Carbohydrate Research, 116 (1983) C1—C4
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Preliminary communication

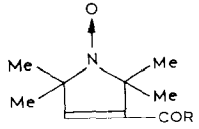
Spin-labeling of polysaccharides by esterification using 3-chloroformyl-2,2,5,5-tetramethylpyrroline-1-oxide*

THOMAS P. MAWHINNEY†, KATHRYN I. FLORINE†, MILTON S. FEATHER†, and DAVID L. COWAN‡

Departments of Biochemistry and Child Health and Physics, University of Missouri, 322-A Chemistry Building, Columbia, Missouri 65211 (U.S.A.)

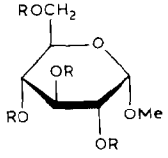
(Received January 7th, 1983; accepted for publication, February 24th, 1983)

Investigations of the paramagnetic resonances of synthetic, organic free-radicals covalently bound to biomolecules have clearly demonstrated that the use of spin labels is an invaluable asset in studying the conformational alterations and mobility of macromolecules, and an aid in elucidating the structure and function of biological membranes. In contrast to the many available reagents for the spin-labeling of lipids and proteins, though, there is a general lack of spin-labeling reagents suitable for carbohydrates. Only recently has this void been filled by methods developed for the spin-labeling of such compounds as sialic acid residues on glycoproteins[1,2], agarose[3], cotton fibers[4,5], and α- and β-glycosides[6]. We report herein a facile procedure for the introduction of covalently bound spin-labels onto dextrans, amylose, xylan, mannan, locust-bean gum, and guar gum *via* an ester linkage by use of 3-chloroformyl-2,2,5,5-tetramethylpyrroline-1-oxide (1).

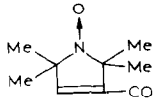


1 R = Cl

2 R = OH



3 R = H,



*This work was supported by a grant (HL-19160) of the Heart, Lung, and Blood Institute, National Institutes of Health, and by a grant (DHHS BRS 5387) of the University of Missouri Medical Center Research Council.

†Department of Biochemistry and Child Health.

‡Department of Physics.

0008-6215/83/$03.00

Reagent **1** (94% yield) in benzene was prepared by treating 3-carboxy-2,2,5,5-tetramethyl-3-pyrroline-1-oxide (2, 0.11 g, 0.59 mmol; Eastman Kodak Co., Rochester, New York 14650) in dry benzene (2.1 mL) and dry pyridine (60 μL) with thionyl chloride (54 μL, 0.70 mmol) under a nitrogen atmosphere in a 3.0-mL reaction vial, as previously described[7,8]. Polysaccharides to be labeled were first dried *in vacuo* for 4 h and at 50°, then dissolved in dry dimethyl sulfoxide (20–30 mL) under nitrogen in a two-necked, round-bottom flask equipped with a rubber septum and an overhead stirrer. Into this solution was slowly added, with moderate stirring, the carbanion reagent [30 mL of dry dimethylsulfoxide and 0.28 mL of 1.2mM sodium methylsulfinylmethanide (dimsyl sodium)][9]. Following this addition, the polysaccharide solution was stirred for 1 h at room temperature, and then the solution of **1** in benzene was slowly injected (≈2.0 min) into the stirred polysaccharide solution. After continued stirring at room temperature for 20 min, the entire reaction mixture was exhaustively dialyzed against running water for 48 h. In order to remove a small amount of adsorbed spin label, the samples were dialyzed against 30% ethanol (3 × 1 L, 8 h each), overnight against distilled water at 55°, and finally lyophilized.

For most studies, 2.0 g of polysaccharide was utilized for each experiment. Only 0.5 g each of locust bean and guar gums were used because of their solubility and very high viscosity in dimethyl sulfoxide.

Methyl β-D-glucopyranoside (2.0 g) was similarly treated, except that instead of being dialyzed, the reaction mixture in dimethyl sulfoxide was poured into benzene (400 mL), stirred for 1 h at room temperature, and then stored for 10 h at 4°. The benzene–dimethyl sulfoxide supernatant solution was removed from the resultant, yellow glycoside syrup and discarded. The syrup was washed several times with cold benzene, dried, and finally subjected to t.l.c. on Silica gel G developed with 8:10:1 (v/v) 1-butanol–acetone–water. Methyl β-D-glucopyranoside and the mixed isomers of methyl β-D-glucopyranoside monosubstituted with the nitroxide spin-label (**3**) exhibited R_F 0.6 and 0.9, respectively.

Anal. Calc. for $C_{16}H_{26}NO_8$: C, 53.33, H, 7.22, O, 35.55; N, 3.88. Found: C, 52.58; H, 6.93; O, 34.62, N, 3.56.

Electron-paramagnetic resonance (e.p.r.) spectra of representative spin-labeled compounds in aqueous solution are shown in Fig. 1. Fig. 1A is the e.p.r. absorption-derivative spectrum of the free radical **2** at room temperature. The three sharp lines produced by nitrogen hyperfine-interaction are typical of a free nitroxide radical rapidly tumbling in solution. Also shown are the e.p.r. spectra of aqueous solutions of dextran (M_r av. 10 500), amylose, xylan, and locust-bean gum derivatives (Fig. 1B–1E, respectively). These spectra are characteristic of covalently bound, and more motionally restricted, nitroxide radicals[10]. In Table I are presented the values of motional-correlation times (τ), which were calculated, assuming isotropic motion, by a method previously described[11].

Significance of the determined correlation times will not be discussed at this time, but it should be noted that these times are considerably shorter than those reported for spin-labeled proteins of comparable molecular weight[10]. This illustrates the greater degree of motional freedom generally associated with polysaccharide molecules. It is noteworthy that the increase in τ value from the free spin-label to the labeled methyl β-D-gluco-

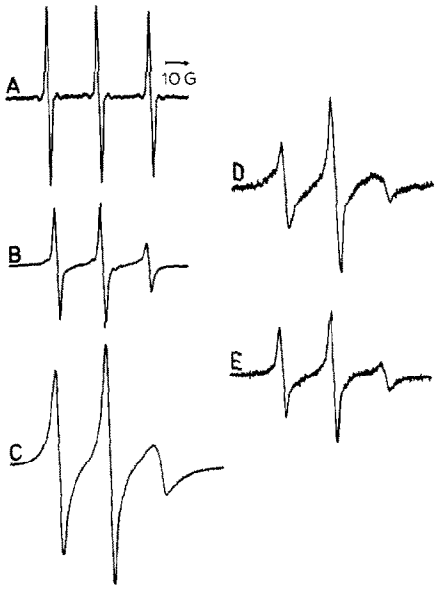


Fig. 1. E.p.r. spectra of spin-labeled compounds. Absorption-derivative spectra were recorded at room temperature for an aqueous solution in a quartz sample-cell with a Varian model V4502 X-band spectrometer, at a modulation frequency of 100 kHz, a power level of 1 mW, and a modulation amplitude of 0.4 G: (A) Free-radical spectrum of 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxide (2) in water; (B) dextran of M_r av. 10 500 (5 mg/mL of water); (C) amylose (10 mg/mL of water); (D) xylan (5 mg/mL of water); and (E) locust bean gum (5 mg/mL of water).

TABLE I

CORRELATION VALUES (τ) FOR POLYSACCHARIDES AND METHYL β-D-GLUCOPYRANOSIDE HAVING 3-FORMYL-2,2,5,5-TETRAMETHYLPYRROLINE-1-OXIDE RESIDUES

Compound	τ (ns)[a]
Derivative of:	
methyl β-D-glucopyranoside (3)	0.10
dextran (M_r av. 10 500)	0.55
dextran (M_r av. 40 000)	0.88
dextran (M_r av. 70 000)	1.00
dextran (M_r av. 151 000)	1.08
dextran (M_r av. 250 000)	1.15
amylose	1.23
xylan	1.88
mannan	1.30
locust bean gum	1.06
guar gum	1.12
3-Carboxy-2,2,5,5-tertramethylpyrroline-1-oxide (2)	0.025

[a]Error ±5%.

pyranoside by a factor of four is consistent with the Stokes- Einstein expression for a tumbling molecule, in which the correlation time is proportional to the cube of the radius of the equivalent hard sphere. It is also noteworthy that a molecular-weight dependence is observed for the correlation times of the spin-labeled dextrans.

Absolute proportions of spin-labeling residues were determined by comparison of the e.p.r. signals of spin-labeled samples with those of standard solutions of **2**, and it was found that the average binding efficiency of **1** with the polysaccharide samples was 10%. This corresponds to ~1 nitroxide spin-label residue per 200 carbohydrate-monomer units. In parallel experiments where the proportion of reagent **1** was varied, it was established that, at this proportion of labeling residue, no contribution to linewidths from spin exchange or dipole- dipole interaction between spins could be observed. A decrease in labeling efficiency was observed with polysaccharide solutions in dimethyl sulfoxide of increasing viscosity.

In summary, a facile procedure for the introduction of a covalently bound spin label into polysaccharides is described. Since the attachment of the nitroxide spin-label is *via* an ester linkage with hydroxyl groups along the polysaccharide molecule, it is not necessary to chemically modify the sample for the binding of the spin label prior to formation of the derivative. In addition, this method permits the preparation of gram quantities of polysaccharide derivatives and requires only dialysis for purification of the sample. Lastly, this procedure may also be applied for the binding of spin labels to methyl glycosides.

REFERENCES

1 J. D. Aplin, M. A. Berstein, C. F. A. Culling, L. D. Hall, and P. E. Reid, *Carbohydr. Res.*, 70 (1979) C9–C12.
2 J. D. Aplin, D. E. Brooks, C. F. A. Culling, L. D. Hall, and P. E. Reid, *Carbohydr. Res.*, 75 (1979) 11–16.
3 J. D. Aplin and L. D. Hall, *Carbohydr. Res.*, 75 (1979) 17–29.
4 R. Marupov and P. Kh. Bobodzhanov, *Biofizika*, 24 (1979) 519–527.
5 R. Marupov, I. Kh. Yusupov, P. Kh. Bododzhanov, V. K. Kol'tover, and G. I. Likhtenshtein, *Dokl. Akad. Nauk SSSR, Ser. Khim.*, 256 (1981) 414–417.
6 N. R. Plessas and I. J. Goldstein, *Carbohydr. Res.*, 89 (1981) 211–220.
7 L. A. Krinitskaya, A. L. Buchachenko, and E. G. Rozantsev, *Zh. Org. Khim.*, 2 (1966) 1301.
8 E. G. Rozantsev, in H. Ulrich (Ed.), *Free Nitroxyl Radicals*, Plenum Press, New York, p. 209.
9 P. A. Sanford and H. E. Conrad, *Biochemistry*, 5 (1966) 1508–1517.
10 C. L. Hamilton and H. M. McConnell, in A. Rich and N. Davidson (Eds.), *Structural Chemistry and Molecular Biology*, W. H. Freeman, San Francisco, 1968, p. 115.
11 T. J. Stone, T. Buckman, P. L. Nordio, and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S. A.*, 54 (1965) 1010–1017.