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THE ENZYMIC SYNTHESIS AND N.M.R. CHARACTERISATION OF SPECIFICALLY DEOXYGENATED AND FLUORINATED GLYCOGENS

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ABSTRACT

The incorporation of several deoxy- and deoxyfluoro-D-glucose analogues into glycogen has been achieved through the action of rabbit muscle glycogen phosphorylase on a number of deoxy- and deoxyfluoro- analogues of α-D-glucopyranosyl phosphate. Time courses for the incorporation of these analogues into glycogen and maltopentaose have been determined, and the introduction of 4-deoxy- or 4-deoxy-4-fluoro-D-glucose units has been demonstrated to terminate after the introduction of one sugar unit per non-reducing terminus. Glycogen analogues containing sugars modified at the 3- and 4-positions have been isolated and characterised by ^{1}H-n.m.r. and ^{19}F-n.m.r. spectroscopy, and the extent of incorporation has been confirmed by integration of the new resonances associated with the incorporated residues. Longitudinal (T_1) relaxation times have been determined for the two ^{19}F-n.m.r. resonances observed for 3-deoxy-3-fluoro-glycogen, and through comparison with the T_1 measured for 4-deoxy-4-fluoro-glycogen, the identity (terminal or internal) of each of these two resonances was determined. Kinetic studies indicate that neither 4-deoxy- nor 4-deoxy-4-fluoro-glycogen can serve as a substrate for glycogen phosphorylase in the direction of glycogen synthesis, proving that these glycogen analogues have been fully substituted. Both of these 4-substituted glycogens are good inhibitors of glycogen phosphorylase.

INTRODUCTION

Analogues of glycogen or starch in which hydroxyl groups have been replaced by other substituents are of considerable interest for several reasons. Firstly, the introduction of suitable reporter groups such as ^{19}F or ^{2}H for n.m.r. spectroscopic studies could be valuable in studies of the conformation and dynamics of the labelled glycogen, both free in solution and bound to another macromolecule. These studies could provide interesting insight into the local dynamics of glycogen as well as information on the modes of the interaction of glycogen with specific binding sites on proteins. Another area of interest is in the preparation of "capped" glycogens in which all the acceptor hydroxyl groups (the 4-hydroxyl group in the case of chain elongation) of the non-reducing terminal sugar residues have been

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replaced by a hydrogen or a fluorine. Such "capped" glycogens will then serve as inhibitors of glycogen synthesis and should prove very useful in mechanistic studies on enzymes such as glycogen phosphorylase and glycogen synthetase. Their substitution for substrate glycogen should allow the formation of conformationally activated, but catalytically incompetent, ternary complexes of the enzyme with both "substrates", which will be useful in studies of these enzymes by physical techniques, as well as in kinetic studies. These compounds may prove particularly valuable in the search for evidence of a glucosyl–enzyme intermediate on these enzymes by means of positional isotope exchange experiments[1,2].

Previous work on the synthesis of modified starch or glycogen analogues has either involved direct chemical modification or, to some extent, enzymic synthesis. Most chemical modification has been at the 6-position, relying upon the greater reactivity of the primary hydroxyl group for the selectivity. Thus, for example, 6-deoxy- and 6-bromo-6-deoxy-amylose have been synthesised, and a study of inclusion complexes has been carried out[3], while 6-deoxy-6-iodo-malto-oligosaccharides were synthesised and used[4] in X-ray crystallographic studies to locate the active site of taka-amylase. 6-Amino-6-deoxy derivatives of amylose have been prepared in a similar fashion[5]. No chemical methods for quantitative and selective substitution of any of the secondary hydroxyl groups of glycogen or amylose have been described, although methods are available for the synthesis of a 4-*O*-methyl-malto-oligosaccharide[6]. However, this analogue is of limited use as a selective enzyme inhibitor due to the bulk of the substituent and the fact that such short oligosaccharides bind only poorly to many of the enzymes involved in glycogen metabolism.

Enzymic approaches to the problem have been limited generally to studies which only involve some demonstration of the formation of modified polysaccharide, and not their isolation and characterisation. For example, a 2-deoxy-glycogen analogue was formed on reaction of glycogen phosphorylase with D-glucal and glycogen[7], and its presence was confirmed by ^{1}H-n.m.r. spectroscopy. The formation of a series of selectively deoxygenated analogues of starch has been claimed[8] upon reaction of potato α-glucan phosphorylase with specifically deoxygenated α-D-glucopyranosyl phosphate analogues, but these were not isolated and characterised. The only report of a fluorinated polysaccharide has been that of a 3-deoxy-3-fluoro-substituted glycogen[9] formed upon incubation of locust tissue extracts with 3-deoxy-3-fluoro-glucose. The 3-deoxy-3-fluoro-substituted glycogen was isolated and characterised by ^{19}F-n.m.r. spectroscopy.

A clean enzymic synthesis of a deoxy- or deoxyfluoro-substituted glycogen requires the use of a purified enzyme (glycogen synthetase or glycogen phosphorylase) and the corresponding modified substrate. Use of glycogen synthetase has the advantage of a more favourable equilibrium constant for glycogen synthesis, but it requires synthesis of a relatively complex substrate. Synthesis of substrate analogues for glycogen phosphorylase is simpler, but the equilibrium constant for glycogen formation is not as favourable, though it does[10] lie in the desired direction (K_{eq} = 3.5) under normal assay conditions. Further, the reaction can be brought to

completion by repeated removal of the product phosphate by dialysis and re-addition of substrate. We have recently synthesised[11,12] the necessary fluorinated and deoxygenated analogues of α-D-glucopyranosyl phosphate and measured their rates of utilisation by rabbit muscle glycogen phosphorylase[13] and potato α-glucan phosphorylase[14] to gain insight into the catalytic mechanisms of these enzymes. In this paper we describe the synthesis, isolation, and n.m.r. characterisation of several specifically deoxygenated and fluorinated glycogen analogues. We also present time courses for incorporation of different analogues, as well as preliminary kinetic studies on the inhibition of the muscle enzyme by the "capped" 4-substituted glycogen analogues.

EXPERIMENTAL

General methods. — The substituted α-D-glucopyranosyl phosphate derivatives were synthesised as previously described[11,12]. Rabbit muscle glycogen phosphorylase b was prepared by the method of Fischer and Krebs[15] using (±)-dithiothreitol (DTT) instead of L-cysteine and recrystallised at least three times before use. Rabbit liver glycogen (type III) purchased from Sigma Chemical Co. was purified on a Dowex-1 [Cl^-]column and assayed by a published method[16]. Maltopentaose was purchased from Wako Pure Chemical Company. All other buffer chemicals and substrates were from Sigma.

Incorporation kinetics. — Time courses for the incorporation of glucose analogues into glycogen and maltopentaose were determined by incubating reaction mixes (0.5 mL) containing large amounts of phosphorylase (5 mg, 300 Units) plus AMP (1mM), the α-D-glucopyranosyl phosphate analogue (10–30mM) and either glycogen (0.16%) or maltopentaose (5mM), at 30° in sealed microcentrifuge tubes. The buffer employed contained potassium chloride (100mM), sodium glycerophosphate (20mM), DTT (4mM), and EDTA (1mM), pH 6.8. Small aliquots were removed at the time intervals indicated in Fig. 1 and assayed for released phosphate using the method of Baginski[17]. This assay is more sensitive than the standard Fiske–Subbarow assay, suffers less from interference from the protein present, and is more suited for use in the presence of very acid-sensitive phosphate esters. Control samples containing all the ingredients except the enzyme were sampled at the same times to assess spontaneous hydrolysis, and the data presented in Fig. 1 have been so corrected. Time courses for the incorporation of 4-deoxy-D-*xylo*-hexose into maltopentaose (Fig. 2), were determined by incubating reaction mixtures (0.5 mL) containing phosphorylase (0.83 mg), 4-deoxy-α-D-*xylo*-hexopyranosyl phosphate (43mM), AMP (1mM), and maltopentaose (2, 5, and 10mM) in the same buffer as above, at 30° in sealed microcentrifuge tubes. Aliquots were assayed as above at intervals over a 24-h period, along with controls for spontaneous hydrolysis.

Glycogen analogue synthesis. — Synthesis of glycogen analogues was achieved by the incubation of reaction mixtures (2.0 mL) containing phosphorylase

(5 mg, 300 Units), AMP (1mM), glycogen (5%), and either 4-deoxy-α-D-*xylo*-hexopyranosyl phosphate (20mM), 4-deoxy-4-fluoro-α-D-glucopyranosyl phosphate (10mM), 3-deoxy-α-D-*ribo*-hexopyranosyl phosphate (20mM), or 3-deoxy-3-fluoro-α-D-glucopyranosyl phosphate (20mM), in the same buffer as above, at room temperature (approx. 22°). After 60 h, the samples were individually dialysed (30 000-dalton cutoff, cellulose membrane) against buffer (500 mL each, two changes) overnight, then they were replaced in tubes, and the same concentrations of AMP and α-D-glucopyranosyl phosphate analogue were reinstated in each mixture, plus an additional 1 mg (60 Units) of phosphorylase to replace enzyme activity lost through denaturation. The sample was then incubated for a further 60 h, and this process was repeated twice more, followed by a final dialysis against a buffer containing sodium glycerophosphate (2mM), EDTA (1mM), and DTT (5mM), pH 7.0. Thus the incubation was performed a total of four times to optimise the incorporation of analogue and essentially ensure total capping by the 4-substituted analogues. Purification of the glycogen analogue from the dialysed mixture was achieved by first loading the sample onto a column (12 mL) containing DE53 cellulose equilibrated in 10mM Tris buffer, pH 8.3. The column was eluted with this same buffer, and fractions (3 mL) were collected and assayed for both phosphorylase activity and for the presence of glycogen. Essentially all the glycogen analogue eluted within the first three fractions while the enzyme was retarded, the latter appearing in the fifth and subsequent fractions. The first three fractions in each case were combined, assayed once more for phosphorylase to ensure no contamination, then applied to a BioGel P2 column (2.6 × 50 cm) equilibrated with deionised water. The column was eluted with water, the effluent being monitored with a refractive index detector. Fractions containing the desalted glycogen analogue were combined and freeze-dried to yield approximately 50 mg of the glycogen analogue in each case.

N.m.r. spectroscopic studies. — ^{1}H-N.m.r. studies were performed on a 400-MHz Bruker spectrometer, while ^{19}F-n.m.r. spectra were determined at 254 MHz on a modified Bruker/Nicolet 270-MHz spectrometer. Samples were dissolved in and lyophilised from D_2O to remove exchangeable protons, then redissolved in D_2O, and acetone (1 μL) was added to serve as an internal standard for the ^{1}H-n.m.r. spectra. ^{1}H-N.m.r. spectra were measured at 95°, the acetone reference being set at 2.23 p.p.m. ^{19}F-N.m.r. spectra were determined on the same samples at 30°, using trifluoroacetic acid as an external standard (δ = 0 p.p.m.) with no proton decoupling. Measurements of T_1 values were performed on the same samples using the progressive saturation method[18].

Study of substituted glycogens as substrates and inhibitors for glycogen phosphorylase. — Assays of phosphorylase activity were performed by measurement of initial rates of glycogen synthesis[19] in a buffer containing potassium chloride (100mM), sodium glycerophosphate (20mM), DTT (4mM), and EDTA (1mM), pH 6.8. Phosphate released was assayed according to Baginski[17], using 1.5 times the normal level of assay reagents to ensure full colour development. Apparent K_m values for glycogen and the disproportionated glycogen were determined by meas-

urement of initial rates at fixed concentrations of α-D-glucopyranosyl phosphate (20mM) and AMP (1mM) and different (0.005–0.5%) concentrations of glycogen. Apparent K_i values for glycogen analogues were determined by measurement of initial rates of glycogen synthesis at fixed concentrations of AMP (1mM), α-D-glucopyranosyl phosphate (20mM) and glycogen (0.98%) in the presence of different concentrations (0.04–0.1%) of the glycogen analogue. Glycogen phosphorylase was used at a concentration of 2–3 μg per reaction mixture, and a reaction time of 5 min was used in all cases.

RESULTS

The time courses for the incorporation of 3-deoxy-D-*ribo*-hexose, 3-deoxy-3-fluoro-D-glucose, and 4-deoxy-4-fluoro-D-glucose into glycogen and/or maltopentaose using rabbit muscle glycogen phosphorylase and the corresponding α-D-hexopyranosyl phosphate analogue are presented in Fig. 1, reaction progress being monitored by the release of inorganic phosphate. Incorporation of 4-deoxy-D-*xylo*-hexose occurs at least two orders of magnitude faster than the foregoing examples, so this sample could not readily be run in parallel. Incorporation of these three sugars is *very* slow, requiring large amounts of enzyme for long time periods. Thus inevitably a considerable loss of enzyme activity occurred making these time courses somewhat inaccurate. Other possible sources of error include spontaneous hydrolysis of substrates and substituted glycogen products. Control experiments were run to estimate the extent of spontaneous hydrolysis of the substrates by measuring the extent of breakdown of the corresponding α-D-hexopyranosyl

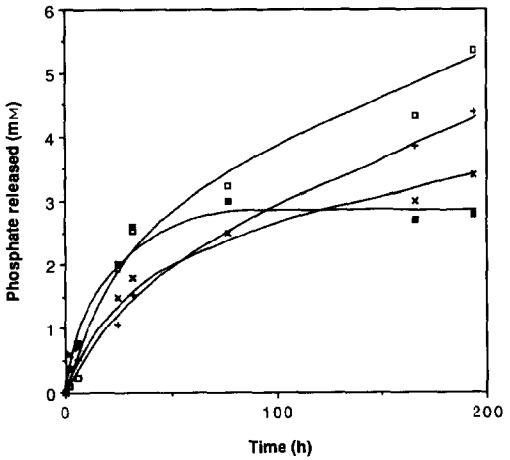

Fig. 1. Incorporation of glucose analogues into glycogen and maltoheptaose. Conditions are as described in the Experimental section with progress being followed by phosphate release. Samples contained the following substrates: (■) 4-deoxy-4-fluoro-α-D-glucopyranosyl phosphate (10mM) and maltopentaose (5mM); (□) 3-deoxy-α-D-*ribo*-hexopyranosyl phosphate (30mM) and maltopentaose (5mM); (+) 3-deoxy-3-fluoro-α-D-glucopyranosyl phosphate (25mM) and maltopentaose (5mM); (×) 3-deoxy-3-fluoro-α-D-glucopyranosyl phosphate (25mM) and glycogen (0.16%).

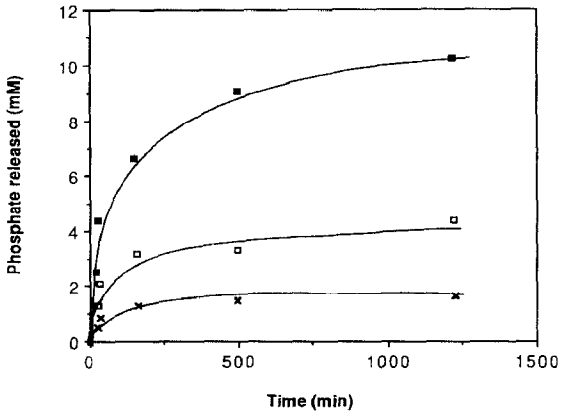

Fig. 2. Incorporation of 4-deoxy-D-*xylo*-hexose into maltopentaose. Samples were prepared as described in the Experimental section and contained the following concentrations of maltopentaose (×) 2mM; (□) 5mM; (■) 10mM. Progress of reaction was followed by release of phosphate.

phosphate analogue under similar conditions. The spontaneous hydrolysis rates were very slow relative to the enzyme-catalysed reactions. For example, the most labile substrate studied over these extended time periods was 3-deoxy-D-*ribo*-hexose[12], and in this case only 0.3mM phosphate was released at the last time point compared to 5mM phosphate released in the enzymic reaction. All data presented in Fig. 1 have been corrected for this small amount of spontaneous hydrolysis. Unfortunately no such control can be run for product hydrolysis, but it should be even slower than hydrolysis of the hexopyranosyl phosphates. Despite these problems, a clear difference exists between the time courses for the 3- and 4-substituted analogues. Clearly the incorporation of 4-substituted analogues results in termination of reaction, while 3-substitution only slows the reaction down. This termination of reaction after the addition of a single 4-substituted sugar per non-reducing chain terminus is best demonstrated and quantified by the data in Fig. 2, which shows time courses for incorporation of 4-deoxy-D-*xylo*-hexose into maltopentaose at three different concentrations of the oligosaccharide.

Attempts to prepare a 4-substituted maltohexaose from maltopentaose and a hexopyranosyl phosphate analogue were unsuccessful since, upon isolation of the oligosaccharide product, it was found that a considerable amount of enzyme-catalysed disproportionation had occurred, resulting in the production of a mixture of high- and low-molecular-weight products. Such disproportionation occurs very rapidly at the concentrations of enzyme employed, once some phosphate has been released[20]. The preparation of substituted glycogen analogues was, however, successful since, although disproportionation still occurs, it does not affect the overall molecular weight, just the distribution of chain lengths. Demonstration of the successful synthesis and isolation of modified glycogens was achieved by means of ^{1}H- and ^{19}F-n.m.r. and kinetic studies described in the following section.

^{1}H-N.m.r. spectra of each of the four substituted glycogens and of glycogen

TABLE I

N.M.R. CHEMICAL SHIFTS (P.P.M.)

Polysaccharide	*δ(Anomeric)*	*δ(Methylene)*	*δ(^{19}F)*[a]
Glycogen	4.98	—	—
	5.37		
3-Deoxy-glycogen	4.98	1.74	—
	5.13	2.38	
	5.27		
	5.37		
3-Deoxy-3-fluoro-glycogen	4.98	—	−116.9 (0.25 s)
	5.34 (s)[b]		−120.8 (0.40 s)
	5.37		
	5.44		
4-Deoxy-glycogen	4.99	1.46	—
	5.38	2.03	
4-Deoxy-4-fluoro-glycogen	4.98	—	−122.2 (0.36 s)
	5.37		

[a] ^{19}F-N.m.r. shifts are quoted to relative external trifluoroacetic acid standard δ(0.0 p.p.m.). Numbers in parentheses are T_1 values. [b](s) = shoulder.

itself were obtained, along with spectra of maltotriose [α-(1→4)-linked] and isomaltose [α-(1→6)-linked]. The chemical shifts of the important resonances in the glycogen analogues are presented in Table I. Spectra were measured at 95° to improve resolution (by decreasing viscosity) and to shift the residual water peak upfield and away from the region of the anomeric protons. Peaks at δ 5.37 and 4.98 p.p.m. are present in all the samples and have been assigned to the anomeric protons of sugar residues involved in α-(1→4)- and α-(1→6)-linkages respectively. This assignment was initially based upon other data[21,22] obtained at different temperatures and was confirmed by measuring spectra of maltotriose and isomaltose as model compounds that contain α-(1→4) and α-(1→6) linkages, under the same conditions. The glycosidic anomeric protons of these two sugars were found to resonate at δ 5.35 and 4.96 p.p.m. respectively, in confirmation of the above assignment. The relative integrations of these two peaks in the polysaccharides, (assuming equal relaxation times, or full relaxation in each case) provide a direct measure of the degree of branching in the sample, as previously pointed out[21,22]. This number is also necessarily equal to the percentage of the sugar residues located at non-reducing termini. The glycogen sample, on this basis, has approximately 9% terminal sugars, in good agreement with previous estimates by this[21] and other[23] methods. ^{1}H-N.m.r. spectra for the 3- and 4-deoxy-glycogen samples are more complex, as anticipated, since they both show resonances between 1.4 and 2.4 p.p.m. due to the methylene ring protons at the deoxygenated carbon, plus additional anomeric proton resonances in the case of the 3-deoxy-glycogen. The only additional features of interest in the ^{1}H-n.m.r. spectra of the fluoro-glycogens are small shoulders on the peak at δ 5.32 in the 3-deoxy-3-fluoro-glycogen sample.

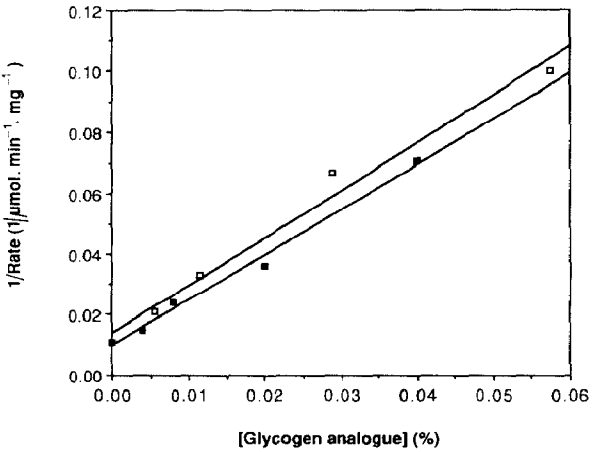

Fig. 3. Dixon plot of the inhibition of phosphorylase b by glycogen analogues. Samples were assayed at constant concentrations of glycogen (0.98%), D-glucose-1-phosphate (20mM) and AMP (1mM); (■) 4-deoxy-glycogen, (□) 4-Deoxy-4-fluoro-glycogen.

^{19}F-N.m.r. spectra were obtained for the 3-deoxy-3-fluoro- and 4-deoxy-4-fluoro-substituted glycogens, the chemical shifts measured being given in Table I. Two resonances are observed for the 3-deoxy-3-fluoro-glycogen, due to the internal and terminal 3-deoxy-3-fluoro-D-glucose residues, while only one is seen for the 4-deoxy-4-fluoro-glycogen since its deoxyfluoro-D-glucose residue can only be terminal. In order to assign the two peaks in the 3-deoxy-3-fluoro-glycogen spectrum, measurements of the spin–lattice relaxation time (T_1) were performed for both samples of fluorinated glycogens. This relaxation time is very sensitive to the degree of motional freedom of the nucleus and should be quite different for the terminal and internal residues. T_1 values of 0.25 and 0.4 sec, respectively, were measured for the large (δ −116.9 p.p.m.) and small (δ −120.78 p.p.m.) resonances from 3-fluoro-glycogen, while a value of 0.36 sec was measured for the 4-fluoro-glycogen (δ −122.2 p.p.m.).

Both purified 4-deoxy- and 4-deoxy-4-fluoro-glycogen were tested as primers in the reaction with glycogen phosphorylase and α-D-glucopyranosyl phosphate to ensure that the substituted analogues were completely "capped". Incubation of phosphorylase with α-D-glucopyranosyl phosphate and normal glycogen (0.12%) led to the expected rate of phosphate release of 54 μmol.min^{-1}.mg^{-1}, while substitution of the glycogen with either 4-deoxy- or 4-deoxy-4-fluoro-glycogen resulted in *no* phosphate release, even after extended reaction times.

These "capped" glycogens were also tested as inhibitors of phosphorylase-catalysed glycogen synthesis. Results obtained are shown in the form of a pair of Dixon plots in Fig. 3. As can be seen, the inhibitors were surprisingly effective, both binding very tightly to the enzyme. Even at a high (0.98%) concentration of glycogen, a concentration of inhibitor of approximately 0.008% is sufficient to reduce the rate of reaction by 50% in each case. Unfortunately, it is not possible to

assign exact K_i values to the inhibitors since the analysis of such numbers is not a trivial matter[20]. Nonetheless, it would appear that they bind some 100-fold more tightly than glycogen itself. The possibility of this strong inhibition being due to time-dependent irreversible inhibition (caused by the glycogen analogues or a contaminant) was investigated by pre-incubation of a high concentration of enzyme with each glycogen analogue for different times, followed by determination of the enzyme activity in a normal assay. No time-dependent inactivation was observed. The possibility that the tight binding might be due to the disproportionation of the glycogen was investigated by synthesis of disproportionated glycogen and measuring a "K_m" value for this sample in parallel with a similar measurement for "normal" glycogen. The two values were essentially identical. Further, in separate experiments, no inhibition of turnover of normal glycogen by disproportionated glycogen was observed.

DISCUSSION

The time course shown in Fig. 1 for incorporation of deoxyfluoro- and deoxy-D-glucose residues into glycogen and maltopentaose are somewhat inaccurate due to the extended reaction times. Nonetheless, they do serve to illustrate the point that, whereas only a single 4-substituted sugar unit can be incorporated per chain, as indicated by the termination of reaction, large numbers of the 3-substituted sugars can be added. The non-linearity of both the time courses for the 3-substituted sugars is probably due largely to the inactivation of the enzyme at these extended times. However, it is likely that the true time course is non-linear in any case since the deoxy- and deoxyfluoro-glycogen products are probably poorer substrates for further elongation by glycogen phosphorylase than is glycogen itself. Thus, rates would be expected to decrease as this product/substrate appears. Previous preliminary ^{19}F-n.m.r. studies on this reaction[13] had shown that no fluoride release occurs upon incubation of the 2-, 3- or 4-deoxy-4-fluoro-α-D-glucopyranosyl phosphates with this enzyme system, so such possible side reactions are not the cause of the non-linearity.

A more accurate assessment of this termination or capping reaction with 4-substituted sugars is given by the data in Fig. 2 where the extent of incorporation of 4-deoxy-D-*xylo*-hexose residues into several different concentrations of maltopentaose, as a function of time, is presented. These data are far more reliable than those in Fig. 1, since the rate of incorporation of 4-deoxy-D-*xylo*-hexose is much greater than that for the other analogues[13]. Thus shorter reaction times can be used, minimising problems due to enzyme denaturation and substrate hydrolysis. Termination of the reaction is clearly seen at each concentration of maltopentaose, with the concentration of sugar incorporated (from phosphate released) being essentially equal to the concentration of maltopentaose initially present.

Successful syntheses of the 3- and 4-substituted glycogen analogues were achieved by repeated treatment of glycogen with glycogen phosphorylase and the

requisite substituted α-D-hexopyranosyl phosphate analogues. The purified products were characterised by n.m.r. spectroscopy and, to a limited extent, through kinetic studies with glycogen phosphorylase. The n.m.r. data are particularly interesting and informative. ^{1}H-N.m.r. spectra of the 3-deoxy- and 4-deoxy-glycogen analogues contain distinctive resonances at high field due to the axial and equatorial methylene ring protons at the deoxygenated carbon. Integration of the peaks in the spectrum obtained for 4-deoxy-glycogen revealed a 1:1:1 relationship between the intensities of the resonances due to the axial methylene, the equatorial methylene, and the α-(1→6)-linked anomeric proton, thus confirming the incorporation of one 4-deoxy-D-*xylo*-hexose residue per branch point, *i.e.*, one residue per non-reducing terminus. The integration of these resonances for 3-deoxy-glycogen revealed a slightly higher degree of incorporation, approximately 1.2 residues per branch point, showing that more than one residue has been incorporated per terminus. A greater degree of incorporation than this should be possible since the reaction does not terminate. However, this would require much higher concentrations of enzyme and much longer reaction times since the incorporation of 3-deoxy-D-*ribo*-hexose residues occurs approximately 100 × more slowly than that of 4-deoxy-D-*xylo*-hexose residues[13].

The anomeric proton region of these spectra is of particular interest. Only two resonances are observed in the spectrum for 4-deoxy-glycogen, that from the α-(1→6) linkages at δ 4.98 p.p.m. and the major peak at δ 5.38 p.p.m. from the α-(1→4)-linked residues. Presumably the anomeric proton resonance from the terminal 4-deoxy-D-*xylo*-hexose residue is coincident with this latter resonance since their relative integration is now 12:1, compared to the ratio of 10:1 in glycogen itself. Four resonances are observed in this region for 3-deoxy-glycogen. Two of these are identical to those in glycogen at δ 5.37 and δ 4.98 (ratio 10:1) and correspond to the α-(1→4)- and α-(1→6)-linked glucose residues. The other two resonances at δ 5.13 and δ 5.27 (relative integration 0.75 and 0.75) presumably arise from the anomeric protons of α-(1→4)-linked internal and terminal 3-deoxy-D-*ribo*-hexose residues, although their assignment (terminal versus internal position) is unclear. Their upfield shifts relative to that of the α-(1→4)-linked anomeric proton of glycogen itself is reasonable since the anomeric proton of 3-deoxy-α-D-*ribo*-hexopyranosyl phosphate resonates 0.16 p.p.m. upfield of that of α-D-glucopyranosyl phosphate[12]. Differences between the shifts of the internal and terminal 3-deoxy-D-*ribo*-hexose anomeric protons are presumably a consequence of the presence or absence of a sugar substituent at the 4-position. Integration of the sum of these two resonances suggests an incorporation of 1.5 deoxy-hexose residues per branch point, a value that is in reasonable agreement with that of 1.2 suggested by the methylene proton integrations.

^{1}H-N.m.r. spectra of the 3- and 4-deoxyfluoro-glycogens are less interesting. Both again show the two anomeric proton resonances due to α-(1→4) and α-(1→6) linkages. In addition, the 3-deoxy-3-fluoro-glycogen spectrum contains two shoulders at approximately δ 5.34 and δ 5.44, the former being the most intense.

These presumably arise from the anomeric protons of internal and terminal 3-deoxy-3-fluoro-D-glucose residues. No such additional resonances are observed in the 4-deoxy-4-fluoro-glycogen sample as the terminal 4-deoxy-4-fluoro-D-glucose anomeric proton resonance is presumably obscured by the major peak at δ 5.37.

The ^{19}F-n.m.r. spectra of the two deoxyfluoro glycogen samples are quite informative. The spectrum for 4-deoxy-4-fluoro-glycogen shows a single resonance, δ −122.2 as expected, due to the terminal 4-deoxy-4-fluoro-D-glucose residues on the glycogen. The 3-deoxy-3-fluoro-glycogen, however, shows two resonances of quite different intensities at δ −116.9 and −120.8, which arise from the internal and terminal residues, the former being the larger. The assignment of these two resonances was an interesting challenge and could not be made on the basis of chemical shifts alone. Assignments were based on the longitudinal relaxation times, T_1, for each of these resonances since these should be very sensitive to the degree of motional freedom of the nucleus in question, and the terminal residue should have considerably greater motional freedom than an internal residue. Further, measurement of the T_1 for 4-deoxy-4-fluoro-glycogen, where the fluorinated sugar *must* be a terminal residue, allows assignment of the two resonances from 3-deoxy-3-fluoro-glycogen since similar T_1 values would be expected for the two terminal residues since they have similar mobilities, and the ^{19}F-nucleus is attached directly to the ring in both cases. Since T_1 = 0.36 s for 4-deoxy-4-fluoro-glycogen, the resonance at δ −120.78 (T_1 = 0.4 s) in the 3-deoxy-3-fluoro-glycogen spectrum must arise from the terminal residue, while the larger resonance at δ −116.9 (T_1 = 0.26 s) must be that of the internal residue. This measurement of faster relaxation for the less mobile resonance was initially surprising, given that the opposite is generally true for macromolecules. However, further investigation showed that this result is quite reasonable since the chain termini of glycogen have mobilities more similar to those of small molecules. This fact was shown in a previous ^{13}C-n.m.r. study of glycogen[24] where a correlation time (τ_c) of ~4 × 10^{-9} s was calculated as the average correlation time for all the sugar residues, based upon T_1 measurements. The correlation time of D-glucose residues in the outer "shell" of glycogen should therefore be less than or equal to this value. Given this information, it is clear that the nucleus in question (^{19}F at 254 MHz) will relax more like a small molecule since $\omega\tau_c < 1$.

It was also initially surprising that the larger peak arose from the internal residue. This finding is, however, also quite reasonable since, once some phosphate has been released into the reaction, disproportionation of the glycogen chains will occur very rapidly at the concentration of enzyme employed. This will result in the addition of normal glucose residues onto the recently added 3-deoxy-3-fluoro-D-glucose residues, ultimately resulting in more internal than terminal 3-deoxy-3-fluoro-D-glucose residues since the transfer of D-glucose itself will occur many times more rapidly than that of 3-deoxy-3-fluoro-D-glucose. Indeed it is interesting to note that, in the previous ^{19}F-n.m.r. characterisation[9] of 3-deoxy-3-fluoro-glycogen extracted from locust tissue that had been previously incubated with 3-deoxy-3-

fluoro-D-glucose, a spectrum is presented with a major peak at δ −121.8 p.p.m. (relative to 0.1M trifluoroacetic acid) and a shoulder approximately 5 p.p.m. downfield, exactly where this second peak would be expected, although no reference is made to this shoulder. The correspondence with the data presented here is gratifying, though it is interesting to note that the relative intensities of these two peaks are inverted. This result is quite reasonable as the major peak should arise from terminal 3-deoxy-3-fluoro-D-glucose residues when the 3-deoxy-3-fluoro-D-glucose is incorporated *via* glycogen synthetase, since no disproportioation occurs with that enzyme.

The isolated "capped" 4-deoxy- and 4-deoxy-4-fluoro-glycogen samples were clearly demonstrated to be incapable of acting as substrates for glycogen phosphorylase in the synthetic direction. This demonstrates that the "capping" was complete. Thus any hydrolysis of polysaccharide which may have occurred during the synthesis was "repaired" by further "recapping" of the newly exposed termini. However, since this poor substrate activity could be the result of poor binding, the capped glycogen samples were also investigated as inhibitors of glycogen phosphorylase-catalysed glycogen synthesis and shown to be surprisingly effective, as is illustrated by the data in Fig. 3. As noted in the Results section, it is not possible to assign meaningful K_i values to these inhibitors because of the difficulties inherent in such analyses. However, it is clear that these inhibitors bind quite tightly, at least 100-fold more tightly than glycogen itself. The origin of this tight binding is not clear and awaits further investigation.

These "capped" analogues should, therefore, act as useful inhibitors of glycogen phosphorylase and glycogen synthetase in mechanistic studies; indeed their tight binding will be quite advantageous to these studies. Experiments are currently underway to use these analogues in a search for glycosyl–enzyme intermediates on both of these enzymes.

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