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The Mechanism of Glycogen Synthetase as Determined by Deuterium Isotope Effects and Positional Isotope Exchange Experiments*

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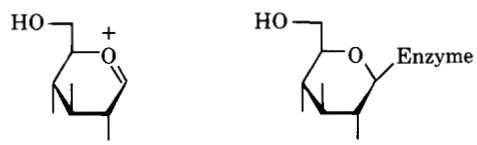
The reaction mechanism for glycogen synthetase from rabbit muscle was examined by α-secondary deuterium isotope effects and positional exchange experiments. Incubation of glycogen synthetase with [β-$^{18}O_2$,αβ-^{18}O]UDP-Glc did not result in any detectable positional isotope exchange from the β-nonbridge position to the anomeric oxygen of the glucose moiety. Glucono-1,5-lactone was found to be a noncompetitive inhibitor *versus* UDP-Glc. The kinetic constants, K_{is} and K_{ii}, were found to be 91 ± 4 μM and 0.70 ± 0.09 mM, respectively. Deoxynojirimycin was a nonlinear inhibitor at pH 7.5. The α-secondary deuterium isotope effects were measured with [1-^{2}H]UDP-Glc by the direct comparison method. The isotope effects on V_{max} and V_{max}/K were found to be 1.23 ± 0.04 and 1.09 ± 0.06, respectively. The inhibitory effects by gluconolactone and deoxynojirimycon plus the large α-secondary isotope effect on V_{max} have been interpreted to show that an oxocarbonium ion is an intermediate in this reaction mechanism. The lack of a detectable positional isotope exchange reaction in the absence of glycogen suggests the formation of a rigid tight ion pair between UDP and the oxocarbonium ion intermediate.

Glycogen synthetase catalyzes the transfer of a glycosyl residue from UDP-glucose to the C-4 hydroxyl group of a growing glycogen primer with the formation of an α1,4 linkage as illustrated below:

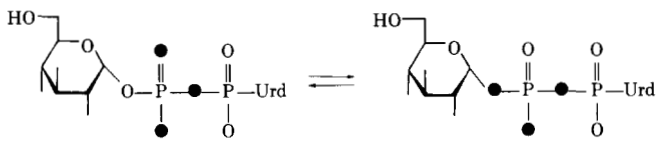
$$\text{UDP-Glc} + [\text{glycogen}]_n = \text{UDP} + [\text{glycogen}]_{n+1} \quad (1)$$

Recent kinetic studies using equilibrium isotope exchange have indicated that glycogen synthetase has a rapid equilibrium random Bi Bi mechanism (1). The stereochemical outcome of the overall chemical reaction is one of retention of configuration at C-1 of the glucose moiety. This observation would strongly suggest that the carbon-oxygen bond of UDP-Glc is completely broken prior to the nucleophilic attack by the incoming glycogen. Probable intermediates formed in such a mechanism could either be an oxocarbonium ion (I) or a covalent glycosyl-enzyme (II) adduct as illustrated below.



(I) (II)

The technique of positional isotope exchange (PIX)[1] has been applied to a variety of enzyme-catalyzed reactions in the search for transient intermediates (2-7). The PIX technique can be used to probe the mechanism of enzymes that direct the attack of a nucleophile at the anomeric center of UDP-Glc. Singh *et al.* (7) synthesized [β-$^{18}O_2$,αβ-^{18}O]UDP-Glc (III) and used this compound to measure the positional isotope exchange rate for the interconversion of a β-nonbridge oxygen and the anomeric oxygen in the reaction catalyzed by sucrose synthetase.



(III) (IV)

The expected exchange reaction by sucrose synthetase did not occur in the absence of fructose. The addition of 2,5-anhydromannitol, an analog for the active conformation of fructose, did not stimulate the positional isotope exchange. This result indicates that either the scissile carbon-oxygen bond is not broken until the fructose is bound to the enzyme or the β-phosphoryl group of UDP is unable to rotate at a kinetically significant rate.

α-Deuterium isotope effects and the tight binding by gluconolactone and deoxynojirimycin have been used previously as evidence in support of an oxocarbonium ion intermediate in the mechanisms of enzymes that involve the transfer of a glycosyl group to some acceptor substrate. The sp^2 character at C-1 and the dominant $^4H_3$ half-chair conformation are thought to be the primary reasons for the inhibition by gluconolactone of enzymatic reactions involving an oxocarbonium ion intermediate (8, 9). Deoxynojirimycin is a powerful inhibitor of α-glucosidase (10). Hosie and Sinnott (9) have proposed a $^{2,5}B$ conformation for the oxocarbonium intermediate in the α-glucosidase reaction. Deoxynojirimycin can adopt this conformation (9). Dahlquist *et al.* (11, 12)

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[1] The abbreviations used are: PIX, positional isotope exchange; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

showed that the nonenzymatic acid-catalyzed hydrolysis of phenyl-β-D-glucopyranoside, which is thought to have a glucopyranosyl cation as an intermediate in the mechanism, has an α-deuterium isotope effect (k_H/k_D) = 1.13; the nonenzymatic base-catalyzed hydrolysis, which is considered to react via an S_N2 mechanism, has an isotope effect (k_H/k_D) = 1.03. The lysozyme mechanism, where k_H/k_D with phenyl-4-*O*-(2-acetamide-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside is 1.11, is consistent with an oxocarbonium ion intermediate or transition state (12).

In this paper, oxygen-18-labeled UDP-Glc has been used to probe for the existence of such an intermediate in the reaction catalyzed by glycogen synthetase under a variety of experimental conditions. Secondary deuterium isotope effects and inhibition studies by transition state analogs were also utilized to elucidate the probable mechanism for this transformation.

MATERIALS AND METHODS

Potassium cyanate was obtained from Alpha Chemical Co. [1-^{2}H] D-Glucose (V) was purchased from MSD Isotopes Co. $KH_2P^{18}O_4$ was synthesized from PCl_5 and oxygen-18-labeled water (97%, Cambridge Isotope Laboratory) according to the method of Risley and Van Etten (13). NMR analysis of the labeled phosphate indicated >95% incorporation of oxygen-18. Nojirimycin was a gift of Professor C.-H. Wong (Texas A&M University). Deoxynojirimycin was synthesized from nojirimycin according to the method of Inouye *et al.* (14). All other reagents were acquired from either Sigma or Aldrich. [β-$^{18}O_2$,αβ-^{18}O]UDP-glucose (III) was synthesized according to the method of Singh *et al.* (7). [1-^{2}H]UDP-Glc (VIII) was synthesized enzymatically from [1-^{2}H]D-glucose according to Scheme I.

Synthesis of [1-^{2}H]D-Glucose 6-Phosphate (VI)—Hexokinase was used to phosphorylate [1-^{2}H]glucose at C-6 with ATP. 10 mM ATP, 5.0 mM [1-^{2}H]glucose, 5.0 mM $MgSO_4$, and 30 units of yeast hexokinase were incubated in a volume of 100 ml containing 20 mM Tris buffer, pH 7.5. The formation of [1-^{2}H]glucose 6-phosphate was monitored spectrophotometrically at various times using glucose-6-phosphate dehydrogenase and NAD^+. The reaction was terminated after 70 min by centrifuging the reaction mixture through a CF-25 Centriflo ultrafiltration membrane cone (Amicon Corp.). The filtrate was diluted to 500 ml and was applied to a Whatman DE-52 anion exchange column (1.5 × 50 cm). The [1-^{2}H]glucose 6-phosphate was eluted using a 2.0-liter gradient from 10 to 200 mM triethylamine/HCO_3^- buffer, pH 7.5. An isolated yield of 477 μmol was obtained (95%).

Synthesis of [1-^{2}H]Uridine Diphosphoglucose (VIII)—[1-^{2}H]UDP-Glc was synthesized by the combined activities of phosphoglucomutase and UDP-Glc pyrophosphorylase. The reaction mixture contained 2.4 mM [1-^{2}H]glucose 6-phosphate, 4.5 mM UTP, 1.0 mM $MgSO_4$, 100 mM Tris buffer, pH 7.5, 0.025 mM glucose 1,6-bisphosphate, 500 units of inorganic pyrophosphatase, 500 units of UDP-Glc pyrophosphorylase, and 1000 units of phosphoglucomutase in a volume of 200 ml. The formation of UDP-Glc was monitored via high performance liquid chromatography. The reaction mixture was quenched by passing the reaction mixture through an Amicon PM-30 ultrafiltration membrane. The filtrate was diluted to 500 ml and was applied to a Whatman DE-52 anion exchange column (1.5 × 50 cm). The [1-^{2}H]UDP-Glc was eluted with a 2.0-liter gradient from 10 to 300 mM triethylamine/HCO_3^-, pH 7.5. The isolated yield was 350 μmol (73%). The proton NMR spectrum of the [1-^{2}H]UDP-Glc indicated that the incorporation of deuterium was >95%.

Positional Isotope Exchange Reactions—Glycogen synthetase was incubated with 1.0 mM [β-$^{18}O_2$,αβ^{18}O]UDP-Glc, 50 mM Hepes, pH 7.5, and 10 mM glucose 6-phosphate in a volume of 12 ml for various lengths of time at 25 °C. The reaction was terminated by centrifugation of the reaction mixture through a CF-25 Centriflo ultrafiltration membrane cone. The filtrate was diluted to 250 ml, and the pH was adjusted to approximately 8.0. The oxygen-18-labeled UDP-Glc was loaded onto a Whatman DE-52 anion exchange column (1.5 × 50 cm) and was eluted with a 2.0-liter gradient from 10 to 250 mM triethylamine/HCO_3^-, pH 7.5. The fractions containing UDP-Glc were pooled and evaporated to dryness. The residue was dissolved in a 3.0-ml solution containing 100 mM EDTA, 150 mM Tris buffer, pH 9.0, and 25% D_2O. Identical experiments were also conducted at pH 7.5 using α-cyclodextrin (0.17 M) in an attempt to induce the PIX reaction by the binding of other ligands to the active site. The distribution of oxygen-18 was determined using ^{31}P NMR spectroscopy as described previously (7).

Inhibition Studies—The incubation medium contained 100 mM Hepes buffer, pH 7.5, 6.0 mM glucose 6-phosphate, 6.0 mM $MgCl_2$, 2.0 mg/ml glycogen, and 0.01 units of glycogen synthetase in a reaction volume of 0.3 ml. The concentration of UDP-Glc was varied from 0.3 to 3.0 mM with variable amounts of glucono-1,5-lactone. The rate of formation of UDP was monitored via high performance liquid chromatography with a Whatman anion exchange column using 0.125 M KH_2PO_4 as the eluting buffer. The data were fit to the equation for noncompetitive inhibition.

$$V = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (2)$$

The inhibitory effect by deoxynojirimycin was measured by the same method using a fixed concentration of UDP-Glc (1.0 mM) and 100 mM Hepes buffer at pH 7.5 or 100 mM MES buffer, pH 6.0.

Measurement of Deuterium Isotope Effects—Secondary deuterium isotope effects of [1-^{2}H]UDP-Glc on glycogen synthetase were determined by the direct comparison of the initial velocities. The initial velocities were measured by coupling the formation of UDP with the reactions catalyzed by pyruvate kinase and lactate dehydrogenase. Reaction mixtures (3.0 ml) contained 100 mM Hepes buffer, pH 7.5, 6.0 mM glucose 6-phosphate, 6.0 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 2.0 mg/ml glycogen, 0.2 mM NADH, 500 units of pyruvate kinase, 100 units of lactate dehydrogenase, and various concentrations of UDP-Glc or [1-^{2}H]UDP-Glc. The reaction was initiated by the addition of 0.05 units of glycogen synthetase and monitored by following the change in the NADH concentration at 340 nm. The isotope effects on V and V/K were obtained according to Equation 3 (15),

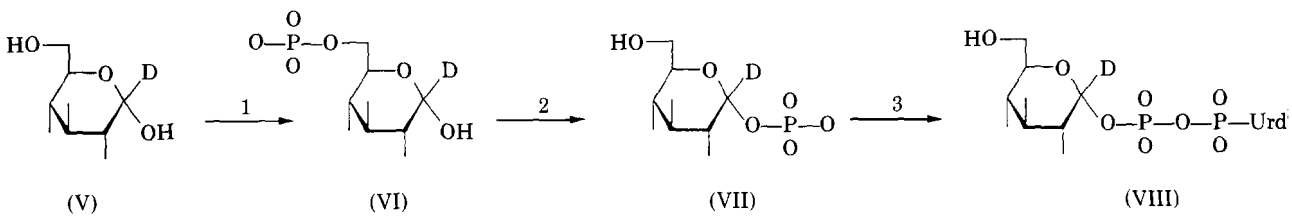
$$V = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (3)$$

where F_i is the fraction of isotope substitution, and E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K, respectively.

^{31}P Nuclear Magnetic Resonance Measurements—^{31}P NMR spectra were obtained on a Varian XL-400 multinuclear spectrometer operating at a frequency of 162 MHz. Typical acquisition parameters were 6000 Hz sweep width, 15 μs pulse width (45°), and broad-band proton decoupling. All spectra were referenced to an internal standard of phosphate at pH 9.0. Up to 10,000 transients were collected and Fourier-transformed.

RESULTS AND DISCUSSION

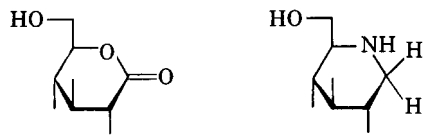
The timing and the sequence of the bond-making and bond-breaking steps in the reaction catalyzed by glycogen synthetase were examined via a combination of PIX, inhibition, and secondary deuterium isotope effect experiments. The PIX



SCHEME 1. **Synthesis of [1-^{2}H]uridine diphosphoglucose.** *1*, hexokinase, ATP, Mg^{2+}; *2*, phosphoglucomutase; *3*, UDPG pyrophosphorylase, UTP, Mg^{2+}.

experiments were designed to determine whether the anomeric carbon-oxygen bond of UDP-Glc was broken prior to the addition of glycogen to the enzyme active site. If the scissile bond of UDP-Glc can be broken in the absence of glycogen, then incubation of [β-$^{18}O_2$,$\alpha\beta$-^{18}O] UDP-Glc with glycogen synthetase would result in the gradual appearance of an oxygen-18 atom in the C-O-P bridging oxygen position. However, this positional isotope exchange reaction would only occur if the β-phosphoryl group of the resulting UDP could rotate freely. The positional isotope exchange reaction can be detected by the appearance of a new ^{31}P NMR resonance for the β-P of UDP-Glc that is 0.015 ppm downfield from the starting material (7). Shown in Fig. 1*A* is the ^{31}P NMR spectrum of [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc (III). The high level of oxygen-18 incorporation at the indicated positions accounts for the observation of only one doublet. Shown in Fig. 1*B* is the ^{31}P NMR spectrum for the β-P of the labeled UDP-Glc (III) that is in isotopic equilibrium with the positionally exchanged UDP-Glc (IV). The difference in the observed chemical shift for the two doublets is due to the larger ^{18}O isotope shift from the β-nonbridging oxygens in III than for the bridging oxygen-18 in IV. Shown in Fig. 1*C* is the β-P of oxygen-18 labeled UDP-Glc (III) that had been incubated with glycogen synthetase for 100 h. No significant exchange of label from the β-nonbridge to the anomeric oxygen position could be detected. The upper limit for the ratio of the V_{max} for the glycogen synthetase reaction relative to the estimated maximal PIX rate is greater than 10,000. Identical results were also obtained when α-cyclodextrin was added to the reaction mixture. The α-cyclodextrin was used as a dead-end mimic of glycogen in an attempt to induce glycogen synthetase into the proper conformational state necessary for C-O bond cleavage. The α-cyclodextrin contains the α1,4 glucosyl linkage but does not have a free hydroxyl at C-4. Therefore, this compound cannot serve as a primer for glycogen synthesis. When 60 mM α-cyclodextrin was included in an assay mixture (glycogen = 1 mg/ml; UDP-glucose = 1.0 mM) the initial velocity of glycogen synthesis was reduced by 64%, and thus α-cyclodextrin is able to bind to glycogen synthetase at high concentrations. Kokesh and Kakuda (16) have previously used α- or β-cyclodextrin to induce a PIX reaction in the starch phosphorylase reaction.

Glucono-1,5-lactone (IX) and deoxynojirimycin (X) were tested as inhibitors of the glycogen synthetase-catalyzed reaction. These compounds have been previously used as transition-state or intermediate-state analogs of the postulated oxocarbonium ion.



(IX) (X)

Fig. 2 shows that glucono-1,5-lactone is a strong noncompetitive inhibitor *versus* UDP-Glc in the reaction catalyzed by glycogen synthetase. The kinetic constants from a fit of the data to Equation 2 are K_{is} = 0.091 ± 0.004 mM and K_{ii} = 0.70 ± 0.09 mM. McVerry and Kim (17) have previously reported the inhibitory effect of glucono-1,5-lactone on the activity of glycogen synthetase and have suggested that an intermediate with the glucosyl moiety in a half-chair conformation exists during the reaction sequence. The inhibition by deoxynojirimycin is more complicated. At pH values higher than 7.5, the Dixon plot (Fig. 3) for the inhibition by deoxynojirimycin is second order rather than first order. This indicates that there may be more than one site on glycogen synthetase that interacts with deoxynojirimycin. However, at pH 6.0 the Dixon plot for the inhibition by deoxynojirimycin is linear. Sevall and Kim (18) have shown that ATP interacts with both the UDP-Glc and glucose 6-phosphate sites in addition to its regulatory site. Deoxynojirimycin probably binds at the glycogen site in addition to the UDP-Glc site.

The secondary deuterium isotope effects exhibited by [1-

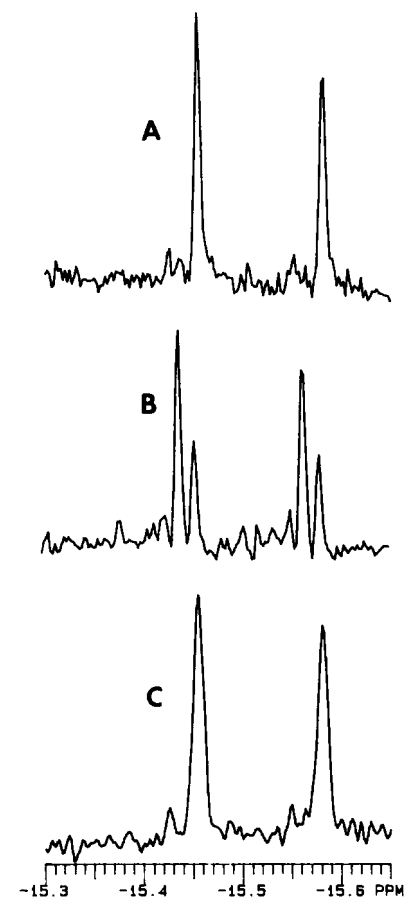


FIG. 1. **^{31}P NMR spectra of the β-P of UDP-glucose.** *A*, [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc; *B*, equilibrium mixture of III and IV; *C*, [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc (III) after incubation with glycogen synthetase for 100 h.

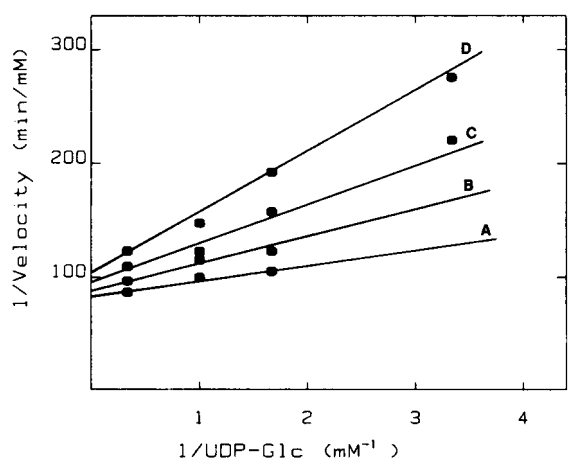


FIG. 2. **The inhibitory effect of glycogen synthetase by glucono-1,5-lactone.** Concentration of gluconolactone: *A*, 0 mM; *B*, 0.05 mM; *C*, 0.10 mM; *D*, 0.20 mM. Additional details are given in the text.

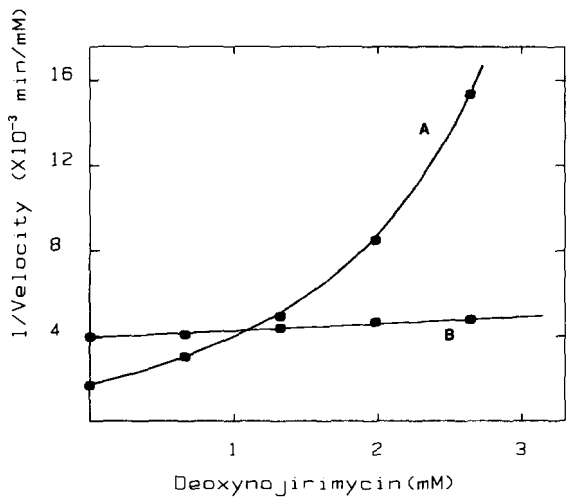


FIG. 3. **Inhibition of glycogen synthetase by deoxynojirimycin.** *A*, pH 7.5; *B*, pH 6.0.

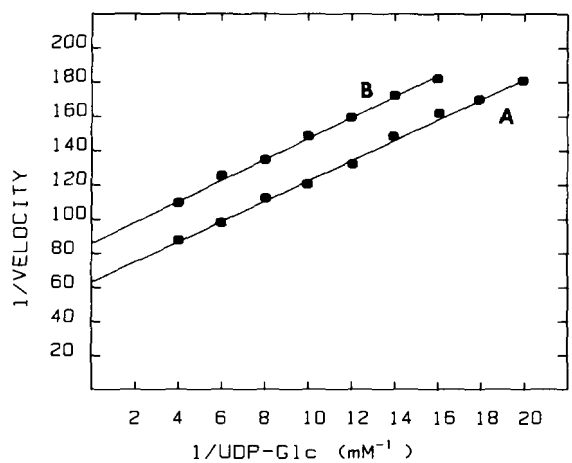
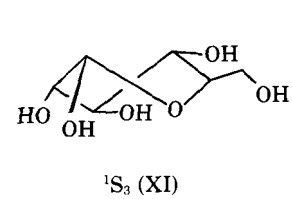


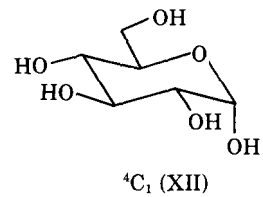
FIG. 4. **Double-reciprocal plot for the deuterium isotope effect by [1-^{2}H]UDP-Glc.** *A*, UDP-Glc; *B*, [1-^{2}H]UDP-Glc.

^{2}H]UDP-Glc were measured in an attempt to determine the degree of bond hybridization change at the anomeric center during enzymatic transformation. The secondary deuterium isotope effects exhibited by [1-^{2}H]UDP-Glc on glycogen synthetase were 1.23 ± 0.04 and 1.09 ± 0.06 for V_{max} and V_{max}/K_M, respectively. The data are illustrated in Fig. 4. The large value of 1.23 for the secondary deuterium isotope effect on V_{max} can be combined with the inhibitory effect by gluconolactone and deoxynojirimycin to suggest that an oxocarbonium ion intermediate is involved in the reaction mechanism.

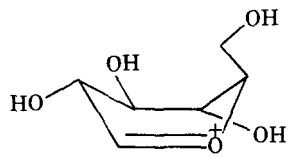
Hosie and Sinnott (9) have reported the α-deuterium isotope effects of aryl α-glucopyranosides and α-D-glucopyranosyl pyridium salts with yeast α-glucosidase; isotope effects of $^DV = 1.01$ and $^D(V/K) = 0.969$ are observed for *p*-nitrophenyl α-glucopyranosides, $^DV = 1.22$ and $^D(V/K) = 1.018$ for the 4-bromoisoquinolinium α-glucopyranosides, and $^DV = 1.15$ and $^D(V/K) = 1.085$ for the pyridium α-glucopyranosides salts (9). The *N*-glucoside substrates have a different ground state conformation, a $^1S_3$ twist type (XI), than the *O*-glucosides which have a normal $^4C_1$ chair form (XII). Hosie and Sinnott (9) have concluded that a noncovalent event, fast in the case of the *N*-glucosides but slow in the case of the *O*-glucosides, precedes the bond-breaking step. Moreover, the inhibition by deoxynojirimycin, which has a ^{2,5}B conformation (XIII), but no inhibition by castanospermine which does not, suggests that the intermediate adopts the ^{2,5}B conformation in the transition state. D-Glucono-1,5-lactone has predominantly a $^4H_3$ half-chair form (XIV), but Walaszek *et al.* (19) found by proton NMR spectroscopy that about 20% of D-glucono-1,5-lactone has a ^{2,5}B conformation in the solution (19).



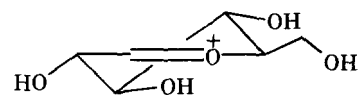
$^1S_3$ (XI)



$^4C_1$ (XII)



^{2,5}B (XIII)



$^4H_3$ (XIV)

The larger α-secondary deuterium isotope effect on V than on V/K suggests that there is a high commitment for catalysis once the substrate has bound to glycogen synthetase (15). This commitment could arise if UDP-Glc is "sticky" (*i.e.* k_{off} is slow relative to V_{max}). Alternatively, a fast, but essentially irreversible, conformational change may precede the kinetically slow bond-breaking step. In conclusion, inhibition of the glycogen synthetase catalyzed reaction by gluconolactone and deoxynojirimycin and the large α-deuterium isotope effect on V_{max} by [1-^{2}H]UDP-Glc strongly supports an oxocarbonium ion intermediate. However, the conformation adopted by this intermediate cannot be deduced from the available data. The lack of a detectable positional isotope exchange reaction in the absence of glycogen suggests the formation of a rigid ion pair between UDP and the oxocarbonium ion intermediate.

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