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Investigation of the Branching Characteristic of Glycogen by Means of Two-Dimensional ^{1}H and ^{13}C NMR Spectroscopy

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Summary. The assignment of ^{13}C and ^{1}H NMR signals of three highly short-chain branched α-*D*-glucan samples from animal tissue (glycogen) of different provenience, dissolved in dimethylsulfoxide, was achieved using two-dimensional H-H and C-H correlated spectroscopy. The results were comparable to those recently obtained for plant derived short-chain branched α-*D*-glucans (amylopectin) and non-branched/long-chain branched α-*D*-glucans (amylose). From these assignments and the integration of pertinent proton signals, the branching degree of the glycogen samples was derived to amount 13.5%. These samples were also analyzed with respect to their degree of polymerization distribution by means of the SEC-DRI/LALLS method. These data and the line broadening of the ^{1}H and ^{13}C NMR spectra of the glycogen samples compared to those of amylopectin and amylose supported the assumption of a broad (homogeneous) distribution of glucose residues in glycogen.

Keywords. Glycogen; Branching; Two-dimensional NMR; SEC-DRI/LALLS method; Degree of polymerization distribution.

Untersuchung der Verzweigungscharakteristik von Glykogen durch zweidimensionale ^{1}H- und ^{13}C-NMR-Spektroskopie

Zusammenfassung. Eine Zuordnung von ^{13}C- und ^{1}H-NMR-Signalen von drei in Dimethylsulfoxid gelösten kurzkettenverzweigten α-*D*-Glucanen aus tierischen Geweben (Glycogen) unterschiedlicher Provenienz konnte mit Hilfe zweidimensionaler H-H- und C-H-Korrelationsspektroskopie erzielt werden. Die Ergebnisse waren vergleichbar zu den kürzlich an pflanzlichen kurzkettenverzweigten α-*D*-Glucanen (Amylopektin) und linearen bzw. langkettenverzweigten α-*D*-Glucanen erhaltenen Zuordnungen. Aus diesen Zuordnungen und der Integration entsprechender Protonensignale konnte ein Verzweigungsgrad dieser Glycogenproben von 13.5% abgeleitet werden. Diese Proben wurden auch mit Hilfe der SEC-DRI/LALLS-Methodik in Hinblick auf deren Polymerisationsgradverteilung untersucht. Diese Daten und die Signalverbreiterung in den ^{1}H- und ^{13}C-NMR Spektren der Glycogenproben verglichen mit jenen des Amylopektins und der Amylose erhärten die Vorstellung einer breiten (homogenen) Verteilung der Glucose-Reste in Glycogen.

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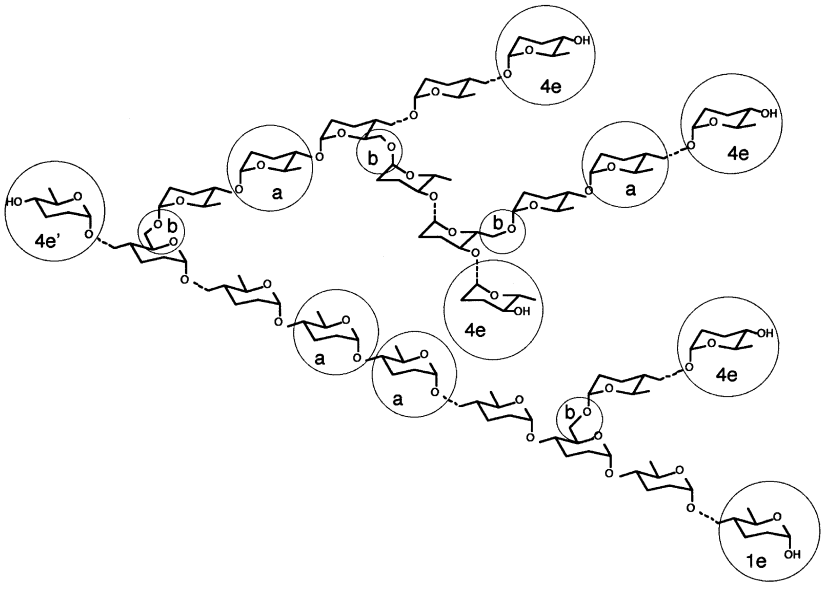

Fig. 1. Schematic structure of a short-chain branched α-*D*-glucan (amylopectin, glycogen); amylose type glucan units within the $\alpha(1\rightarrow4)$ linked glucose chains (a), branching units (b), non reducing terminal glucose units with free 4-OH group (4e, 4e′), and the reducing terminal glucose with its non bonded anomeric carbon atom (1e)

Introduction

Glycogen is the fundamental storage polysaccharide of animals and humans [1]. It is a homo-polymer consisting of α-*D*-glucose monomers. These monosaccharide residues are linked by $\alpha(1\rightarrow4)$ glycosidic bonds in a linear way with more or less $\alpha(1\rightarrow6)$ glycosidically linked branches. The degree of polymerization and the extent of branching varies with the source of the material but, in general, strongly depends on the energetical and historical background of the glycogen containing system. About 8–10% of branched glucose constitute typical values [2] (Fig. 1). From the view of history, the non-branched $\alpha(1\rightarrow4)$ linked *D*-glucans (amylose type) and the $\alpha(1\rightarrow4)$ linked plus $\alpha(1\rightarrow6)$ branched *D*-glucans (amylopectin, glycogen) have strictly been distinguished. For physicochemical analyses, however, a classification into non-branched, long-chain branched, and short-chain branched glucans, including transition forms, is thought to be more convenient. Thus, aside from their strongly differing molecular masses, the glycogens closely resemble the structural branching aspects of amylopectin [3]. According to the *Whelan* model, which assumes random branching, glycogen forms an irregular structure [4] and exists in solution as a more or less compressed random coil as illustrated in Figs. 2 and 3.

Polysaccharides such as α-*D*-glucans are of increasing interest. On the one hand they represent components of renewable resources (starch of green plants), and on the other hand they participate in animal and human metabolism (glycogen). In addition, carbohydrates and polysaccharides are ubiquitously available as low-cost raw material. Thus, investigation of their molecular background for technological and physiological properties is reasonable. Carbohydrates and polysaccharides

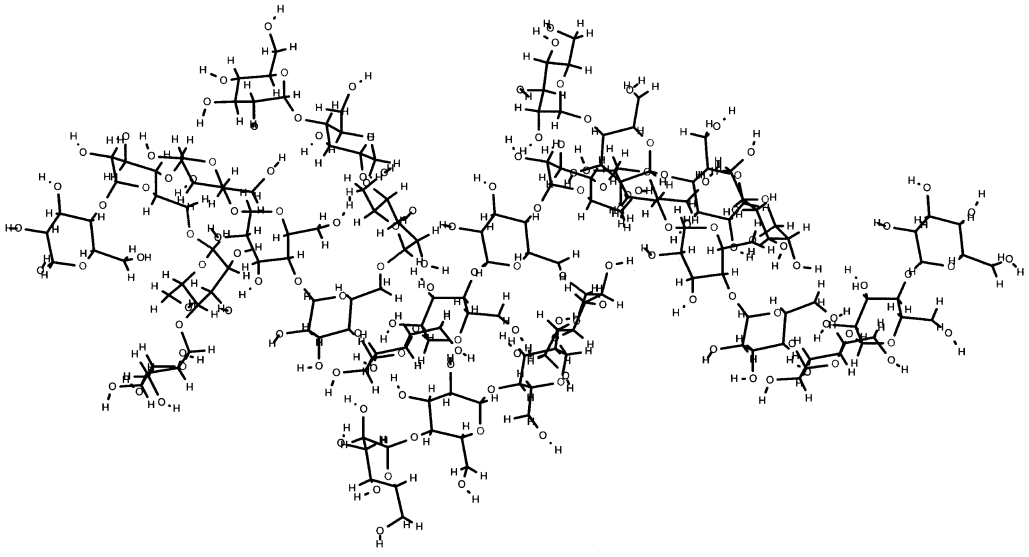

Fig. 2. Wire frame model of a typical glycogen fragment (drawn from the non-reducing end at the left side → reducing end at the right side)

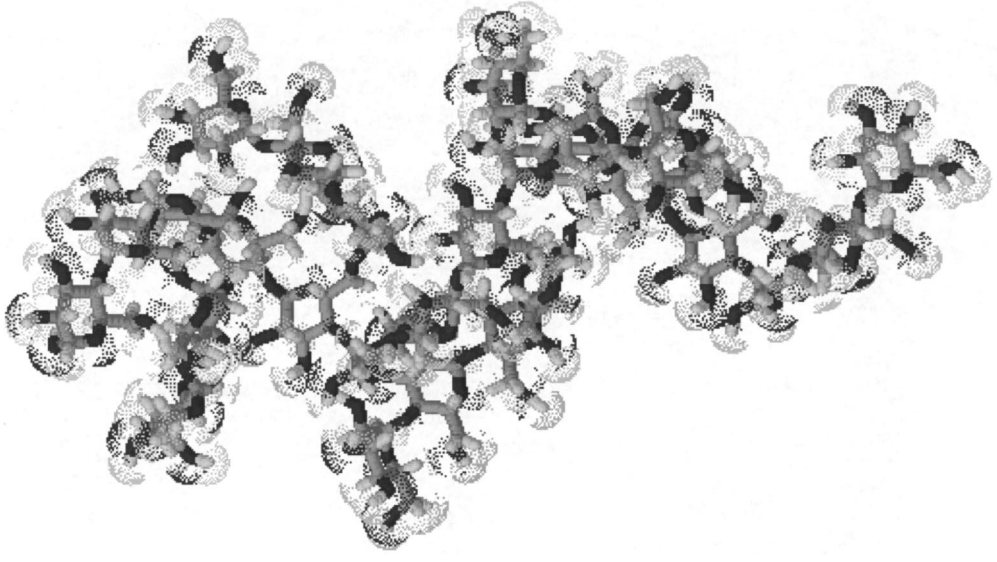

Fig. 3. Ball and stick model of the glycogen fragment depicted in Fig. 2 indicating the solvatation radii of the oxygen atoms

represent the dominant class of the total biomass annually assimilated. They amount to approximately $1–4 \cdot 10^{11}$ tons of dry matter, primarily produced by photosynthesis, with carbohydrates and polysaccharides totalling about 75%.

Although carbohydrates and polysaccharides are produced in such huge volumes, knowledge about the correlation between conformation, molecular structure, and functionality of these polymers is rather diffuse. The main reason for

this lack of information is the high variability of polysaccharides on the molecular level, in particular in degree of polymerization distribution, in branching characteristics, and in complex interactive properties.

Recently, ^{1}H and ^{13}C NMR spectroscopy has been applied successfully to the examination of structural details of short-chain branched glucans and short-chain branched glucan derivatives [5, 6]. ^{1}H and ^{13}C NMR provides the advantage of being a non-destructive technique, and thus the obtained results will not be affected by incomplete substitution (for following fragment analysis by MS) and/or non-specific hydrolysis of $\alpha(1\rightarrow6)$ branching linkages (for following glucan chain-length determinations by HPLC) [7]. Accordingly, ^{1}H and ^{13}C NMR spectroscopy is used in the present investigation to probe the branching aspect of glycogen.

Results and Discussion

Branched glucose residues in glycogen are $\alpha(1\rightarrow4)$ linked in the main chain with the same kinds of chains connected $\alpha(1\rightarrow6)$ glycosidically as branches at the C6 position (cf. Figs. 1–3). As discussed in a recent study [5], all these units 4e and b (Fig. 1) which are slightly different from each other depending on their individual position within the molecular frames (it should be kept in mind that within an ensemble of such molecules even of the same molecular mass there will not be two molecules of identical structure!) will be more or less indistinguishable viewed from the standpoint of chemical shifts. However, these small structural differences will contribute to the *distributions of shifts* which will be reflected in the line shapes of the various signals.

It should be noted that – with respect to the very high molecular mass of the glucan biopolymer – the numbers of the 1e and 4e′ fragments of glycogen are

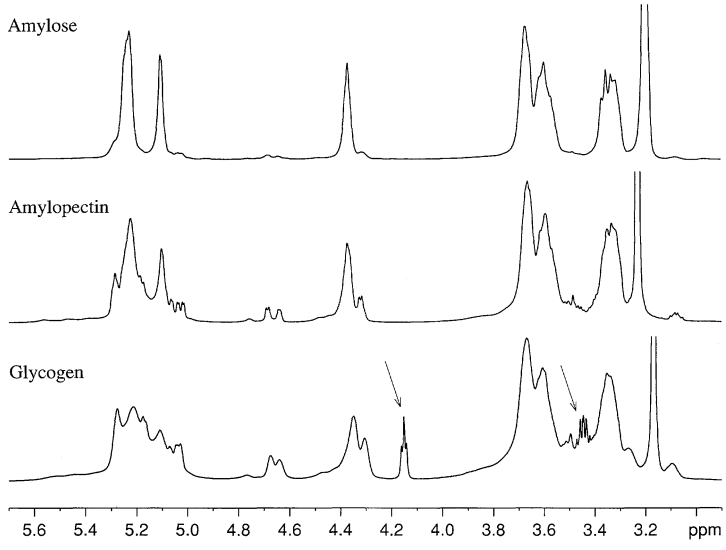

Fig. 4. ^{1}H NMR spectra of amylose (non-branched/long-chain branched α-*D*-glucan), amylopectin (short-chain branched α-*D*-glucan), and glycogen (**3**, highly short-chain branched α-*D*-glucan) dissolved in *DMSO*-d_6 (with arrows indicating ethanol contamination signals)

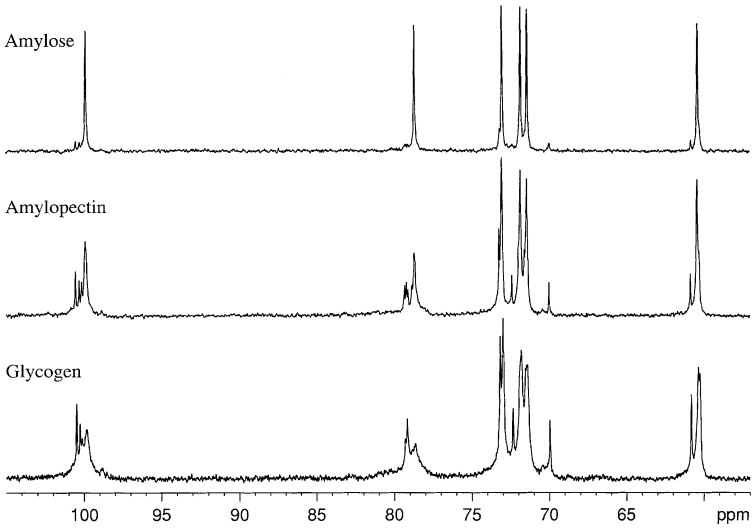

Fig. 5. ^{13}C NMR spectra amylose (non-branched/long-chain branched α-*D*-glucan), amylopectin (short-chain branched α-*D*-glucan), and glycogen (**3**, highly short-chain branched α-*D*-glucan) dissolved in *DMSO*-d_6

negligible (*i.e.* they will be present at less than 0.01% of all the glucan moieties and thus result in signals in their NMR spectra which are much below the detection limits) compared to the amylose type linearly connected a-units or the 4e-type glucan fragments. Due to the dendritic structure of amylopectin [1, 5] and glycogen [4], the amylose type terminal group 4e′ becomes indistinguishable from the terminal groups 4e that result from branching and, consequently, the number of branchings becomes equivalent to the number of the terminal groups labeled 4e.

First, the ^1H and ^{13}C NMR spectra of three glycogen preparations (**1**, rabbit liver, type III; **2**, bovine liver, Type IX; **3**, bovine liver) dissolved in *DMSO*-d_6 were recorded. Their spectra were found to be virtually identical within experimental error. However, compared to the spectra of amylopectin, and even more to those of amylose recorded under the same conditions, significant line broadening was observed for all signals of glycogen (cf. Figs. 4, 5). Apart from this effect, the chemical shifts of amylopectin and glycogen matched. Secondly, the corresponding signal assignments of the latter were advanced as reported earlier [5] by means of two-dimensional gradient enhanced H-C COSY and H-H DQF COSY spectra as shown in Figs. 6, 7, and 8. They proved to be identical with the assignments obtained for amylopectin [5].

As, particularly in the ^{13}C spectra of the short-chain branched glucan glycogen, line broadening was observed (Figs. 4, 5) it was necessary to clarify whether this observation originated from differences in molecular weight (degree of polymerization) distribution or from structural differences on the molecular level. Therefore, the degree of polymerization distributions for the three glycogen samples was determined absolutely by means of size exclusion chromatography combined with detection of mass and scattering intensity. The degree of polymerization distributions of the three samples primarily differed in the

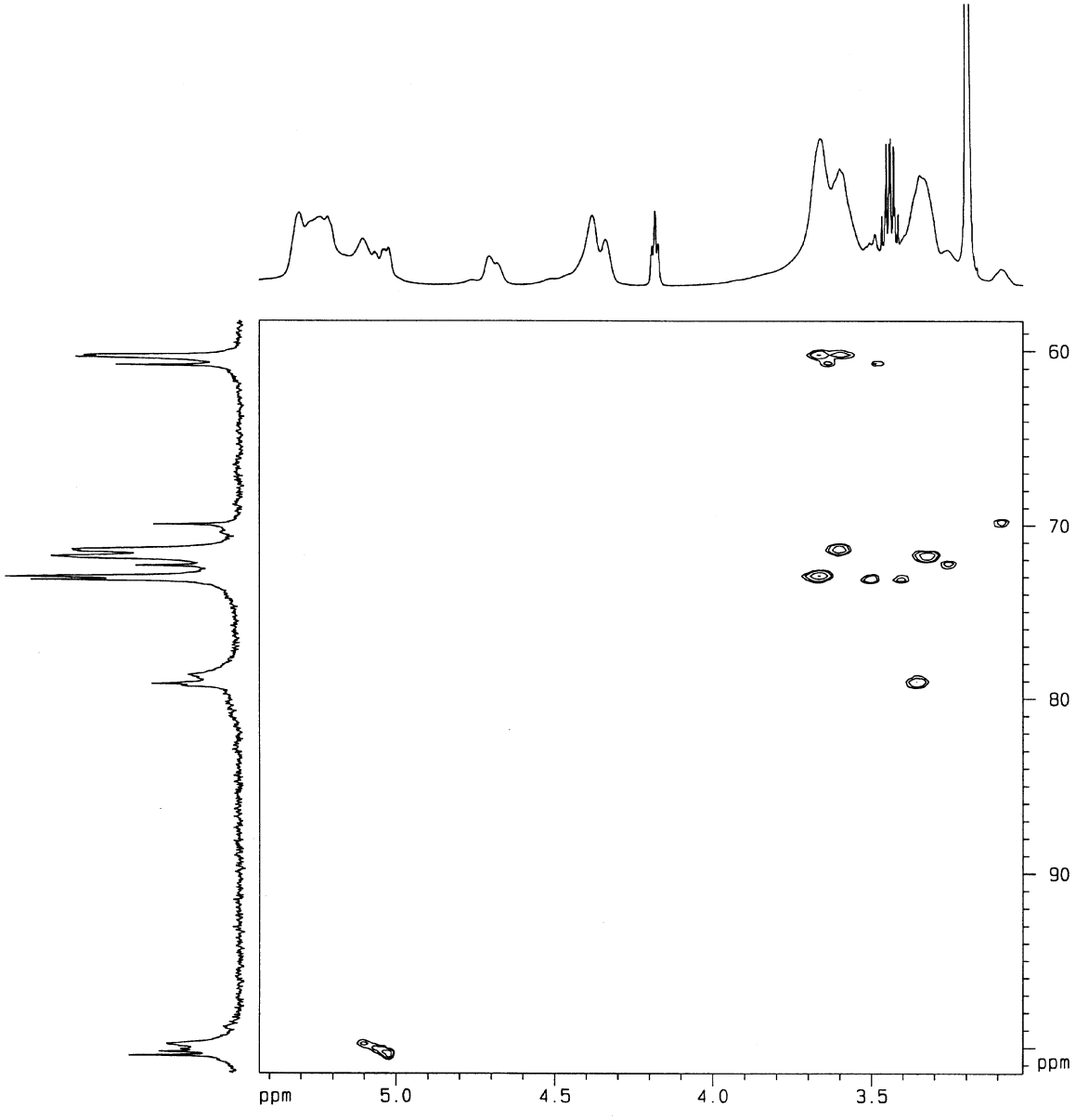

Fig. 6. 2D H, C-COSY spectrum of glycogen **3** dissolved in *DMSO*-d_6 (for the assigned shift values, cf. Fig. 8)

percentage of low-*dp* glucans which comes up as tailing, in particular for the glycogen sample **2**, in the mass distribution (Fig. 9), and becomes more obvious in the plot of molar fractions (Fig. 10). For glucans with $dp > 3000$ Glc, the composition of components is more or less identical for the three samples. However, different to the glycogens **1** and **3**, glycogen **2** consists of a low- and a high-*dp* population. Nevertheless, these differences in the degree of polymerization distributions cannot be judged to be responsible for the observed line broadening in the NMR spectra, since in this case the spectra should have shown different line

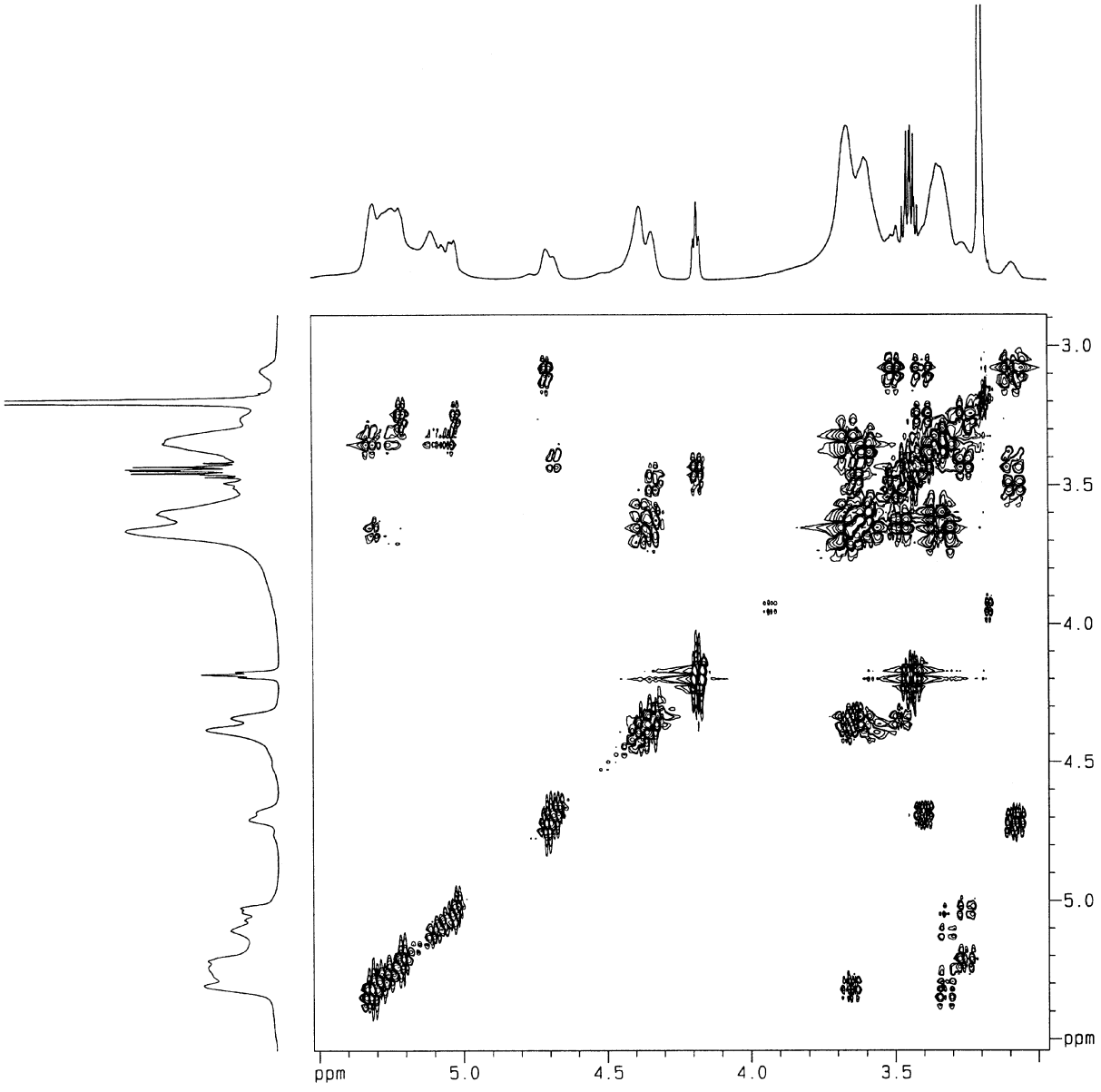

Fig. 7. 2D H, H-DQF-COSY spectrum of glycogen **3** dissolved in *DMSO*-d_6 (for the assigned shift values, cf. Fig. 8)

broadening for the three glycogen samples. Consequently, the main reasons for line dispersion are differences in the molecular structure of amylose and amylopectin on the one hand and glycogen on the other hand. Thus, a broad (homogeneous) distribution of non-branched Glc(a)- and branched Glc(b)-residues (cf. Fig. 11: x, x′, x″, . . ., y, y′, y″, . . ., z, z′, z″, . . .) for glycogen can be deduced from these data.

The pronounced structural heterogeneity of glycogen, as compared to amylopectin, could be explained with an extreme and homogeneous short-chain branched structure. Although both glycogen and amylopectin are classified as short-chain branched glucans, the difference causing the increased line broadening

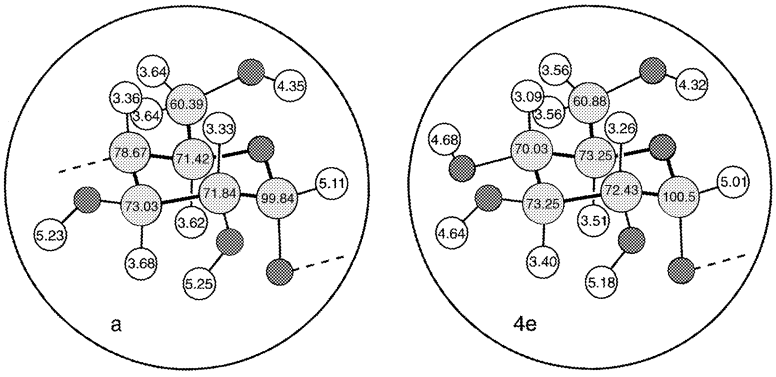

Fig. 8. ^{13}C NMR and ^{1}H NMR signal assignments of the glucan units a and 4e (cf. Fig. 1) of glycogen dissolved in *DMSO*-d_6

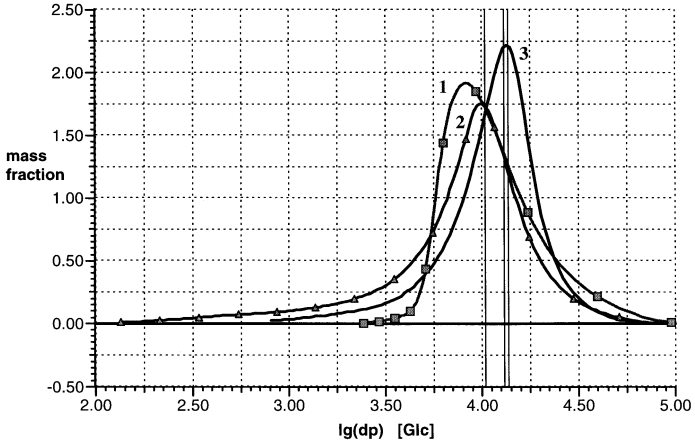

Fig. 9. Differential degree of polymerization distributions: mass fractions of glycogen components of **1** (–□–), **2** (–Δ–), and **3** (—); the distributions were normalized to area = 1.0; weight average degrees of polymerization: $dp_w(\mathbf{1}) = 13770$ Glc, $dp_w(\mathbf{2}) = 10430$ Glc, $dp_w(\mathbf{3}) = 13010$ Glc; mean weights of the molecular masses: $M_w(\mathbf{1}) = 2231000$, $M_w(\mathbf{2}) = 1689000$, $M_w(\mathbf{3}) = 210800$

of the glycogen NMR spectra is due to a larger number of branched Glc residues in slightly differing surroundings. As a consequence it may be concluded that the functionality of α-*D*-glucans in native systems obviously is controlled by (slight) variations in the branching pattern. Variation of intensity of short-chain branching causes a variation of interactive properties: modified inter- and intramolecular polymer/polymer- and polymer/solvent interactions as indicated in Fig. 3 by the solvent accessibility spheres. Such a control mechanism seems reasonable as it is well known that branched glucans dissolved better in water than non-branched and/or long-chain branched glucans. Finally, although up to now only roughly known, interactive properties are closely correlated with the functionality of glucans in living systems.

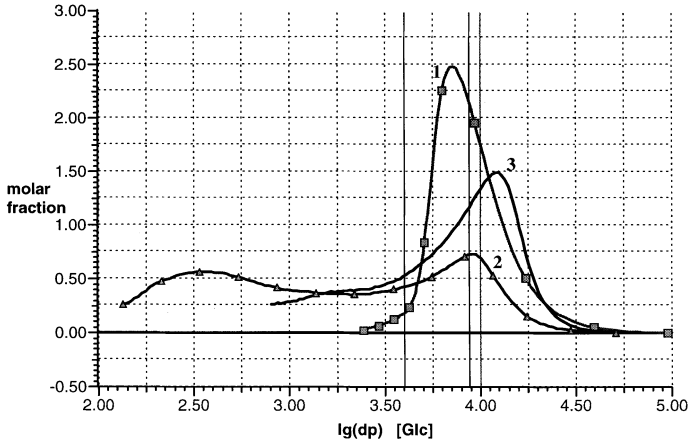

Fig. 10. Differential degree of polymerization distributions: mass fractions of glycogen components of **1** (–□–), **2** (–Δ–), and **3** (—); the distributions were normalized to area = 1.0; number average degrees of polymerization: $dp_n(\mathbf{1}) = 9980$ Glc, $dp_n(\mathbf{2}) = 4010$ Glc, $dp_n(\mathbf{3}) = 8750$ Glc

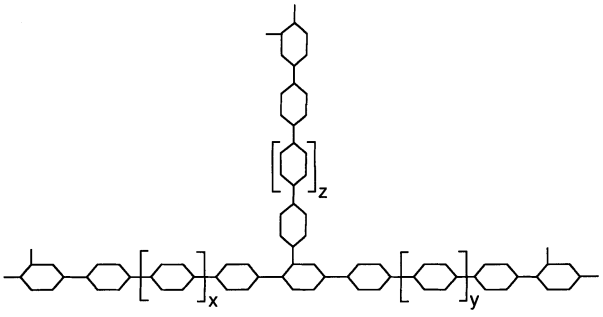

Fig. 11. Schematic drawing of the various linear and branching units of amylopectin and glycogen

This result of a higher structural heterogeneity of glycogen may be also rationalized from the physiological differences which are characteristic for the two storage carbohydrates amylopectin and glycogen. Whereas amylopectin is biosynthesized and then deposited without any further degradative intervention [3], every glycogen molecule has a history of various synthesis and degradation events governed by the availability of glocose in serum according to its involvement in the flow equilibrium of its metabolism [2]. Thus, amylopectin will be rather uniform judged from the structural standpoint of branching characteristics, whereas the glycogen structure will be rather heterogeneous or chaotic in this respect.

The branching degree of glycogen was derived to amount 13.5% by means of an integration of the overlapping 6-OH(a) + 6-OH(e) + 3-OH(e) or, alternatively, 3-OH(a) + 2-OH(a) + 1-CH(a) + 3-OH(e) signals and comparison with the intensity of the resolved signal of 4-OH(e) at 4.68 ppm. It was found to be identical within experimental error for the three samples **1–3**. Thus, as in the case of amylose and amylopectin, this true value was found to be systematically higher than the value of 8–10% derived for glycogen from degradation methodology [2]. This high branching degree taken together with the broad distribution of different

individual glucan units nicely corroborated the designation of glycogen as a highly short-chain branched polymer which thus displays a unique dendritic structure.

Experimental

Glycogen samples **1** (Rabbit liver, type III) and **2** (bovine liver, Type IX) were obtained from Sigma, **3** (bovine liver) from Fluka. Native amylopectin from waxy maize was of commercial origin (Wachsmaisstärke alkaligewaschen, Agrana Stärke G.m.b.H. Gmünd, Austria), and amylose was a product of Serva (Lösliche Stärke). All samples were utilized without any further purification or fractionation. According to their NMR spectra, **2** and **3** contained traces of ethanol.

The NMR samples were prepared by dissolving 30.0 mg of the respective material in 0.5 ml *DMSO*-d_6 (99.95% D, Uetikon) under an argon atmosphere at 60°C for 45 minutes in 5 mm quartz sample tubes. The spectra were recorded at 332±1 K on a Bruker DRX 500 instrument at a proton frequency of 500.13 MHz and a carbon frequency of 125 MHz under non spinning conditions. 2D (H, H and H, C COSY) experiments were executed using the gradient enhanced phase sensitive H, H-DQF-COSY technique [8] (4096 data points in F2, 1024 data points in F1; 4 scans; 16 dummy scans; 2976.2 Hz spectral width in both dimensions; phase sensitive (TPPI); apodization function: sinebell squared) and the inverse HC-COSY strategy (*ge*-HMQC [9]; 4096 data points in F2, 512 data points in F1; 8 scans; 32 dummy scans; 2976.2 Hz spectral width in F2 and 11340 Hz in F1; phase sensitive (TPPI); apodization function: sinebell).

Degree of polymerization (molecular weight) distributions and average values of degree of polymerization (molecular weight) were determined absolutely by means of size exclusion chromatography with an aqueous eluent (0.05 *M* NaCl) combined with dual detection of separated components with respect to their mass and scattering intensity at a low scattering angle (SEC-DRI-LALLS method, [10]). The size exclusion chromatography system consisted of a series of prepacked TSK columns (pre-column + PWM + PW6000 + PW5000 + PW4000 + PW3000; each of them $l = 300$ mm, $id = 7.5$ mm) of TosoHaas (Japan) and was run at a constant flow rate of 0.83 ml/min by a HPLC pump (constametric III, TSP, USA). Mass detection was achieved with an interferometric refractometer (Optilab 903 R, Wyatt Technology, USA), scattering intensity was monitored at a scattering angle of $\theta = 5°$ by means of a low angle laser light scattering device (KMX-6, TSP, USA). Data acquisition was managed by the CODAwin software package, data interpretation and documentation was performed with the CPCwin software package (both: *A. H.* group, Graz Austria).

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