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PROBING ACCEPTOR SPECIFICITY IN THE GLYCOGEN SYNTHASE REACTION WITH POLYMER-BOUND OLIGOSACCHARIDES*

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ABSTRACT

Polymers having maltose and maltotriose side-chains were synthesized by attaching 4-carboxy-2-nitrobenzyl 4-*O*-α-D-glucopyranosyl-β-D-glucopyranoside or 4-carboxy-2-nitrobenzyl *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-*O*-β-D-glucopyranoside, respectively, to aminoethyl-substituted polyacrylamide gel beads. Subsequently, the two polymers, and analogous polymers having D-glucose and cellobiose side-chains, served in a comparative study as acceptors in the glycogen synthase (UDP-D-glucose: glycogen 4-α-glucosyltransferase, EC 2.4.1.11) reaction. Highest transfer (4.2%) was observed for the polymer bearing maltotriose groups. The bound saccharides were then removed by irradiation (>320 nm), and examination of them demonstrated that α-D-glucosyl oligomerization in the glycogen synthase reaction had occurred.

INTRODUCTION

Numerous studies (e.g. refs. 1–10 and works cited therein) have been concerned with the necessity of a primer, and primer requirements, in glycogen biosynthesis and more explicitly in the glycogen synthase reactions. Glycogen itself serves as a primer, and it was proposed that the additional—so called "unprimed"—glycogen synthase reaction may involve an acceptor glucoprotein[3,4,10]. The existence of this glucoprotein is debated[6,7], as no covalent bond has been demonstrated between the carbohydrate moiety and the protein. Most results may be alternatively explained by the formation of a trichloroacetic acid-insoluble glycogen precipitate and a small amount of glycogen bound noncovalently to proteins—particularly to enzymes related to glycogen metabolism. Furthermore, such glucoproteins were not demonstrated by conversion into glycogen to be genuine glycogen precursors.

Related to the primer issue is the ability to elicit *de novo* synthesis of glycogen. Thus, the *de novo* synthesis reported by many (cited in refs. 6 and 7) is questioned

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as being most probably due to a small amount of a tightly-bound primer[5–7].

Acceptors of lower molecular weight have also been studied in the glycogen synthase reaction[11–13]. In two cases, saccharide products resulting from the addition of a glucose unit were reported, but without significant formation of higher, homologous oligosaccharides.

In the present work, the synthesis of suitable maltose and maltotriose derivatives, and use of the technique of enzymic oligosaccharide synthesis on insoluble polymer supports[14], were aimed at determining the extent of transfer, based on incorporation of glucose. In each instance, the polymer was considered a useful handle suitable for "fishing out" even low yields of products, all of which must arise from *de novo* synthesis.

RESULTS AND DISCUSSION

Two glycosides, 4-carboxymethyl-2-nitrobenzyl 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-β-D-glucopyranoside (**1**) and 4-carboxymethyl-2-nitrobenzyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)-*O*-2,3,6-tri-*O*-acetyl-α-D-glucopyranoside (**2**), were synthesized by Koenigs–Knorr condensation of methyl 4-hydroxymethyl-3-nitrobenzoate and the corresponding glycosyl bromides. Following deacetylation and using a combination of α-D-glucosidase and β-D-glucosidase, the anomeric linkage to the aglycon was determined in both cases to be β; optical rotation and migration in t.l.c. (Table I), n.m.r., and analytical data supported the proposed structures. The protecting groups were removed from compounds **1** and **2** and the resulting (unisolated) carboxylic derivatives were condensed with aminoethyl-substituted polyacrylamide gel beads (H_2N-Ⓟ) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, with subsequent *N*-acetylation, to yield compounds **4** and **5**, respectively. Compounds **3** and **6** were prepared as before[14]. As polymers **3–6** are light sensitive, release by irradiation was used to determine the attached saccharide in each instance.

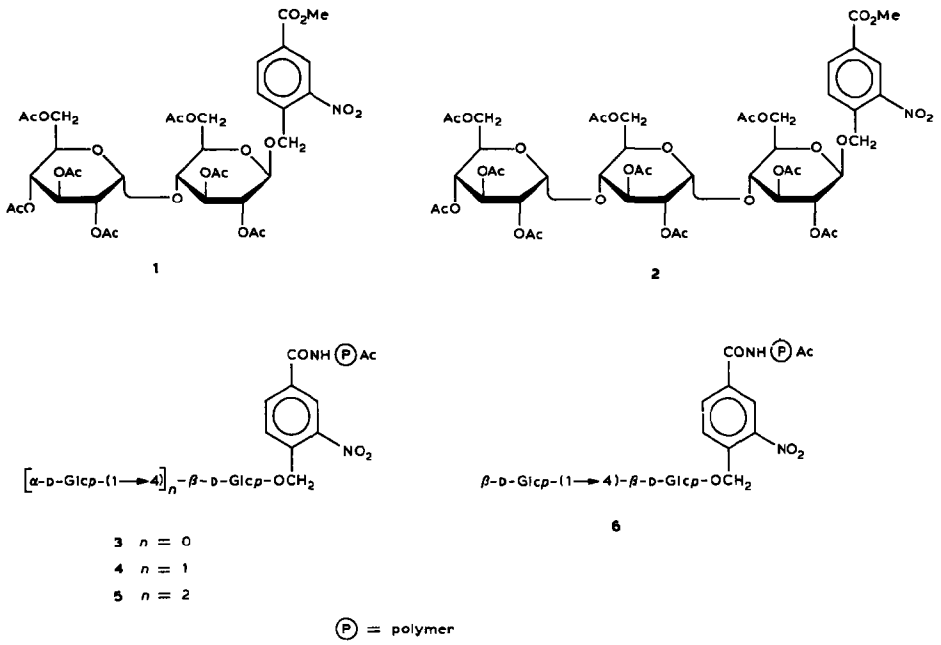
Polymers **3–6** served as acceptors in the glycogen synthase reaction, giving

TABLE I

T.L.C. AND OPTICAL ROTATION OF GLYCOSIDES OF THE MALTOSE SERIES

Compound	R_2^g *nitrobenzylalcohol*	$[\alpha]_D^g$
Methyl 4-hydroxymethyl-3-nitrobenzoate[13]	0.90	0
4-Carboxymethyl-2-nitrobenzyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside[13]	0.75	−24.6 ±1.9 (0.22)
Compound **1**	0.58	+23.6 ±1.1 (0.54)
Compound **2**	0.36	+46.9 ±0.7 (1.3)

[a]Ethyl acetate–petroleum ether, 1:1 (v/v). [b]Degrees, measured in chloroform at 25°, concentrations (percentage) are given in parentheses.



rise to polymers **7–10**, respectively. The incorporation of D-glucose from radioactively labeled UDP-D-glucose into these polymers (**3–6**) was determined from the radioactivity bound (Table II). The efficiency of transfer correlated well with the structure of the acceptor. Thus, the polymer (**6**) having cellobiose attached had the same, low acceptor efficiency as that (**3**) having β-D-glucose attached. Sequential addition of α-(1→4)-glucopyranosyl groups results in improved transfer to the maltose derived polymer (**4**) and a further improved transfer to the maltotriose-derived polymer (**5**), analogous to the reported homogeneous reactions of maltose and maltotriose[11,13]. It is pertinent that, in the present technique, although the transfer was low (up to 4.23%, 233.134 c.p.m./g), there was no difficulty in isolating the products (**7–10**) and determining the extent of transfer.

Irradiation of polymer **9**, followed by counting of radioactivity in the reacted polymer (**11**), indicated that 35% of the counts remained—in variance from the high yields of photochemical release observed in previous work. Paper chromatography of the residue obtained after lyophilization of the water washings showed 5% of the counts to migrate like maltotetraose (**12**). In this instance, reduction by sodium borohydride followed by acid hydrolysis showed the absence of labelled glucose at the reducing end—an observation compatible with transfer of glucose units by glycogen synthase to the non-reducing end. Of the total radioactivity, 44% migrated in chromatography slower than maltotetraose, a considerable proportion (**13**) staying next to the origin. Treatment of such origin material (**13**) with amyloglucosidase (which is indicative of α-D-glucopyranosyl linkages, normally present in

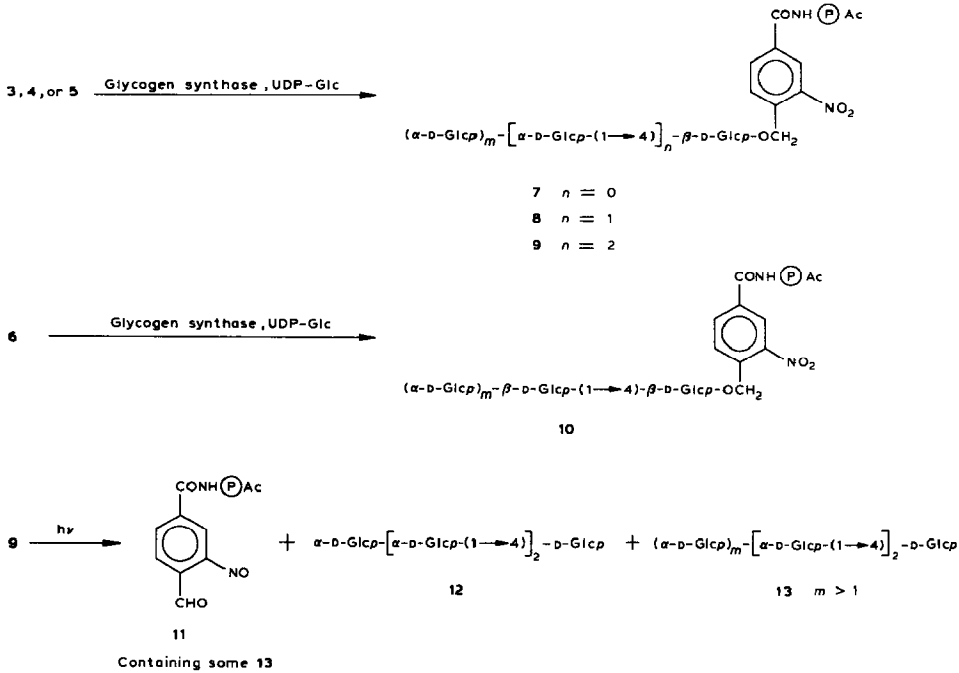
TABLE II

GLYCOGEN SYNTHASE-CATALYZED INCORPORATION[a] OF D-GLUCOSE INTO ACCEPTOR POLYMERS

	Experiment 1		*Experiment 2*	
	μmol/g	*% transfer*	*μmol/g*	*% transfer*
Compound **3**	0.11	0.36	0.033	0.11
Compound **4**	0.076	1.01	0.042	0.56
Compound **5**	0.22	4.23	0.048	0.92
Compound **6**			0.0056	0.093

[a]Details of incubation conditions are described in the experimental section.

maltodextrins and glycogen), releases glucose. Most of the radioactivity remaining on polymer **11** was also released by amyloglucosidase. Although not yet supported by direct structural evidence, the current results are compatible with the formation of higher α-D-glucopyranosyl oligomers. Consequently, further structural and kinetic experiments are planned with a highly purified glycogen synthase preparation.



The ease in which products are isolated by this polymer technique[14–16] is noteworthy. It permits efficient comparison of acceptor specificity by employing the same acceptors, and is capable of demonstrating *de novo* synthesis.

EXPERIMENTAL

General. — Materials, methods and equipment were as described[14]. Enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO 63178) unless otherwise mentioned. UDP-D-[U-1^{14}C]Glucose was purchased from the Radiochemical Centre (Amersham, Bucks, HP75LL, U.K.). The enzymes used were: α-D-glucosidase (EC 3.2.1.20, from rice), a product of Makor Chemicals, Jerusalem, Israel; β-D-glucosidase (emulsin, EC 3.2.1.21, from almonds), a product of Fluka, CH-9470 Buchs, Switzerland; glycogen synthase (UDP-D-glucose: glycogen 4-α-glucosyltransferase, EC 2.4.1.11, from rabbit muscle); and amyloglucosidase (glucoamylase, exo-(1→4)-α-D-glucosidase, (1→4)-α-D-glucan glucohydrolase, EC 3.2.1.3, from *Rhizopus*). T.l.c. was performed on precoated Silica gel $60F_{254}$ sheets (E. Merck D-6600 Darmstadt, F.R.G.), and compounds were detected by viewing under u.v. light or by spraying with sulfuric acid. Column chromatography was performed on Silica Gel 60 (0.063–0.2 mm, Merck).

4-Carboxymethyl-2-nitrobenzyl 2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-β-D-glucopyranoside (**1**). — This compound was prepared, by analogy to a literature procedure[17], with the following quantities: 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-α-D-glucopyranosyl bromide[18] (13 g, 30.4 mmol), methyl 4-hydroxymethyl-3-nitrobenzoate[14] (2.8 g, 13.3 mmol), calcium sulfate (1.3 g), calcium carbonate (2.5 g), nitromethane (75 mL), and silver perchlorate (3.2 g). Following filtration through a Celite filter and evaporation, the residue was applied to a column of silica gel (120 g, 2.5 cm in diameter) eluted with 1:1 (v/v) ethyl acetate–petroleum ether (12-mL fractions). Unreacted methyl 4-hydroxymethyl-3-nitrobenzoate (0.71 g) was recovered from fractions 14–20. Compound **1**, emerging in fractions 34–72, was applied to a second column of silica gel (150 g, 3 cm in diameter) eluted with the same solvent mixture (15-mL Pure, crystalline compound **1** was recovered from fractions 56–74 (1.7 g, 21% yield) and recrystallized as needles from ethyl acetate–petroleum ether, m.p. 110°; ^{1}H-n.m.r. (80 MHz) includes: δ 8.65 (d, 1 H, *J* 2 Hz, aromatic), 8.21 (q, 1 H, *J* 2 Hz, *J* 8.5 Hz, aromatic), 7.71 (d, 1 H, *J* 8.5 Hz, aromatic), 3.93 (s, 3 H, OCH_3), and 2.12, 2.10, 2.04, 2.02, and 2.00 (total 24 H, OAc).

Anal. Calc. for $C_{35}H_{43}NO_{22}$: C, 50.66; H, 5.22. Found: C, 50.71; H, 5.24.

4-Carboxymethyl-2-nitrobenzyl O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1→4)-O-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**2**). — *O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)-*O*-2,3,6-tri-*O*-acetyl-α- and β-D-glucopyranose[19,20] (0.75 g) was dissolved with stirring in 45% (w/v) hydrobromic acid in acetic acid (3 mL). Stirring was continued for 2 h at room temperature, the mixture was poured into ice, extracted with cold chloroform, and the extract washed with ice–water. The chloroform solution was dried (calcium chloride) and evaporated *in vacuo*. The residue was condensed with methyl 4-hydroxymethyl-3-nitrobenzoate (0.4 g) as described for compound **1**. Fractionation was conducted

on a column of silica gel (70 g, 1.5 cm in diameter), that was eluted with 1:2 (v/v) ethyl acetate–petroleum ether (130 mL) followed by 1:1 (v/v) ethyl acetate–petroleum ether (10-mL fractions). Compound **2**, pure by t.l.c. (last solvent mixture) was recovered as a yellowish oil from fractions 47–63 (140 mg, 9%); ^{1}H-n.m.r. (80 MHz) includes: δ 8.66 (d, 1 H, J 2 Hz, aromatic), 8.25 (m, 1 H, aromatic), 7.82 (d, 1 H, J 8.5 Hz, aromatic), 5.09 (benzylic CH_2 on top of ring protons), 3.99 (s, 3 H, OCH_3), and 2.12, 2.10, 2.09, 2.06, 2.03, and 2.02 (total 30 H, OAc).

Anal. Calc. for $C_{47}H_{59}NO_{30}$: C, 50.5; H, 5.3; N, 1.3. Found: C, 50.0; H, 5.6; N, 1.1.

Anomeric linkage to aglycon. — An acetylated glycoside: 4-carboxymethyl-2-nitrobenzyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside[14], compound **1**, or compound **2** (0.25 mL, 5.5 mM) in 0.1M methanolic sodium methoxide was kept overnight at 4°. The solution was made neutral with M acetic acid and evaporated *in vacuo*. Each sample was dissolved in water (0.25 mL), α-D-glucosidase (1 unit in 25 μL) was added, and the mixture was incubated for 3 h at 37°. (a) β-D-Glucosidase (0.25 units in 25 μL) was added to one-half of the volume of each sample and the incubation was continued for 2 h at 37°. (b) Water (25 μL) was added into the remaining half of each sample and incubation was continued for 2 h at 37°. T.l.c. in 1:1.7 (v/v) ethyl acetate–petroleum ether demonstrated the presence of methyl 4-hydroxymethyl-3-nitrobenzoate in all the β-D-glucosidase reactions (a); it was not observed in the controls (b).

Attachment of saccharides. — The polymer used for condensation with glycosides was aminoethyl-substituted polyacrylamide gel in bead from[14,21] (Ⓟ-NH_2, 3.8 meq. NH_2/g). 2-Nitro-4-(*N*-ⓅAc-carboxyamido)benzyl β-D-glucopyranoside (**3**, 31 μeq. glucose/g, release upon irradiation) and 2-nitro-4-(*N*-ⓅAc-carboxyamido)-benzyl 4-*O*-β-D-glucopyranosyl-β-D-glucopyranoside (**6**, 6 μeq. cellobiose/g, release upon irradiation) were prepared as described[14]. 2-Nitro-4-(*N*-ⓅAc-carboxamido)benzyl 4-*O*-α-D-glucopyranosyl-β-D-glucopyranoside (**4**, 7.5 μeq. maltose/g, release upon irradiation) and 2-nitro-4-(*N*-ⓅAc-carboxamido)benzyl (4-*O*-α-D-glucopyranosyl)-4-*O*-α-D-glucopyranosyl-β-D-glucopyranoside (**5**, 5.2 μeq. maltotriose/g, release upon irradiation) were prepared from compounds **1** and **2**, respectively as described[14] for compound **6**.

Glycogen synthase reaction. — Incubation conditions were based on those of Salsas and Larner[12]. In experiment 1, the polymer (60 mg) was preincubated for 20 min, with stirring, at 30° in a mixture (2.0 mL) containing UDP-D-[^{14}C]glucose (1mM, 1,059,700 c.p.m./μmol), tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl, 50mM, pH 7.8), ethylenedinitrilo(tetracetic acid) (EDTA, 5mM, 1,4-dithio-DL-threitol (5mM), D-glucose 6-phosphate (10mM), and sodium sulfate (2 mM). Glycogen synthase (50 mU in 0.28 mL) was added and the incubation was continued for 6 h at 30°. The polymer was isolated by filtration, washed extensively with 5% aqueous sodium chloride and water (until no radioactivity was released in the washings), and lyophilized.

In experiment 2, the polymer (containing 197 μmol of bound saccharide/mL)

was incubated in a mixture containing a lower concentration of UDP-[^{14}C]-D-glucose (250μM; 1,254,000 c.p.m./μmol). Glycogen synthase (23 mU in 0.13 mL) was added to the mixture (1.0 mL), and the incubation and isolation were as described in experiment 1.

Glycogen synthase activity (I + D) was determined by the incorporation of D-[^{14}C]-glucose into glycogen[22]. Glycogen synthase I activity was 27.5% of the stated values.

Estimation of the amount of sugar released from the polymer by irradiation. — The polymer (10–15 mg) was suspended with stirring in water (1.0 mL) in a closed Pyrex test-tube, and irradiated (20 h) in an RPR-100 apparatus (Rayonet, the Southern New England Ultraviolet Company, Hamden, Connecticut 06514) with RPR 3500A lamps. Following filtration, the released saccharide was determined in the filtrate by the phenol–sulfuric acid test[23].

Release of saccharides. — The polymer was suspended in water (100 mg/20 mL) and irradiated as described for the determination of sugar released. The reacted polymer was removed by filtration, washed with warm water, the filtrate and the washings were lyophilized, and the residue subjected to descending paper chromatography (Whatman No. 3MM paper; 3:5:1:3 pyridine–1-butanol–benzene–water, upper phase; markers were stained by silver nitrate[24]).

Amyloglucosidase digestion. — Material at the origin (**13**, 1,400 c.p.m.) obtained from compound **9** following irradiation, paper chromatography, extraction with water, and lyophilization was dissolved in 0.1M sodium acetate buffer (pH 4.9, 2.0 mL). Amyloglucosidase (6 mg) was added and the mixture was incubated[25] for 2 h at 55°. The mixture was deionized by filtering through Amberlite IR-120 (H^+) and Amberlite 45 (OH^-) resin, lyophilized, and subjected to paper chromatography (compare release of saccharides); the radioactivity appeared almost quantitatively at the D-glucose marker.

The irradiated polymer (**11**, 2,583 c.p.m.) was treated similarly with amyloglucosidase, releasing 90% of the radioactivity. Examination of the deionized solution by paper chromatography showed that 87% of the radioactivity appeared in the area ranging from glucose to maltotetraose. This area was extracted with water (5 mL), lyophilized, and subjected once again to the aforementioned amyloglucosidase treatment. Paper chromatography following lyophilization of the deionized solution gave rise to one radioactive peak (60% of the original counts) at the D-glucose marker.

Qualitative reducing-end determination. — Sodium borohydride (10 mg) was added to a solution of compound **12** (1,005 c.p.m.) in water (1 mL). After 16 h at room temperature, the solution was treated with Amberlite IR-120 (H^+), evaporated *in vacuo*, and methanol (2 × 5 mL) was evaporated from the flask. The residue was boiled under reflux for 2 h in 0.5M sulfuric acid in 3:1 water–1,4-dioxane, the resulting solution was diluted with water (5 mL), made neutral by stirring with barium carbonate, filtered through Celite, Amberlite IR-120 (H^+) and Amberlite 45 (OH^-), evaporated, and subjected to descending paper chromatog-

raphy (Whatman No. 3MM paper; 2:1:2 ethyl acetate–pyridine–water, upper phase, markers were stained by silver nitrate[24]). Radioactivity was not detected at the glucitol marker, whereas it was present (260 c.p.m.) at the glucose marker.

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