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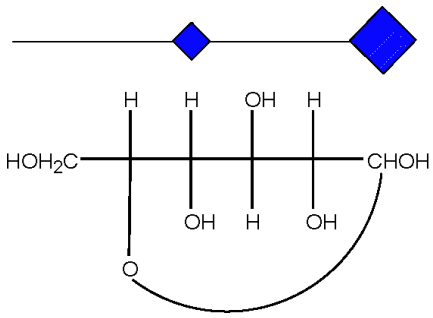

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Isotope Effect-Mapping of the Ionization of Glucose Demonstrates Unusual Charge Sharing

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Abstract: Isotopic substitution is known to affect kinetic rate constants and equilibrium constants in chemistry. In this study, we have used tritium substitution and high pH to probe the glucose $\rightleftharpoons$ glucose$^-$ + H^+ equilibrium. Passing partially ionized mixtures of [^{3}H]- and [^{14}C]glucose over anionic exchange resin has permitted the detection of subtle differences in pK_a. We have found that, at pH 11.7 in an anionic exchange system, [^{3}H]glucose always elutes ahead of the [^{14}C]glucose, and we report isotope effects of 1.051 ± 0.0007, 1.012 ± 1.0005, 1.014 ± 0.0004, 1.024 ± 0.0003, 1.014 ± 0.0004, and 1.015 ± 0.0014 for [1-^{3}H]-, [2-^{3}H]-, [3-^{3}H]-, [4-^{3}H]-, [5-^{3}H]-, and [6,6-$^3H_2$]glucose, respectively, as compared to either [2-^{14}C]- or [6-^{14}C]glucose. The elevated isotope effects at H1 and H4 imply unusual charge sharing in anionic aqueous glucose. Base titration of ^{13}C-chemical shift changes demonstrates the dominance of pyranose forms at elevated pH, and ab initio isotope effect computations on gas-phase glucose anions are presented.

Introduction

In chemistry and enzymology, isotope effects are used regularly to observe transition states and equilibria and to determine rate-limiting steps. Several isotopomers may be recruited to give a perspective on the process under examination, but, typically, the reaction involves transformation at one moiety or the breaking of one bond. However, a position-specific multiple isotope effect study can also be used to understand the localization of a global process. One example is a study of isotopic fractionation of nonionized benzoic acid under reverse-phase chromatography attributed to regiospecific hydrophobic or van der Waals interactions with the stationary phase.[1] Similar studies of hydrophobicity by retention time isotope effects would form another example, except that the substrates used were invariably perdeuterated.[2,3] To our knowledge, a multiple isotope effect study has not been performed to characterize the behavior of the acidic groups in a polyprotic molecule such as glucose.

Carbohydrates are among the most studied biological molecules, including important early findings of sugar mutarotation as a function of pH (refs 4−8). Titration of hydroxide solutions with carbohydrates showed the existence of at least two and possibly three ionizable groups in aqueous glucose.[9] More recently, from studies of aldose−ketose isomerizations at alkaline pH, it was concluded that some hexose anions could be stabilized by favorable ion−hydroxyl interactions;[10] because the mannose anion was seen to be one of the more stable ions, its charge would be shared by the alternating equatorial−axial−equatorial OH1−3 groups in the β-anomer. In glucose, presumably, charge cannot be shared stably between these three equitorial hydroxyls in the same way (Figure 1). Carbohydrate complexes with alkali and alkaline-earth metals have been studied with implications for sugar symport,[11−13] and while crystallography has provided structural information about sugar-alkali metal hydroxide adducts,[14,15] the solution complexes appear to be understood only to the level of stoichiometry.

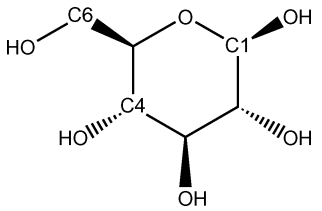

Figure 1. β-D-Glucopyranose.

In this paper, we have generated the glucose anion under high-pH conditions and used anion-exchange chromatography resin to perform isotopic fractionation. We have found that [1-^{3}H]-glucose can be separated to near-baseline purity from [^{14}C]-glucose, but fractionation for other tritium labels is not as large. We have performed a pH titration of ^{13}C NMR chemical shifts in natural abundance over the basic range to show the dominance of β-glucopyranose and α-glucopyranose at high pH, and we show calculations of isotope effects which aid in interpreting the HPLC data. We conclude that variation in the retention time

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isotope effects reflects the extent to which each hydroxyl is ionized in the β-glucopyranose anion at pH 11.7 and that this anion carries its charge primarily on O1 and secondarily on O4.

Materials and Methods

^{3}H- and ^{14}C-Labeled Glucose. All glucose or radiolabeled glucose was purchased from either Amersham Radiochemicals (Piscataway, NJ) or Sigma Chemicals (St. Louis, MO), except for the [4-^{3}H]glucose, which was synthesized in a procedure adapted from that of Söderman and Widmalm.[16,17]

HPLC. High-pH anion-exchange chromatography was performed on an HPLC system purchased from Dionex Corp. (Sunnyvale, CA), using a CarboPac PA1 column (4 mm × 250 mm) and isocratic aqueous NaOH pH 11.7 (5 mM NaOH). Peaks were visualized in real-time using pulsed-amperometric detection (PAD), but the isotope effect data reported herein were obtained by injecting a 20 μL mixture of [^{14}C]-glucose and [^{3}H]glucose with a 50 μL Hamilton syringe. Next, 6-s fractions around the expected elution time were collected and analyzed for both tritium and ^{14}C content; 10 μL of each fraction was added to 1 mL of H_2O and 10 mL of Liquiscint (National Diagnostics, Atlanta, GA) scintillation fluid and counted for 1 min in a Searle Delta 300 instrument. The first moment of the elution peak of each isotope was calculated according to

$$\text{mean} = \frac{\sum(\text{time of fraction i} \times \text{cpm in fraction i})}{\text{total cpm}}$$

where each fraction was assigned the time at the midpoint of its 6-s span. These were divided (time ^{14}C/time ^{3}H) to obtain the retention time isotope effect. Because solute travel velocities scale linearly with the percent of time spent in the mobile phase, the observed isotope effects directly represent the fraction of sugar ionized.

Chemical Shift Titration with pH. First, 300 mg of unlabeled sugar was dissolved in 600 μL of D_2O, and the pH was titrated with 10 N NaOH and concentrated NaOH. ^{1}H-uncoupled ^{13}C NMR spectra were then obtained using a 300 MHz Bruker instrument and uncorrected for deuterium content at the following pH values: 8.3, 9.0, 10.4, 11.7, 12.1, 12.4, 12.6, 12.8, 13.0, 13.25, and 13.7.

Computations. Glucopyranose anomers in the gas phase were energetically minimized using Gaussian[18] software at the density functional level of theory (b3pw91/6-31g**). Each molecule was ionized by removal of a proton from a free hydroxyl, minimized again at the same level of theory, and then subjected to frequency calculation in Gaussian. Deuterium and tritium isotope effects were calculated for each model with respect to the neutral anomer via IsoEff98.[19] The frequency scaling factor used was 0.9561 as suggested in ref 20. No attempt to model the ^{13}C NMR chemical shifts was made computationally.

Results

Experimental. HPLC. Glucose elution at 13.5 min is seen as the most prominent feature in the top panel of Figure 2. The chromatograph follows a typical injection of the 20 μL sample and demonstrates both the solvent front at approximately 1.7 min and a baseline waver at 18 min, corresponding to the onset of the column-washing phase. Other artifacts either were not radioactive or were significantly different in retention time from the main glucose peak.

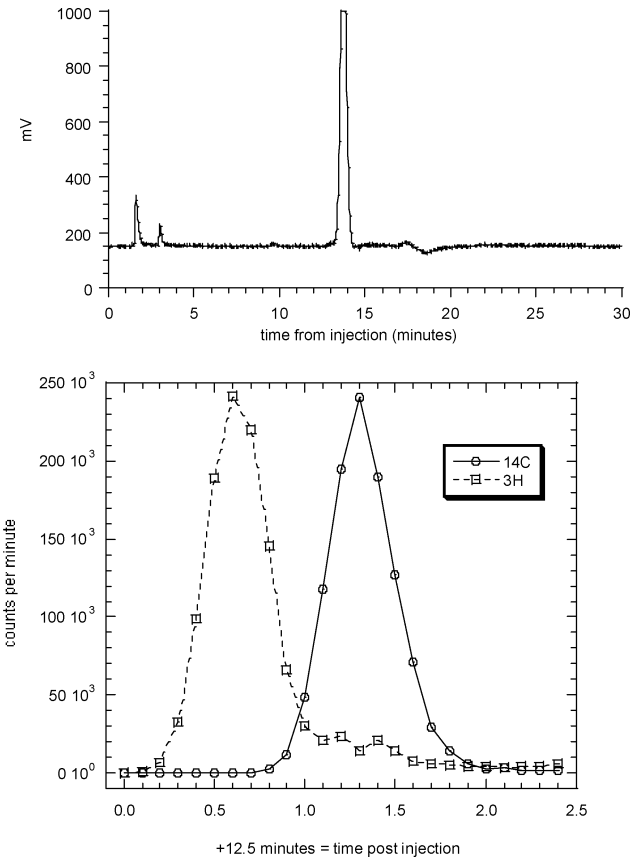

Figure 2. Typical HPLC chromatographs. The top panel shows detection by pulsed amperometry, and the bottom panel demonstrates near-baseline separation of isotopes for the co-injection of [1-^{3}H]glucose and [6-^{14}C]-glucose.

Expansion of fractions about the glucose peak for the simultaneous injection of [1-^{3}H]- and [6-^{14}C]glucose demonstrates near-baseline separation of isotopes (lower panel, Figure 2). The results for each backbone hydrogen substitution in glucose are summarized in Table 1.

It is significant that isotope effects are observed for all substitutions. In all cases, the tritiated material was seen to elute before the [^{14}C]glucose. The largest separations of 1.057 ± 0.0007 were obtained for tritiation at H1 and of 1.024 ± 0.0003 for tritiation at H4. All other effects (with tritium at H2, H3, H5, and H6) were similar (1.012−1.015). It was possible to obtain measurements within a small experimental error while keeping the total number of injections between three and seven replicate experiments. This accuracy arises from the simultaneous injection of isotopes, yielding an internal control, and to the large number of data points (8−10) comprising each isotopic elution.

We also attempted the observation of deuterium isotope effects by co-injecting unlabeled- and [1-d]glucose and scanning for two peaks in the resultant pulsed-amperometry chromatogram. Unfortunately, the deuterium isotope effects are subtle enough to prevent useful line shape analysis for peak decon-

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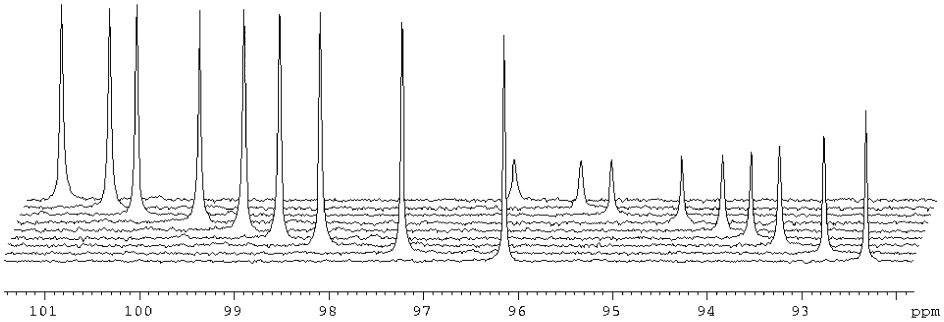

Figure 3. Titration of the C1β and C1α chemical shift change with pH. The closest spectrum corresponds to pH 9.0, with C1β and C1α at 96.2 and 92.3 ppm, respectively. Moving away from the reader are spectra at progressively higher pH: 11.7, 12.1, 12.4, 12.6, 12.8, 13.0, 13.25, and 13.7.

Table 1. Retention Time Isotope Effects (RTIE) for Glucose and High-pH Anion-Exchange HPLC

labels	isotope effect[a]	std. dev.	*n*
[1-^{3}H] + [2- or 6-^{14}C]glucose	1.057	0.0007	3
[2-^{3}H] + [2- or 6-^{14}C]glucose	1.012	0.0005	7
[3-^{3}H] + [2- or 6-^{14}C]glucose	1.014	0.0004	7
[4-^{3}H] + [2- or 6-^{14}C]glucose	1.024	0.0003	4
[5-^{3}H] + [2- or 6-^{14}C]glucose	1.014	0.0004	4
[6,6-^{3}H$_2$] + [2-^{14}C]glucose	1.015	0.0014	4

[a] Calculated as the first moment of elution peak (^{14}C)/first moment of elution peak (^{3}H).

volution. Isotopic observation by scintillation counting does not suffer from this limitation.

Chemical Shift Titration with pH. We used ^{13}C NMR to visualize the aqueous equilibrium of glucose as a function of pH. Chemical shifts for all ^{13}C spins were found to vary smoothly as a function of pH (C1β and C1α titrations illustrated in Figure 3). The data for all nuclei are given in Table 2.

Several features of the pH titration deserve note. First, the ratio of β- to α-pyranose anomers increases by a factor of 4 as the solution is basified from pH 8.3 to 13.7. Second, chemical shift changes are seen for each nucleus in both anomers, with the largest chemical shift change belonging to the C1β and C1α spins, followed by C2β, C2α, and C5α. Only one nucleus in each anomer undergoes negative shift with increasing pH, but the nuclei are different: C3β or C2α.

For samples between pH 10.4 and pH 14, separate pH measurements before and after the NMR spectrum was taken (20−30 min) demonstrate a variable acidic shift by up to 0.16 pH unit. Further, the small chemical shift changes for those spins caused significant errors in most pK_a fits for our titrations. Despite these errors, the pK_a values were consistent with published values of 12.1 and 13.85.[9]

Computations. Deprotonation of 2-Propanol. A sample calculation of deprotonation in model compounds can serve to reveal electronic changes giving rise to isotope effects. With 2-propanol, a secondary alcohol resembling a generic hydroxyl group in glucose, we calculate the isotope effect on the equilibrium of ionization to be 1.36 for the tritiation of the central CH bond. The basis of this effect is illustrated in Figure 4. Hydroxyl deprotonation increases the ability of oxygen lone pairs to hyperconjugate into the antibonding (σ^*) orbital of the geminal CH bond. This $n \rightarrow \sigma^*$ hyperconjugation lowers the bond order of the CH bond with respect to the protonated molecule, permitting the CH hydrogen atom to "feel" a vibrationally more loose environment. It is well accepted that lighter isotopes prefer vibrational laxity, while their heavier

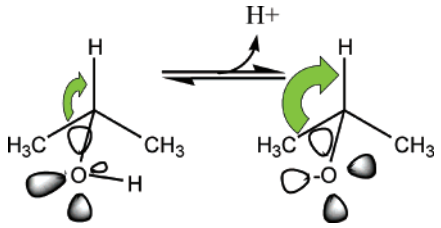

Figure 4. Hyperconjugation increases with deprotonation.

counterparts prefer tighter environments. Therefore, the alteration of vibrational environment between two states at equilibrium can be probed by looking for isotopic enrichment in either state, by definition, the isotopic perturbation of an equilibrium constant. Incidentally, the methyl CH bonds antiperiplanar to the central CH bond in our example are subject to a normal isotope effect as well, but they are 4 times smaller in magnitude.

Isotope Effects in Glucose Ionization. High level computations on glucose are summarized in Table 3. In each anomer, represented in the gas phase, the free hydroxyl moieties were individually deprotonated, and fractionation factor changes were computed for the backbone hydrogen atoms. Reading the second column, for instance, demonstrates that deprotonating the 2-hydroxyl group in α-glucose causes normal isotope effects at all backbone hydrogen atoms, with the largest effect by far coming at the (geminal) H2 atom. In reading rows, we can see that each isotope substitution has the greatest effect on the acid dissociation constant when it is closest to the deprotonated group, and in this way we expect isotope effects upon deprotonation to report on the proximity to the deprotonation site in glucose.

The 3-anion is not listed in calculated isotope effects for ionization (Table 3). Minimization of the initial 3-alkoxide models caused the migration of the 4-hydroxyl proton to form OH3 for both anomers. The low relative free energies of the 4-alkoxide are seen in the table.

Ionization of any glucose hydroxyl causes significant isotope effects at all positions, not only the geminal CH. This is important in interpreting the experimental isotope effects, where significant isotope effects are seen at all positions, and it is most noticeable in the [5-^{3}H] row of isotope effects. The ring oxygen O5 in cyclic glucopyranose is never free to ionize, yet labeling H5 perturbs the acid dissociation constant of any other hydroxyl group. Finally, the [6,6-^{3}H$_2$] isotope effect is comparable to other isotope effects for ionization of the 4-hydroxyl, but it is surprisingly large for ionization of the 6-hydroxyl, an unusual result. Changes in the HO6−O6−C6−C5 torsional angle can lead to significant isotope effects,[21] but this is not operant here; the gas-phase 6-hydroxyl always points to its partner, whether

Table 2. Titration of Chemical Shift Change with pH by Addition of 10 N NaOH

		^{13}C-signal											
pH	(β/α)	C1β	C1α	C5β	C3β	C2β	C3α	C5α	C2α	C4α	C4β	C6β	C6α
13.70	3.84	101.08	96.28	76.63	75.52	77.18	73.82	74.20	70.29	70.74	70.86	61.46	61.29
13.25	3.25	100.56	95.56	76.52	75.53	76.87	73.67	73.75	70.52	70.55	70.69	61.39	61.19
13.00	2.97	100.22	95.21	76.57	75.62	76.76	73.71	73.61	70.55		70.70	61.46	61.23
12.80	3.00	99.53	94.44	76.37	75.63	76.31	73.45	73.04	70.89	70.31	70.47	61.30	61.07
12.60	2.57	99.03	93.97	76.32	75.70	76.03	73.35	72.77	71.06	70.22	70.38	61.27	61.03
12.40	2.33	98.61	93.65	76.26	75.76	75.80	73.29	72.56	71.18	70.15	70.31	61.24	61.00
12.10	2.14	98.15	93.31	76.22	75.82	75.54	73.22	72.35	71.30	70.09	70.22	61.20	60.99
11.70	1.93	97.27	92.81	76.13	75.97	75.03	73.14	72.03	71.49	70.00	70.07	61.15	60.95
11.40	1.79	97.13	92.79	76.20	76.06	75.01	73.21	72.07	71.58	70.06	70.11	61.20	61.00
10.40	1.62	96.24	92.37	76.11	76.01	74.44	73.05	71.74	71.65	69.91	69.86	61.06	60.92
9.00	1.58	96.15	92.34	76.13	76.00	74.39	73.05	71.72	71.66	69.91	69.85	61.06	60.92
8.30	0.98	96.15	92.34	76.14	76.00	74.39	73.05	71.72	71.66	69.91	69.85	61.06	60.92
						Fit Parameters							
δ_{min}		96.24	92.41	76.12	76.01	74.44	73.06	71.77	71.64	69.92	69.87	61.07	60.93
$\Delta\delta$		4.93	4.33	0.59	−0.54	2.77	0.86	2.71	−1.48	0.94	0.99	0.39	0.46

Table 3. Calculated Isotope Effects on Ionization (b3pw91/6-31g**)[a]

	α			
label	1	2	4	6
1-^{3}H	1.36	1.09	1.06	1.07
2-^{3}H	1.16	1.45	1.03	1.03
3-^{3}H	0.96	1.06	1.09	1.01
4-^{3}H	1.04	1.03	1.26	1.07
5-^{3}H	1.05	1.04	1.06	1.11
6,6-^{3}H$_2$	1.07	1.06	1.22	2.95
rel. free energy (kcal/mol)	0.0	14.4	2.4	39.8
	β			
	1	2	4	6
1-^{3}H	1.36	1.08	1.02	1.05
2-^{3}H	1.07	1.33	1.04	1.02
3-^{3}H	1.01	1.11	1.09	1.01
4-^{3}H	1.02	1.03	1.27	1.06
5-^{3}H	1.07	1.04	1.06	1.10
6,6-^{3}H$_2$	1.06	1.06	1.23	3.00
rel. free energy (kcal/mol)	5.2	14.6	3.9	41.4

[a] The 3-anion could not be generated in the gas phase. Minimization always deprotonates O4 to make the H3-hydroxyl.

4-hydroxyl or 4-hydroxide (structures may be found in the Supporting Information).

Discussion

Evidence of Pyranose Forms at High pH. Smooth titration of C1β or C1α peaks shown in Figure 3 indicates a slow equilibrium (<300 s^{-1}) between low-pH anomers but rapid equilibrium between each of these and one of the two high-pH species. Neither of the latter corresponds to the chemical shift of acyclic aldehyde (207 ppm) or aldehydic hydrate (90.3 ppm) quantitated previously.[22] Were these signals due to furanose forms, they would appear in slow equilibrium with the pyranoses, as the rate-limiting step for pyranose−furanose exchange (ring-opening) is identical to that for pyranose−pyranose exchange. Rapid exchange to furanose forms would also be revealed by splitting of the β peak (and α) and smooth transition of each daughter peak to the position of each of the two high-pH signals. The high-pH peaks are therefore most likely due to the ionized pyranose anomers, which would be in rapid equilibrium with the neutral forms.

Ionic versus Size-Exclusion Retention. Isotope effect measurements on the chromatography of glucose under alkaline conditions have been performed (see ref 23); however, in that paper, only the [1-d] and [2-d] equilibrium isotope effects were measured. The conclusion that the observed isotope effects are due to ionization finds precedent in the literature. The pK_a of a basic group is invariably increased by the introduction of vicinal hydrogen isotopes (see refs 24−26). This was seen as accelerated elution of isotopomers of *N*-methyl labeled benzazepines during reversed-phase chromatography;[27] increased pK_a or basicity of the amino group led to a greater proportion of charged molecules, which elute more quickly under these conditions. The largest effects on chromatographic mobility are seen where the solute population is partially charged.

The nature of the interaction between glucose and the CarboPAC PA1 column at high pH is predominantly that of ionic-exchange. The lowest pK_a of glucose at 25 °C is approximately 12.09,[9] and the eluent in the present study was pH 11.7. Therefore, a significant proportion of the glucose molecules passing over the stationary phase will possess the negative charge required to interact with and be retained in the instrument beyond solvent front elution.

Size-fractionation can be a concern in the elution of small molecules over any resin. However, even though isotopic differences in bond length (and molecular extent) do exist, the proximity to a bulky group has been shown to suppress fractionation.[1] We would expect the extracyclic methylene to partially shield H4 from direct interaction with the stationary phase, giving rise to decreased fractionation at that position relative to H2 or any of the other positions. Instead, we see greater fractionation. This does not eliminate the possibility of size-exclusion fractionation, masked by a greater ionization effect, but any size-fractionation isotope effects are mixed with significant isotope effects resulting from ionization even at distal sites (see Table 3). Therefore, we judge the contribution of size-

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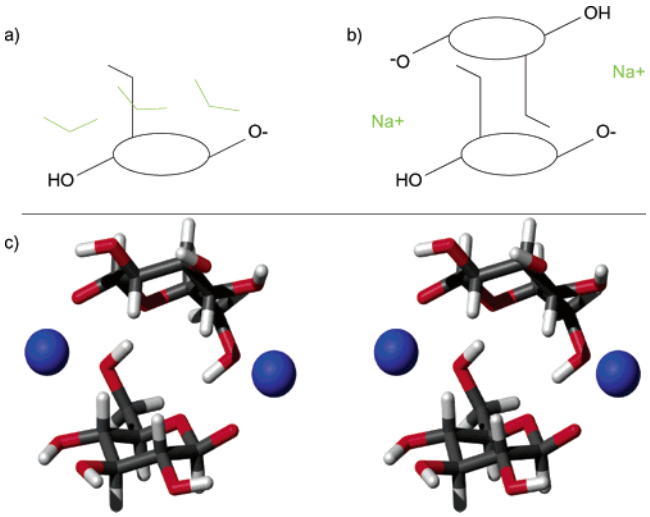

Figure 5. In aqueous solution, the acidic O1 and O4 communicate either through a bridge of water molecules (these are shown in red, panel a) or to the conjugate site on a complexed glucose molecule, stabilized by coordinating sodium ions (panels b and c). Panel c shows a stereoview of one such possible complex.

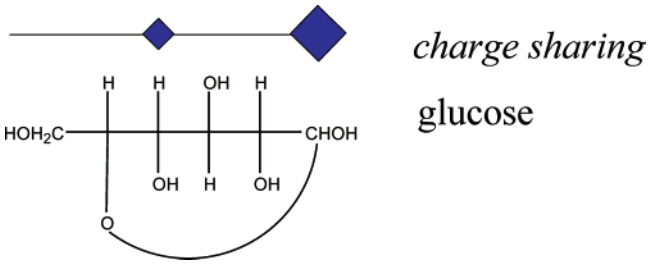

Figure 6. Proposed charge sharing in aqueous ionized glucose at pH 11.7.

fractionation isotope effects as negligible in the current observations. Hydrophobic fractionation is unlikely on the same grounds.

Uneven Charge Sharing at High pH. The hypothesis of uneven charge sharing (see Figure 6) requires differences in hydroxyl pK_a's. Previous workers have demonstrated the presence of at least two ionizable groups in the many carbohydrates;[9] the first two values for glucose reported are 12.09 and 13.85. At least the lower pK_a can be seen to come from only a single deprotonation, as the number of hydroxides bound per glucose at pH = pK_a = 12.1 in that reference is 0.5 and not 1.0. The present data were taken at pH 11.7, close to the first pK_a but over 2 pH units from the second. The latter ionization is unlikely to be observed in this study, implying that the ionization presently observed derives from a single deprotonation of each glucose molecule with pK_a of 12.1.

Several observations support the idea that ionization occurs primarily at OH1 in glucose and secondarily at OH4. First, the largest HPLC isotope effects are seen at these positions. Isotope effect calculations show that, with the exception of the primary alcohol at C6, all backbone hydrogens are equally sensitive to geminal deprotonation. In fact, the extreme sensitivity of H6 atoms virtually eliminates the possibility of their participation in ionization, because no effect is seen experimentally. Next, pH titration of chemical shifts reveals by far the largest $\Delta\delta$ (ppm) for C1. Finally, gas-phase computations on these ionic forms demonstrate the enthalpic preference for deprotonation at OH1 and OH4. Of these, the experimental isotope effects are the most convincing.

Let us begin with the assumption that the pK_a of O1 is slightly lower than that for O4, with respect to the first ionization of a glucose molecule. Subtracting a "background" of 0.14 from our isotope effects leaves 1.040 and 1.010 for O1 and O4, respectively. We showed in Table 3 that H1 is more sensitive to ionization at O1 than H4 is to ionization at O4 by a factor of 1.5. This leaves about 2.6 times as many glucose molecules ionized only at O1 as those ionized only at O4, at pH 11.7, corresponding to pK_a values of 12.2 and 12.7, respectively.

It is clear that deprotonation of one hydroxyl must raise the pK_a of the other group, as the overall pK_a of 12.1 for glucose corresponds to a single deprotonation as mentioned before. The question remains as to how such inhibition may occur. Should we assume that electrostatics govern the process, especially given the very polar solvent and sodium cations? If not, then we must propose an apparatus for the transmittal of information from one site to the other: O1, O4, and some intermediary atoms or molecules. Such an effect would certainly be distance-dependent, and it could mimic an electrostatic-type decay with distance. As a corollary, this arrangement can be thought of as a collective acid, where anion burden is shared between several atoms each with partial charge.

Two models for the charge sharing include a three or four molecule water bridge acting as a collective acid (Figure 5, panel a) or a complex of stoichiometry $2\text{Glucose}^{-1}\cdot 2\text{Na}^{+}$. In the ion-pair model, the second sugar molecule is rotated with respect to the first by 180° about two orthogonal axes and arranged so that the normal to each (approximate) sugar plane is made parallel and is nearly overlying (Figure 5, panels b and c). The latter model is probably more likely, and such a complex would juxtapose both OH4 against OH1′ and OH1 against OH4′. Deprotonation and binding of each Na^{+} would then be shared between each of the two hydroxyls in each of two sites per dimer. One caveat to this particular suggestion is that alkali metal hydroxide adducts of sugars have regularly been isolated in the literature, and the glucose:NaOH pair typically precipitates in a molar ratio of 2:1.[13] However, molar concentrations of hydroxide can lower the ratio to a minimum near 1:1, and the solution complex structure does not necessarily coincide with the solid complex structure.

Implications for Mutarotation. Three main processes must occur in the ring-opening of a glucopyranose form: deprotonation at O1, protonation at O5, and bond-breakage between C1 and O5. On the other hand, at high pH, pyranose anions are stable molecules, with ionic burden carried predominantly at the anomeric oxygen (present results). By Occam's razor, we expect the anomeric deprotonation under alkaline conditions to present the ring-opening reaction with an activated substrate. It is assumed that the ionic form is not a dead-end state but rather on-path to ring-opening in aqueous solution, and this point certainly awaits verification by transition-state analysis at high pH.

Conclusions

The isotope effects reported for anionic-exchange HPLC retention time represent slight differences in the pK_a of glucopyranose hydroxyl groups. Most of the charge in ionized glucose at pH 11.7 is carried by OH1 with some participation by OH4 (Figure 6). Further, β-glucopyranose dominates macroscopic observables at high pH by a factor of 2−4. In this

way, any contribution of α-glucopyranose to the observed isotope effects will be upstaged by the nature of things in the β-sugar.

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Note Added after ASAP Publication: Tables 1 and 3 contained errors in the version published on the Web 6/6/2003. The version published 6/10/2003 and the print version are correct.

Supporting Information Available: Cartesian coordinates for the ionized glucose molecules (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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