations.

The program for the Hewlett Packard calculator is listed on the three pages following Fig. B.1.
Fig. B.1 (A) ESR spectrum of SL-ghosts, (B) digitized spectrum of (A), (C) the first and second integrations of the digitized spectrum (B). The dotted line is the actual first integration and the solid line the rezeroed first integration. The second integration is of the rezeroed first integration along with its numerical value.
OPERATION

1. Set up the plotter
   position the spectrum on the plotter bed as shown in Fig. B.2

2. Load the program

3. When 'P_1'
   move the digitizing site (in pen # A stall) with the cursor controls on
   the plotter to position P_1 as defined in Fig. B.2 and press ENTER on
   plotter

4. When 'P_2'
   move the digitizing site with the cursor controls to position P_2 as
   defined in Fig. B.2 and press ENTER on plotter

5. When 'X min, max, Y min, max'
   enter 55 RUN/STOP, 89 RUN/STOP, -1 RUN/STOP, 0 RUN/STOP
   this sets the scale for the plotter

6. When 'LOAD'
   a. enter 1 (means YES) RUN/STOP if plotting a stored spectrum from tape
      and go to step 8
   b. enter 0 (means NO) RUN/STOP if digitizing an ESR spectrum

7. Digitizing the spectrum
   the digitizer pen goes to point (0,0) on the plotter.
   a. move the digitizer site onto the spectrum by moving the vertical
      cursor controls
   b. press ENTER on the plotter, digitizer pen automatically moves an
      increment along the x-axis
   c. repeat 7a and b until 150 points have been digitized

8. When 'NEW PAPER'
   replace spectrum with blank paper then press RUN/STOP

9. The digitized spectrum is plotted with pen #1

10. When 'STORE'
    a. enter 1 RUN/STOP and then the file # RUN/STOP if the spectrum is to
       be stored
    b. enter 0 RUN/STOP if the spectrum is not to be stored

11. Plotter proceeds to plot the first integration with pen #1

12. When '4 ZERO PTS'
    move digitizer site over the first of the four zero points (lowest x)
    and press ENTER on plotter, repeat for the other three points, the four
    points are printed after the last one has been entered

13. Press RUN/STOP to plot the rezeroed first integration with pen #2
14. When 'OK'
   a. enter 0 RUN/STOP if the rezeroed integration unsatisfactory and repeat step 12 and 13
   b. enter 1 RUN/STOP if rezeroed integration is good, this integration is automatically replotted

15. When 'STORE'
   a. enter 1 RUN/STOP and the file # RUN/STOP if want the rezeroed integration to be stored
   b. enter 0 RUN/STOP if don't want the integration stored

16. after step 15 the plotter automatically plots the second integration with pen # 3 and prints its numerical value.

Fig. B.2 Placement of the ESR spectrum onto the plotter bed and the location of $P_1$ and $P_2$. 
Illustrated in this and the following Appendix are two examples of lectin interactions, the fetuin:PNA system and WGA affinity purification. Fetuin contains six sialo-oligosaccharide chains, of which three are binding sites for PNA when the sialic acid is removed. Fetuin is thus an excellent model to study for the neuraminidase, galactose oxidize spin-labeling reaction. WGA was purified by a variety of methods and raises questions concerning the selectivity of the isolation method.

C.1(a) Fetuin modification

Fetuin (Type IV from Sigma) was iodinated by the lactoperoxidase method (see Chapter 4, section 4.2(d)) which resulted in an activity of $4.10 \pm 0.6 \times 10^8$ cpm/mg. Free $^{125}$I was separated from $^{125}$I-fetuin on a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column. This was done before each use to ensure no free $^{125}$I interfered with the experiment.

Fetuin was desialylated with neuraminidase (*Vibrio cholerae*) at 0.1 U/ml in 0.05 M sodium acetate pH 5.5, 0.154 M NaCl, 9 mM CaCl$_2$, 0.025% azide at 37°C for 51.5 hours.

Fetuin was spin labeled by the neuraminidase, galactose'oxidase method as in Aplin et al (1979) producing NAGO SL-fetuin. This method was found to release $73 \pm 3\%$ of the bound sialic acid.
C.1(b) Peanut agglutinin affinity column

The procedure is that of Cuatrecasas (1970). Five grams of wet Sepharose 4B (Pharmacia) were modified with 0.3 g of CNBr dissolved in 0.4 ml of acetonitrile diluted 1:1 with water. The CNBr activated Sepharose was suspended in 10 ml of 0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.3 which also included 0.2 M gal, and the PNA added (Miles-Yeda, Rehovot, Israel or Sigma) (1 mg/ml in the buffer with 0.2 M gal). This was stirred gently for two hours at room temperature and then packed into polystyrene columns (Isolab Inc., Akron, OH) of 6 ml capacity and washed.

The amount of PNA coupled to the beads was determined as the amount added reduced by the amount recovered in the washing solutions. The resultant column had 0.7 mg PNA/ml Sepharose.

C.2 Results

PNA Fetuin interactions

Addition of 16 µg (6 x 10^6 cpm) fetuin in PBS/azide to the 5 ml column of PNA sepharose, which bound desialylated RBCs, resulted in 33% of the fetuin nonspecifically bound to the column (protein could not be removed with 0.2 M gal, 1 M NaCl or ethylene glycol). No other binding to the PNA column was observed either for fetuin, neuraminidase treated fetuin (with no detectable bound sialic acid) or NAGO SL-fetuin even if allowed to equilibrate for 15 min to ensure maximum binding at 4°C.
Addition of PNA lectin (2 mg in 400 µl plus 18 mg SL-fetuin (SP = 0.03) in 1 ml, both in tris buffer) produced a precipitate. This precipitate was washed 3 times in the tris buffer (each wash being 2 ml), with each successive wash decreasing the amount of material in the pellet (SP = 0.13 for the pellet and 0.03 for the solution). Analysis of the pellet on SDS PAGE showed it to contain PNA and fetuin (data not shown).

C.3 Discussion

Fetuin is a fetal calf serum sialoglycoprotein which bears six oligosaccharide types. Three are N-glycosidically linked and three O-glycosidically linked (Nilsson et al, 1979; Baenziger & Fiete, 1979). The removal of sialic acid exposes the gal beta 1-3galNAc residue of the O-glycosidically linked oligosaccharides, a strongly binding sugar unit for PNA (Goldstein & Haynes, 1978). This O-glycosidically linked oligosaccharide is also believed to be present on glycophorin A (15/molecule, Anstee, 1981, Furthmayr, 1981 and Lisowska et al, 1980), the major sialoglycoprotein of the red cell membrane.

NAGO SL-fetuin has already been studied by Aplin et al (1979) so the modification of the system was not analyzed here.

Spin labeling fetuin by the NAGO method produced no alterations as detected by SDS PAGE (data not shown). Since not all (73%) of the sialic acid was removed by the neuraminidase, the possibility arose of populations of fetuin heterogeneous with regard to sialic acid content and thus spin labeling. One would then expect that not all of the NAGO SL-fetuin would bind to a PNA affinity column.
The PNA column made (0.7 mg PNA/ml) was found to bind desialylated red cells in a manner which was inhibited or reversed with 10 mM gal in PBS. The same preparation was found not to bind any of the above mentioned fetuin or modified fetuin fractions, however. A high degree of nonspecific binding occurred which was difficult to reverse. Sepharose is known to allow some hydrophobic interactions (Lotan et al, 1977) which may explain this result.

Lotan et al (1977) used a high capacity, charge free, non-leaching polyacrylic hydrazide sepharose column, with 5 mg PNA/ml column, and obtained some desialylated fetuin binding. It is known (at least for wheat germ affinity columns) that clusters of receptors determine whether or not binding will occur (Yamamoto et al, 1981). The lectin density of the column also appears to influence binding (Bhavanadan & Katlic, 1979), with low density columns binding only high clusters of binding sites (such as are present on red cells) and the binding of lower density clusters (such as fetuin) occurring only when there is a high lectin density on the gels. Dulaney et al (1979) list several variables which appear to be important in the binding of glycoproteins to lectins (such as temperature, time of contact, pH and ionic strength). Conditions chosen here were considered optimal by Lotan et al (1977) so the most likely explanation for the lack of binding is that of the PNA density of the sepharose column. The desialylated red cells have a high density of receptor sites available and bind to the PNA column. The desialylated fetuin (spin labeled or not) may not have bound because the receptor density and lectin density were too low. It is also possible that the nonspecific binding (perhaps due to the impurities in the fetuin) interfered with specific binding, sterically hindering access to these specific sites (unfortunately, the nonspecifically
saturated gels were not tested for their ability to bind desialylated red cells).

Solutions studies support the hypothesis that it is the lectin density of the PNA affinity column which is the factor dictating the binding since the SL-fetuin will bind to PNA in solution even though not completely desialylated (presumably because the local density of PNA or fetuin is higher than on the gel). Of the 12 possible gal residues to be spin labeled on desialylated fetuin, only three are optimal for PNA. About 30% of the sialic acid remained on the molecules, probably decreasing the ratio of SL to optimal PNA binding site, yet this NAGO SL-fetuin still interacted with PNA in solution.
APPENDIX D

D.1 WGA Purification

D.1(a) Fast protein liquid chromatography (FPLC)

The FPLC system (Pharmacia) includes a gradient programmer GP-500, two P-500 pumps and a single path monitor UV-1 set at 280 nm, whose output was displayed on a Hewlett Packard 7100 B strip chart recorder. The column used was a Pharmacia mono S HR5/5 cation exchange column bearing \(-\text{CH}_2\text{-SO}_3^-

This column was equilibrated with 0.05 M sodium acetate, 0.05 M NaCl, 0.025% azide at pH 4.3 (buffer A) which was also the buffer in which WGA was dissolved. Buffer B was identical to buffer A except the NaCl was raised to 0.45 M. The program used was 0% buffer B for the first 15 minutes followed by 100% buffer B for 3 minutes then 0% buffer B for the last 3 minutes. For the first 15 minutes, the flow rate was set at 0.5 ml/min, to allow WGA to bind to the column. After buffer B was applied, the flow rate was increased to 2 ml/min and remained at this rate for the rest of the run.

The column was re-equilibrated with buffer A for 3 minutes of the run, making the column ready for another WGA sample. A maximum of 10 mg WGA (in 500 µl buffer) can be loaded on the column per run. After the WGA runs were finished, the column was washed with 0.1 M NaOH to elute any remaining protein.
D.l(b) CM-Sepharose CL-6B

The procedure used was that of Kronis & Carver (1982), a modification of LaCelle (1979). To a 190 ml (2 x 73 cm) column of CM-Sepharose CL-6B (Pharmacia) equilibrated in 0.05 M NaCl, 0.05 M sodium acetate, pH 4.3 (in 0.025% NaN₃) was added 5 ml of 37 mg/ml WGA in the same buffer and the column washed with this buffer. The eluent was monitored on a LKB Uvicord Type 4701A at 253.7 nm and the WGA was eluted with 0.45 M NaCl in the pH 4.3 acetate buffer. The column was subsequently washed with 0.1 M NaOH to remove residual protein.

D.l(c) Chitin column

The chitin column (in 0.01 M tris HCl, pH 8.5, 1 M NaCl, 0.025% NaN₃) was prepared according to Bloch & Burger (1974), using chitin (crab shells) purchased from Sigma.

D.l(d) Ovomucoid column

The ovomucoid column was prepared according to Marchesi (1972) using ovomucoid type III-0 trypsin inhibitor from Sigma: 974 mg of ovomucoid was dissolved in 50 ml 0.2 M sodium bicarbonate, pH 8.6, in 0.02% NaN₃ and added to 38 ml of Sepharose CL-4B (Pharmacia) which had been CNBr activated by the addition of 10 g of CNBr in 65 ml 2 M sodium carbonate.

The coupling efficiency was 10%, providing 1.2 mg/ml ovomucoid Sepharose. The WGA was loaded onto the column (1 x 18 cm) and the column
washed in 0.05 M phosphate buffer, pH 7, 0.25 M NaCl and 0.025% NaN3. The affinity bound WGA was then eluted with 0.1 M acetic acid (pH = 2.9).

D.2 RESULTS

D.2(a) SDS PAGE

A sensitive measure of WGA impurity was obtained when iodinated WGA preparations (as described in Chapter 4 section 4.2(d)) were run on SDS PAGE. Sigma WGA lot # A9C-97A0 was iodinated via the lactoperoxidase/H2O2 method, yielding an activity of 8.533 x 10^6 cpm/mg WGA. A 10% Laemmli SDS PAGE (Appendix A) was run on a 25 µg sample of iodinated WGA (reduced with dithiothreitol) mixed with 100 µg of cold WGA of the same lot (Fig. D.1). Only 58% of the 125I label was found to be associated with the WGA peak, indicating about 40% impurity. Vector WGA, iodinated by the Iodo-bead method and purified on the CM-Sepharose column, was 97% pure, according to the SDS PAGE profile (Fig. D.1).

Iodinated WGA (Sigma WGA (lot 90F-9515) 6.44 x 10^7 cpm/mg) had a microtitre the same as unmodified Sigma WGA (agglutinated 2% red cells down to 5 µg/ml). On a 10% Laemmli gel about 70% of the radiolabel associated with the WGA peak (Fig. D.2). Addition of red cells (final concentration of 1%) to a 3.0 µg/ml solution (in PBS) resulted in only 19% of the 125I being lost from the supernatant bound within red cell pellet. SDS PAGE of the WGA solution before and after addition of red cells, revealed that one major peak had disappeared. The maximum activity had shifted from gel slices 13-18 to 27-30 (Fig. D.2).
Fig. D.1 SDS PAGE (12% laemml) of reduced WGA, sliced at 1 mm intervals and counted for \( ^{125}I \). (A) impure Sigma WGA (6 \( \mu \)g, lot # 49C-9740) iodinated by the lactoperoxidase method. (B) pure Vector WGA (10 \( \mu \)g) iodinated by the Iodo-bead method, shown here for comparison.
Fig. D.2 SDS PAGE (10% Laemmli) of unreduced $^{125}$I WGA. Gels sliced at 1 mm intervals and counted for $^{125}$I. (A) stock $^{125}$I Sigma WGA (4 $\mu$g) and (B) the supernatant $^{125}$I WGA (0.2 $\mu$g) after exposure to red cells.
D.2(b) Affinity columns

Purification of the Sigma WGA (lot # 90F-9515) was attempted using a chitin and an ovomucoid affinity column. Table D.1 tabulates the results of the affinity columns with WGA, stating the amount of WGA loaded and the amount eluted. Fig. D.3 shows the SDS PAGE of these various fractions.

**TABLE D.1**

% BINDING OF IODINATED SIGMA WGA TO CHITIN OR OVOMUCOID COLUMNS

<table>
<thead>
<tr>
<th>Affinity column used</th>
<th>Chitin column¹</th>
<th>Ovomucoid column²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WGA loaded (mg)</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>% unbound</td>
<td>35</td>
<td>95</td>
</tr>
<tr>
<td>% affinity eluted³</td>
<td>5.4</td>
<td>1.2</td>
</tr>
<tr>
<td>% non-specifically bound⁴</td>
<td>56</td>
<td>3.8</td>
</tr>
</tbody>
</table>

¹the 19 ml column has a capacity of 185 mg WGA (Bloch & Burger, 1974)
²the 18 ml column (1.2 mg ovomucoid/ml) has a capacity of 11 mg WGA (Marchesi, 1972)
³For the chitin column, affinity WGA eluted with 0.05 M HCl
⁴For the ovomucoid column, affinity WGA eluted with 0.1 M acetic acid
⁵Iodinated Sigma WGA which could not be eluted.

Analysis of the various fractions by SDS PAGE showed that the affinity purified WGA of the chitin column didn't bind the material which bound to red cells while the ovomucoid column did (all the radiolabel was situated in the top 25% of the gel, where the high molecular weight component which bound red cells was situated (Fig. D.3)).
Fig. D.3 SDS PAGE (10%, pH 6.1) of several column fractions of $^{125}$I Sigma WGA reduced. (A) the ovomucoid column fractions, sliced at 1 mm intervals and counted for $^{125}$I (cpm/slice) (except gel 3 where four gels were sliced at two mm intervals, the slices in register combined and counted for $^{125}$I which resulted in a decrease in resolution because the slices were thicker and perfect alignment among gels was impossible) and (B) the chitin column fractions stained with coomassie blue and scanned at 595 nm. (1) 88 $\mu$g of WGA before being added to the columns, (2) the WGA fraction which didn't bind to the column and (3) the WGA which bound to the column and was eluted with (A) acetic acid or (B) HCl.
D.2(c) Cation exchange chromatography

The method which proved to be best for purifying WGA from commercial sources was cation exchange chromatography. Two methods were employed to purify WGA, a CM-Sepharose CL-6B (bearing COO⁻ groups) and FPLC, using mono S HR 5/5 (-CH₂SO₃⁻) cation exchange column. Tables D.2 and D.3 tabulate the results of both types of column chromatography and Fig. D.4 the resultant SDS PAGE gels of Vector WGA from the cation exchange column fractions. No salt gradients were used to separate the lectin into its isolectins as in Kronis & Carver (1980), since the individual isolectins are less soluble than when combined and the binding of each isolectin was indistinguishable by nmr, circular dichroism, fluorescence and binding to Chinese hamster ovary cells (LaCelle, 1979).

The fraction which eluted in high salt was considered the purified WGA. Any fractions not associated with the high salt (pure) fraction were considered impure. The protein that eluted with NaOH was always yellow and also considered an impurity.

After the WGA was iodinated in the acetate pH 4.3 buffer, it was separated from free ¹²⁵I and purified simultaneously on the CM column. Vector WGA iodinated and run down the column gave the same results as in Table D.3 (93% pure).
### TABLE D.2

**CM-SEPHAROSE CHROMATOGRAPHY OF COMMERCIAL WGA**

<table>
<thead>
<tr>
<th>Source</th>
<th>Vector total recovered</th>
<th>Vector titre (µg/ml)</th>
<th>Sigma² total recovered</th>
<th>Sigma² titre (µg/ml)</th>
<th>EY total recovered</th>
<th>EY titre (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>7</td>
<td></td>
<td>4.2³</td>
<td>500</td>
<td>6</td>
<td>134</td>
</tr>
<tr>
<td>% unbound</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% eluted by 0.45 M NaCl</td>
<td>92</td>
<td>7</td>
<td>92</td>
<td>18</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>% eluted by 0.1 M NaOH</td>
<td>8</td>
<td>147</td>
<td>3.6</td>
<td>100</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>% recovered</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. minimum agglutination concentration
2. all fractions were yellow
3. altered absorption spectrum, maxima at 320 nm and 282 nm

### TABLE D.3

**FPLC CHROMATOGRAPHY OF VECTOR AND SIGMA WGA**

<table>
<thead>
<tr>
<th>Source</th>
<th>Vector total recovered</th>
<th>Vector titre (µg/ml)²</th>
<th>Sigma¹ total recovered</th>
<th>Sigma¹ titre (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>13</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>% unbound</td>
<td>0.4</td>
<td>38</td>
<td>7.7</td>
<td>60</td>
</tr>
<tr>
<td>% eluted by 0.45 M NaCl</td>
<td>97</td>
<td>13</td>
<td>86³</td>
<td>25</td>
</tr>
<tr>
<td>% eluted by 0.1 M NaOH</td>
<td>2.4</td>
<td>25</td>
<td>6</td>
<td>72</td>
</tr>
</tbody>
</table>

1. fraction eluted with 0.45 M NaCl on CM-Sepharose of Table 4.3
2. minimum agglutination concentration
3. still yellow at 2 mg/ml
Fig. D.4 SDS PAGE of various Vector WGA fractions (50 µg) from cation exchange columns, reduced and stained with coomassie blue. (A) 15% laemmli gels of the FPLC fractions, (B) 10%, pH 6.1 gels of CM-Sepharose fractions. (1) the initial Vector WGA, (2) WGA which eluted with high salt and (3) protein eluted with 0.1 N NaOH. The numbers above the protein bands are the calculated molecular weights (+ 2000 daltons).
D.3 General discussion

Cation exchange chromatography detected impurities in commercial preparations, (the fraction which did not bind to the columns at all and the yellow pigment eluted with NaOH) (Tables D.2 and D.3). LaCelle (1979) found an impurity which did not bind to a CM-Sepharose column which lowered the binding of Sigma WGA to CHO cells. This impurity was felt to be toxic to the CHO cells. In the present work, only 0.4% of the total failed to bind to the CM or mono S columns (Vector) (Tables D.2 and D.3). It agglutinated red cells at about three times the concentration of purified WGA. For Sigma WGA, this fraction comprised about 7-8% of the protein and had comparable binding to the purified fraction (Tables D.2 and D.3).

The yellow pigment has also been referred to as an impurity. This may be a misnomer since it does agglutinate cells, although at a higher concentration (140 compared to 7-20 μg/ml for pure WGA). Analysis of this fraction shows a complex mixture of proteins on SDS PAGE. At least four bands are present, ranging from 7000 to 28000 daltons (Fig. D.4). One major fraction had a molecular weight of 16000 ± 2000 daltons, very close to that of WGA (18000 ± 2000 daltons). This pigment was also seen by Bouchard et al (1976). LeVine et al (1972) found that combining it with WGA greatly enhanced the solubility (at neutral pH) of WGA.

Thus impurities may aid in solubilization of WGA and also bind to cells.

Bassett (1975) claims that the isolation method chosen selects a WGA fraction with a characteristic solubility, the amino acid composition
reflecting the solubility. Nagata & Burger (1974) state WGA is slightly soluble in water while Bouchard et al (1976) find WGA soluble up to 20 mg/ml water. All stock solutions of WGA were soluble up to 20 mg/ml in PBS. Some Sigma preparations were equally soluble in water. Other preparations (e.g. BRL) precipitated out when dialysed against water; Vector WGA precipitated (at 20 mg/ml) in 0.05 M acetate, 0.05 M NaCl, pH 4.3, if left for 4 hours at room temperature.

Table D.4 lists eight different reported amino acid compositions for WGA, all normalized to a molecular weight of 18000 (except for Wright et al, 1984, who use 20600). Some amino acids are consistent while others appear to vary considerably (aspartic acid varies from 13.6 to 18/monomer, glutamic acid from 15.7 to 19/monomer).

WGA is known to contain four isolectins (I, Ia, Ib, and II) (Rice & Etzler, 1975; LaCelle, 1979), the tetraploid strain containing no isolectin I (Rice, 1976) and the hexaploid strains (the source usually used for WGA) varying in their relative amounts (Rice, 1976; Lotan et al, 1973), thus explaining the amino acid variability.

Addition of red cells to iodinated WGA (Sigma WGA (lot 90F-9515)) resulted in only 19% of the $^{125}$I being lost from the supernatant bound within red cell pellet. SDS PAGE of the WGA solution before and after addition of red cells, revealed that one major peak had disappeared (Fig. D.2). The ovomucoid affinity column also appeared to bind this protein fraction but the chitin column did not (Fig. D.3 and Table D.1).

The isolation method chosen selects a WGA. Perhaps, like many biological entities, co-factors are required to perform its function in vivo (which are mainly unknown for lectins). Glucosaminidases, proteases and
TABLE D.4
AMINO ACID COMPOSITION OF WGA

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nd = not determined
1 and 11 are isoelectins WGA 1 and WGA 11

aNormalized to a molecular weight of 18000 for the monomer except reference 10 which has a molecular weight of 20600

reference
1. Shaper et al, 1973
2. Bassett, 1975
3. Nagata & Burger, 1974
4. Allen et al, 1973
5. Bouchard et al, 1976
7. Rice & Etzler, 1975
8. LaCelle, 1979
9. Wright, 1981
high molecular weight contaminants could bind chitin columns (Bloch & Burger, 1974) and poor recoveries were found using ovomucoid columns that had been used previously (Bhavanandan & Katlic, 1979). Brown et al (1976) isolated a mitogen from wheat germ on a chitin column, Nagata & Burger (1974) co-isolated a trypsin inhibitor along with WGA and WGAs of varying solubility and impurities have been shown. Perhaps, it is these impurities which aid WGA in its function (in Chapter 4, impure Sigma binding to SL-ghosts produced the broadest spectra).

Just as the PNA:fetuin system showed that the density of the lectin or receptor is important in its selectivity, the WGA studies show that the isolation procedure of the lectin may also be important.
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Appendix A


Appendix C

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