Carbohydrate Research, 220 (1991) 1–9
Elsevier Science Publishers B.V., Amsterdam

Assignment of the ^1H chemical shifts of glycogen

Li-Hsin Zang, Alistair M. Howseman, and Robert G. Shulman
Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510 (U.S.A.)

(Received November 2nd, 1990; accepted for publication March 22nd, 1991)

ABSTRACT

Assignments of nearly all the ^1H chemical shifts of glycogen are made by 2-D ^1H–^1H homonuclear and ^{13}C–^1H heteronuclear COSY. We demonstrated that it is possible to obtain well-resolved 2-D n.m.r. spectra for a large molecule like glycogen. The seven nonequivalent protons of the glucose residues in the α-(1→4)-linked chains, and of those at the nonreducing ends, were completely assigned. Distinct chemical shifts for H-1 and H-2 immediately adjacent to the α-(1→6) bonds at the branch points were also determined. Several modifications of previous ^{13}C chemical shift assignments were made from the heteronuclear 2-D n.m.r. data.

INTRODUCTION

Glycogen is the principal storage form of carbohydrate in mammals. The metabolism of glycogen has been extensively studied in liver, kidney, and muscle. ^{13}C-N.m.r. provides a useful measurement of glycogen *in situ*, as demonstrated by studies of perfused organs, intact animals[1,2] and, most recently, humans[3]. A recent ^1H-n.m.r. investigation of glycogen in solution showed well-resolved spectra, while comparison of the glycogen intensities with those from the glucose formed by glycogenolysis indicated close to 100% n.m.r. visibility[4]. The glycogen ^1H resonances are all within approximately 1 p.p.m. of the water signal. Although it was possible to assign several glycogen resonances by comparison with those of model disaccharides[4], the majority of the lines are incompletely resolved, even at 500 MHz, and remained unassigned. It was therefore necessary to determine the proton chemical shifts of glycogen by 2-D n.m.r., as described in this paper.

Glycogen is composed of α-(1→4)-linked chains of glucose residues having α-(1→6)-linked branch points approximately every 13 residues[5]. The form of the branching is well known[5]. In accord with previous studies on oligosaccharides, the chemical shifts of the ^1H and ^{13}C atoms of the glucose residues in glycogen are expected to be influenced by the presence of glycosidic bonds. In terms of this bonding the glucose units can be divided into three groups: (I) mid-chain residues involved in two α-(1→4) glycosidic linkages, (II) residues at the nonreducing ends of chains, with C-4 and H-4 not subject to influence by neighboring linkages, and (III) those with α-(1→4) and α-(1→6) linkages at branching points, where a pair of glucose residues are included per branching point. The group I residues are most abundant. In this paper we report complete proton chemical shift assignments for glycogen, obtained using 1-D and 2-D n.m.r. methods at 360 MHz. In addition, results of ^{13}C–^1H heteronuclear 2-D COSY

0008-6215/91/$ 03.50

measurements have allowed more complete assignments of the ^{13}C-n.m.r. spectra of glycogen.

METHODS

Type III rabbit liver glycogen was purchased from Sigma Chemical Co. The molecular weight of the glycogen was estimated by the company to be $>10^6$. The preparation was further purified by dialysis in water to remove ethanol, and dried by centrifugation under vacuum at room temperature. A solution of 50 mg mL^{-1} in D_2O was made for ^1H-n.m.r. investigation. All measurements were performed on a Bruker AM spectrometer at 360 MHz at 55°.

A proton 2-D COSY experiment was performed to assign the proton chemical shifts *via* their J connectivities. The COSY spectrum was collected in quadrature with 1024 points in t_2 and 256 points in t_1, zero-filling being employed in the latter direction. The sweep width in both dimensions is 1100 Hz. 32 Scans were accumulated for each t1 value with a repetition time of 3.3 s.

A heteronuclear, natural abundance, carbon–proton 2-D COSY experiment completed the data collection. A glycogen solution of 100 mg mL^{-1} concentration was prepared for this measurement. The pulse sequence used is $90°(^1H)$–$(t_1)/2$–$180°(^{13}C)$–$(t_1)/2$–Δ_1–$90°(^1H)$–$90°(^{13}C)$–Δ_2–^{13}C FID (^1H broadband decoupling). The refocusing delays Δ_1 and Δ_2 were set to $1/(2J_{C,H})$ and $1/(3J_{C,H})$, respectively, in order to observe both CH and CH_2 protons. The sweep widths of F_1 (^1H) and F_2 (^{13}C) are 1100 and 4800 Hz, respectively. 1024 Points were acquired in t_2, and 64 points were collected in t_1 with zero filling in the data processing. 512 Scans were averaged for each t_1 value with a repetition time of 1.3 s.

We denote the six nonequivalent carbon and seven nonequivalent protons of group I glucose residues in unbranched chains by C-1(1→4), H-1(1→4), C-2, H-2, C-3, H-3, C-4, H-4, C-5, H-5, C-6, H-6a, and H-6b. The addition of primes to the notation (*e.g.* C-4′ and H-4′) indicates nuclei of the nonreducing glucose residues (group II) at the end of chains. For the group III residues at branching points, C-1(1→6), H-1(1→6), C-6(6→1), H-6a(6→1), and H-6b(6→1) are used to denote the nuclei immediately involved in α-(1→6) glycosidic bond formation. The same notations as for group I are used for the rest of the corresponding nuclei in group III, assuming that they have the same chemical shifts as those of group I. When any of these nuclei yield distinct chemical shifts they are further specified [*e.g.* H-2(1→6)].

RESULTS AND DISCUSSION

Our assignments start from the published values of the chemical shifts of glucose and the relevant glucobioses and the J coupling constants of the intra-glucose ^1H–^1H couplings[6,7]. These are summarized in Table I.

Fig. 1 shows the fully relaxed ^1H spectrum of glycogen taken at 55°. The signals downfield of water are from H-1(1→4) (5.38 p.p.m.) and H-1(1→6) (4.98 p.p.m.). These

TABLE I

Proton chemical shifts and *J* coupling constants of α-glucose and glucobioses in D_2O

Sugar	*Chemical shifts, coupling constants*[a]						
	H-1 ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5}$)	*H-5* ($J_{5,6a}$)	*H-6a* ($J_{5,6b}$)	*H-6b* ($J_{6a,6b}$)
α-Glucose[b]	5.21 (3.6)	3.52 (9.5)	3.72 (9.5)	3.41 (9.5)	3.83 (2.8)	3.83 (5.7)	3.75 (12.8)
Maltose[c]	5.41 (3.8)			3.65 (10)			
Isomaltose[c]	4.99 (3.8)				(4.8)	3.96 (2.0)	3.77 (11.4)

[a] Chemical shifts are quoted in p.p.m. from internal sodium 2,2,3,3-tetra-deuterio-4,4-dimethyl-4-silapentanoate (TSP) as reference. Coupling constants are given in Hz. [b] Data from ref. 6. Shifts are converted to TSP standard. [c] Data from ref. 7. Values are for the protons bound to carbons involved in glycosidic linkages.

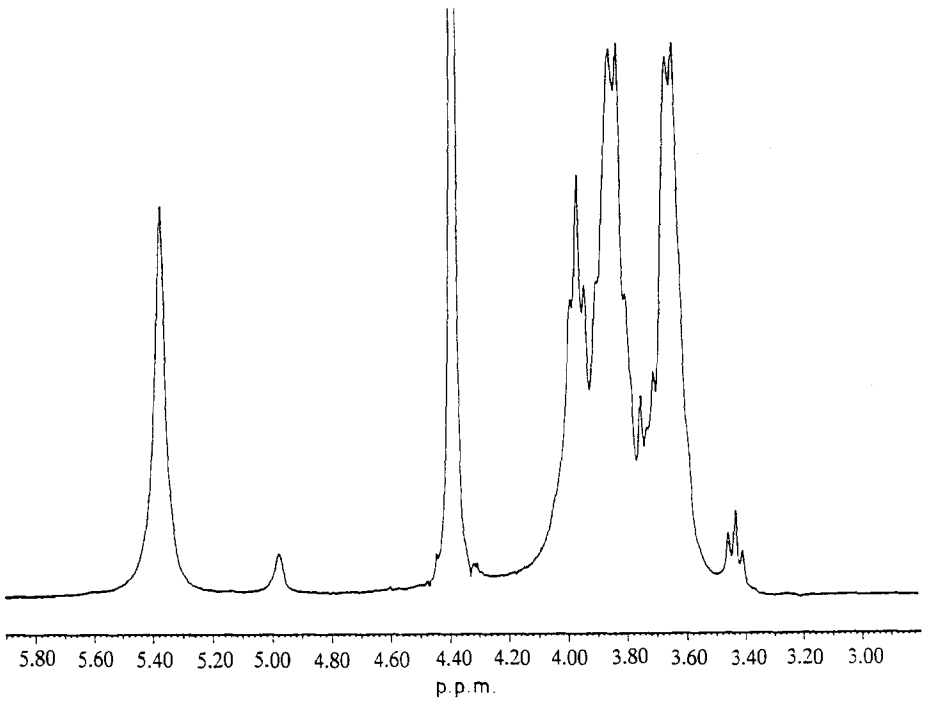

Fig. 1. Fully relaxed 360 MHz proton n.m.r. spectrum (4 scans) of glycogen in D_2O (50 mg mL^{-1}) at 55°. The spectrum was acquired using a 90° pulse with a repetition time of 6 s.

assignments are made in accord with the maltose and isomaltose data in Table I. We made the n.m.r. measurements at 55° because ^{1}H and ^{13}C resonances of glycogen are better resolved at higher temperatures, especially the H-1(1→6) peak. A 50% reduction in the peak area of H-1(1→6) [relative to the H-1(1→4) peak] resulted when the temperature was lowered from 60 to 37° (See Table II). This is probably due to motional restriction at the branching point. Note that at the higher temperatures the H-1(1→6) intensity relative to H-1(1→4) levels off at 8–9%, which approaches the H-4′ intensity. The two should be equal if H-1(1→6) starts chains and H-4′ terminates them. At 37° the H-1(1→6) intensity is reduced by about 3% from its high temperature value. This suggests that the ^{1}H-n.m.r. visibility of glycogen at 37° is slightly below 100% (ref. 4). On the basis of the relative intensities we estimate that the reduction of the H-1(1→6) intensity at 37° means that the H-1 resonance of glycogen is about 97% visible at this temperature.

The six other protons are resonant in the region upfield from H_2O. Fig. 2 shows the ^{1}H homonuclear COSY spectrum of glycogen at 55°, taken at 360 MHz. We start by considering the H-1(1→4) resonance at 5.38 p.p.m., which is presumably the sum of H-1(1→4) and H-1′. The proton–proton scalar couplings $J_{1,2}$ and $J_{1',2'}$ yield cross peaks which identify the H-2 and H-2′ diagonal peaks. The cross peak of the H-1(1→6) resonance with the H-2(1→6) resonance is shifted from that of H-1(1→4) linked to its H-2. The fraction of glucose residues at the branch points which are also the end of a chain is presumably very small, *i.e.*, these units are not primed. We therefore attribute the slight change of the chemical shift to a change in chemical environment at H-2(1→6) due to the α-(1→6) linkage. Hence all three classes of H-2 peaks have slightly different chemical shifts. However, the H-2 and H-2′ diagonal peaks are not separated by this procedure.

Next consider the resonances from the group II glucosyl units. In Fig. 2b, if we start from the previously assigned H-4′ peak at 3.44 p.p.m., well resolved cross peaks originating from scalar $J_{3',4'}$, $J_{4',5'}$, and $J_{4',6a'}$ allow us to determine the chemical shifts of H-3′, H-5′, and H-6a′ unambiguously. The $J_{4',6a'}$ cross peak was tentatively assigned

TABLE II

Relative signal areas of H-1(1→4), H-1(1→6), and H-4′ measured at 360 MHz at various temperatures

Temp. (°)	*Integrated signal areas (ratio)*[a] *H-1(1→4):H-1(1→6):H-4′*
37	1.00 : 0.053 : 0.11
45	1.00 : 0.0680 : 0.12
50	1.00 : 0.0741 : 0.11
55	1.00 : 0.077 : 0.12
60	1.00 : 0.079 : 0.12
65	1.00 : 0.085 : 0.11

[a] Results are averages from two sets of data. Sample preparation as described in the caption to Fig. 1.

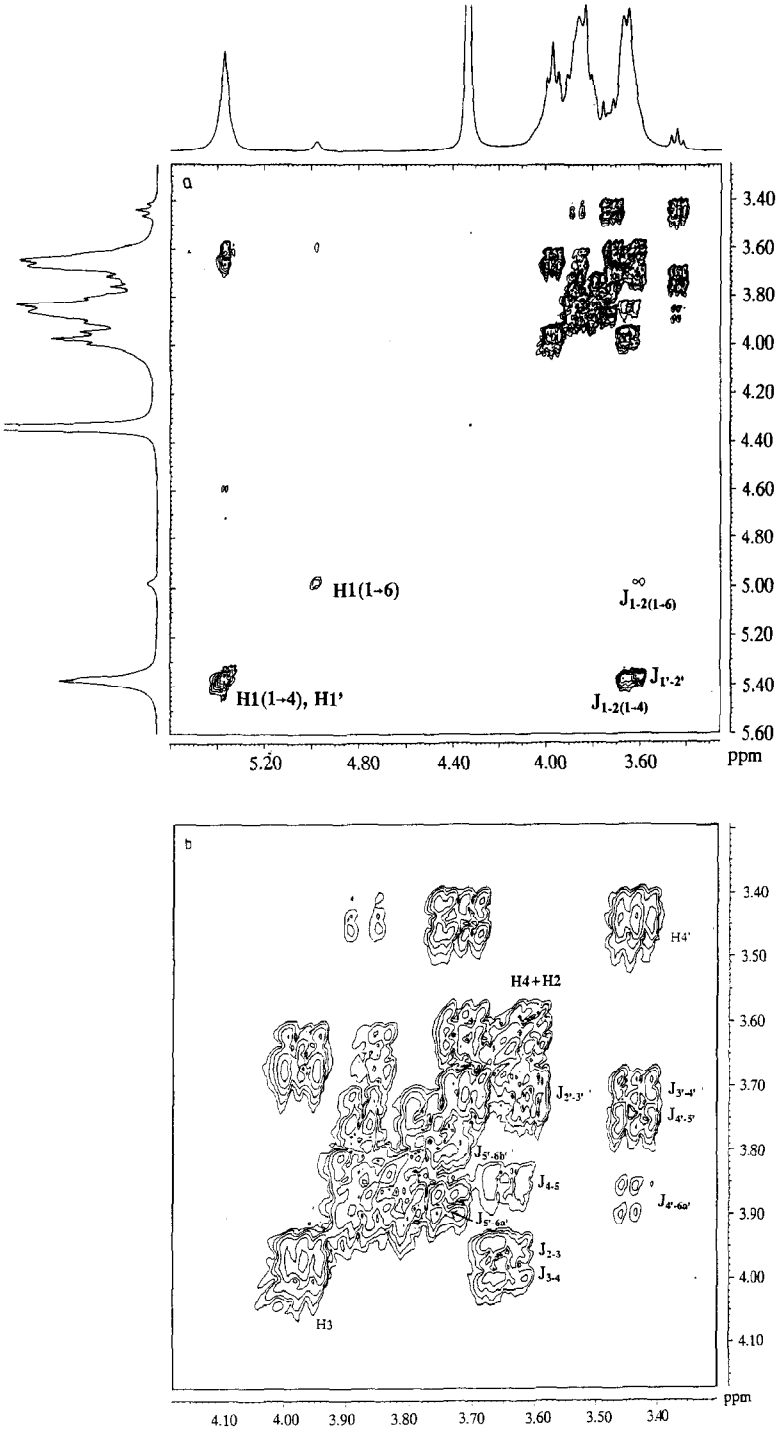

Fig. 2. (a) Homonuclear proton correlation spectrum of glycogen in D_2O (50 mg mL^{-1}) at 55°. The high resolution reference spectrum of glycogen under the same experimental conditions is plotted on the left and top. (b) Regional enlargement of (a).

(Fig. 2b), although its coupling constant was expected to be < 1 Hz. This detection is probably due to the long acquisition times used in both t_1 and t_2 (~ 0.4 and 0.1 s), since the measured T_2 of H-1 is about 30 ms[4]. The purpose of using these acquisition times is to compensate for the loss of digital resolution for the necessary spectral width of 1100 Hz, and to detect the small couplings $J_{1,2}$ and $J_{1',2'}$ (~ 3 Hz). Furthermore, the T_2 value for the group II nuclei is most likely larger than that for groups I and III, judging from the narrower linewidth of H-4′ (Fig. 1), because of the greater motional flexibility of group II residues. Therefore, the long acquisition time should ensure complete detection of the connectivities of group II nuclei, especially $J_{1',2'}$.

It is difficult to make similar assignments for group I glucose residues from the COSY spectrum due to the overlap of their cross peaks. For this reason, we acquired the ^{13}C–^1H heteronuclear COSY spectrum shown in Fig. 3. In this measurement, ^1H chemical shifts are assigned through the one-bond proton–carbon J couplings, by taking advantage of the better spectral resolution of ^{13}C-n.m.r. and using the known carbon chemical shifts of glycogen[8]. This allowed us to identify all the ^1H resonances for group I residues. The result shows that the chemical shift of H-4 determined from the $J_{C,H}$ cross peak of C-4 is almost coincident with that of H-2. The $J_{C,H}$ cross peak for C-3 suggests a chemical shift for H-3 of 3.97 p.p.m. If we return to the ^1H–^1H COSY in Fig. 2b, we can now trace the cross peaks $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$, where $J_{2,3}$ and $J_{3,4}$ are coincident. This in turn leads to the assignment of H-2 and H-4 overlapping in the vicinity of 3.65 p.p.m., and of H-5 at 3.85 p.p.m., which is consistent with the ^{13}C–^1H COSY result of 3.84 p.p.m.

We have labelled the H-2′, H-3′, H-4′, and H-5′ peaks in Fig. 3b on the basis of our assignment from the homonuclear COSY (Fig. 2). Using these ^1H assignments, we can use the heteronuclear COSY data to improve upon the original assignments of the ^{13}C spectrum of glycogen[8]. We note that the original assignments of the C-2 and C-5 peaks should be interchanged. Furthermore we are now able to identify for the first time the resonances from C-2′, C-3′, and C-5′.

The $J_{C,H}$ cross peaks from C-6 in Figure 3b indicate that the chemical shift range for the two H-6 protons is between 3.74 and 3.96 p.p.m. In the homonuclear COSY spectrum the region of H-6a,b and H-6a′,b′ and their associated cross peaks is severely overlapping. We also note that the weak coupling condition of $J << \Delta\delta$ is not valid for these peaks. This makes accurate assignment of this region rather difficult, although we can draw certain conclusions.

We can identify a cross peak in Fig. 2b between H-5′ and one of the H-6′ protons and assign this proton a shift of 3.9 p.p.m. We tentatively assign another cross peak to the second H-6′, yielding a chemical shift of 3.8 p.p.m. These assignments are based upon the H-6a,b shifts assigned by heteronuclear COSY (Fig. 3b), and the supposition that the chemical shifts of the protons bound to C-6 in group I are similar to those in group II. The absence of any other cross peaks at these shifts in the ^{13}C–^1H COSY spectrum and of any unassigned sharp lines in the 1-D carbon spectrum implies most strongly that the chemical shifts of C-6 are also independent of position, except in the case of C-6(6→1) at a branch point. The C-6(6→1) resonance is broadened in the 1-D

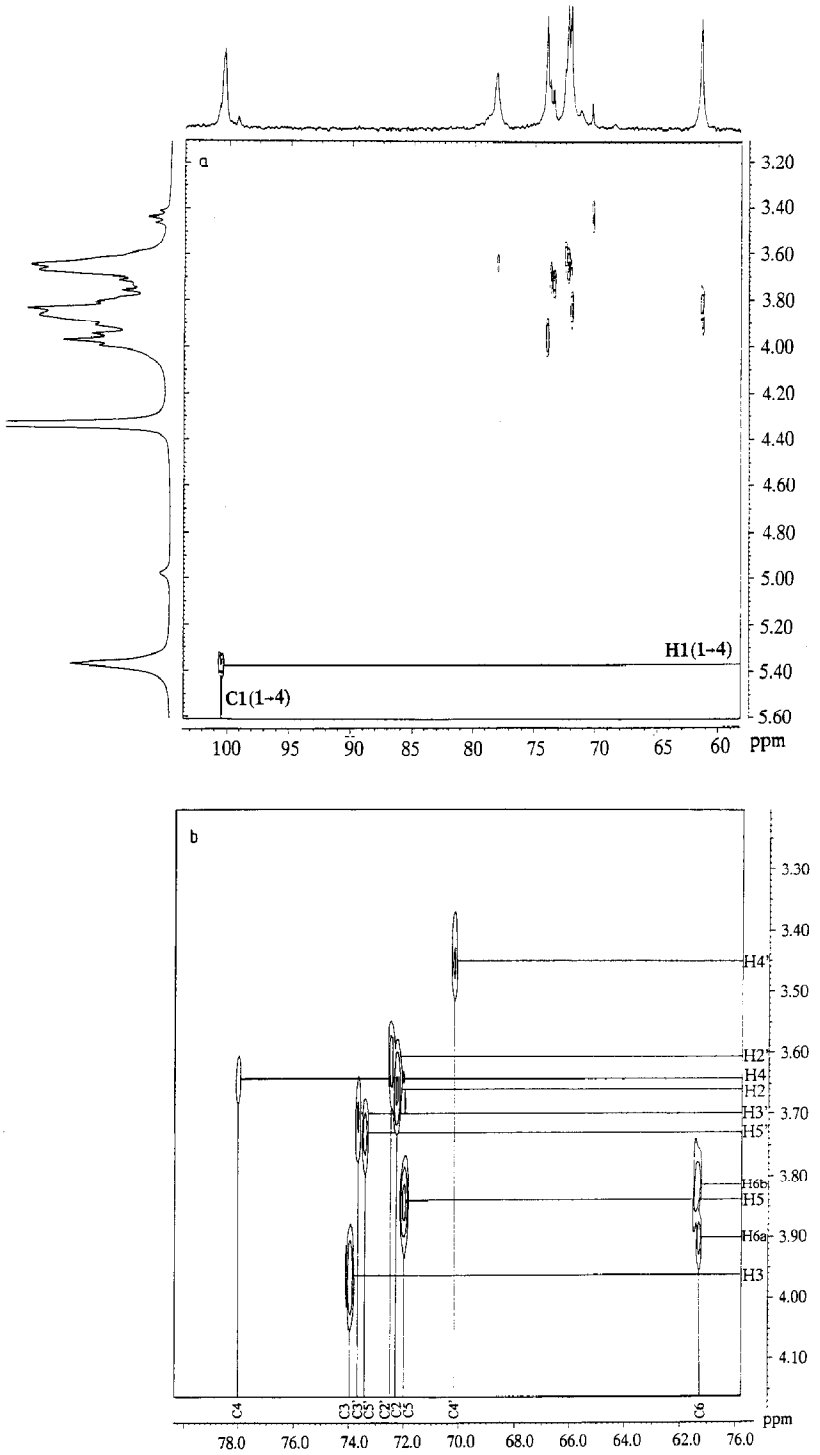

Fig. 3. (a) Heteronuclear carbon–proton correlation spectrum of glycogen in D_2O (100 mg mL^{-1}) at 55°. The high resolution reference spectra of carbons and protons measured under the same conditions are plotted on the top and at the left, respectively. (b) Regional enlargement of (a).

spectrum[8] and is below the level of detection in our ^{13}C–^{1}H COSY spectrum. Consequently, we were unable to identify the $J_{C,H}$ cross peaks originating from C-6(6→1). Therefore, the chemical shifts of H-6a,b(6→1) at branching points remain unassigned.

The almost complete assignment of proton and carbon chemical shifts for glycogen is summarized in Table III. The results show that compared with α-D-glucose, the resonances of H-1(1→4) and H-4 are shifted 0.17 and 0.23 p.p.m. downfield due to the α-(1→4) glycosidic linkage, while that of H-1(1→6) is shifted 0.23 p.p.m. upfield due to the α-(1→6) glycosidic linkage. These observations are similar to those reported for the glucobiose model compounds (Table I). Changes in ^{1}H chemical shifts observed for other nuclei in group I residues *vs.* α-D-glucose are relatively small except for H-3, where a downfield shift of 0.25 p.p.m. is observed. This suggests that the chemical environment of H-3 undergoes a unique change in the glycogen molecule. On the other hand, resonances from H-4′ and H-3′ at nonreducing ends remain the same as those in α-D-glucose. Although H-6a,b(6→1) are not assigned at this time, changes might be expected from the formation of α-(1→6) bonds. However, comparison between the chemical shifts of H-6a,b and H-6a′,b′ in glycogen and H-6a,b(6→1) in isomaltose (Table I) shows a great similarity, suggesting that the resonances of H-6a,b(6→1) at the branching points may not shift by very much, unlike that of H-4. It will be interesting to see the difference upon the assignment of H-6a,b(6→1) in the future.

In summary, the proton chemical shifts of glycogen have been assigned using 2-D homonuclear and heteronuclear COSY measurements. Improvement on carbon chemical shift assignments has been made on the basis of the 2-D heteronuclear COSY data. We have demonstrated that glycogen having a high molecular weight and short T_2 (~30 ms)[4] can yield well-resolved 2-D spectra, which allows accurate chemical shift assignments.

TABLE III

Proton and carbon chemical shifts of glycogen at 55°, measured at 360 MHz

Position	*Chemical shifts (p.p.m.)*	
	^{1}H	^{13}C
1,1′(1→4)	5.38	100.5[a]
1(1→6)	4.98	
2(1→4)	3.66	72.3
2(1→6)	3.62	
2′	3.62	72.5
3; 3′	3.97; 3.72	74.0; 73.7
3; 4′	3.64; 3.44	78.0; 70.1
4; 4′	3.85; 3.73	72.0; 73.4
5; 5′	3.85; 3.73	72.0; 70.1
6a,a′; 6b,b′	3.9; 3.8	61.3

[a] Data from ref. 8.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health Grant DK-27121.

REFERENCES

1 G. I. Shulman, D. L. Rothman, Y. Chung, L. Rossetti, W. A. Petit, Jr., E. J. Barrett, and R. G. Shulman, *J. Biol. Chem.*, 1263 (1988) 5027–5029.
2 R. A. Shalwitz, N. V. Reo, N. N. Becker, A. C. Hill, C. S. Ewy, and J. J. H. Ackerman, *J. Biol. Chem.*, 264 (1989) 3930–3934.
3 G. I. Shulman, D. L. Rothman, T. Jue, P. Stein, R. A. DeFronzo, and R. G. Shulman, *New England J. Med.*, 322 (1990) 223–228.
4 L. Zang, D. Rothman, and R. G. Shulman, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 1678–1680.
5 L. Stryer, *Biochemistry*, 3rd edn., Freeman, New York, 1988, pp. 449–466.
6 K. Bock and H. Thøgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
7 A. De Bruyn, M. Anteunis, and G. Verhegge, *Bull. Soc. Chim. Belg.*, 84 (1975) 721–734.
8 L. O. Sillerud and R. G. Shulman, *Biochemistry*, 22 (1983) 1087–1094.
9 K. Bock, B. Meyer, and J. Thiem, *Angew. Chem.*, 90 (1978) 472–478.