Spin-Labeled Carbohydrates

THOMAS GNEWUCH and GEORGE SOSNOVSKY*

Department of Chemistry, University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53201

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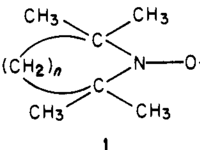
I. Introduction

Stable free radicals, as exemplified by the nitroxyl radical of the general structure 1, have been employed



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to study a variety of biological and medical problems. The first study involving nitroxyl radicals dates back to a paper in 1965 that dealt with the application of nitroxyls as molecular probes of enzyme active sites and of membrane structure and function.[1]

The nitroxyl radical moiety is usually attached by covalent bonds to a target molecule, and changes in its electron spin resonance (ESR) spectrum can provide information about the environment of the radical in the solid state as well as in solution. Such molecules are said to be *spin labeled.*

A number of books and reviews devoted to different aspects of the technique have appeared since 1968.[2-19] Several of these are concerned with the synthesis and chemistry of nitroxyls.[8,9,13-16,18] Other articles concentrate on biological applications of ESR and spin labeling.[5,6,10-14,16,17] The reports *Electron Spin Resonance* annually cover literature on the spin-labeling technique.

While a great deal of attention has been centered on spin labeling of lipids and proteins, it has been only within the last 10 years that a significant amount of work on carbohydrates has been reported. To date, no comprehensive review exists on this topic, and it is hoped that this review will fill the void.

II. Scope and Limitations

This review is organized according to classes of carbohydrates. It begins with monosaccharides and then continues with disaccharides and the polysaccharides, including cyclodextrins. The discussion then centers on glycoconjugates or combinations of carbohydrates with other materials such as glycoproteins, immunoglobulins, nucleosides, and nucleotides. Finally, there is a section on possible future uses of spin-labeled carbohydrates.

Throughout the review emphasis is placed on chemically well-defined compounds. Tables of physical constants and ESR spectral data are given where possible. This type of information is usually available for the mono- and disaccharide derivatives. The discussion of the spin-labeled polysaccharides and glycoconjugates often does not include many well-defined materials, and in these cases the emphasis is more on the theoretical and practical applications.

The literature has been searched manually and by computer through Oct. 1985.

III. Nomenclature

Several nomenclature systems have been used to describe the stable free radical of type 1. The *nitroxide* term used by *Chemical Abstracts* in its listings, and unfortunately adopted by many researchers in this field, is really a misnomer since these compounds are not oxides of nitrogen. They contain a N–O bond with a lone electron and can be depicted by the resonance structures

or in a simplified form

The correct name to describe these radicals should be *nitroxyl.*

The following nitroxyls are used often as starting materials (Figure 1). The reader may refer to these structures while reading the review. They are characterized as five- or six-membered rings containing *gem*-dimethyl groups on the α-carbon atoms. The correct

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Thomas Gnewuch was born in Fond du Lac, WI. His academic record includes a B.S. degree from Georgetown University (1960), M.S. degree from Iowa State University (1963), and Ph.D. degree from Indiana University (1966). His doctoral thesis under Professor Ernest Wenkert was concerned with alkaloid total synthesis. Since that time he has held teaching or research positions at Marquette University, The Medical College of Wisconsin, The Upjohn Co., the University of Missouri, the University of Wisconsin—Stout, and the University of Wisonsin—Milwaukee. His recent research interests include carbohydrate chemistry, chemical carcinogenesis, and the synthesis of site-specific agents, useful in both cancer treatment and NMR imaging.

George Sosnovsky has been Professor of Chemistry at the University of Wisconsin—Milwaukee since 1967. He received his Ph.D. from the University of Innsbruck in 1948. After graduation, his experience included research at CSIRO and ICI, Australia, 1949–1956; Postdoctoral Research Associate, University of Chicago, 1956–1959; Senior Scientist, IIT Research Institute, Chicago, 1959–1963; Associate Professor, IIT, Chicago, 1963–1966; and Special Senior Research Fellow of Public Health Service at the University College, London, and the University of Tübingen, 1967–1968. He was the editor of *Synthesis*, *International Journal of Methods in Synthetic Organic Chemistry*, 1969–1985. He was regional director for the National Foundation for Cancer Research, 1980–1985. He has authored about 130 publications and a book "Free Radical Reactions in Preparative Organic Chemistry", Macmillan, 1964. His research interests are focused on medicinal chemistry, specifically (a) the structure–activity relationship of anticancer drugs and syntheses and biological evaluation of new anticancer drugs and (b) contrast-enhancing agents for NMR imaging in diagnostic assessment of tumors.

nomenclature for the spin label **2** is 2,2,6,6-tetramethylpiperidine-1-oxyl, with the *-oxyl* ending. For more details concerning nomenclature, see ref 18.

Several abbreviations are used throughout the review. The descriptions of ESR spectra include the following terms: a_x, the hyperfine coupling constant of atom x;

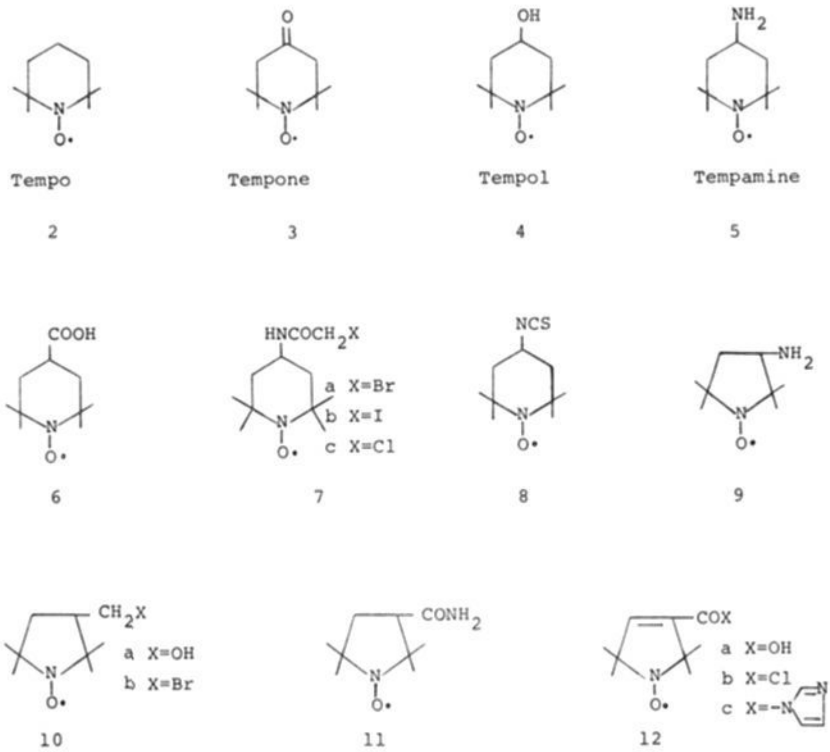


Figure 1. Structures of most frequently used nitroxyls.

g, the **g** value tensor of the nitroxyl ($\simeq$2.00); h_+, h_0, and h_-, the peak heights of the low-field, center, and high-field lines, respectively, of a triplet; τ_c, the rotational correlation time. For NMR spectra the following terms are used: δ, chemical shift; $J_{x,y}$, the coupling constant between hydrogens x and y.

With chemical schemes the following abbreviations are used routinely: s, second; min, minute; h, hour; d, day; rt, room temperature; Tr, trityl or triphenylmethyl group; Ac, acetyl group; Bn, benzyl group; Bz, benzoyl group; Dnp, 2,4-dinitrophenyl group; DNS, dansyl or 5-(dimethylamino)-1-naphthalenesulfonyl group;

SCHEME I

13 + **14** —(1) Ag_2CO_3, $CaSO_4$-$CHCl_3$; (2) NaOMe-MeOH→ **15**

SCHEME II

16 —Ac_2O-py, 25 °C, 14 h, 46%→ **17** —Ac_2O-HCl, 5 °C, 3 d, 66%→ **18** —**10a**, $Hg(CN)_2$, $CHCl_3$, 25 °C, 48 h→ —NH_3-MeOH, 5 °C, 24 h, 20%→ **19**

TABLE I. Spin-Labeled Monosaccharides

compd	method	yield, %	mp, °C	temp	$[\alpha]_D$, deg (c)[a]	anal.	ESR no. of lines	ESR a_N	other spectra[b]	ref
15	1	7.4	na[c]		na					20
19	1	20	semicryst amorphous solid		na	+[d]			I	21
20	1	70	142–145		na	na	3[e]	14.5	M	22, 24
26a	1	60	174–176		na	+				25
26b	1	58	164–165		na	+ (H off)				25
28a	1	17	amorphous		na	+				25
28b	1	21	amorphous		na	+ (C off)				25
31	1	59	na		na	na				28
32	1	na	na		na	impure				28
36	2	57	85	22	−0.5 (6[f])	na				30
37	2	78	110	22	+5.5 (1.8[f])	na				30
38	2	72	105	22	+3.4 (5[f])	na				30
39	2	85	108	22	6.6 (6[f])	na				30
41	2	na	na		na	na				30
47	2	40	170	22	−5.5 (1.4[f])	na				30
49	2	na	na	na		+ (C off)				31
57	3a	na	syrup	na	na					24
			73–77	na		+	3		M, I	27
59	3a	79	153–155	na		+	5[e]	15	N, M	24
61	3a	60		na		+	3[e]		M, I	27
64	3a	53	62–65	na			3[e]	14.5	M	22, 24
66	3ai	74	40–41	na		+				34
69	3ai	44	78(s)–98	na		+	3	15.2		37
		72								
70	3bi	52	139	25	+6.8 (1.6[f])	na				37
73	3bi	47	167	25	−100.9 (1.1[f])	na				37
76	3bi	36	na		na	+	3		I, M	27
78	3bii	65	na		na	(+) (C, N off)				25
80a	3bii	57	190–192		na	+				25
80b	3bii	59	na		na	+ (C, N off)				25

[a] Concentration. [b] N = NMR, M = MS, I = IR, U = UV. [c] na = not available. [d] + = microanalysis is correct. [e] DMF. [f] $CHCl_3$.

Me_2SO, dimethyl sulfoxide; dimsyl sodium, dimethyl sulfoxide carbanion; DCC, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; DMF, dimethylformamide; THF, tetrahydrofuran; py, pyridine.

IV. Monosaccharides

Since this treatise is primarily a chemical review it was decided to organize this section by reaction types. In some cases they are subdivided for greater clarity. The spin-labeled compounds are assembled in Tables I–III and are grouped by reaction type. The tables include physical constants, yields, and ESR data, where available, for each compound.

A. Reaction Types

1. Glycosidations

Formally these reactions can be considered as oxygen alkylations. However, since they are such characteristic reactions of sugars, they are included in this section instead of section 2. This reaction by definition is concerned with spin labeling at the carbon-1 (C-1) position of the sugar ring.

The first report of a spin-labeled sugar was that of the tritiated β-D-glucoside **15** (Scheme I).[20] This compound was formed in low yield by the classical Koenigs–Knorr glycosidation procedure involving acetobromoglucose (**13**) and the tritiated hydroxyl radical **14**, which was prepared by sodium borotriteride reduction of ketone **3**. The intermediate product was deacetylated by mild alkaline hydrolysis. No analytical results were given for either the intermediate or final product.

TABLE II. Spin-Labeled Monosaccharides: Nitrone Alkylations Using Method 4[39]

compd[a]	ESR[b] a_N	a_{Me}	$a_{H'}$	$a_{H''}$
95	14.8	11.9		
96	15.1	11.8		
97	14.4	12.0		
98	14.5	12.0		
99	15.3	12.9		
100	14.4	11.2		
101	14.8	12.7	2.7	
102	15.5	12.5	2.8	
103	14.8	12.0	2.4	
108	14.4		14.4	
109	14.1			
110	14.3			
149	12.3[c]		12.3	8.7
150	7.1[d]		5.3	

[a] The yields, melting point, and $[\alpha]_D$ values were not reported. [b] Solvent is benzene except where indicated. [c] CH_2Cl_2. [d] CCl_4.

Compound **15** was isolated by paper chromatography. This compound was designed as a spin-labeled substrate for β-galactosidase and β-galactosidase permease.[20]

Several amino sugars were spin labeled at C-1 by Wien et al.[21] *N*-Acetyl-α-D-glucosamine (**16**) was converted to acetochloroglucosamine (**18**) by acetylation to **17**, followed by displacement of the acetoxyl at C-1 with chloride ion (Scheme II). Compound **18** was condensed with spin label **10a**, with mercuric cyanide as catalyst. The intermediate was selectively deacetylated with methanolic ammonia, yielding **19**.[21]

An important contribution to the synthesis of spin-labeled sugars has been made by Gagnaire and Odier.[22-24] Their work encompasses monosaccharides,[22,24] disaccharides,[24] polysaccharides,[22,24] and cyclodextrins.[24]

TABLE III. Spin-Labeled Monosaccharides: Nitronyl Nitroxyls (C) and Imino Nitroxyls (D) Using Method 5[42]

compd[a]	yield, °C	mp, °C	temp	$[\alpha]_D$, deg (c)[b]	ESR[c] a_N-1	a_N-3	a_H-1
114c	69	syrup		na		7.5[e]	1.2
114d	48	syrup	23	-24 (2)	9.4	4.1	1.8
115c	76	syrup				7.5[e]	1.1
115d	59	syrup	23	-32 (0.8)	9.4	4.1	1.9
116c	16	133.8-135.1				7.0[e]	
116d	84	99-101	21	-121 (0.5)	8.7	4.4	
117c	85	109.4-112.7				7.3[e]	0
117d	66	68.6-69.5	23	+122.2 (0.45)	8.8	4.3	0
118c	95	129.5-133.0				7.3[e]	2.1
118d	49	84.5-85.1	22	-220 (0.6)	9.5	4.0	1.6
119c	65	95.3-97.8				7.3[e]	0
119d		not isolated in pure state			8.8	4.2	0
120c	88	syrup				7.6[d,e]	1.5
120d	62.8	syrup	21	0 (1.3)	9.1	4.4	1.5
121c	58	114.3-115.2				7.15[e]	0
121d	59	80.4-82.2	20	+193 (0.8)	9.0	4.2	0
122c	62.7	137.7-142.3				7.3[e]	0
122d	48	99.6-101.0	20	+112.7 (0.8)	9.1	4.15	0
123c	56.6	syrup				7.2[e]	0
123d	14	syrup			8.8	4.3	0
124c	66	syrup				7.5[e]	1.5, 3.2
124d	56	65.6-67.5	22	+4.1 (1.0)	8.7	4.25	0
125c	73	156-158				7.3[e]	2.3
125d	77	139.7-140.2	26	+39 (1.2)	10.0	4.3	1.8
126c	60.6	syrup				7.16[e]	
126d	53	syrup	22	-120.8 (1.1)	8.5	4.25	
127c	40.3	syrup				7.53[e]	0
127d	63	syrup	24	-87.2 (0.4)	8.2	4.09	0

[a] The microanalyses of all compounds listed are correct. UV, IR, and mass spectral data are given for all compounds. [b] Concentration. [c] Solvent is benzene except where indicated. [d] H_2O. [e] a_N-1 = a_N-3 for these compounds.

SCHEME III

13 + **12a** (Ag_2CO_3, benzene-py, 60 °C, 24 h, 70%) → **20**; **13** (Ag_2CO_3, H_2O, acetone) → **21**; **21** + **12b** → **20**

SCHEME IV

22a: R^1 = OBn, R^2 = H; **b**: R^1 = H, R^2 = OBn + **23** (*i*-Pr_2NEt, $Et_4N^+Br^-$, molec sieve, CH_2Cl_2, rt, 2 d) →

24a: R^1 = OBn, R^2 = H (88%); **b**: R^1 = H, R^2 = OBn (84%) (H_2, $Pd(OH)_2$, atm *P*, rt, 20 h) →

25a: R^1 = OH, R^2 = H (83%); **b**: R^1 = H, R^2 = OH (95%) (30% H_2O_2, Na_2WO_4, EDTA, H_2O, rt, 4 d) →

26a: R^1 = OH, R^2 = H (50%); **b**: R^1 = H, R^2 = OH (60%)

In the monosaccharide series synthetic sequences were devised so that a spin label could be incorporated selectively at different positions. The final compounds and most intermediates were characterized by mass and ESR spectra and microanalyses. The NMR spectra were obtained on the corresponding hydroxylamines (>N-OH) by the reduction of the nitroxyls (>N-O•) with zinc-acetic acid.

Substitution at C-1 was effected by the reaction of **13** with nitroxyl acid **12a** to give the product **20** (Scheme III).[22,24] Alternatively, compound **20** was prepared from 2,3,4,6-tetraacetyl-β-D-glucose (**21**; prepared from **13**) and the acid chloride **12b**. Compound **20** was reduced to its hydroxylamine (>N-OH) analogue, and its NMR spectrum consisted of a doublet for H-1 (δ 5.77 (J_{12} = 7 Hz)), which is characteristic of a trans diaxial relationship between the hydrogens on carbons 1 and 2.[22] This result proved that the first reaction proceeded with inversion of configuration and the latter with retention of configuration.

Several spin-labeled α- and β-glycosides with free hydroxyl groups were prepared by Plessas and Goldstein[25] using known methods. Preparation of the α-glycosides was accomplished by the reaction of the

SCHEME V

13: R^1 = OAc, R^2 = H
27: R^1 = H, R^2 = OAc

28a: R^1 = OH, R^2 = H (21%)
b: R^1 = H, R^2 = OH (17%)

29

2,3,4,6-tetrabenzyl-α-D-glycopyranosyl bromides (**22a,b**) with **23** to give **24a,b** (Scheme IV). This reaction proceeds with retention of configuration.[26] Catalytic hydrogenolysis removed the benzyl groups to yield **25a,b**, which were oxidized by a known procedure to give the nitroxyl radicals **26a,b**. The authors state that the reaction of **22** with the spin label **4** was not successful for the synthesis of **26** because the nitroxyl was reduced to an amine during the hydrogenolysis step.[25]

The corresponding β-glycosides were synthesized by a direct reaction of the acetobromoglycosides **13** and **27** with nitroxyl **4** to give intermediates that were deacetylated to give the unblocked spin-labeled glycosides **28a,b** (Scheme V).[25] The glycosidation of **13** was effected with silver carbonate–iodine in dry chloroform while the reaction of **27** was mediated by mercuric cyanide in dry nitromethane. It was stated that these glycosidation reactions always gave some ortho ester **29**, but no evidence was provided to substantiate this observation.[25] The best conditions found for the formation of β-glycosides were either (1) silver carbonate in ethanol-free chloroform containing a trace of iodine for 18 h at room temperature or (2) mercuric cyanide in dry nitromethane. Orthoester analogues could be obtained in high yield by the use of mercuric cyanide in dimethylformamide.

Generally, the yields for these reactions are low even under these "best" conditions. Similar results were obtained by us.[27] Support for the assigned structures of the labeled galactosides was provided by their hydrolysis with α- and β-D-galactosidases.[25] Thus, coffee bean α-D-galactosidase completely hydrolyzed the α-D-galactoside analogue **26a** at 37 °C in 12 h. The β-D-galactosidase from *Escherichia coli* gave complete hydrolysis of the β-D-galactoside **28a**.

The synthesis of two thiogalactosides **31** and **32** was reported.[28] The starting material tetra-*O*-acetyl-β-D-thiogalactose (**30**; Scheme VI) was converted to the thiogalactosides by the procedure of Černý and Pacák.[29] S_N2 displacement of the appropriate halide labels **10b** and **7b**, followed by mild alkaline hydrolysis, produced the spin-labeled thiogalactosides **31** and **32**. There is doubt about the identity of these products since no microanalysis is given for **31** and the analysis for **32** is unsatisfactory.[28]

SCHEME VI

SCHEME VII

36 (57%) 37 (78%) 38 (72%) 39 (85%)

2. Alkylations

In this subsection are included methods for the alkylation of sulfur, nitrogen, or oxygen. Carbon alkylations are covered separately under nitrone alkylations (subsection 4).

In a paper was reported the alkylation of sulfur, nitrogen, and oxygen atoms of monosaccharides by the use of spin-labeled *s*-triazines.[30] Starting with 2,4,6-trichloro-*s*-triazine (cyanuric chloride) it is possible to substitute one, two, or three chloro groups with nitroxyl moieties or various combinations of labeled and nonlabeled groups. In this paper the authors chose a few examples to show the possibilities.[30] The mono- and dioxygen compounds **33** and **34** were prepared by the reaction of cyanuric chloride with **4** and the mono-

nitrogen compound **35** by the reaction with **5**. In the following structures (**36**–**47**) the spin-labeled side chains are abbreviated as OSl and NHSl. The reaction of thio sugar **30** with 1 or 0.5 equiv of **33** and **35** gave the monosubstituted (**36**, **38**) and disubstituted (**37**, **39**) products, respectively (Scheme VII).

The reaction of OSl compound **33** proceeded more rapidly than that of the NHSl analogue **35**. For example, the reaction of the blocked amino sugar **40** with **33** gave **41** in 53% yield while the reaction with **35** was unsuccessful.[30] Similarly, the reaction of hexylamine with **33** to give **42** proceeded under milder conditions than was required for the reaction with **35** to give **43**.

The reaction of D-glucosamine was reported to proceed readily with cyanuric chloride to give **44**, but no reaction was observed with **35**.[30] Since it was mentioned that **33** is more reactive than **35**, it is puzzling that no reaction of D-glucosamine with **33** was attempted that would give the water-soluble spin-labeled amino sugar **45**.

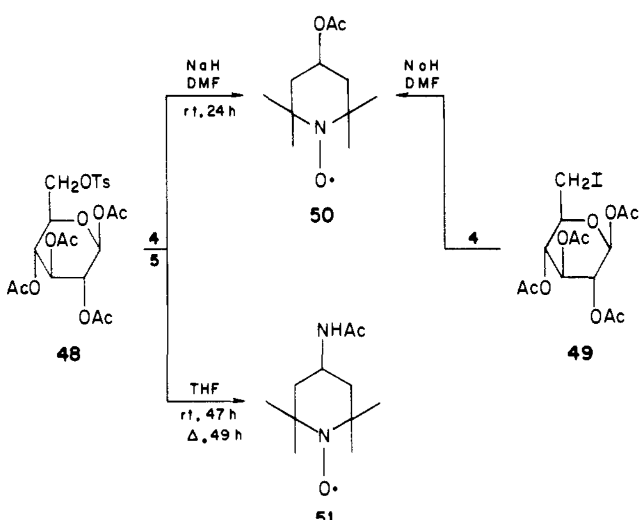
Finally, there was presented one example in which two different groups were substituted for the two chlorine atoms of **33** (Scheme VIII).[30] The blocked galactose derivative **46** was reacted with **33** to yield an intermediate that was reacted in situ with *n*-hexylamine to give the product **47**. Obviously, a great variety of products such as **47** could be envisioned.[27] The spin-labeled triazine ring can serve as a link between two carbohydrates or a carbohydrate and an amino acid, peptide, or protein. In addition, the solubility of such combinations could be adjusted by retaining or step wise eliminating the blocking groups.[27]

The spin-labeled *s*-triazines also have been used to label various polysaccharides, and this topic will be discussed in section VI.

Another approach to spin labeling of sugars by alkylation would be through nucleophilic substitution of good leaving groups. Attempts to prepare sugars spin

SCHEME VIII

SCHEME IX



labeled at C-6 by S_N2 reaction of either the tosylate **48** or the iodide **49** were unsuccessful (Scheme IX).[27] Reactions with nitroxyls **4** or **5** led to their acetate derivatives **50** and **51**, respectively, and some starting materials. This result appears to be an interesting example of a transacetylation reaction.

An attempt was made to alkylate the nitrogen of an amino sugar by reductive amination (Scheme X).[27] However, the reaction of D-glucosamine **52** with keto radical **3** in the presence of sodium cyanoborohydride in methanol did not yield the desired product **53**. Instead, a complex mixture of polar materials was obtained.[27]

3. Acylations

a. At Oxygen. Such acylations have been carried out at the C-1 (see **20**), C-3, C-2 + C-3, and C-6 positions.

C-3 acylation was effected by the reaction of 1,2,5,6-diisopropylidene-α-D-glucose (diacetone-D-glucose, **54**) with **12b** to give **55** (Scheme XI).[24,27]

A monosaccharide diradical spin-labeled at the C-2 and C-3 positions was synthesized as shown in Scheme XII.[24] Thus, methyl-4,6-*O*-benzylidene-α-D-gluco-

SCHEME X

SCHEME XI

SCHEME XII

SCHEME XIII

SCHEME XIV

SCHEME XV

pyranoside (**57**), prepared from methyl-α-D-glucoside (**56**), was reacted with 2 mol equiv of the acid chloride **12b**, yielding the analogue **58**. The ESR spectrum at room temperature consisted of five lines (a_N = 15 G), which indicates exchange coupling between the two radical moieties.[24]

By the same approach the nitroxyl **12b** was attached to C-3 of the 2-deoxy-D-glucoside **59** to give the labeled analogue **60** (Scheme XIII).[27]

The C-6 position was labeled selectively as illustrated in Scheme XIV.[22,24] The reaction of methyl-β-D-glucoside (**61**) with trityl chloride–pyridine gave the 6-trityl ether (not shown). The secondary hydroxyls were then acetylated, followed by mild acid hydrolysis of the trityl ether to produce **62**. The reaction of this suitably blocked derivative with the acid chloride **12b** yielded the desired spin-labeled analogue **63**.

A method for nonspecific acylations of all the hydroxyl groups in mono- or polysaccharides was reported.[31-33] For example, the reaction of methyl-α-D-glucoside (**56**) with dimsylsodium, followed by the reaction with the nitroxyl acid chloride **12b**, gave **64** (Scheme XV).[31] However, no molar ratios of reactants or yield of product was given. The microanalysis of the isolated spin-labeled product agreed with a monosubstitution of the hydroxyl groups.[31] It appears that the product is a random mixture of 2-, 3-, 4-, and 6-labeled positional isomers as shown. This method has the advantage of simplicity without tedious chemical modification of the sugar. It has the disadvantage of a complete lack of specificity of covalent binding. The application of this reaction to polysaccharides will be discussed in section VI.

Phosphorylative Spin Labeling. The phosphorylative nitroxyl labeling of the blocked D-glucose was achieved by two related methodologies as shown in Scheme XVI. Thus, the reaction of acetone-D-glucose (**54**) with phenyldichlorophosphine gave **65** which, in turn, was reacted with **4** to yield **66**.[34] Analogously, the reaction of **54** with either chloridate **67** or imidazolide **68** gave the 3-substituted spin-labeled phosphate derivative **69**.[35] The reduction of **69** to the corresponding hydroxylamine derivative was achieved with ascorbic

SCHEME XVI

54 + $PhPCl_2$ $\xrightarrow{Et_3N, ether}$ 65 $\xrightarrow{4, Et_3N, ether}$ 66

54 + 67 $\xrightarrow{Et_3N, dioxane, 44\%}$ 69

68 + 54 $\xrightarrow{Et_3N, dioxane, 72\%}$ 69

SCHEME XVII

40 + 6 $\xrightarrow{DCC, CH_2Cl_2, 0\ °C, 1\ h; 20\ °C, 16\ h}$ 70 (52%) + 71 (21%)

72 + 5 $\xrightarrow{DCC, CH_2Cl_2, 0\ °C, 1\ h; 20\ °C, 16\ h}$ 73 (47%) + 74 (12%)

SCHEME XVIII

5 + 75 $\xrightarrow{THF, rt, 54\ h; \Delta, 8\ h; 36\%}$ 76

acid.[36] Unfortunately, the important deblocking step was not attempted.

b. At Nitrogen. Amide Formation. In a short paper Evelyn and Hall[37] described the synthesis of two monosaccharides **70** and **73** spin labeled on either amino or carboxyl groups, using dicyclohexylcarbodiimide (DCC) as the coupling agent (Scheme XVII). The starting sugars **40** and **72** were blocked so that the reaction could take place only between the –COOH and $-NH_2$ groups. The examples demonstrate that the amino and carboxyl groups can be on either the sugar or the nitroxyl moiety in the starting materials. In addition to the desired amide products there was obtained some of the *N*-acylureas **71** and **74**, which are common contaminants in reactions involving DCC. The removal of the blocking groups of compounds **70** and **73** to give water-soluble analogues, which could be useful for biological studies, was not reported.

An alternate approach to this amide formation is envisaged by the reaction of 1,2-*O*-isopropylidene-glucono-3,6-α-D-furanolactone (**75**) with **5**, leading to the 6-amide derivative **76** (Scheme XVIII).[27]

The amide of glucosamine **52** containing the acyl function of nitroxyl **12** was included in a series of nitroxyl spin probes used to study the bioenergetics of cells.[38] No synthetic details were reported. The half-time for full equilibration of this amino sugar across membranes of halobacterial vesicles was 15 h as compared to half-times of less than 100 ms for more lipophilic nitroxyls such as **2–6**.[38]

Urea Formation. Labeled sugars containing a thiourea group, **78** and **80a,b** were prepared from the

SCHEME XIX

77 + 8 $\xrightarrow[\Delta,\ 1\ h,\ 65\%]{EtOH}$ 78

79a: R^1 = OH, R^2 = H
b: R^1 = H, R^2 = OH

+ 8 $\xrightarrow[\Delta,\ 1/2\ h]{EtOH}$

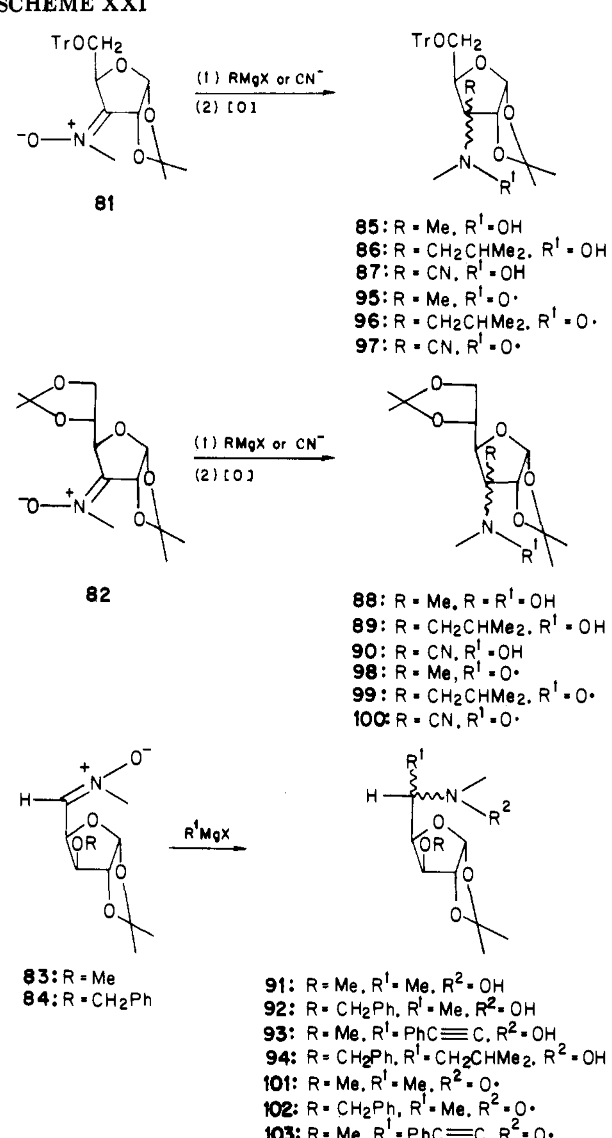
80a: R^1 = OH, R^2 = H (57%)
b: R^1 = H, R^2 = OH (59%)

SCHEME XX

A $\xrightarrow[\text{or } CN^-]{RMgX}$ B $\xrightarrow{[O]}$ C

Nu = CN, R

SCHEME XXI



nitroxyl isothiocyanate 8 (Scheme XIX).[25] Compound 77 was prepared by reduction of (*p*-nitrophenyl)-β-D-glucoside (128). This method appears to be a convenient route to some unblocked monosaccharide derivatives. The benzene ring in 78 can be considered as a spacer separating the nitroxyl from the sugar moiety.

4. Nitrone Alkylations

Tronchet et al.[39] accomplished the syntheses of a series of spin-labeled amino sugars following the general route of Scheme XX. Thus, aldehydo or keto sugars were converted to aldo nitrones or keto nitrones **A** by previously described methods.[40,41] These starting materials underwent nucleophilic additions with either Grignard reagents or cyanide ion to give the corresponding desoxyhydroxylamino sugars **B**. The latter, in turn, were oxidized (air, periodic acid, lead dioxide) to the sugar nitroxyls **C**.

Four blocked furanose sugars **81–84** were converted to the desoxyhydroxyamino derivatives **85–94**, which were oxidized to the corresponding nitroxyl derivatives **95–103** (Scheme XXI). The reactions leading to the β series (**85–94**) were reported to be generally stereoselective and sometimes stereospecific.[39] Solutions of these intermediates oxidize in air to the nitroxyl **C** series, however better yields were obtained with either periodic acid or lead dioxide. These radicals were described as stable for several hours at room temperature, eventually reverting to the nitrone structure. In Table II are shown the sugar nitroxyls with their ESR spectral parameters. However, although no microanalyses or other spectral data were reported, they were said to support the assigned structures.[39]

The nucleophilic addition reaction of nitronyl nitroxyl **104**[42] with ethylmagnesium bromide yielded the mono- (**105**) (26%) and di- (**106**) (24%) addition products (Scheme XXII).[39] The reaction of **104** with methylmagnesium iodide gave only a 13% yield of the dialkylated product **107**. These hydroxylamine sugars were oxidized to the nitroxyls **108–110**.

5. Heterocycle Formation

Interesting work was described by Tronchet et al.[42-44] in which the nitroxyl radical is incorporated directly into the carbon skeleton of sugars. This approach, as

SCHEME XXII

(1) RMgX
(2) [O]

104

105: R = Et, R^1 = H, R^2 = OH
106: R = Et, R^1 = Et, R^2 = OH
107: R = Me, R^1 = Me, R^2 = OH
108: R = Et, R^1 = H, R^2 = O•
109: R = Et, R^1 = Et, R^2 = O•
110: R = Me, R^1 = Me, R^2 = O•

SCHEME XXIII

sugar—CHO + 111 → B (A)
air or PbO_2
B′ E
$NaNO_2$
C D
112a, R = OH
b, R = O•
113

illustrated in Scheme XXIII, begins with the reaction of aldehydo sugars **A** with 2,3-bis(hydroxyamino)-2,3-dimethylbutane (**111**) to give the corresponding 1,3-dihydroxyimidazolines **B**.[45] The yields of the **B** compounds ranged from 27 to 89%.[42] A contaminant in these reactions was the monohydroxyimidazoline **E**, but its presence could be reduced greatly by the use of anhydrous solvents. The crystalline compounds **B** in solution could be air oxidized rapidly to the **B′** structures.[42] The ESR spectra of solutions of **B** consisted

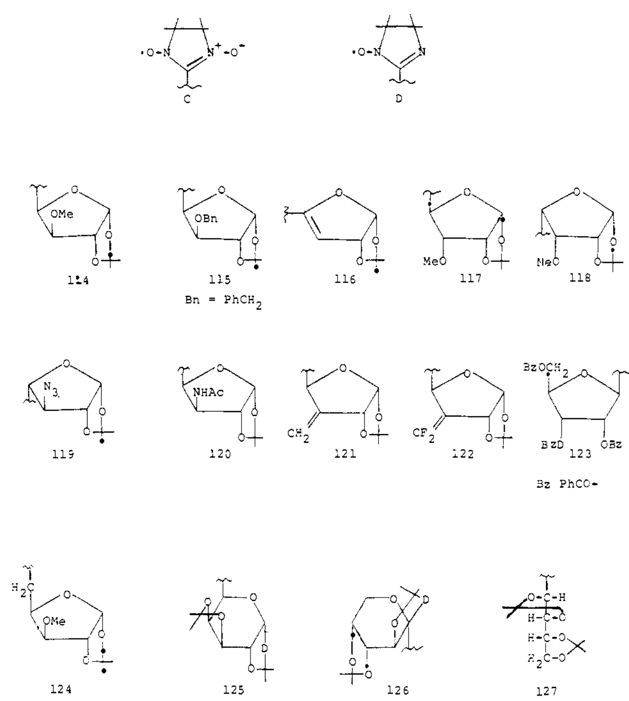


Figure 2. Monosaccharides containing the nitronyl nitroxyl (C) and imino nitroxyl (D) structures.

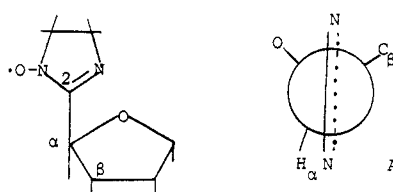


Figure 3. Stereochemical aspects of the **C** and **D** structures. The **A** structure represents the eclipsed conformation.

of a doublet (a_H 20.3 G) of triplets (a_N 14.6 G) in accord with structure **B′**. After standing for 12 h in solution in the presence of air, **B′** was oxidized further to **C**. The structure of the intermediates **B′** was proven by the synthesis of the deuterated compound **112a**, which oxidized spontaneously to **112b**.[42] The ESR spectrum of **112b** consisted of a triplet of triplets (a_D 2.9 G, a_N 14.6 G), confirming the structure of **B′**.

A convenient synthesis of the nitronyl nitroxyl structure **C** was accomplished by lead dioxide oxidation of **B**.[42] The crystalline **C** compounds are violet and are stable indefinitely at −20 °C. A byproduct (5–18%) of this oxidation reaction was the nitronyl nitroxyl **113**. If **B′** was subjected to UV irradiation with a mercury vapor pressure lamp, the doublet of triplets of **B′** was transformed to a triplet corresponding to a nitroxyl radical of unknown structure.[42]

The imino nitroxyls of structure **D** were prepared by sodium nitrite reduction of the **C** compounds.[42] These nitroxyls are stable orange crystalline compounds. Table III contains physical, spectral, and analytical data for the **C** and **D** structures of the monosaccharides **114–127**. The point of attachment of the heterocycle to the sugar moiety is shown in Figure 2.

The ESR spectra of the nitronyl nitroxyls **114–127 C** and imino nitroxyls **114–127 D** were analyzed in detail.[42] For the former the two nitrogens are equivalent and $a_{N1} = a_{N3}$ while for the latter the intensity of a_{N1} is double that of a_{N3}. An interesting aspect of this work

SCHEME XXIV

128 → ($h\nu$, *i*-PrOH, KOH) 130; 128 → ($h\nu$, MeOH) 129

was the correlation of a_H values of the sugar α proton with conformations about C-2 of the imidazoline ring of the **C** and **D** series (Figure 3). Comparison of the calculated values of a_H for different conformations about the glycosidic bond with the observed a_H values from their ESR spectra led to the conclusion that an eclipsed conformation (**A**) is the most stable.[42] Also the configuration and steric requirements of substituents on C-β of the sugar moiety favored an eclipsed conformation. There was a correlation between the $J_{2,\alpha}$ values from the NMR spectra of the dihydroxyimidazoline **B** series and the a_H values from the ESR spectra of the nitronyl nitroxyl **C** series.[42] For compounds with an axial substituent on C-β (**114**, **115**, **120**) there were high values for $J_{2,\alpha}$ and a_H while for compounds with equatorial substituents on C-β (**117**, **121**, **122**) the values for $J_{2,\alpha}$ and a_H were low.[42]

6. Photochemical Reactions

In concluding this section on spin-labeled monosaccharides mention should be made of a novel preparation of nitroxyl and nitro anion radicals from (nitrophenyl)-β-D-glycosides.[46] Thus, when (*p*-nitrophenyl)-β-D-glycoside (**128**) was irradiated with UV light in methanol for a prolonged period, the product was the nitroxyl radical **129** (Scheme XXIV).

The mechanism for this reaction was assumed to be the same as that which was determined earlier for nitrobenzene[47] (eq 1–4):

$$\mathrm{PhNO_2} \xrightarrow{h\nu} \mathrm{PhNO_2}^{*} \qquad (1)$$

$$\mathrm{PhNO_2}^{*} + \mathrm{RCH_2OH} \longrightarrow \mathrm{Ph\dot{N}O_2H} + \mathrm{R\dot{C}HOH} \qquad (2)$$

$$\mathrm{Ph\dot{N}O_2H} + \mathrm{R\dot{C}HOH} \longrightarrow \mathrm{Ph\overset{+}{N}(OH)(^{-}O)\!-\!CHR(OH)} \qquad (3)$$

$$\mathrm{Ph\overset{+}{N}(OH)(^{-}O)\!-\!CHR(OH)} \longrightarrow \mathrm{PhN(\cdot O)\!-\!CHR(OH)} + \cdot\mathrm{OH} \qquad (4)$$

An analysis of the ESR spectrum of compound **129** revealed that the glycosidic bond is preserved in the nitroxyl radical **129**.[46] Additional support for this contention came from the fact that irradiation of *p*-nitrophenol in methanol produced no nitroxyl radical. The same type of nitroxyl radical was obtained for the (*m*-nitrophenyl)glucoside as well as for the para and meta isomers of the corresponding galactosides.[46] In contrast, the ortho isomer corresponding to **129** could not be isolated because of rapid hydrolysis of the glycosidic bond.

Irradiation of **128** in strongly alkaline 2-propanol solution rapidly produced the nitro anion radical **130**.[46] This radical could be generated more effectively by electrolysis. In the latter case the ESR spectra of the ortho, as well as those of the meta and para isomers, could be obtained. There was a small coupling (0.3–0.4 G) between the N and H-1 of the sugar in the case of the para isomer.[46] This coupling was used to detect the electron-donating or electron-withdrawing effects of para substituents. The hyperfine coupling constant a_N from the ESR spectra was substituted into the Hammett equation

$$\log (a_N^{*}/a_N^{H}) = \rho\sigma_x$$

where a_N^{*} represents the nitrogen coupling constant of the para-substituted nitrobenzene anion in different solvents, a_N^{H}, the nitrogen coupling constant for nitrobenzene, ρ the solvent constant, and σ_x the substituent constant. As an example, $\rho = -0.295$ in acetonitrile. For galactose and glucose the calculated σ values are −0.15 and −0.13, respectively, as compared with σ for −OMe (−0.16) and −OH (−0.42). The conclusion drawn from these results is that the glycosidic group has an electron-donating effect similar to that of the OMe group and, therefore, represents an ether-type linkage.[46]

V. Disaccharides

A number of reactions discussed for monosaccharides have been applied also to disaccharides. Table IV contains physical and spectral data for these compounds.

Gagnaire and Odier[24] extended their synthetic approaches, discussed in section IV, to maltose and cellobiose. The 6,6′ spin-labeled derivatives were prepared by the same sequence as that used for the preparation of the 6-labeled glucose analogue **63**. Thus, commercial octaacetylcellobioside (**131**) and maltoside **132** were

131: R = Ac, R¹ = Ac, R² = Ac
133: R = H, R¹ = H, R² = Me (β)
135: R = Ac, R¹ = H, R² = Me (β)

132: R = Ac, R¹ = Ac, R² = Ac
134: R = H, R¹ = H, R² = Me (β)
136: R = Ac, R¹ = H, R² = Me (β)

reacted with hydrogen bromide–methanol to yield acetobromocellobiose and acetobromomaltose, respectively (α configuration). The methyl-β-glycosides of these bromo derivatives were prepared by the reaction with silver carbonate in methanol. Removal of the acetate moieties was accomplished by mild alkaline hydrolysis using a catalytic amount of sodium methoxide in methanol, yielding the unblocked derivatives **133** and **134**. These derivatives, in turn, were converted

TABLE IV. Spin-Labeled Disaccharides

compd	method	yield, %	mp, °C	$[\alpha]_D$	anal.	ESR a_N (no. of lines)	$a_{H'}$	$a_{H''}$	$a_{H'''}$	$a_{H''''}$	ref
137	3	77	105–110	na[e]	+[f]	(3)	13.0[a]				24
138	3	80	102–105	na	+	6.5[a] (5) rt; (5) 135 °C; 13.0[a] (3) −40 °C.					24
139	3	75	78–85	na	(+) off	13.0[a] (3)					24
142	1	32		na	+	na					21
145	1	55	amorphous	na	+	na					25
159a	4	na	na	na	na	13.8[b]	9.7	6.2			39
159b		na	na	na	na	13.7	6.9	3.6			39
160a	4	na	na	na	na	14.4[c]	9.3	6.1			39
160b		na	na	na	na	13.5[b]	4.5	4.5			
161a	4	na	na	na	na	14.4[b]	8.9	6.5			39
161b		na	na	na	na	13.2	3.8	3.8			
162b	4	na	na	na	na	13.5[b]	4.4	4.4			39
163	4	na	na	na	na	13.9[b]	17.6	6.7			39
164	4	na	na	na	na	na					39
169	4	na	na	na	na	13.1[d]	4.2	4.2			39
170	4	na	na	na	na	12.8[b]	4.2	4.2			39
174	4	na	na	na	na	13.4[b]	9.5	7.7			39
177	4	na	na	na	na	13.9[b]	17.6	6.4			39
183	4	na	na	na	na	13.1[b]	18.1	2.2	0		39
185	4	na	na	na	na	12.4[c]	12.4	12.4	7.7	7.7	48

[a] DMF. [b] CCl_4. [c] $CHCl_3$. [d] Benzene. [e] na = not available. [f] + = microanalysis is correct.

to the 6,6′-dihydroxy analogues **135** and **136** by blocking the 6,6′ primary alcohol groups with trityl chloride–pyridine to give the trityl ethers, followed by acetylation of the secondary alcohol groups with acetic anhydride–pyridine and then careful cleavage of the trityl ethers with acetic acid–water (4:1) at 50 °C for no longer than 5 h. The authors stated[24] that attempts to cleave the trityl ethers with either hydrogen bromide–acetic acid or with aqueous acetic acid for prolonged periods of time led to products resulting from acetyl migration from the 4′-position to either the 6- or 6′-positions.

Acylation of **135** and **136** with the spin label **12b** in pyridine yielded the 6,6′-labeled cellobioside **137** (77%) and maltoside **138** (80%), respectively.[24]

137, R = Ac
139, R = Me

138, R = Ac
140, R = Me

The methyl ether analogue of cellobioside **139** was prepared also.[24] As described previously, the 6,6′-positions of **133** were blocked by tritylation (trityl chloride–pyridine, 70 °C, 16 h), followed by methylation of the secondary hydroxyls (methyl iodide–silver oxide–dimethylformamide, 16 h) and detritylation (acetic acid–water (4:1), 50 °C, 6 h) to give **135** (R = Me, R′ = H, R^2 = Me(β)] (80%). Acylation of this material with **12b** in pyridine (room temperature, 16 h) yielded the desired product **139** (75%). The methyl ether analogue of maltoside **140** could not be synthesized because the tritylation of **134** produced a mixture of di- and two mono- (6 and 6′) tritylated products. The alcohols resulting from detritylation of this mixture were not purified to the point where they could be reacted with the spin label **12b**. It was stated[24] that the 6′-position of **134** was more reactive toward tritylation than the 6-position.

The ESR spectra of the spin-labeled cellobiosides **137** and **139** were identical.[24] They consisted of three lines with a_N = 13 G, which is characteristic of a diradical with no exchange interaction. There was no evidence of dipolar coupling in the frozen state. It was a different story for the spin-labeled maltoside **138**. Its ESR spectrum at room temperature consisted of five lines with a_N = 6.5 G. The line intensities were characteristic of an average exchange coupling. Raising the temperature to 135 °C produced a quintuplet (enhanced exchange) while lowering the temperature to −40 °C produced three lines (no coupling) with a_N = 13 G.[24] No dipolar coupling was observed for diradical **138** in a frozen state.

The differences in the ESR spectra of the two derivatives **137** and **138** reflect the difference in configuration of the glycosidic bonds: β (1→4) for cellobiose and α (1→4) for maltose.[24] The variations in the ESR spectra of the maltoside analogue **138** with temperature were explained by subtle conformational changes. These changes probably occur also with **137** but were not observed by ESR.[24]

Wien et al.[21] synthesized a chitobiose analogue **142** spin labeled at the C-1 position (Scheme XXV). The procedure was the same as that for compound **19**. Thus, acetochlorochitobiose (**141**) was reacted with **10a**, using mercuric cyanide as catalyst. Deacetylation with alcoholic ammonia produced **142** in 32% overall yield. Both **19** and **142** must be mixtures of optical isomers due to the asymmetric center on the pyrrolidine ring.[21]

SCHEME XXV

141 + 10a → (1) $Hg(CN)_2$-$CHCl_3$, rt, 72 h; (2) NH_3-MeOH, 5 °C, 24 h → 142

SCHEME XXVI

143 + 4 → (1) Ag_2CO_3-I_2, molec sieve, $CHCH_3$, dark, rt, 18 h; (2) NaOMe-MeOH →

144, R = Ac
145, R = H

SCHEME XXVII

146: R = H, R^1 = OH
147: R = Ac, R^1 = OAc
148: R = Ac, R^1 = OH
149: R = H, R^1 = O•
150: R = Ac, R^1 = O•

→ 159–164 (R^3 = O•)

153: R^3 = OH, R^2 = Ph
154: R^3 = OH, R^2 = C≡CPH
155: R^3 = OH, R^2 = C≡CPPH$_2$
156: R^3 = OH, R^2 = C≡CP(O)PH$_2$
157: R^3 = OH, R^2 = H
158: R^3 = OH, R^2 = D

Compound **19** and the disaccharide analogue **142** were found[21] to bind to the active site of lysozyme. The broadening of ^{1}H NMR lines was used to calculate distances of up to 20 Å between the nitroxyl radical electron and the proton of interest.[21] The paramagnetic nitroxyl radical broadens the resonance of the C-2 proton on histidine-15, providing a measure of the distance from subsite D to histidine-15.[21]

Plessas and Goldstein[25] applied their synthetic approach on monosaccharides to maltose. Acetobromomaltose (**143**) was reacted with nitroxyl **4** to give **144** which, in turn, was deacetylated to **145** (Scheme XXVI). The yield of the glycosidation reaction was poor, as was the case for the monosaccharide series.

In an extension of their work on sugar nitrones Tronchet et al.[39] prepared several disaccharides in which the intermediate glycosidic oxygen bridge is replaced by an hydroxyimino group. A model reaction was used to test the synthetic feasibility (Scheme XXVII). Thus, the reaction of the 3-hydroxyamino sugar **146** with the aldehydo sugar **151** yielded the nitrone **152**. This intermediate was reacted with various Grignard reagents to give **153–156** and with borohydrides to give **157** and **158**. Oxidation of **153–158** produced the unstable nitroxyl **159–164**. The acetate derivative (**147**) of **146** could be selectively deacetylated to **148**. Oxidation of **146** and **148** by periodic acid produced the nitroxyls **149** and **150**, respectively. Although only ESR spectral data were reported for these spin-labeled disaccharides it was stated that microanalyses and other spectral data (UV, IR, MS) supported the assigned structure.[39]

Compound **146** was reacted with several reducing sugars to produce the disaccharides containing an hydroxyimino group as the bridging unit (Scheme XXVIII).[39] As is shown for the reaction of **146** with **165** the intermediate is considered to be a nitrone arising from a nucleophilic attack of the hydroxylamine group on the open-chain aldehyde form of **165**. This nitrone then is trapped by nucleophilic addition of the free OH group to re-form the cyclic sugar **166**. Acetylation produced the diacetate **167**, which was selectively deacetylated to **168**. Oxidation of **166** and **168** with either lead dioxide or periodic acid yielded the nitroxyls **169** and **170**, respectively. Analogously, the reaction with the sugar derivative **171** produced **172–174** and with **21** the compounds **175–177**.[39] Compound **166** has the β while **172** has the α configuration, demonstrating the influence of a steric hindrance in the reducing sugar on the approach of the nitrogen to the plane of the ring.[39]

A second approach to these unusual disaccharides is shown in Scheme XXIX.[39] The reaction of the reducing sugar **178** with hydroxylamine formed an aldose oxime **179** which is in equilibrium with the cyclic form **179a**. The latter then was reacted with an aldehydo sugar such as **180** to give an intermediate which was reduced in situ with sodium borohydride and acetylated to yield a crystalline **181**. This compound could be hydrolyzed to **182** and oxidized to the nitroxyl **183**. Alternatively, the aldehydo sugar **180** could be reacted

SCHEME XXVIII

146 + 165 →

166: R = H, R' = OH
167: R = Ac, R' = OAc
168: R = Ac, R' = OH
169: R = H, R' = O•
170: R = Ac, R' = O•

146 + 171 →

172, R = OH
173, R = OAc
174, R = O•

146 + 21 →

175, R = OH
176, R = OAc
177 R = O•

first with hydroxylamine followed by borohydride reduction to give the hydroxylamino derivative **184**. The reaction of **184** with **178** followed by acetylation produced the same compound **181**.[39]

An analysis of the ESR spectra of these mono- and diglycosyl nitroxyls is given (Table IV).[39] Compound **185** was obtained from earlier work.[48] The hyperfine coupling constants a_H–$a_{H''''}$ represent the hydrogens on the carbons α to the nitroxyl group. They are very sensitive to the nature of the adjoining group. In the cases of compounds **159–162**, which were separated into

SCHEME XXIX

178 —(H_2NOH)→ 179 ⇌ 179a

180 —(1) H_2NOH, (2) $NaBH_4$→ 184

179a —(1) 180, (2) $NaBH_4$, (3) Ac_2O→

184 + 178, Ac_2O →

181, R = OAc
182, R = OH
183, R = O•

two epimers **a** and **b** by chromatography, there is a clear differentiation in the size of the a_H values for the epimers.[39] In contrast, the coupling constants of the ^1H NMR spectra are not sufficiently different to distinguish the two epimers.

For the diglycosyl nitroxyls **169**, **170**, **174**, **177**, **183**, and **185**, the values of the a_H constants are sensitive to the glycosidic bonds of the adjacent sugar moieties.[39]

185

The suggestion was made that it should be possible to incorporate such spin-labeled disaccharides into cellular membranes in order to obtain useful information about their biological environment through analysis of the a_H values from their ESR spectra.[39]

VI. Polysaccharides

A. Introduction

In recent years, the technique of spin labeling has been applied to polysaccharides in an increasing number of cases. These biopolymers comprise many useful industrial starting materials including cellulose, starch, gums, and muscilages from plants and seaweeds, and pectins. They are also important constituents of membranes such as glycolipids and glycoproteins.

Analysis of the ESR spectra of spin-labeled polysaccharides can provide valuable information about their structures in solution as well as in the gel and sol

state. Particular attention has been paid to the gel state.[49,50] Polysaccharide gels have biological functions in plant cell walls, in animal fluids and connective tissues, and in the bacterial capsule. They are used industrially in foodstuffs, cosmetics, paper, and textiles. In the laboratory agar and Sephadex are familiar media for gel filtration and gel electrophoresis. Sephadex derivatives are used also for ion-exchange chromatography and agar gels for bacterial culture.

The chemistry of polysaccharide derivatization has been reviewed.[51,52] For the purposes of this review the spin-labeling reactions are broadly classified as (1) *specific* and (2) *nonspecific*. The *specific* methods involve the blocking of certain hydroxyl groups and allowing reactions to occur only at specific positions of the monomer units as well as choosing reactions that occur predominantly, if not exclusively, with primary alcohol, amino, or carboxyl groups. Primary alcohol groups can be either selectively oxidized to aldehydes or converted to trityl ethers and tosyl esters. Amino groups can undergo reductive amination with aldehydes and ketones, or selective acylations, or reactions with carboxyl groups, in the presence of carbodiimide, to form amides. Carboxyl groups also can be activated by conversion to various esters, hydrazides, etc., which in turn can be converted to spin-labeled derivatives. The *nonspecific* methods include reactions that can take place with a variety of chemical groupings such as alkoxide formation using dimsylsodium followed by acylation, alcohol or amino substitutions on labeled *s*-triazine dichlorides, and periodate oxidation forming dialdehydes that can undergo reductive amination.

After completion of the reactions the reaction mixtures are normally purified by extensive dialysis against either water or a buffer. The dialysate is monitored by ESR until no more unreacted spin label can be detected. Often the remaining spin-labeled polysaccharide is purified further by gel filtration on Sephadex. Even with this purification, there sometimes is found physically trapped (not covalently bound) spin label which adds complexity to the resulting ESR spectra.

Under the best of conditions there is usually less than 100% substitution of the desired group(s) in a given polysaccharide. In addition to the usual variables of concentration, time, and temperature of monomer chemistry there is the complication of accessibility of particular groups in a polymer chain. Folding and aggregation of chains in solution can result in interior *pockets* or *cores* that are less accessible to the reagents. As a result, entrapment of spin label reagents can occur. For these reasons it is important to determine the degree of substitution (d_s) for polysaccharide derivatives. This determination is done usually by the method of ESR double integration. A standard curve of concentration vs. line amplitude is plotted for the spin label reagent used in a given study. The concentration of the spin label in the polysaccharide then can be determined from the curve and knowledge of the quantity of starting polymer. Results are given as percentages or numbers of spin labels per monomer subunits, i.e., 0.01 or 1 in 100.

In order to make this section more useful to the reader the spin-labeling reactions are classified on the basis of specificity. Under each grouping is discussed the particular labeled polysaccharides. In Table V the

Cellulose
→4)-β-D-Glc-(1→4)-β-D-Glc(1→

Alginic Acid
→4)-β-D-ManA-(1→4)-α-L-GulA-α(1→

Starch: Amylose + Amylopectin

Amylose
→4)-α-D-Glc-(1→4)-α-D-Glc-(1→

Amylopectin
→4)-α-D-Glc-(1→4)-α-D-Glc
1|α
6|
→4)-α-D-Glc-(1→4)-α-D-Glc-(1→

D-Gal
1|α
6|
→4)-β-D-Man$_m$-(1→4)-β-D-Man$_n$(1→

Guran: m/n = 1.8
Locust bean gum: m/n = 4.3

Xanthan Gum
→4)-β-D-Glc-(1→4)-β-D-Glc(1→
3|α
D-Man
2|β
D-GlcA
4|β
D-Glc⟩ Pyr

Agarose
→3(-β-D-Gal-(1→4)-α-L-3,6-Anhydro-Gal-α(1→

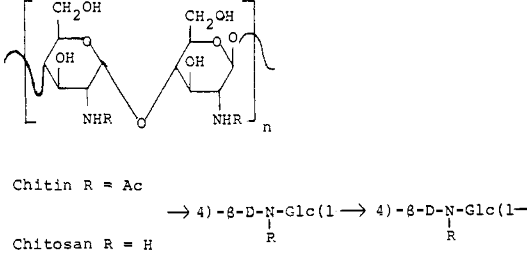


Figure 4. Partial structures of polysaccharides discussed in section VI.

polysaccharides are grouped by name, and under each name there is provided a listing of spin-labeling methods used, yields, and specifics of their ESR spectra.

The polysaccharides that have been used for spin labeling are represented by the following chemical

TABLE V. Spin-Labeled Polysaccharides[e]

compd		method	yield d_s	τ_c, ns	ESR		ref
					a_N	no. of lines	
cellulose							
188		1a	0.50	na[d]	14.75–15	3 (rt)[a]	22, 24
					14.5	3 (100 °C)	
					25, 32	3 (−100 °C)	
191		1a	na	na	14.5–14.8	3 (rt)[a]	22, 24
					14.5	3 (100 °C)	
					25, 35	3 (−120 °C)	
196		1a	na	na		3 (rt)[a]	24
					13	3 (130 °C)	
					25, 30	3 (−140 °C)	
206		1bix	0.08	0.091		3[b]	54, 55
210	d_0 0.05	2a	0.01	na	two nitroxyl populations		54, 55
	d_0 0.19	2a	0.063	na	two nitroxyl populations		
	d_0 0.34	2a	0.065	na	two nitroxyl populations		
211b	d_0 0.85	2b	0.28	na		3[c]	54, 60
212b	d_0 0.80	2b	0.09	na		3[b] some bound label indicated	54, 60
235		2d	0.0062	na		3[b]	68
					two nitroxyl populations		
236		2d	na	na		3[b]	68
244		2fi	0.00011	na		na	30
249		2fii	0.000048	na		na	30
251		2fiii	0.000089	na		na	30
(carboxymethyl)cellulose							
199		1biv	0.003	17		3[b]	54, 55
220		2d	na	na		na	66
Sephadex G-50							
245		2fi	0.00037	na		na	30
250		2fii	0.00024	na		na	30
252		2fiii	0.00048	na		na	30
agarose							
216	hydrated ppt	2c	0.00091	1.85		3[b]	62, 63
216	sol form	2c		0.3		3[b]	
216	gel form	2c		0.92		3[b]	
230		2d	na	1.4		3[b]	62, 67
233		2d	na	0.4		3[b]	62, 67
247		2fi	0.00021	na		na	30
amylose							
217		2c	0.05	na		na	64
239		2e	0.02	1.23		3[b]	31
253		2g	na	na		15[b]	69
254		2g	na	1.1		3[b]	69
amylopectin							
218		2c	0.025–0.036	na		3[b]	64
alginic acid							
197		1bi	0.01 (DCC)	na			54, 55
			1.0 (EDC)	15		3[b]	
197	d_0 0.80–0.85	1bii	0.15			na	54, 55
	d_0 0.50–0.60	1bii	0.10	33		na	
200	d_0 0.76	1bv	0.21	na		3[b]	54, 55
201		1bvi	0.05	20		3[b]	54, 55
202	d_0 0.12	1bvii	0.02	31		3[b]	54, 55
209	d_0 0.10	2a	0.05	51		3[b]	54, 55
	d_0 0.44	2a	0.13	57		3[b]	
213		2c	0.03–0.04	0.011		3[b]	54, 55
219		2d	0.01–0.04	na		na	65
xanthan gum							
198		1bi	0.43	0.061		3[b]	54, 55
198		1biii	0.05	na		3[b]	
246		2fi	0.00053	na		na	30
guar gum							
243		2e	0.005	1.12		na	31
248		2fi	0.0011	na		na	30
locust bean gum							
242		2e	0.005	1.06		na	31
guran							
203		1bviii	0.6–0.7	na		na	54, 56
mannan							
241		2e	0.005	1.30		na	31
xylan							
240		2e	0.005	1.88		na	31
dextrans **238**							
M_r 10 500		2e	0.005	0.1		na	31–33
M_r 40 000		2e	0.005	0.55		na	
M_r 70 000		2e	0.005	1.0		na	
M_r 151 000		2e	0.005	1.08		na	

TABLE V (Continued)

compd	method	yield d_s	τ_c, ns	ESR a_N	ESR no. of lines	ref
M_r 250 000	2e	0.005	1.15		na	
chitin						
214	2c	0.04	2		3^b	58
chitosan						
207	1bx	0.45	na		3^b	58
208	1bx	0.80	na		3^b	59
215	2c	0.50	35		3^b br	58
chitosan analogues						
204	1bviii	0.35	68	two nitroxyl populations		54, 58
205	1bviii	0.15	43			54, 58

[a] DMF. [b] H_2O. [c] $CHCl_3$. [d] na = not available. [e] Key: d_s = degree of substitution; d_o = degree of oxidation; τ_c = rotational correlation time; rt = room temperature; DCC = *N,N'*-dicyclohexylcarbodiimide; EDC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

structures (Figure 4). Below each structure is a short-form letter notation that is used in the literature as a space-saving device.

B. Reaction Types

1. Specific Reactions

a. Blockage of Specific Hydroxyl Groups. Labeling at C-1. This task was accomplished by following the same procedure that was used for the preparation of labeled glucose derivatives.[22,24] Thus, acetobromocellulose (**187**) was prepared by the reaction of com-

186: R = H(OAc), R' = OAc(H)
187: R = Br, R' = H
188: R = H, R' = O–C(=O)–(nitroxyl)

mercial cellulose acetate (**186**) with 40% hydrogen bromide–acetic acid. This unstable intermediate **187** was reacted with **12a** to give the cellulose derivative **188** labeled only at the terminal glucose unit. The β configuration was assumed by analogy with the reaction of acetobromoglucose giving **20**.

Labeling at C-6. The substitution at the C-6 position of cellulose was achieved by the same synthetic pathway as that used for the preparation of the glucose analogue **63**.[22,24] Commercial cellulose acetate (**186**) was tritylated (**189**), the tritylated derivative was completely

189 R = Ac, R' = Tr
190 R = Ac, R' = H
191 R = Ac, R' = CO–(nitroxyl)

acetylated, and the trityl groups were removed by 40% hydrogen bromide–acetic acid, giving **190**. The reaction of this material in portions with the spin reagent **12b** followed by acetylation of any unreacted 6-hydroxyl groups gave the spin-labeled cellulose derivative **191**. This product was shown to contain a maximum of one spin label for every five glucose subunits.[53]

Labeling at C-2 + C-3. In order to prepare a cellulose containing a large number of spin labels the 2- and 3-positions of each glucose unit were labeled.[24] Cellulose acetate (**186**) was completely deacetylated (**192**), the 6-positions were tritylated (**193**), and the remaining free hydroxyls at C-2 and C-3 were esterified with the spin label **12b** (**194**). Acid hydrolysis of the trityl groups (**195**) followed by acetylation produced the final product **196**. Compound **196** was shown to be a mixture of 2- or 3-mono- and 2,3-disubstituted derivatives.

192: R = H, R' = H, R² = H
193: R = H, R' = Tr, R² = H
194: R = CO–(nitroxyl), H, R' = Tr, R² = CO–(nitroxyl), H
195: R = CO–(nitroxyl), H, R' = H, R² = CO–(nitroxyl), H
196: R = CO–(nitroxyl), Ac, R' = Ac, R² = CO–(nitroxyl), Ac

The ESR spectra of the three spin labeled cellulose derivatives **188**, **191**, and **196** were different.[24] The 1-substituted derivative **188** could be distinguished from the 6-substituted **191** by comparison of their three-line spectra. The high-field line of both compounds was greatly reduced, but in **188** the low-field line was of lower intensity than the middle line. To explain this difference the authors considered that the triacetates of cellulose exist in solution as a rigid rod. The nitroxyl fixed in the β-anomeric position is parallel to the axis of the rod while the radical in position 6 is perpendicular to the axis. In the case of the 2,3-labeled cellulose **196** both the high-field and low-field lines were greatly reduced and broadened. This result was interpreted by assuming that the nitroxyl moieties of **196** are the

least mobile of the three cellulose compounds.[24]

Also included in this work is a study of the effect of temperature (130 to −140 °C) on the ESR spectra of these three cellulose analogues.[24] At the temperature extremes they exhibited either three sharp (high-temperature) or three broad (low-temperature) lines. At intermediate temperatures some additional side bands appeared which the authors attributed to some unbound spin label **12a** trapped within the core of the polymer.

b. Reaction of Acid, Amines, and Primary Alcohols. This part is subdivided according to the position of the spin label on the polysaccharide subunits. The abbreviated structures show the site of the main chemical reaction for each method. The reader may refer to the more complete structures at the beginning of this section to visualize better the location of the spin label. Below each procedure is listed the polysaccharides spin labeled by that procedure.

Labeling at C-6. Procedures 1bi–1biii were used to prepare the 6-amide analogues of polysaccharides. The

i. —COOH + **5** → (DCC/DMF or EDC/acetone-H_2O, pH 6.5, 18 h) —CONH—(N—O•)

alginic acid analogue **197**,[54,55] xanthan gum analogue **198**[54,55]

ii. —COOR + **5** → (DMF, 14 h) —CONH—(N—O•)

alginic acid analogue **197**[54,55], R = $CH_2CH(OH)CH_3$

iii. —COOH + **5** → (11 h) —CONH—(N—O•)

xanthan gum analogue **198**[54,55]

alginic acid analogue **197** was prepared by two different methods.[55] In the carbodiimide-mediated procedure the use of the water-soluble reagent EDC produced a 10 times greater yield than did DCC (Table V). In the other procedure two samples of the propylene glycol ester of alginic acid with different degrees of esterification were condensed with the amine radical **5** to yield compound **197**.

The 6-amide derivative of xanthan gum **198** was prepared by two different methods also.[55] In the first procedure the reagent EDC was used. In the second procedure the reaction was carried out by shaking a heavy paste prepared from xanthan gum, amino radical **5**, and three drops of water.

The 6-hydrazide of (carboxymethyl)cellulose was prepared and reductively aminated with the keto label **3** to obtain the spin-labeled hydrazide **199**.[54] In the same fashion the ester of alginic acid was transformed into its hydrazide analogue **200**.[55]

iv. —CH_2COOH → (H_2NNH_2) —$CH_2CONHNH_2$ → (**3**, $NaBH_3CN$, pH 7, 15 h) —$CH_2CONHNH$—(N—O•)

carboxymethyl cellulose analogue **199**[54,55]

v. —COOR → (H_2NNH_2, rt) —$CONHNH_2$ → (**3**, $NaBH_3CN$, H_2O) —CONHNH—(N—O•)

alginic acid analogue **200**[54,55], R = $CH_2CH(OH)CH_3$

A partially dehydrated alginic acid sample was esterified with Tempol (**4**) to yield the spin-labeled analogue **201**.[55]

vi. —COOH + **4** → (acetone-H_2O, pH 5-7) —COO—(N—O•)

alginic acid analogue **201**[54,55]

The reaction of propylene glycol alginate with dimethylbiguanidine hydrochloride yielded a *s*-triazine intermediate which was reductively aminated with **3** to give the analogue **202**.[55]

vii. —COOR + Me_2NC(=NH)NHC(=NH)NH_2 → (THF-MeOH, 0 °C, 20 min, rt, 4 d) → ($NaBH_3CN$, H_2O, 13 h)

alginic acid analogue **202**[54,55], R = $CH_2CH(OH)CH_3$

Hall and Yalpani[56] used D-galactose oxidase to selectively oxidize the galactose polysaccharide guaran (D-galacto-D-mannan) in phosphate buffer at pH 7. This selective oxidation resulted in a conversion of the 6-CH_2OH to the 6-CHO on the *galactose* units only. The intermediate aldehyde (not isolated) was subjected to reductive amination with Tempamine (**5**) to give the spin-labeled guaran **203**. Similarly, two branched chitosan derivatives[57] were treated with galactose oxidase followed by reductive amination with **5** to produce the water-soluble-labeled derivatives **204** and **205**.[58]

Labeling at C-2 + C-3. The commercially available cellulose carbonate was reacted with the amino radical **5** in a phosphate buffer (pH 7) to give the spin-labeled material **206**.[55]

viii. $-CH_2OH \xrightarrow{[O]} -CHO \xrightarrow[NaBH_3CN,\ H_2O]{5} -CH_2NH-$(TEMPO)

guaran analogue 203[54,55] chitosan analogue 204 and 205[54,58]

203 204 205

R = NH–(TEMPO)

ix. (cyclic carbonate) + 5 $\xrightarrow{\text{phosphate buffer}}$ –OCONH–(TEMPO), –OH

cellulose analogue 206[54,55]

Labeling of the Amino Groups by Reductive Amination. The compound 207 was prepared in the solvent system methanol–acetic acid.[58] A greater yield of this analogue was obtained by reductive amination of its *N*-sulfate derivative which was soluble in a phosphate buffer at 50 °C. It is this latter compound that is included in Table V. Chitin could not be spin labeled by this procedure, using a lithium chloride–dimethylacetamide solvent system.[58]

x. $-NH_2 + 3 \xrightarrow{NaBH_3CN} -NH-$(TEMPO)

chitosan analogues 207[58] and 208[59]

As part of a series of branched-chain water-soluble chitosan derivatives the spin-labeled analogue **208** was prepared by reductive amination[59] (see also **204** and **205**).

2. Nonspecific Reactions

This section is organized on the basis of reaction type rather than on the location of the spin label in the polysaccharide.

a. Labeling by the Periodate Oxidation–Reductive Amination Procedure. It was possible to stop the periodate oxidation at different stages, giving products with different degrees of oxidation (d_o) (Table V). Each of these intermediates was then reductively aminated with **5** to give products with different degrees of substitution (d_s). The evidence for the diradical **A** (Figure 5) rather than the monoradical **B** was provided

207 208

by a dipolar coupling in the ESR spectra even at low overall d_s levels.[55] This coupling would occur most likely between two spins on the same sugar (**A**) rather than on different chains (**B**).

(–OH, –OH) $\xrightarrow{NaIO_4}$ (CHO, CHO) + $\xrightarrow[NaBH_3CN]{5}$ CH_2HN-(TEMPO), CH_2NH-(TEMPO)

alginic acid 209[54,55] cellulose 210[54,55]

b. Labeling by the Me$_2$SO Oxidation–Reductive Amination Procedure. Recently 3-oxo- and 2-oxo-cellulose derivatives, **211a** and **212a**, respectively, were prepared by a selective oxidation of cellulose, using acetic anhydride in the dimethyl sulfoxide–paraformaldehyde system.[60,61] The reaction of 6-*O*-tritylcellulose yielded the 2-oxo derivative **212a** selectively under these conditions whereas the reaction of unblocked cellulose gave only its 3-oxo analogue **211a**. This surprising result was explained by a blocking of the C-2 and C-6 hydroxyl groups through reversible formation of hydroxymethyl and poly(oxymethylene)ol moieties that allowed a selective oxidation of the secondary hydroxyl at the C-3 position.[61] In the case of **212a** the bulky trityl group at C-6 probably prevented oxidation of the C-3 hydroxyl due to steric hindrance. These ketone products then were reductively aminated with Tempamine (**5**), producing the spin-labeled materials **211b** and **212b**.[60]

(–OH, –OH) $\xrightarrow[Me_2SO-(HCHO)_n]{Ac_2O}$ (=O, –OH) + $\xrightarrow[MeOH]{5,\ NaBN_3CN}$ H–C–NH–(TEMPO), –OH

cellulose 211 and 212[54,60,61]

The presence of the large trityl group in **212a** probably caused the lower d_s value of **212b** than that of **211b**

Figure 5. Possible structures of the product obtained from the periodate oxidation–reductive amination sequence.

(Table V).[60] The trityl group also had an effect on the solubility properties of these two products. The completely unblocked analogue **211b** was water soluble and insoluble in organic solvents while the tritylated analogue **212b** had the reverse solubilities.[60]

211a, R = C=O 212a, R = C=O

b, R = CHNH– b, R = CHNH–

c. Labeling by Halo Ketone Alkylation. This procedure is capable of alkylating all hydroxyl and amino groups of these polysaccharides. An example of this labeling is illustrated for the analogues of chitin **214** and chitosan **215**.[58]

–OH + 7a–c →(base) –OCH2CONH–

alginic acid **213**[54,55] chitin **214**[58], chitosan **215**[58], agarose **216**[62,63], amylose **217**[64], amylopectin **218**[64]

214: R = H, CH2CONH– N–O·; R' = Ac

215: R,R' = H, CH2CONH– N–O·

d. Labeling by Cyanogen Bromide Activation of Alcohol Groups. In a discussion of this method it was stated that there was little cross-linking between chains, which occurs sometimes with water-soluble carbodiimides, Woodward's Reagent K, or cyanuric chloride.[66] The evidence presented for the lack of cross-linking included (a) practically no change in polymer (**220**) molecular weight after spin labeling and (b) no differences in line widths or chemical shifts between the labeled and unlabeled samples in their ^1H and ^{13}C NMR spectra. It was assumed that the label was randomly attached to oxygen moieties along the cellulose chain.[66] Quite the contrary conclusion was reached by Aplin[62] for the cyanogen bromide activation of hydroxylic polymers. It was proposed that the reaction involving pairs of neighboring hydroxyls may occur in either intra- or intermolecular fashion, the latter giving rise to cross-linkages.[62]

–OH + CNBr →(pH 11) –OCN + RNH2 →(EDC) –OC(=NH)–R

alginic acid **219**,[65] R = NH–

(carboxymethyl) cellulose **220**[66], R = NH–

agarose (Sepharose 4B) **221–234**[62,67]

221, R = NH–

222, R = NHCH2CONHR·[a]

223, R = NH(CH2)3CONHR·[a]

224, R = (NHCH2CO)2NHR·[a]

225, R = NH(CH2)5CONHR·[a]

226, R = (NHCH2CO)3NHR·[a]

227, R = (NHCH2CO)4NHR·[a]

228, R = (NHCH2CO)5NHR·[a]

229, R = (NHCH2CO)6NHR·[a]

230, R = NH–

231, R = NHCH2CONHR·[b]

232, R = NH(CH2)3CONHR·[b]

233, R = NH(CH2)5CONHR·[b]

234, R = NH(CH2)10CONHR·[b]

cellulose **235**[68] R = NH–

cellulose **236**[68] R = NH(CH2)5CONH–

wood **237**[68] R = NH–

[a]R· = [b]R· =

e. Labeling by Alkoxide Ion Acylation. This method gives very random labeling of the hydroxyl groups.

–OH →(CH3SOCH2⁻Na⁺, Me2SO, rt, 1 h) –O⁻Na⁺ + 12b →(benzene, Me2SO, rt, 20 m) –OC(O)–

dextrans (various molecular weights) **238**,[31-33] amylose **239**,[31] xylan **240**,[31] mannan **241**,[31] locust bean gum **242**,[31] guar gum **243**[31] (see also Scheme XV)

f. Labeling by s-Triazine Alkylation. Adam and Hall[30] extended the use of s-triazine derivatization to

polysaccharides (see also Schemes VII and VIII).

i. ⊢OH + 33 → ⊢O–(chlorotriazinyl)–O–(TEMPO)

cellulose powder **244**, Sephadex G50 **245**, xanthan gum **246**, agarose **247**, guar gum **248**[30]

ii. ⊢OH + 34 → ⊢O–(triazinyl bis-TEMPO-oxy)

cellulose powder **249**, Sephadex G50 **250**[30]

iii. ⊢OH + 35 → ⊢O–(chlorotriazinyl)–NH–(TEMPO)

cellulose powder **251**, Sephadex **252**[30]

g. Labeling with Miscellaneous Reagents.[69]

i. ⊢OH + (imidazoline nitroxide)–CH_2Cl $\xrightarrow[\text{rt, 4 d}]{Ag_2O,\ Me_2SO}$ ⊢O–CH_2–(imidazoline nitroxide) **253**

ii. ⊢OH + **8** $\xrightarrow[\text{rt, 3 d; 40 °C, 2 h}]{Me_2SO}$ ⊢OC(=S)–NH–(TEMPO) **254**

amylose **253**, **254**[69]

C. ESR Studies

ESR spectral studies of spin-labeled polysaccharides have provided valuable information about the microenvironment of these biopolymers in solution as well as in the gel and sol states.

In a number of instances the ESR spectra of labeled polysaccharides were complex and seemed to indicate the presence of two nitroxyl populations, one more mobile and probably located at the exterior surface, the other less mobile and located in interior pockets or cores, resulting from folding and/or aggregation of polymer chains.[55,58]

Spin labeling has been used to study the rheological properties of polysaccharides. This technique is useful for studying conformational changes of these biopolymers in solution, affecting properties such as viscosity and gel–sol transitions. It gives a better indication of the microenvironment around the polymer than changes in optical rotation, which only detect the macroscopic transitions at the melting and gelling temperatures.

The microviscosity of various polysaccharides in solution (as measured by the τ_c of mixtures of sugar and spin labels **5** and **9**) was compared with the macroviscosity measured in a viscometer.[62,65] For sucrose there was a definite increase in τ_c of **5** with increasing concentration of sucrose (up to 240% w/v sucrose in water at 28 °C). This result indicates a restricted nitroxyl motion. By contrast there was no such increase of τ_c for solutions of either sodium alginate, starch, xanthan gum, or locust bean gum. This difference was explained by "solvent pockets" which effectively trap the spin label **5** and remove it from interaction between polymer chains, the latter being responsible for the macroscopic viscosity.[65]

When label **9** was covalently bound to alginic acid by the cyanogen bromide method (2d) there was a marked reduction in nitroxyl mobility.[65] Also no solvent pockets were apparent because neither increasing the concentration of the labeled alginate nor adding sodium alginate appreciably changed the ESR line shape. The labeled material **219** formed a gel on addition of calcium ions, demonstrating that the spin label had little effect on the overall polymer conformation.[65]

The same approach was used to study the sol → gel transformation of agarose.[62,63] Thus, when the concentration of spin label **5** in water was increased, there appeared a spin exchange line broadening in the ESR spectrum. These ESR spectral changes in water were reproduced when the same concentration of **5** was suspended in 3% agarose gel. This result supports the concept of solvent pockets in the gel that allow the spin label the same motional freedom as in free solution.[63] The size of the gel pores is too large to effect the translational differences of the label. Again covalent labeling of agarose with **7c** (method 2c) provided information about polymer conformation.[63] From Table V (compound **216**) it can be seen that the τ_c values decrease in the order (a) hydrated precipitate, (b) gel, (c) sol forms. Also there was a decrease in the mobility of the spin-labeled agarose in the order (a) hydrated precipitated form, 298 K, (b) precipitated form, 77 K, (c) precipitated form, 298 K, after freeze-drying.[63] Thus, there appears to be definite relationship between polysaccharide solvation and mobility.

It is apparent from Table V that the τ_c values for the sol, gel, and solid forms of agarose are similar. This similarity was explained by assuming that the solvation of the spin label in all three states was identical because it was attached only to the surface of the solid and was not trapped within the gel matrix.[63] The possibility of an entrapment of **5** in the gel matrix was eliminated because the mobility of the spin label attached after the gelation process was roughly comparable to that before gelation.[63] The assumption of equal solvation was shown to be an approximation by use of the spin probe–spin label technique.[70] Addition of the paramagnetic nickelous ions (Ni^{2+}) caused complete elimination of the nitroxyl signal in the sol form, but there remained some signal in the hydrated precipitate and gel forms.[63] This result indicated that there were some nitroxyl populations that were sterically hindered from spin-exchange broadening with Ni^{2+} ions.

The dependence of τ_c on temperature for the gel–sol–gel cycle, plotted as an Arrhenius curve, revealed a clear hysteresis.[63] Arrhenius plots of τ_c gel and τ_c sol

for a single hysteresis cycle as a function of absolute temperature indicated a large change below the melting point during the heating cycle beginning near 320 K. Within one hysteresis cycle it was not possible to return to τ_c values as large as those prevailing at the start of the experiment. Also, there can be marked differences in mobilities of polymer molecules in gels of different states of organization as well as between gels at the same temperature but with different immediate histories.[63] Thus, the τ_c of a gel directly after setting (at 303 K) was about one-seventh of that at the same temperature allowed to stand at room temperature overnight.[63] These results can be considered to depend on microscopic changes in the gel structure[63] as well as on changes from a threefold double helix of the solid state to larger aggregates in the gel state arising from interhelix packing and formation of bundles.[49,71]

Some interesting ESR results were obtained for the spin-labeled chitin **214**, chitosan **207** and **215**, and their water-soluble analogues **204**, **205**, and **208**.[54,58] The τ_c value for the chitin derivative **214** (Table V) was considerably smaller than those obtained for the chitosan derivatives. This result was attributed to the fact that chitin was labeled under heterogeneous conditions in which the d_s was lower (0.04), but the label was attached mostly to the surface of the biopolymer.[58] Consequently this surface labeling allows more rotational mobility for the spin label while the polymer motion ($\tau_c > 100$ ns) would make no contribution on the ESR time scale. However, in the case of the chitosan analogues **207**, **215**, **204**, and **205**, the overall tumbling rate includes the motion of the large polymer chain, within either a gel matrix or solution aggregate, and so the observed τ_c values are larger.[58]

Compound **207** is less mobile than **215** because the C-6 position of **215** enjoys more motional freedom than the 2-amino function of **207**. Also, the nitroxyl group of **215** is freer to rotate because of the spacer of three atoms separating it from the polymer chain.[58]

Compound **205** has a lower τ_c value (more mobile) than **204**, and this result was explained by their different structures in solution.[58] Compound **205** has a 1→6 glycosidic linkage in the side chain which causes it to be more extended and less sterically restricted, allowing greater motional freedom for the spin label. Because of the 1→4 linkage in the side chain of **204** it was argued that there is a greater tendency for twisting and entanglement of side chains, resulting in a restricted motion for the label.[58] These differences in side-chain conformations between **204** and **205** were suggested earlier on the basis of scanning electron microscopy (SEM) studies of the diamagnetic solid precursors.[72]

Studies with gels of compound **207** revealed a complex ESR spectrum that seemed to indicate two nitroxyl populations.[54,58] When **207** was subjected to a water–methanol–water solvent cycle over a 2-week period, the ESR changed to a simple three-line spectrum.[58] In this case the mobile nitroxyl population completely predominated, indicating a structural reorganization allowing more spin label to have access to the solvent.

Application of the spin probe–spin label method to these derivatives provided more support for the concept of two nitroxyl populations.[58] When the centerfield line width vs. the Ni^{2+} ion concentration was plotted for compounds **207** and **205**, there was a rapid linear increase in line broadening at low Ni^{2+} concentrations, then a rounding off to a point of little or no change at concentrations above 10 mM. This latter state would seem to indicate that some nitroxyl groups are not accessible to exchange interaction with Ni^{2+} ions. Compounds **215** and **204** lost their nitroxyl signals completely at much lower concentrations of nickelous ions, supporting the idea of a greater nitroxyl accessibility to solvent for these two derivatives than for **207** and **205**.[58]

ESR studies have provided information about other aspects of polysaccharide structure in solution.[31-33] A series of polysaccharides were randomly labeled by the alkoxide ion acylation method (2d).[31] As can be seen from Table V there is a molecular weight dependence on the rotational correlation time τ_c of the spin-labeled dextrans **238**.[31-33] As the molecular weights increase, the τ_c values increase, and thus the rotational mobility of the nitroxyl label decreases. It was reported that these τ_c values represent an average between a relatively rapid motion of the ends of the polymer chain and a slower segmented reorientation of the entire polymer chain.[32,33]

ESR was also used to study the depolymerization by α-amylase of the spin-labeled amylose derivatives **253** and **254**.[69] A plot of the amplitude of the h-line vs. time for various concentrations of α-amylase indicated a dependence on enzyme concentration and a drop-off in rate with inhibition by the product. It was stated[69] that these changes were dependent on the motion of the entire polymer, rather than on the motion of either chain segments or of the label relative to the chain.

Information about the distribution of nitroxyls in the guaran analogue **203** (Table V) was obtained from the dipolar coupling contribution to the ESR line width.[54,56] The spectral parameter d_1/d at 77 K[73] (where d_1 = the total intensity of the outermost lines and d = the intensity of the central line) can be used to calculate the mean nearest-neighbor distance between spins (r).[73,74] The value of r for guaran was 1.36 nm (±5%), which implied that the D-galactosyl groups are distributed in blocks,[56] in agreement with the results of a theoretical analysis of the kinetics of periodate oxidation of guaran.[75] The latter study indicated that the D-galactosyl groups are arranged in small blocks of two to four units, attached to the D-mannosyl residues on the chains.[75]

Attention also has been devoted to the use of spin labels for the study of surface properties of polysaccharides.[64,67,68] One approach has been to attach a nitroxyl label to a polysaccharide surface by spacers of varying length and then to measure the τ_c values from their ESR spectra in aqueous solution. An investigation of agarose used as a support matrix for affinity chromatography involved the synthesis of spin-labeled compounds **221–234**.[67] The ESR spectra of the products were sharper with increasing numbers of atoms (n) separating the nitroxyl moiety from the agarose surface, and the τ_c values decreased, approaching a limiting value of 0.3 ns for large values of n.[67] Obviously, there is less steric hindrance from the solid support as the spacer is elongated, resulting in increased motional freedom for the nitroxyl. For compounds with no spacer such as **230**, evidence was presented for two types of reaction sites: open (lower τ_c) and masked

(higher τ_c).[67] The open ligand attachment sites would be located in regions of single-stranded, unassociated chains, and the masked sites would be formed by associations of chains. Such interpretations of ESR spectra are subject to error. As was pointed out[67] the line shapes for **230** could be the result of an anisotropic rotational reorientation of a spatially isotropic distribution of spin labels.[76]

Similar results were obtained for cellulose powder and wood samples spin labeled by the cyanogen bromide activation method (2d).[68] Again, there were reported differences in the ESR spectra of the cellulose analogues with no spacer (**235**) and those with a long spacer (**236**). For example, the addition of the nickelous ion ($Ni(H_2O)_6^{2+}$) completely broadened the ESR signals of **235**. Replacement of the nickelous ion by the ferricyanide ion ($Fe(CN)_6^{4-}$) nearly completely broadened the signals of **236** but produced no change in the ESR line shape of **235**. It was hypothesized that there are two types of reactive sites on the surface of the cellulose analogue **235**.[68] Two different reactive sites seemed to be indicated also when these materials were reacted with different reducing agents.[68] Thus, sodium ascorbate and sodium dithionate completely reduced the nitroxyl signals of **235** and **236** immediately. In contrast, the addition of ferrous ion ($Fe(H_2O)_6^{2+}$) caused a complete reduction of the nitroxyl signal of **236** but only a partial reduction of **235**.

These results could be interpreted in the following way. A nitroxyl linked to the polysaccharide surface by no spacer, i.e. **230** and **235**, can exist in both more accessible and less accessible reactive sites.[68] In contrast, the nitroxyls linked by long spacers, i.e. **236**, are completely accessible to all the reagents listed. Such a nitroxyl appears to be insensitive to surface geometry and/or does not become involved in the less accessible reactive sites.[68]

The authors state that the pore diameters of the different spin-labeled cellulose powders might play a large role in the accessibilities of different hydrated metal ions to the nitroxyl moieties.[68] In addition, a positive interaction between the hydration sphere of the ferrous and nickelous ions with the cellulose hydroxyl groups may occur during their penetration into the cellulose matrix. Both of these factors could explain the inability of the larger ferricyanide ion to broaden the nitroxyl signal of compound **235**. Other variables to be considered are ion size, charge, hydrophilicity, and counterion character.[68]

Another study on surface properties of polysaccharides was concerned with the adsorption of neutral polysaccharides on a negatively charged solid (calcite).[64] As part of this study spin-labeled amylose **217** and amylopectin **218** were prepared. These labeled polymers were formed by the reaction of their alkoxide salts with the nitroxyl **7b** (method 2c).

To determine the adsorption characteristics of these materials, the unlabeled polysaccharides first were hydrolyzed in dilute hydrochloric acid for varying lengths of time to form monomers of different molecular weights.[64] Adsorption isotherms of these materials were established on a 100 g/L solid dispersion. From the plateaus of the Langmuir adsorption isotherms the maximum weight of polymer adsorbed per gram of solid (Q_s) was calculated. Using an area of 25 Å^2 per monomeric unit, the weight of polymer required to cover the solid (Q_{max}) was calculated to be 1.9 mg/g. The ratio Q_{max}/Q_s is assumed to be the maximum value of p, the fraction of monomer units in the form of trains.[64]

Following this procedure, aqueous suspensions of the spin-labeled amylose and amylopectin fractions adsorbed on calcite were analyzed by ESR. These spectra were recalculated on the basis of model spectra for nitroxyl **7b** in the free form (in dimethyl sulfoxide at 20 °C) (polymer loops) and in the bound form (in glycerol–water (3:1) mixture at −65 °C) (polymer trains). From these spectra values of p were calculated and found to be in qualitative agreement with the values obtained with the unlabeled polysaccharides.[64]

There were distinct differences between the two polysaccharides.[64] Thus, for amylose there was no variation of Q_s with molecular weight and also a large p value, indicating that this biopolymer formed a flat layer on the solid surface of the calcite. In contrast, amylopectin gave decreasing values of Q_s (increasing p) with decreasing molecular weights, indicating that this polymer is adsorbed as a flat layer at low molecular weights and as loops at higher molecular weights. It was concluded, therefore, that molecular structure modifies the mechanism of adsorption.[64]

In addition to the spin labels covalently bound to polysaccharides, research on films of nitroxyls and polysaccharides was reported.[77] Films of acylated chitins, containing one to 12 carbon atoms in the acyl portion, and Tempol (**4**) were prepared and their ESR spectra recorded at low and high temperatures.[77] The correlation time τ_c at higher temperatures (fast motion) was calculated by using the equation developed by Kivelson[78] while that at lower temperatures (restricted motion) was calculated by using the method of Freed et al.[79] The activation energies of the spin probe **4** were determined by means of Arrhenius plots. Plots of log τ_c vs. the number of carbon atoms in the acyl groups were then constructed. At 0 °C there was a steady decrease of τ_c with increasing side chain length. At 135 °C the τ_c values were nearly equal for side chains with carbon numbers up to three, followed by an abrupt decrease beginning with side chains of carbon number four. This spin probe method revealed that the side chain region became more mobile with an increase in side chain length.[77]

The activation energies for the rotational motion of **4** were calculated to be about 3 kcal mol^{-1} in the low-temperature range and from 4 to 6 kcal mol^{-1} in the higher temperature range.[77] These activation energy ranges were said to indicate two motional processes: oscillations (low temperature) and rotations (high temperature).[77] In the latter range the activation energies decreased with increasing chain length, except for acetylchitin (chain length of two), which had a low value. This anomaly seemed to have some relationship to the wetting characteristics and abnormally long blood clotting times on films of acetylchitin compared to those on films of other acyl chitins.[77] Acylated chitins have been examined as materials for blood contact surfaces.[77]

In a recent article was described a study of amylose in solution, using a combination of electron spin resonance, ultracentrifugation studies, and fluorescent depolarization measurements.[80] The results are used to support a random-coil structure for amylose consisting

DTNB:βCD Tempo:βCD Tempol:βCD

Figure 6. Structures of inclusion complexes of spin labels and β-cyclodextrin.

of helical segments, with each segment containing more than 100 monomeric units.

VII. Cyclodextrins

Cyclodextrins (CD) are cyclic oligosaccharides consisting of six, seven, or eight D-glucopyranose units (α,β,γ-cyclodextrins) linked by α (1→4) bonds with a central cavity of 6–10 Å which can complex various moieties such as metal ions and a variety of organic compounds.

This complexation or host–guest interaction has been studied by a variety of techniques, including the ESR spectroscopy of nitroxyl radicals.[81-86] Nitroxyl radicals **2–4**, **11**, and **255** form inclusion complexes in aqueous

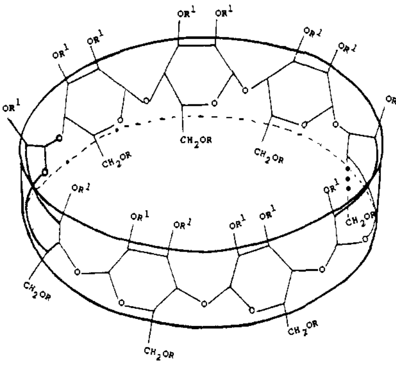
255

solutions with β- and γ- but not with α-cyclodextrins.[81] The central cavity of α-cyclodextrin probably is too small to form such inclusion complexes. The isotropic hyperfine coupling constants a_N of **4** and **11** when incubated with β-cyclodextrin as well as those of **2** and **3** when incubated with either β- or γ-cyclodextrins showed small decreases. The a_N values were interpreted to indicate a hydrophobic environment for the –NO· groups.[81] There was considerable line broadening and a decrease in the h-line when these radicals were mixed with β- or γ-cyclodextrins. The correlation times τ_c for the five radicals were about 0.1 ns in the presence of β-CD and about 0.01 ns in the absence of β-CD.[81]

The equilibrium constants for the complex formation between β-CD and DTBN (di-*tert*-butylnitroxyl), Tempo (**2**), and Tempol (**3**) were reported,[82,83] and the structures proposed for these complexes are as shown in Figure 6.

It was possible to isolate a 1:1 molar ratio complex of β-CD and nitroxyl **3** as a solid precipitate from aqueous solution and characterize it by melting point, IR spectrum (not shown or discussed), and combustion analysis (C is 2% off).[81] Its ESR spectrum was completely different from that of a 1:1 ground mixture of the two components, in support of a true inclusion compound. However, a precipitate of nitroxyl **2** and β-CD had essentially the same ESR spectrum as a solid 1:1 mixture of the two compounds. This result led to the conclusion that it was not a true inclusion complex.[81]

Studies of spin-label-induced NMR relaxation of cyclodextrins provided a more detailed picture of the



264 R=R¹=H

265 R=Tr, R¹=H; 80% m.p. 200-205°

266 R=Tr, R¹=Ac; 80% m.p. 200-205°

267 R=H, R¹=Ac; 80%

268 R= (spin-labeled pyrroline carbonyl), R¹=Ac 60% m.p. 100-105°

Figure 7. Preparation of a spin-labeled β-cyclodextrin (CD). The dimensions of the β-CD are exaggerated for the sake of clarity. The ring consists of a hydrophobic cavity and primary and secondary hydroxyl groups projecting above and below the ring plane, respectively.

host–guest interactions.[84-86] Tempo (**2**) and several paramagnetic metal ions of the lanthanide series were used. Thus, inclusion of **2** caused line broadening of resonances of the H-3 and H-5 protons of β-CD. It is known that these protons are directed toward the interior of the cavity (see structure **264**). Also, the proton spin lattice relaxation time (T^1) values for H-3 and H-5 were decreased significantly with increasing nitroxyl concentrations. Both observations support the notion that these protons lie at the binding site for the nitroxyl radical.[84] The dissociation constant K_D of the **2**–β-CD complex equals 3.9×10^{-3} M, and the amount of the complexed **2** is about 5%. Of the lanthanide cations tested gadolinium(3+) produced line broadening in the ^{1}H NMR spectra but no differentiation in the CH resonances. The addition of either europium(3+) or lanthanum(3+) cations produced no shifts or line broadening in the ^{1}H NMR spectra of cyclodextrins.[84-86]

Other studies on interactions between nitroxyls and cyclodextrins were reported.[87-89] The French workers investigated the association of 10 radicals with α- and β-cyclodextrins. In addition to compounds **2–4**, a series of bicyclic oxazolidin-*N*-oxyl derivatives (**256–262**) were studied.[87] Nitroxyls **2** and **256–262** formed inclusion complexes with β-CD and compounds **256**, **257**, and **259** with both α- and β-cyclodextrins. In a separate paper, Ohara[81] showed conclusively that nitroxyls **3** and **4** also form inclusions with β-CD. An analysis of the ESR spectra of solutions of diradical **262** alone and combined with β-CD in dimethyl sulfoxide–water (1:1) allowed calculations of the τ_c values of 0.1 ns for free **262** and 1.9 ns for the complexed **262**.[87] In addition, the equilibrium constant for the complex formation was calculated, and from this result was determined the following thermodynamic factors: $H_{assoc} = -12 \pm 2$ kcal/mol; $S_{assoc} = -30 \pm 3$ cal/(deg mol).

256 257 258

259 260 261

262

A diradical **263** related to **262** was used to demonstrate a chiral recognition by a cyclodextrin.[88] When β-CD and **263** were mixed in a solution of water–dimethyl sulfoxide (1:1), an inclusion complex was formed. Addition of (+)-fenchone to a solution of this complex produced a greater reduction in the ESR signals corresponding to **263** than did addition of (−)-fenchone. This result indicated that (+)-fenchone is complexed more strongly by β-CD than is (−)-fenchone.

263
3″(*R*),1″(*R*) (D)
3″(*S*),1″(*S*) (L)

Optically active isomers of **263** were prepared starting from (*R*)-(+)-3-methylcyclohexanone and the racemic 3-methylcyclohexanone.[89] The synthesis with the former yielded only one of the possible epimers 3″(*R*),1″(*R*) (D) while the latter gave a racemic mixture (3″(*R*),1″(*R*) (D) and 3″(*S*),1″(*S*) (L)). Studies of the ESR spectra of their inclusion complexes with β-CD and calculations of the corresponding K_{eq}, ΔH, and ΔS values revealed that the D isomer had a better fit in the central cavity than the L isomer. Furthermore, it was demonstrated that the chiral recognition is based on the 3-methylcyclohexane moiety.

A report has appeared concerning competitive inclusions between Tempol (**4**) and the various chlorinated acetic acids for a composite gel made from β-CD and starch in a 1:2 ratio and cross-linked by epichlorohydrin.[90]

To date, there have been only a few reports of nitroxyl radicals covalently linked to hydroxyl groups of cyclodextrins. A review of cyclodextrin derivatization has appeared recently.[91] Gagnaire and Odier followed the same approach as used for the spin labeling of mono-, di-, and polysaccharides.[24] The primary hydroxyls at C-6 of β-CD **264** were blocked first as trityl ethers (**265**); then, the remaining hydroxyls at C-2 and C-3 were acetylated (**266**). The trityl ethers were hydrolyzed with acetic acid (**267**), and the resulting C-6 hydroxyls reacted with the spin label **12b** to yield the hepta radical **268**. A satisfactory microanalysis based on seven nitroxyl radicals was obtained. The ^{1}H NMR spectrum revealed the presence of five nitroxyls attached to the C-6 positions and two at the C-3 positions. This result was attributed to a migration of two acetyl groups from C-3 to C-6 during detritylation.[24] Acetylation of the final intermediate **267** with deuterated acetyl chloride allowed the assignment of the C-6 protons in the NMR of the completely acetylated cyclodextrin and thus the position of the spin label at C-6. The variations of the ESR spectrum with temperature were qualitatively the same as those obtained for maltose labeled in the 6,6′-positions.[24]

TABLE VI. Reaction of Spin-Labeled Cyclodextrin 269[94]

compd	K_D, mM	k_2,^{a} s^{-1}	k_3,^{a} s^{-1}	τ_c,^{b} ns
269				0.035
β-cyclodextrin:	0.75 ± 0.06^a	6.9×10^{-3}		0.334
269 complex	−0.60 ± 0.2^b			
270			3.2×10^{-5}	0.504

^{a}pH 9.7. ^{b}pH 5.75.

Van Etten and co-workers have shown[92] that both α- and β-cyclodextrins, unlike glucose or methylglucoside, cause large increases in the rate of hydrolysis of meta-substituted phenyl acetates. Interestingly, this rate enhancement was not found when either γ-CD or para-substituted phenyl acetates were used. On the basis of these findings the proposal was made that these large rate enhancements are the result of a precise fit of the substrate ester in the cavity of the cyclodextrin, which allows strong interactions with the secondary hydroxyls of the cyclodextrin.[92] This reaction was considered a model for the so-called Michaelis complex proposed for enzyme-catalyzed hydrolysis reactions.[93] A kinetic scheme (eq 5–7) was proposed to explain this reaction (S = substrate and CD = cyclodextrin).

$$\mathrm{S + CD} \underset{K_D}{\rightleftharpoons} \mathrm{S{-}CD} \quad (5)$$

$$\mathrm{S{-}CD} \xrightarrow[k_2]{} \mathrm{S'CD + P_1} \quad (6)$$

$$\mathrm{S'CD} \xrightarrow[k_3]{} \mathrm{CD + P_2} \quad (7)$$

Some interactions between the spin label **269** and β-CD were used to elucidate the mechanism of this model reaction.[94]

269, R = *m*-nitrophenyl
270, R = β-CD
271, R = -H

The inclusion complex formed between nitroxyl **269** (S) and β-CD was used as a model for the Michaelis complex (S–CD) (**5**).[94] The constants K_D and k_2 for the reactions of this complex were determined at pH 9.7 through measurement of the *m*-nitrophenolate ion (P_1) in the presence of varying amounts of excess β-CD (see Table VI). Preparation of the acylated cyclodextrin S′CD (**270**) (**6**) was achieved by the reaction of nitroxyl

SCHEME XXX

IO_4^- ; 5, $NaBH_3CN$

272 N-acetylneuraminic acid (NeuAc)

273 (n = 1 or 2)

269 with β-CD, according to a procedure of Van Etten et al.[95] and purified by G-10 Sephadex chromatography. The rate constant k_3 for the deacylation of 270 to 271 (P_2) (7) was determined by the analysis of the ESR spectrum at pH 9.6 (Table VI).

The rotational correlation time τ_c for the inclusion complex (0.334 ns) (Table VI) was much closer to that of the nitroxyl covalently linked to β-CD 270 (0.504 ns) than it was to that of the free nitroxyl 269 in solution (0.035 ns).[94] This result suggests that the R^1 group of the acyl function, i.e. the nitroxyl moiety of 269, is greatly immobilized in the cyclodextrin cavity. Thus, there is support for the structure of the Michaelis complex proposed for enzyme-catalyzed hydrolysis reactions.[93]

VIII. Glycoproteins

The discussion in this section will be limited to the spin labeling of the carbohydrate portions of these biopolymers.

All investigations so far reported[96-101] have involved the selective labeling of terminal N-acetylneuraminic acid (sialic acid) residues (structure 272). Periodate oxidation selectively cleaves the three-carbon side chains to give a mixture of C-7 and C-8 aldehydes (Scheme XXX). This mixture is reductively aminated with Tempamine (5) in the presence of sodium cyanoborohydride to give the spin-labeled glycoprotein of general structure 273. Purification is achieved usually by dialysis and/or gel filtration through Sephadex G-50, as is used with polysaccharides (section VI). Under the proper conditions, the periodate oxidation affects only the sialic acids of these biopolymers.[99]

The first reported glycoprotein to be spin labeled was probably glycophorin.[96] The polypeptide component of glycophorin stretches across the entire cell membrane, and the polysaccharide head group extends outward into the solution. The latter group is considered to have an important role in cellular recognition events.[96] A labeled glycophorin was incorporated into various lipid bilayer structures to mimic the membrane environment of the glycoprotein. The τ_c value (0.96 ns) changed negligibly for the bilayers which were fluid (e.g. phosphatidylcholine), semirigid (extracted erythrocyte lipid), rigid (dipalmitoylphosphatidylcholine), or negatively charged (phosphatidylserine). The use of a bilayer of egg phosphatidylcholine coated with Dextran T 500, human γ-globulin, and BSA (bovine serum albumin), which mimics cell surface materials that could crowd the oligosaccharide head group, reduced somewhat the mobility (τ_c = 1.21 ns) of the spin label attached to the sialic acids in glycophorin. These results support the idea that the binding of the polypeptide chain within the membrane has a negligible effect on the conformational mobility of the polysaccharide chain on the exterior of the cell.[96] The fact that the value of a_N (16.75) for all the systems tested remained that of a totally aqueous environment was interpreted to mean that the oligosaccharide head group did not interact with hydrophobic lipid or protein groups.[96]

TABLE VII. Glycoprotein Spin Labeled with 5[62]

matl[a]	method	τ_c, ns extpl	τ_c, ns Stokes law
fetuin	EDC coupling to $-CO_2H$	1.55	14.4
	periodate oxidation–reductive amination	0.75	
asialofetuin	galactose oxidase–reductive amination	0.52	
	periodate oxidation–reductive amination	1.20	
	EDC coupling to $-CO_2H$	1.38	
BSM	EDC coupling to $-CO_2H$	1.83	≥300
	periodate oxidation–reductive amination	0.35	
asialo-BSM	galactose oxidase–reductive amination	0.49	
	periodate oxidation–reductive amination	0.35	
human erythrocytes	EDC coupling to $-CO_2H$	0.52	
	periodate oxidation–reductive amination	1.0	
BSA	EDC coupling to $-CO_2H$	2.98	-20.0
HSA	EDC coupling to $-CO_2H$	2.90	20.0

[a] BSM = bovin submaxillary mucin; BSA = bovine serum albumin; HSA = human serum albumin; EDC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

Glycophorin is known to be the receptor on human erythrocytes for the lectin WGA (wheat germ agglutinin).[96] When WGA (4 mg/mL) was added to a solution of labeled glycophorin, the τ_c increased to 4.77 ns, showing a drastic reduction in nitroxyl mobility. When the value of τ_c was plotted as a function of WGA concentration, a sigmoid curve was produced, indicating that the binding process exhibits a positive cooperativity.[96] The maximum point of the curve corresponded to total receptor site occupancy. This result suggested that the binding of the lectin to its receptor, terminal N-acetylneuraminic acid residue, causes a conformational change in the receptor which increases the amount of binding.[96]

Aplin et al.[62,97] used the periodate oxidation–reductive amination sequence on a serum glycoprotein (fetuin), a mucin (bovine submaxillary mucin BSM), and human erythrocytes. These conditions caused the labeling to occur on both the sialic acid and galactose units. When *Vibrio cholera* neuraminidase and D-galactose oxidase were added together to the glycoproteins, the sialic acids were removed and the $-CH_2OH$ groups of the terminal galactose units oxidized to $-CHO$ which, in turn, were reductively aminated with Tempamine (5). The estimated yields of labeling were 20–30% for the periodate

oxidation and 5% for the neuraminidase + D-galactose oxidase treatment. In Table VII are listed these glycoproteins and their desialylated (asialo) analogues prepared by the two reaction routes. The τ_c values were considered to reflect an unexpectedly high mobility of the nitroxyl spin label in these biopolymers.[97]

Labeling of the sialic acid residues in these glycoproteins has been accomplished also by the reaction of the carboxyl groups with Tempamine (5) in the presence of the water-soluble carbodiimide EDC.[62,98] The general structure of the labeled sugar is shown in 274.

CH2OH, OH, OH, AcHN, O, CONH, N—O•, O—(sugar units)n—polypeptide, OH H

274

This amidation reaction was applied also to a mucin (BSM), a serum glycoprotein (fetuin), control groups containing anionic groups of noncarbohydrate origin (bovine and human serum albumins—BSA and HSA, calf thymus DNA), tissue sections from human colon, and human erythrocytes membrane components.[98] The sialic acid containing materials also were desialylated by neuraminidase and then spin labeled. These asialo materials possessed significant background signals in the ESR which implies that this labeling procedure was nonspecific. The labeling occurred also at the amino acid side chain carboxyls of the polypeptide chain and perhaps at the phosphates of lipid head groups. As can be seen from Table VII the τ_c value for asialofetuin (1.38 ns) was almost equal to that for fetuin (1.55 ns), and the τ_c for BSA (2.98 ns) and HSA (2.90 ns) were similar to that for fetuin. This result, it was speculated,[98] indicates that the position of the label (whether on a carbohydrate chain of long or short length or attached to an amino acid side chain carboxyl) did not affect appreciably the correlation time and thus the nitroxyl mobility. It is clear, however, that such a nonspecific labeling provides little useful information about the structure of glycoproteins in solution.

A detailed study was done on the spin labeling of terminal sialic acid groups of glycoproteins and glycolipids in erythrocyte membranes.[99,100] Periodate-activated erythrocyte ghosts were reductively aminated with nitroxyl 5. If the reductive aminations were carried out without prior oxidation with periodate, then there was negligible incorporation of the label, indicating that only sialic acids were spin labeled by this procedure.[99] The apparent τ_c value was 0.84 ns. The extent of sialic acid labeling was estimated from the blockage of tritium incorporation by sodium borotriteride ($NaBT_4$) with prior spin labeling of the glycoprotein. A radioactivity profile of SDS–polyacrylamide gel electrophoresis slices revealed that the label 5 had blocked the tritium incorporation into all sialoglycoproteins normally labeled by $NaBT_4$ and to a roughly proportional amount. Thus, all of the surface sialoglycoproteins appeared to be labeled, whereby glycophorin, the most heavily sialyated component of the erythrocyte membrane, bound the majority of the spin label.[99] About 40% of the sialic acids was labeled. It was also shown that about 30% of the total ESR signal intensity could be attributed to the spin-labeled gangliosides, a class of glycolipids.[99] No nonspecific labeling of phospholipids was found. In a recent report it was stated that less than 10% of the glycolipids was spin labeled.[100]

The periodate oxidation–reductive amination method was used to spin label terminal sialic acids of two glycopeptides isolated by extensive pronase digestion of human serotransferrin and Cohn fraction IV.[101] The glycopeptides contained either two (bichain) or three (trichain) carbohydrate chains (see structures 275 and 276). At 20 °C the rotational correlation time τ_c was 0.63 ns, and there was a temperature dependence of τ_c from 0 to 50 °C. An Arrhenius plot of τ_c vs. absolute temperature (K^{-1}) allowed the calculation of the rotational activation energy equal to 6.9 ± 1.0 kcal/°C.

α-NeuAc(2-6)-β-Gal(1-4)-β-GlcNAc(1-2)-α-Man(1-3)
β-Man(1-4)-β-GlcNAc(1-4)-
α-NeuAc(2-6)-β-Gal(1-4)-β-GlcNAc(1-2)-α-Man(1-6)

275

α-NeuAc(2-3)-β-Gal(1-4)-β-GlcNAc(1-4)
α-NeuAc(2-6)-β-Gal(1-4)-β-GlcNAc(1-2)-α-Man(1-3)
β-Man(1-4)-
α-NeuAc(2-6)-β-Gal(1-4)-β-GlcNAc(1-2)-α-Man(1-6)

276

An increase in temperature of the glycopeptides above 20 °C resulted in an increased ratio of the first two lines, h_+ and h_0, of the ESR spectra. This enhancement was attributed to spin–spin interactions that originate from collisions between nitroxyls.[102] A plot of h_+/h_0 vs. temperature for the two glycopeptides resulted in a ratio greater than 1 above 35 °C. This increase in h_+/h_0 was attributed to intramolecular collisions between the chains because a 60-fold dilution of the samples did not alter the ratio with temperature.[101] The trichain material (276) had the greater increase in the h_+/h_0 ratio, and this result was explained by the collisions between the supplementary chain and the two main chains of the carbohydrate portion. If only one terminal sialic acid of one chain was spin labeled, then the h_+/h_0 was always less than 1 at all temperatures because of a lack of intramolecular collisions.[101]

When the bichain glycopeptide (275) was frozen at −17 °C, the apparent τ_c was found to be greater than 100 ns, which is a measure of the intramolecular motion of the chain independent of the fast rotation of the nitroxyl radical. Similar results were obtained with the trichain glycopeptide 276.[101]

The addition of the lectin concanavalin A (con A) decreased slightly the mobility of the spin probe (τ_c = 1.10 ns at 20 °C) in the bichain but not in the trichain materials.[101] Con A is thought to bind to the *β-Glc-NAc (1→2)-α-Man* residue because the addition of methyl-α-D-mannoside, which causes a dissociation of the complex, reversed the τ_c to its original value without lectin. In spite of the fact that the con A binding site is several residues removed from the terminal sialic acids, which are spin labeled, there still was observed a decrease in the mobility of the nitroxyl moiety. It was concluded that this transmission effect is attributable to some rigidity in the polysaccharide chain.[101]

IX. Immunoglobulins

For the purposes of this review the emphasis is placed on investigations dealing with spin labeling of the

Figure 8. Schematic drawing of human immunoglobulin G(IgG). It is composed of two heavy chains (H) and two light chains (L) joined together by four disulfide bonds. Each chain is divided into regions of constant (C) and variable (V) amino acid composition. The darkened area is the hinge region. The immunoglobulin can be considered to be made of several homologous regions or domains (C_H1, C_H2, C_H3, C_L, V_H, V_L). Each domain is stabilized by intrachain disulfide bonds and has one or more functions. The antigen binding sites are located in the variable domains of light and heavy chains. For IgG the polysaccharide side chain (CHO) is attached to the C_H2 domain. For all immunoglobulin classes the light chains are similar and are of either κ or λ types. The heavy chains are specific for each class: γ(IgG), α(IgA), μ(IgM), δ(IgD), ϵ(IgE).[139]

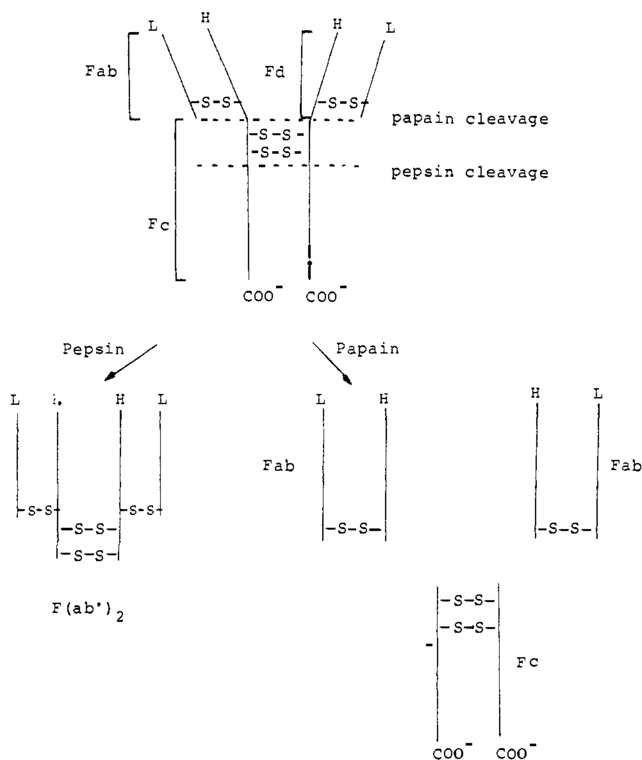


Figure 9. Fragments obtained by enzymatic cleavage of IgG. Fc (fragment, crystallizable) contains the carbohydrate component. Fab (fragment, antigen binding) contains the antigen binding sites.

carbohydrate portions of immunoglobulins. A review article by Nezlin and Sykulev[103] covers the literature to 1982, and includes papers dealing with labeling of the protein components.

A. Antibodies

The spin-labeling procedures for immunoglobulins (Ig) are generally the same as those for glycoproteins (section VIII). Thus, limited periodate oxidation is followed by reductive amination with Tempamine (**5**) to label the carbohydrate portion. Although this methodology introduces the label mainly into the terminal *N*-acetylneuraminic acid (sialic acid) residues, there is some nonspecific labeling of nonterminal sugar units since prior desialylation with neuraminidase does not block all incorporation of the spin label.[104] The protein component is labeled usually with the nitroxyl-*s*-triazine (**35**) or its pyrrolidine analogue. The ESR spectra of immunoglobulins belonging to different classes and labeled with the same spin label do not differ significantly.[103] The spin label to protein molar ratios for various immunoglobulins that were spin labeled on the carbohydrate groups are as follows: IgG, 1.0–1.8; IgE, 8; IgM, 30.[103] There is a higher carbohydrate content in IgE and IgM than in IgG.

A diagramatic structure for immunoglobulin G (IgG) is shown in Figure 8. The carbohydrate chain (CHO) is attached to the C_H2 domains of IgG. A comparison with Figure 9 reveals that the carbohydrate chain is located in the Fc region of IgG. Each of the Fab and Fc fragments (Figure 9) consists of four compact globules or domains stabilized by intrachain disulfide bridges (Figure 8) and bound to each other by less compact segments of polypeptide chains.

It is evident from Figure 9 that spin labeling of the carbohydrate component should give important information about the mobility of the carbohydrate chain as well as the mobility of the Fc region relative to the rest of the macromolecule. The specific labeling of the Fc region was reported.[104-107] Timofeev et al.[106] labeled human IgG and its papain Fc carbohydrate fragments with nitroxyl **5** and rabbit IgG and its peptic Fab′ and $F(ab')_2$ protein fragments with **35**. A study of the change of ESR spectra with increasing solution viscosity at constant temperature allowed an estimation of the mobility of the macromolecule relative to the rotational mobility of the spin label.[106] Well-defined outer wide lines as well as sharp inner lines appeared with increasing viscosity. According to an hypothesis of Käiväräinen et al.,[107,108] such patterns in ESR spectra can be explained by the existence of the spin label in restricted and rapid rotational states, respectively. More specifically, these two components of the ESR spectra are the result of the Fab segments of the macromolecule existing as two conformers: A (less mobile) and B (more mobile).[107] These two conformers exist because of Brownian motion of the domains. The A conformer corresponds to the state in which the distance between the domains of the light and heavy chains is the smallest possible while the B conformer is the state in which the distance is at a maximum.[107] On the basis of this model, the rotational mobility of a spin label attached to the Fab region would depend on its attachment to the A (slower) or B (faster) conformers.

This hypothesis was supported by spin labeling of different amino acid residues of immunoglobulin proteins.[109] ESR spectra corresponding to weakly immobilized and strongly immobilized spin labels were obtained.[109] Also halving of the spin-labeled dimers of L chains caused the more immobilized A component of the ESR spectra to disappear.[109] It was demonstrated that the two components of the ESR spectrum are not the result of the spin label's attachment to two different

sites on the IgG molecule but rather to an equilibrium between the A and B states of the spin label.[107]

The values of the distance between the outer wide lines (or maximum hyperfine splitting) were plotted vs. the temperature to viscosity (T:η) ratio.[106] The measurements were made at constant temperature (0 °C), and the solution viscosity was increased by the addition of sucrose. The linear part of the resultant isotherm was extrapolated to its interception with the y axis, i.e. extrapolation to infinite viscosity. The closer is the point of interception to the calculated rigid limit, the greater is the restriction of the mobility of the spin label.

The isotherms for human IgG and its Fc fragment were linear throughout, and its intercept was very close to the rigid limit.[106] This result was interpreted to mean that with increasing viscosity the label attached to the polysaccharide chain is immobilized at the same rate as the entire macromolecule.[106] In order for this immobilization to occur, the carbohydrate chain must be attached very closely to the protein component.[106] Support for this conclusion comes from X-ray data which indicate that the Fc polysaccharide chain is closely attached to the C face of the C_H2 domain.[110] Furthermore, ^{13}C NMR[111] and X-ray[112] data corroborate the concept of a rigid polysaccharide chain fixed on the protein surface and covering either nonpolar amino acid residues or hydrophobic side chains of polar amino acids of the C_H2 domains.

By way of contrast, the isotherm for rabbit IgG and its Fab′ and F(ab′)$_2$ fragments showed a linear portion (immobilization of the protein) followed by a sharp rise near the y axis (immobilization of the spin label) with increasing viscosity.[106] The y intercept was lower than that for the Fc component. Thus, the nitroxyl attached to the protein component has a greater mobility than that attached to the carbohydrate.

Mathematical manipulation of these plots[113-115] allowed calculation of the τ_c values.[106] The τ_c values calculated for rabbit IgG and its Fab′ and F(ab′)$_2$ fragments spin labeled on protein were 26, 21, and 25 ns, respectively.[106] These results were in qualitative agreement with the values of the rotational relaxation time (ρ_h) obtained from fluorescent polarization studies of a DNS–IgG conjugate (60 ns) and the DNS–Fab fragment (64 ns),[116,117] and are considerably smaller than the ρ_h value (220 ns) calculated for a rigid sphere of the same molecular weight (50 000).[116] These results lead to two conclusions: First, the lower values of both τ_c and ρ_h support a model of the IgG macromolecule that has an appreciable flexibility because of Brownian rotation of its subunits. This rotation could be attributed to the flexibility of the polypeptide chains connecting the domains of the Fab region. Second, the similarity of the τ_c[106] and ρ_h[116,117] values for IgG and its Fab fragments indicates a relatively compact structure for the Fab component.[106,117]

By contrast, the τ_c values for IgG and its Fc fragment labeled at carbohydrate were 11 and 12 ns, respectively.[106] The value for DNS–Fc was 37 ns.[117] The fact that both the τ_c and ρ_h values for the Fc fragments were considerably smaller than those for the Fab fragments indicates that the Fc component of IgG has a looser, less compact structure.[105,117] Support for this conclusion comes from X-ray data that indicate that the Fc fragment is not compact since the C_H2 domains are widely separated from each other.[110]

From these studies there arises a dynamic picture of the IgG macromolecule. A rigid polysaccharide chain is attached to a looser, less compact Fc subunit which, in turn, is attached to a more compact Fab subunit. The overall IgG structure has appreciable flexibility because of Brownian motion of the subunits, arising from the flexibility of the polypeptide chains connecting the various domains.

Human myeloma immunoglobulin M(IgM) was spin labeled in the same manner as IgG.[103,118-120] IgM contains five carbohydrate chains, one of which is present in the Fab region. Evidence was presented that the label **5** is attached to the carbohydrate chain located in the (Fc)$_5$ fragments and not to the Fab fragment.[118] Labeling the Fc regions of IgM, its 7S subunit (IgM$_{7S}$), and (Fc)$_5$ fragments yielded nearly identical τ_c values, ranging from 6.5 to 7.5 ns.[103] The ESR spectrum consisted of two components as a result of increasing the viscosity at constant temperature. This result could be attributed to the binding of nitroxyl **5** to two types of carbohydrate chains in IgM, one more mobile the other less mobile.[118] The fact that the τ_c values for **5** attached to IgG and IgM were similar indicates that the label in IgM was bound to a carbohydrate chain of one of the Fc domains possessing appreciable flexibility.[118]

The spin labeling of IgM with the pyrrolidine analogue of **35** resulted in a τ_c value of 50 ns that was much less than that calculated for a rigid sphere of the same molecular weight (300 ns).[120] Again, this result indicates an overall increased mobility of the Fab component in IgM. The same conclusion was obtained from fluorescence polarization studies.[120] A comparison of τ_c values for Fab fragments isolated and included in immunoglobulin molecules reveals that this subunit has an increasingly restricted mobility in the order: Fab–F(ab′)$_2$–IgG–IgA–IgM.[118]

Incorporation of a spin label into the protein component of human myeloma immunoglobulin A (IgA) produced the same results as for IgM.[120] The τ_c value for IgA labeled with **35** was 32 ns.[119] The corresponding ρ_h value from fluorescent polarization studies was 33 ns,[120] far less than the calculated value of 62 ns for a hydrated sphere with the same molecular weight and partial volume of IgA.[121] Fluorescent polarization studies of DNS–Fab conjugates of IgA[122] produced a value of ρ_h = 25 ns, which was very similar to that for the DNS-IgA conjugate (33,[120] 26 ns[122]). These results point to an increased mobility for the Fab subunits in IgA.[120] Both the protein and carbohydrate components of IgA1 and IgA2 were spin labeled.[123] The Fc fragment of IgA1 appears to have a rigid structure. The labeled oligosaccharide chains of IgA2 were shown to possess great rotational mobility while some of these chains of IgA1 had restricted mobility because of a close attachment to the protein component.[123]

A study of human immunoglobulin E(Yu)(IgE(Yu)) spin labeled on protein with **35** revealed that IgE possesses segmental flexibility, but it is less than that of IgG.[124] The τ_c values of the spin label relative to that of the macromolecule were 60 ns for IgE and 35 ns for IgG. These values were considerably lower than those calculated on the assumption of a rigid sphere, i.e. 117 and 96.5 ns, respectively.[124] Heating solutions of labeled

IgE and IgG increased the mobility of the spin label in the B state and had no effect on the mobility in the A state.[124] This result is in agreement with the concept that the A conformer represents an inflexible state of the immunoglobulins.[107] The proposal was made that the lower segmental flexibility of IgE is caused by an additional bonding between Fab segments which results in their joint rotation around the Fc segment.[124] This peculiarity of the IgE structure agrees with the fact that the IgE antibodies do not form a precipitate after combining with the antigen.[124] The carbohydrate group of IgE(Yu) was spin labeled.[105]

B. Antigen–Antibody Complexes

Some intriguing results have come from studies of antigen–antibody interactions by analysis of the ESR spectra of their spin-labeled carbohydrate fragments. As can be seen from Figures 8 and 9 the antigen binding site is located on the Fab region. The reaction of labeled anti-human IgG antibody with its antigen (papain hydrolysate of IgG)[105] and rabbit anti-Dnp antibody with its antigen (ϵ-Dnp-lysine)[104] produced no changes in the ESR spectrum of the spin label in the Fc region. The binding of ϵ-DNP-lysine to rabbit anti-DNP antibody is not affected by spin labeling of the Fc region since fluorescent titration still indicated six binding sites per antibody molecule with an average association constant of 10^8 (M^{-1}).[104]

Limited aggregation of spin-labeled nonimmune rabbit IgG by protein A (a cell wall protein *S. aureus* that binds to the Fc region) did not affect the ESR spectrum, although the labeled IgG was still capable of a complement fixation in the presence of protein A.[104] Also, the binding of Dnp–BGG to the spin-labeled anti-Dnp antibodies produced a precipitate whose ESR spectrum was interpreted to indicate considerable mobility for the nitroxyl label.[104] Apparently even with precipitate formation the nitroxyl label **5**, bound to the carbohydrate chain on the C_H2 domain of the Fc region, possessed a rotational mobility independent of the protein. It was suggested that the binding of Dnp–BGG by IgG does not cause a large conformational change at the C_H2–C_H2 domain interface.[104] Either glycoprotein M or its antibody were labeled with **7b** at pH 7 and 9.[125] There was reported no change in τ_c values during immune reaction, indicating that the nitroxyl's mobility in solution was not affected significantly during the immunological reactions.[125]

These results are in contrast to those obtained in experiments in which the spin label was bound to the protein component of the IgG antibody.[107–109] For example, antibodies spin labeled on protein with either **35** or **7b** were prepared against immunoglobulins or other substances.[107] The ESR spectra of the resulting complexes between labeled antibodies and antigens indicated an increase in the less mobile **A** conformer.[107] The τ_c values of the spin label increased, corresponding to a decreased rotational mobility of the label attached to the protein of the Fab segment. This apparent conformational change was the same for all antibodies and was not dependent on the particular antigen or its site of attachment to the immunoglobulin molecule.[107,108] This increase in τ_c values occurred for several combinations, including donkey antibodies + human IgG, rabbit antibodies + bovine IgG, and rabbit antibodies + hemoglobin (Hb).[107]

Another set of experiments involved the preparation of antibodies against the protein spin labels **35** or **7b** followed by their reaction with antigens spin labeled with either **35** or **7b**.[107] The combinations of donkey antibody + human IgG-35, rabbit antibody + Hb-7b, and pepsin hydrolysate ($F(ab')_2$) of rabbit antibody + Hb-7b were used.[107] In these cases the resultant ESR spectra indicated a decrease in the less mobile **A** conformer and an increase in the more mobile **B** conformer. These results were attributed to an increased flexibility in the variable (V) region sites where the spin-labeled antigen was bound.[107] The important concept that changes in the heavy- (H) chain domains of the Fab region could be transmitted to the heavy chains of the Fc region on antigen binding (Figure 9) was proposed.[107] This transmission effect could account for the decreased flexibility of polypeptide chains joining domains following the formation of antibody–antigen complexes.[107]

Other studies on the changes of immunoglobulin structure during the complex formation with haptens (antigens) were reported.[126,127] The interaction of the hapten ϵ-DNS–lysine with anti-DNS IgG (spin labeled on protein with either **35** or **7b**) caused a slow change in the ESR spectrum characteristic of a conformational change in the IgG macromolecule.[127] This change occurred for several minutes after the binding of the hapten to the antibody. The nitroxyl label did not quench the fluoresence of the ϵ-DNS–lysine, and therefore, it was concluded that the change in the ESR spectrum was not caused by a close contact between the hapten and the spin label.[127] Also the hapten binding did not change the τ_c value of 26 ns for the spin-labeled IgG antibody.[127]

A further investigation of this change involved the interaction of the hapten ϵ-DNS–lysine with pig anti-DNS antibodies spin labeled at the carbohydrate moiety.[128] The ESR spectrum of the resulting complex again was characteristic of a conformational change, with the τ_c value different from that before binding.[128] It was shown that binding of the hapten occurred in the Fab region of these antibodies.[128] These two results when combined support the concept that hapten binding at the Fab region causes a conformational change which is transmitted through the macromolecule to the Fc region, causing a change in rotational mobility of the nitroxyl spin label.[103,107]

Mention was made of a study of a complex formation between a spin-labeled polysaccharide antigen from pneumococcus SII and an excess of rabbit antihuman IgG antibody.[103] There was a change in the ESR spectrum characteristic of a reduced mobility of bound spin label. This change in mobility probably occurred because of an immobilization of a part of this large antigen in the antigen combining site.[103]

Some information was reported concerning the separation of the two Fab antigen sites in IgG (Figure 8).[128] IgG antibodies against spin-labeled hemocyanin were prepared. Papain and pepsin-mediated hydrolyses of portions of the labeled IgG produced Fab and $F(ab')_2$ fragments, respectively. The ESR spectra of solutions of spin-labeled hemocyanin and antibody IgG and its $F(ab')_2$ and Fab fragments yielded τ_c values of 32, 31, and 18 ns, respectively.[128] The lower value for the free Fab fragment demonstrated that its motion is relatively

restricted in the more complex systems of F(ab′)$_2$ and IgG. It was possible to calculate the distance between the spins attached to the F(ab′)$_2$ fragment from the enlarged central line (dipole–dipole interaction) of its ESR spectrum.[128] At pH 6.3 and 25 °C this distance was found to be 15.2 Å. Thus, spin–spin interaction occurs between Fab′ subunits of the F(ab′)$_2$ complex in the absence of the Fc component at pH 6.3. There was no dipole widening in the ESR spectrum of the intact IgG–antigen complex, which indicated that the Fc component stabilizes the Y-shape of IgG and prevents interaction between the Fab subunits.[128] This interaction of the Fab′ subunits of F(ab′)$_2$ fragments occurs only at pH 6.3, which is the isoelectric point.[128] When the pH is above or below this point, the interaction between the Fab′ subunits is prevented by electrostatic repulsions. The distance between the antigen combining sites in the F(ab′)$_2$–antigen complex is similar to the Stokes radius (15–20 Å) of the domains of immunoglobulin peptide chains.[128] Therefore, one can infer that the antigen combining sites are located in one of the two domains (V_H and V_L) (Figure 8).[128]

X. Nucleosides and Nucleotides

Most of the spin labels on nucleosides and nucleotides have been attached to the pyrimidine and purine bases.[16,129] This section will be limited to those molecules in which the spin label is covalently attached to the carbohydrate moiety.

A. Nucleosides and Mononucleotides

Nucleosides and nucleotides were labeled in the 2′- or 3′-positions of the ribose sugar by the reaction with the imidazole spin label **12c**.[130] Reactions of the nucleoside and nucleotides in water with **12c** in tetrahydrofuran at 60 °C for 10 min and then at room temperature for 5 h yielded the monoradicals nucleoside and nucleotide 5-mono-, di-, and triphosphates (**277a–d**). There was obtained also a 14% yield of a diradical with the nitroxyl located on either 2′- or 3′-positions and on the 5′-phosphate. There was no acylation of the amino group in the purine moiety by this procedure.[130]

277a, X = H (75%)
b, X = PO_3^- (60%)
c, X = $P_2O_6^{2-}$ (45%)
d, X = $P_3O_9^{3-}$ (40%)

When the nitroxyl acid chloride **12b** was used for acylation, there was obtained a mixture of O- and N-acylated products.[131] Thus, the reaction of adenosine 5′-monophosphate (**278a**) with **12b** produced the diradical **279a** with a radical on either the 2′- or 3′-position while the reaction of adenosine 3′-monophosphate (**278b**) yielded the triradical **279b** (Scheme XXXI).[131] The alternate diradical structure containing nitroxyls on both the 2′- and 3′-positions was eliminated from consideration by the absence of any spin exchange coupling in the ESR spectrum.[131] Analogously, the reaction of **12b** with cytidine 3′-monophosphate (**280a**) and cytosine (**280b**) gave the diradicals **281a** and **281b**, respectively.

SCHEME XXXI

278a: R = R′ = H, R^2 = PO_3^{2-}
b: R = R^2 = H, R′ = PO_3^{2-}

279a: R = R′ = H, R^2 = PO_3^{2-}
b: R = R^2 = CO-(nitroxyl), R′ = PO_3^{2-}

280a: R = PO_3^{2-}, R′ = H
b: R = R′ = H

281a: R = PO_3^{2-}
b: R = H

Selective O-deacylation of **279** and **281** was accomplished by the reaction with 1 M sodium hydroxide solution at a pH greater than 12 for 10–15 min. This procedure yielded the spin-labeled *N*-acyl derivatives **282a,b** and **283a,b**, respectively (Scheme XXXII).[131] The compounds were characterized by UV, IR, and ESR spectra, but no microanalyses were given.[131]

Spin-labeled adenine and adenosine 5′-mono-, di-, and triphosphates (**277a–d**) were used to study the self-association of these purine bases in aqueous solution.[132,133] When unlabeled ATP was added to a solution of the labeled ATP (**277d**) in 1 M sodium chloride at pH 8,

SCHEME XXXII

279a,b $\xrightarrow[\text{pH 12}]{\text{base}}$

282a: R=H, R′=PO_3^{2-} (80%)
b: R=PO_3^{2-}, R′=H (83%)

281a,b $\xrightarrow[\text{pH 12}]{\text{base}}$

283a: R=PO_3^{2-}
b: R=H

the ESR spectrum was characteristic of a nitroxyl radical with a reduced rotational mobility, corresponding to an increase of τ_c from 0.10 to 0.51 ns.[132] This result is a reflection of increased associations between the nucleotide bases. Interestingly, addition of unlabeled uridine to the spin-labeled uridine caused no change in the τ_c values.[132] Apparently there is little or no association between the pyrimidine bases in aqueous solution.

The self-association phenomenon was attributed to a stacking of the purine bases one on top of the other in neutral solution.[132,133] In an acidic medium at pH 4 the τ_c values for **277b–d** increased in the presence of unlabeled ATP, to 0.68 ns.[132] This result was explained by an association between the positive ammonium ion of the purine base and the negative phosphate ion of an adjacent ribose sugar.[132,133]

At neutral pH the association constants K_a of the adenyl nucleotides (AMP, ADP, ATP) **277b–d** were significantly greater than that for adenosine (**277a**).[133] This result could be explained by a stabilization of water structure by the phosphate anion.[133] The association constants of AMP, ADP, and ATP were almost independent of the nucleotide phosphate chain length and were equal to 9.7 ± 0.3 M^{-1} in 0.1 M sodium chloride at pH 7.5.[133] The explanation for this finding was that the phosphate anions are at their maximum distance apart in solutions of this ionic strength and the differences in electrostatic attraction among the nucleotides have little effect on self-association.[133] When the pH was changed to 4, the K_a values nearly doubled.[133] This result was a reflection of the previously described base–phosphate interaction.

The thermodynamic factors ΔG, ΔH, and ΔS for the nucleotide self-association were determined from the relationship of K_a with temperature[133] which, in turn, was determined from the dependence of the rotational frequency of the spin-labeled nucleotide on the concentration of the unlabeled nucleotide at different temperatures. The ΔH and ΔS values for adenyl nucleotide self-association were −4.2 kcal/mol and −9 eu, respectively,[133] and the corresponding values for adenosine were −4.8 kcal/mol and −15 eu. It is evident that a more favorable entropy (ΔS) factor for nucleotide self-association is responsible for their increased free energy values. The important factor is most likely an increase in the solvent entropy arising from a decrease in hydration during the self-association process.[133] The authors state that nucleotide association may play a role in intracellular regulation.[133]

B. Polynucleotides

This study on spin labeling and self-association has been applied to polynucleotides.[134-136] The structure of the complex which is formed between the poly U and trideoxyadenylic acid, $d(pA)_3$, was studied by the use of the spin-labeled derivative S-$d(pA)_3$ (**284**).[134] In this case the amino nitroxyl is attached to the 5′-terminal phosphate group. It was prepared by the reaction of Tempamine (**5**) with a diphenyl chlorophosphate activated oligonucleotide.[137]

S—$d(pA)_3$ ≡ ·O—N(tetramethylpiperidinyl)—NHpdApdApdA
284

S—$(pU)_8$ ≡ ·O—N(tetramethylpiperidinyl)—NH—pU—$(pU)_7$
285

The ESR spectrum of the complex S-$d(pA)_3$·**2**·(poly U) appeared to contain two different signals, with one having broadened h_+ and h_- lines. This observation was used to support a model which predicted an antiparallel orientation of the two nucleotides resulting in 5′,5′ contacts between radicals and limiting the radical's mobility.[134a]

As a further test of this model a complex between spin-labeled octauridylate, S-$(pU)_8$ (**285**), and poly A was studied. The ESR spectrum contained only a reduced h_- line. This result was interpreted to support a parallel orientation of these nucleotides in which only 3′,5′ radical contacts are possible.[134]

Poly U, poly C, and poly A were spin labeled with the imidazolyl derivative **12c** which selectively acylated the 2′-hydroxyl of the ribose sugars (**286**).[135] The spin label,

286

located as it is on the 2′-position, is sensitive to changes in polynucleotide conformation while at the same time it is not interfering with centers which are responsible

TABLE VIII. Spin-Labeled Polynucleotides[135]

matl[a]	yield, % spin label per poly N	τ_c, ns	ΔE, kcal/mol
poly A	1.5 ± 0.2	3.21	7.5 ± 0.2
poly C	1.7 ± 0.2	2.53	6.9 ± 0.2
poly U	4.2 ± 0.2	1.24	5.7 ± 0.2

[a] A = adenosine; C = cytosine; U = uridine.

for hydrogen bonding and stacking interactions of bases. This noninterference concept was based on the nearly identical UV and CD spectra of the spin-labeled and -unlabeled polynucleotides.[135]

The extent of spin labeling was found to be independent of the nature of the base and was determined entirely by the rigidity of the secondary structure of the polynucleotide.[135] The yields, correlation times (τ_c), and activation energies (ΔE) of the spin-labeled polymers are shown in Table VIII.

The ESR spectra of these labeled polynucleotides were complex, and some interesting results were obtained from studies of the variation of the h_0 to h_+ ratio with pH for these polymers.[135] At neutral pH and room temperature poly A and poly C are single-stranded helical structures while poly U is disordered. As the acidity was increased to pH 5.8, a sharp cooperative effect occurred as evident from the curve of h_0/h_+ vs. pH for poly A and poly C.[135] This effect represents the transition from a single- to a double-stranded structure. In the case of poly U there was only a horizontal line for the pH range 3.5–9, indicating a disordered structure. At pH 4 the spectra of poly A and poly C consisted of two types of signals: wide outer lines, corresponding to immobile spin labels located in the double-helical areas, and narrow inner lines, corresponding to more mobile labels attached to the end nucleotide residues.[135]

As can be seen from Table VIII poly U possesses a better reactivity with spin label **5**. The rotational mobility of its attached nitroxyl radical is greater (lower τ_c value), and the activation energy of its nitroxyl's motion is lower than those of poly A or poly C. All these results support a disordered structure for poly U.[135]

Plots of temperature vs. the h_0 to h_+ ratio for different pH values provided useful information about the behavior of these biopolymers in solution.[135] At pH 4 the protonated double-helical poly A produced a sigmoid- (S-) shaped temperature-dependence curve, indicating a cooperative melting process. For the single-stranded poly A at pH 7.5 there was an exponential decrease in the h_0 to h_+ ratio with increasing temperature. These temperature curves must be interpreted with caution because they include changes in the rotational mobility of the spin label independent of any structural change in the polynucleotide. From the graph at pH 7.5 an ESR "melting temperature" (T_M^{SP}) of 50 °C was obtained, which differed significantly from the UV "melting temperatures" (T_M) of 76 and 78 °C for spin-labeled and -unlabeled poly A, respectively.[135] These results reveal that the nitroxyl spin label lowers the T_M because of some destabilization of the polynucleotide structure.[135]

A study was conducted on the complex formation between the spin-labeled poly U and the pyrimidine bases uridine and cytosine.[136] In this case the binding was much weaker than that with the purine bases. Plots of τ_c vs. concentration of the nucleosides U and C revealed a positive cooperativity of binding.[136] Thermodynamic parameters were calculated from binding isotherms. The equilibrium constants for nucleation (K') and for chain growth (K) of complexes of poly U with uridine and cytidine were about a 100-fold lower than those for the complexes formed with purine nucleosides.[136]

ESR studies of the interaction of spin-labeled polynucleotides with rat liver ribosomes were reported recently.[138]

XI. Future Work

As revealed in this review spin-labeled carbohydrates have been used for a variety of purposes. Much future work could be based on the types of studies discussed herein.

The monomeric sugars have been involved in research on the structures of enzymes,[21] lectins,[142] and epimers,[39] the properties of the glycosidic bond,[46] and the bioenergetics of cells.[38] They have been proposed also as probes for cell membrane structure.[39]

Spin labeling of polysaccharides has been used to study their viscosity,[62,65] gel–sol transitions,[54,58,62,63,65] structural features,[31–33,54,56,58,80,143] surface properties,[64,67,68,70] enzymatic depolymerization,[69] and distribution of different sugar subunits.[54,56] A spin-labeled cyclodextrin was used to test a model of an enzyme-catalyzed reaction.[94]

In the area of glycoconjugates, spin labeling of the carbohydrate portion has revealed details about their chain structure in glycoproteins[96,101] and immunoglobulins,[103,106,118] transmission effects between carbohydrate and protein components in glycoproteins[96,101] and immunoglobulins,[103–105,107,126,127] and the structure and self-association in solution of nucleotides[132,133] and polynucleotides.[134–136] No doubt, these and related areas will be pursued over the coming years.

Another area of research worthy of attention would be the synthesis of *unblocked* spin-labeled monosaccharides. These would be of value in biological and medical research (NMR imaging for example) because of their expected water solubility. Consequently, there is a need for simple, high-yield reactions to give spin-labeled unblocked carbohydrates. Preferably, the label should be attached to the 2- or 3-positions, which do not seem to have important binding to the cellular membrane. In many instances the C-1 glycosidic and C-6 positions should remain free.[27] If blocked analogues are employed in the synthesis, methodologies should be developed which would permit the removal of the protecting moieties under mild conditions without destruction of the nitroxyl radical. In this regard silylated sugar derivatives could prove to be of use.[27]

XII. Summary

A comprehensive review of the spin labeling of carbohydrates, covering the literature through Oct. 1985, is presented. All classes of carbohydrates are discussed in terms of chemical synthesis, chemical and physical data, and some interpretations of ESR spectra. Important information is presented about the structures of monosaccharides, disaccharides, polysaccharides,

cyclodextrins, glycoproteins, immunoglobulins, nucleosides, and nucleotides.

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XIII. Addenda

For reviews see ref 140–142.

The following spin-labeled glucosamine analogues were used as haptens to study the combining site of anti-streptococcus group A antibodies.[143]

A series of spin-labeled β-D-galactopyranosides were synthesized as analogues of the natural inducer of the *E. coli* lactose operon allolactose.[144]

R	R'	R²
NO_2	Ac	OAc
H	Ac	OAc
NO_2	Ac	H
NO_2	H	OH
H	H	OH
NO_2	H	H

These imino nitroxyl compounds were prepared by the same sequence of reactions as used by Tronchet et al.[42] (see Scheme XXIII). These compounds are analogues of (*o*-nitrophenyl)-β-D-galactopyranoside and -fucopyranoside, which are described as a neutral effector and an anti-inducer of the *lac* operon, respectively.[144] In an extension of earlier work,[39] Tronchet et al. reported[145] that the nuclophilic addition of dialkyl phosphites to the sugar nitrone **83** (R = H, Me) and its C-3 isomer (see Scheme XXI) yielded a series of α-deoxy-α-(*N*-hydroxy-*N*-methylamino) sugar phosphonates. These *N*-hydroxy derivatives were oxidized by air to the corresponding nitroxyls. At certain concentrations, both the NMR spectra of the hydroxylamines and the ESR spectra of the corresponding nitroxyl radicals could be recorded. The ESR spectra of the nitroxyl derivatives were used to discriminate between the *R* and *S* epimers. Apparently, in solution the following equilibrium exists

$$\text{sugar—N—OH} \rightleftharpoons \text{sugar—N—O}\cdot$$

It was suggested[145] that under these conditions the couple might be applicable as a probe to biological studies. Oxidation of the *N*-hydroxy derivatives with DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) resulted in a cyclic nitrone arising from the condensation of the C-3 OH group with an intermediate "methylene nitrone".[145]

The incorporation of a nitroxyl spin label at the reducing end of dextran using the reductive amination method has been reported recently by Yalpani and Brooks[146] (see ref 31–33 for the nonspecific labeling of dextrans).

Several reports concerning the use of spin labeling of carbohydrates to study lectin binding to cell membranes have appeared recently. Goldstein et al.[147] have reported on the binding characteristics of the spin-labeled glycosides **26b**, **78**, and **80b**[25] with the tetrameric α-D-galactosyl (GS-I-B_4) and *N*-acetyl-α-D-galactosaminyl (GS-I-A_4) binding isolectins from *Griffonia simplicifolia* seeds. Farmer and Butterfield[148] have used a perdeuterated [^{15}N]tempamine spin label to selectively monitor cell surface sialic acids of human erythrocyte membranes (see ref 99) as well as the effects of wheat germ agglutinin (WGA) on the motion of terminal sialic acids of membrane glycoconjugates. Sharom and Ross[149] have used spin-labeled sialic acid and galactose residues of pig lymphocyte plasma membrane to study their interaction with specific lectins. A differential restriction in oligosaccharide motion following lectin binding was attributed partially to the sugar specificity of the lectins.[149] A concentration dependence of the lectin-induced spin-label immobilization suggested a cooperative binding between lectins and their receptors[149] (see ref 96 for similar conclusions).

Ebel et al.[150] determined the equilibrium constant for the association of 2,2,5,5-tetramethylpyrrolidin-1-oxyl and β-CD by monitoring by ESR spectroscopy the kinetics of the reduction of the nitroxyl by the ascorbate anion in the presence of varying concentrations of β-CD in phosphate-buffered solution. A stopped-flow technique was used[151] to study the rates of the ascorbic acid reduction of DTBN (di-*tert*-butylnitroxyl), tempo (2), and Tempol(4) in the presence of β-cyclodextrin.

Spin labeling of the Fc fragment of a IgG1 antibody provided evidence for flexibility within the C_H3 domains.[152]

XIV. References

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