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SEPARATION OF SUGAR ANOMERS BY AQUEOUS CHROMATOGRAPHY ON CALCIUM- AND LEAD-FORM ION-EXCHANGE COLUMNS

APPLICATION TO ANOMERIC ANALYSIS OF ENZYME REACTION PRODUCTS

JOHN O. BAKER* and MICHAEL E. HIMMEL
Biotechnology Branch, Solar Fuels Research Division, Solar Energy Research Institute, 1617 Cole Boulevard, Golden, CO 80401 (U.S.A.)
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SUMMARY

Analytical useful separations of the α- and β-anomers of five economically important monosaccharides (glucose, xylose, galactose, mannose and arabinose) can be obtained by totally aqueous chromatography at 1.5°C on a commercially available calcium-form ion-exchange column, the Bio-Rad HPX-87C. Such analyses are expected to be very important in determining the mechanisms of action of different enzymes converting the polysaccharides found in woody biomass to monomeric units fermentable to fuel alcohol. Aqueous chromatography on a similar column having a different metal counterion, the lead-form Bio-Rad HPX-87P, separates the anomers of glucose but fails to separate the anomers of the other four sugars that are anomerically resolved by the calcium column. This counterion-dependence is shown to arise not from a lack of chromatographic selectivity by the lead-form column, but to the substantially higher rate of mutarotation of the sugars in the presence of the lead-form packing material. Analysis of the shapes of the elution profiles yields estimates of the effective rate constants for mutarotation of glucose on the calcium columns; the observed perturbation of the values of k_α and k_β with respect to those measured in water at the same temperature in turn suggest a possible mechanism for the anomeric separation. A third column, the calcium-form Bio-Rad HPX-42C, which has a more open resin structure, shows promise for the separation of the products of enzyme reactions converting polysaccharides to disaccharides (such as maltose and cellobiose) rather than monosaccharides.

INTRODUCTION

The α- and β-anomers of monosaccharides have been shown to be separable by chromatography at low temperature on ion-exchange resins using either aqueous-organic eluent systems as first reported by Ramnas and Samuelson[1] in 1974, or pure water as eluent as described by Goulding[2] in 1975. The systems using aqueous-

organic mixtures as eluent[1,3–7] are, in general, capable of somewhat better resolution than are the systems using pure water, due to the decreased rate of mutarotation in systems having decreased concentrations of water[8], and especially if advantage is taken of the antifreeze properties of such systems in order to operate below the freezing point of water[1]. For certain applications, however, there are good reasons for preferring purely aqueous systems. One such application is the determination of the anomeric configuration of the products resulting from enzymatic hydrolysis of glycosidic bonds, for instance, the conversion of polymers such as starch and cellulose to monomeric glucose[9–13]. In such determinations, it is desired that a relatively high concentration of product be generated before any sizeable percentage of the product has changed its anomeric configuration through mutarotation. To this end, rather high concentrations of enzyme are used, in order to provide rates of generation of product much greater than the rate of subsequent mutarotation of the product in solution. If aliquots from such reaction mixtures are injected directly onto a chromatographic system using an aqueous–organic mixture as eluent, there is a high probability that the protein will precipitate on the column. Procedures are available for avoiding such an undesirable result by removal of protein from the sample prior to chromatography, but in view of the desirability of speed and operational simplicity in such determinations and also the desirability of avoiding uncertainties concerning the quantitative transfer of sugars in extraction steps, a chromatographic procedure allowing direct injection of reaction mixture aliquots is distinctly advantageous. As will be shown in this report, several commercially available high-performance liquid chromatography (HPLC) columns, with calcium- or lead-form sulfonated polystyrene packing materials and pure water as eluent, are found to separate sugar anomers efficiently and to permit direct injection of reaction mixture aliquots containing substantial amounts of protein.

Although the separation of sugar anomers by aqueous chromatography has been noted in several connections during recent years[14–19] it has for the most part been treated either as a curiosity or, more frequently, as an undesirable effect to be minimized in the analysis of sugars. In the last few years, however, increasing interest in the conversion of woody biomass to fermentable sugars, (and thence to fuel alcohol) has resulted in increased interest not only in the mechanisms of enzymes capable of converting glucose polymers to monomers[9,20], but also in the mechanisms of enzymes capable of hydrolyzing glycosidic bonds involving xylose, galactose, mannose and arabinose[21], which are other important constituents of woody biomass. One of the important questions to be answered concerning the mechanism of a given reaction of this sort is whether the configuration about the anomeric carbon of the susceptible bond is retained or inverted during the hydrolysis reaction. It appeared to us that in answering this question a direct aqueous LC method not requiring any sample pretreatment would compare very favorably with other methods of anomeric analysis currently available.

Determination of the anomeric configuration of the glucose product during enzymatic hydrolysis has traditionally been performed either by polarimetry or by gas–liquid chromatography (GLC) of the trimethylchlorosilane-treated sugars. The optical rotation methods suffer from being non-specific, in that this method indicates the additive optical rotatory power of the entire solution (including that of optically-active aglycones liberated) which renders impractical the analysis of many complex

enzyme reaction mixtures. The use of glycosyl fluorides as substrates reduces the number of complications in polarimetric studies, in that the aglycone liberated is not optically active, but this approach suffers from the disadvantage that a non-natural substrate is being used[12]. GLC, on the other hand, requires the cumbersome procedure of silanation to allow volatilization of the sugar components. Recently, NMR has also been used to estimate anomeric composition. This method, however, requires large concentrations of reaction mixture components[9].

We report in this study the development of a rapid method for anomeric analysis of five sugars (glucose, xylose, galactose, mannose and arabinose) using a calcium-form cation-exchange column (Bio-Rad HPX-87C) and deionized water as eluent, and of one sugar (glucose) using a lead-form column (Bio-Rad HPX-87P). In addition, results are presented that indicate a third column, the Bio-Rad HPX-42C, may be useful in anomeric analysis of the products of those enzyme reactions that liberate disaccharides, rather than monosaccharides, from carbohydrate polymer chains.

EXPERIMENTAL

Instrumentation

The Beckman HPLC system used in these studies consisted of a Model 100A pump, Model 421 controller and Model 210 injection valve. A Hewlett-Packard high-sensitivity RI detector (Erma Optical Works, Japan) was used routinely at an attenuation of 1 ×. The data were recorded on a Shimadzu Model CR3-A integrator with RS-232C transmission to a Tektronix graphics computer. A custom flask was made for the mobile phase which allowed the water eluent to be continuously boiled during chromatography in order to achieve low dissolved gas levels. All columns (HPX-87C, HPX-87P, and HPX-42C, each 300 × 7.8 mm) were purchased from Bio-Rad, as were the 30-mm length calcium- and lead-form guard columns (Carbo-C and Carbo-P, respectively).

The pre-column and column for each system were encased in a water jacket and controlled at various temperatures with a Forma Model 2006 refrigerated water circulator. The column flow-rate used for routine analysis was 0.3 ml/min. A 20-μl sample loop was used for all injections.

Materials and methods

All sugars and sugar derivatives used as standards were obtained from Sigma. Anomeric compositions of the sugars as received were confirmed, or determined, using a Polyscience SR-6 polarimeter. The enzyme substrates maltohexaose, salicin, and *p*-nitrophenyl-α-D-glucopyranoside, as well as the enzymes: amyloglucosidase (1,4-α-D-glucan glucohydrolase; E.C. 3.2.1.3) from *Aspergillus niger*, almond β-glucosidase (β-D-glucoside glucohydrolase; E.C. 3.2.1.21) and yeast α-glucosidase (α-D-glucoside glucohydrolase; E.C. 3.2.1.20) were purchased from Sigma. The β-glucosidase was received as a lyophilized, essentially salt-free powder; the other two enzymes were received as suspensions in 3.2 *M* ammonium sulfate. Each enzyme was dialyzed for a total of 36–40 h against two changes of the reaction buffer (700-fold volume excess) before being used in the assay. Specific reaction conditions are given in figure legends.

Computer-modeling of the chromatographic separations was carried out by means of two BASIC programs for the Tektronix 4052A computer, which was equipped with a TransEra Model 6400 auxiliary memory unit and a Hewlett-Packard 7470A plotter. One of the programs, that used to estimate band-broadening parameters, was a slight modification of a peak-deconvolution program developed earlier by Dr. M. P. Tucker for use in studying the size-exclusion chromatography of lignin fractions[22]. Copies of both this program, and the program used to evaluate the effects of on-column mutarotation, are available from the authors upon request.

RESULTS AND DISCUSSION

When operated at 1.5°C with deionized water as eluent at a flow-rate of 0.3 ml/min), the calcium-form HPX-87C provides analytically useful separation of the anomers of all five of the monosaccharides tested (Fig. 1). In sharp contrast, the lead-form HPX-87P, with packing material identical to that of the HPX-87C except for choice of metal ion, gives useful separation of only one sugar, D-glucose. The failure of the lead-form column to give useful anomeric separation of four of the sugars is not due to a lack of selectivity for the α- and β-anomers, but is traceable, instead, to the fact that at a given temperature all five of the sugars mutarotate more rapidly on the lead column than on the calcium-form column. For both of the columns, the elution profile for a given sugar has three major components: one peak

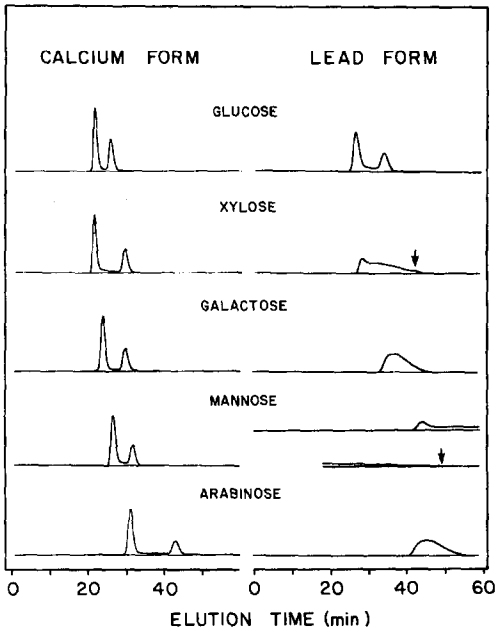

Fig. 1. Separation of sugar anomers by aqueous chromatography on calcium- and lead-form ion-exchange columns. Chromatograms on the left were run on the calcium-form Bio-Rad HPX-87C, those on the right, on the lead-form Bio-Rad HPX-87P. For both columns, operating temperature was 1.5°C; flow-rate was 0.3 ml/min. Sample size: 100 μg in 0.02 ml, detection by refractive index deflection.

each for the α- and β-anomers, consisting of sugar molecules that have retained the same anomeric configuration throughout transit of the column, and between these peaks a "reaction zone"[23,24] representing the elution of sugar molecules that have changed anomeric configuration (mutarotated) at least once during passage through the column. Since the sugar molecules that have mutarotated while on the column have spent part of the time on the column as α-anomer and part as β, their average velocities and their elution positions will obviously be intermediate between the average velocities and the elution positions, respectively, of the two anomers. For the calcium-form column, the "reaction zones" are relatively small, resulting in near-baseline separation for the anomers of all five sugars. For the lead-form column, however, the reaction zones are considerably larger with respect to the total area under the elution profile; in fact, for all of the sugars other than glucose, the "reaction zone" represents the major portion of the injected material. The small vertical arrows in Fig. 1 indicate the locations on the chromatograms of small remnants, visible at lower attenuation, of the peaks representing the more-retained anomer. No such separate peaks are discernible for galactose and arabinose on the lead column. Here almost all of the injected molecules have mutarotated at least once while on the column, and have, therefore, been transferred from the anomer peaks to the reaction zone. Even at 1.5°C, the lead-column elution profile for these two sugars is well on the way to being transformed into the single peak seen at higher temperature.

Figs. 2 and 3 show the temperature-dependence of the elution of these sugars from the calcium- and lead-form columns, respectively. Branching of the curves indicates splitting of the sugar peaks into separate anomer peaks at low temperature. It should be noted, however, that the merging of the plots for the separate anomers, as shown here, does not necessarily indicate that the anomers elute at the same position at this temperature; instead, the temperature at which the plots merge is merely the temperature above which the "reaction zone" (now becoming a "reaction peak") has increased at the expense of the two anomeric peaks to such an extent that the two peaks can no longer be distinguished. Another point to be noted in connection with Figs. 2 and 3 is that the order of elution of the different sugars (as estimated from weighted average elution times for the two anomers) appears to be dependent upon the metal counterion selected. With calcium, the order is glucose, xylose, gal-

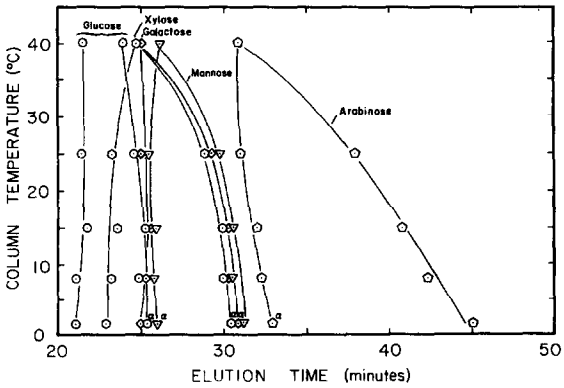

Fig. 2. Temperature dependence of separation of sugar anomers by aqueous chromatography on the calcium-form HPX-87C. Flow-rate, 0.3 ml/min; sample size, 100 μg in 0.02 ml.

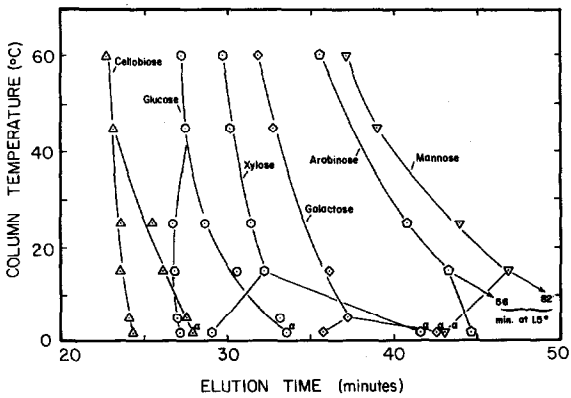

Fig. 3. Temperature dependence of separation of sugar anomers by aqueous chromatography on the lead-form HPX-87P. Conditions as in Fig. 2.

actose, mannose, arabinose; with lead as counterion, the order becomes glucose, xylose, galactose, arabinose, mannose. Similar differences in elution order have been noted for the single sugar peaks eluted from these two columns at an operating temperature of 85°C[17].

Inasmuch as the sugar samples have undergone some mutarotation between injection and elution, the observed profile can be only an estimator of, rather than an exact determination of, the anomeric composition of the sample at injection. In order to evaluate the usefulness of the elution profile as an estimator of the original state, one must have a logical procedure for apportioning the area under the elution profile among the two peaks and the reaction zone. Two methods that have been used to divide such profiles are "peak reflection" and the use of simple vertical "cuts" in the profile[25]. Peak-reflection techniques assume symmetrical peaks; consequently such techniques do not work well for systems such as the ones under consideration here, for which we have found, by studying the elution patterns of 1-O-methyl glycosides, that even non-mutarotating solutes elute in peaks that have a slight, but definite asymmetry and noticeable tailing. The use of vertical cuts to divide the two peaks from the reaction zone appears to involve a somewhat arbitrary selection of positions for the cuts. The criteria for selection of these positions are often not stated explicitly, but presumably are based upon the apparent bandwidth of the peaks and the rate of change of the slope of the profile. It appeared to us that more reliable estimates of peak and zone areas would be obtained from a protocol that took into account the actual (or, at least, the theoretically expected) distribution of the mutarotated material along the elution coordinate. Accordingly, we undertook a two-part computer modeling study designed to predict the shape of the "reaction zone" that lies between the anomeric peaks and can be presumed to underly both of them to some extent.

It should be noted at the outset that the protocol to be developed here is designed for use only under conditions such that the chromatography time is sufficiently short in comparison with the half-lives for mutarotation of both anomers that, of those molecules that mutarotate at least once while on the column, only a

very small fraction will mutarotate more than once. From simple kinetic and chromatographic considerations, it can be shown that the fraction of once-mutarotated molecules that will mutarotate more than once while travelling through the column, represented by F_2, is given by eqn. 1.

$$F_2 = \frac{\alpha 2_{\text{total}} + \beta 2_{\text{total}}}{\alpha 1_{\text{total}} + \beta 1_{\text{total}}} \tag{1}$$

where $\alpha 1_{\text{total}}$, $\alpha 2_{\text{total}}$, $\beta 1_{\text{total}}$ and $\beta 2_{\text{total}}$ are defined as follows:

$$\alpha 1_{\text{total}} = \beta_0(1 - e^{-k_\beta t_\beta}) \tag{2}$$

$$\beta 1_{\text{total}} = \alpha_0(1 - e^{-k_\alpha t_\alpha}) \tag{3}$$

$$\alpha 2_{\text{total}} = \alpha_0(1 - e^{-k_\alpha t_\alpha}) - \alpha_0(e^{-k_\beta t_\beta})/\left(\frac{k_\beta t_\beta}{k_\alpha t_\alpha} - 1\right)(e^{[(k_\beta t_\beta / t_\alpha)} - {}^{k_\alpha]t_\alpha} - 1) \tag{4}$$

$$\beta 2_{\text{total}} = \beta_0(1 - e^{-k_\beta t_\beta}) - \beta_0(e^{-k_\alpha t_\alpha})/\left(\frac{k_\alpha t_\alpha}{k_\beta t_\beta} - 1\right)(e^{[(k_\alpha t_\alpha / t_\beta)} - {}^{k_\beta]t_\beta} - 1) \tag{5}$$

In eqns. 1–5, α_0 and β_0 represent the concentrations of α- and β-anomer, respectively, in the sample injected onto the column at time $t = 0$. $\alpha 1$ refers to those molecules injected as β that have mutarotated once to become α-anomer. A subsequent mutarotation of $\alpha 1$ results in reversion to a β-form designated as $\beta 2$. $\alpha 1$ and $\beta 2$ thus represent, respectively, "first- and second-generation" mutarotated material. $\alpha 1_{\text{total}}$ and $\beta 2_{\text{total}}$ simply refer to the total quantity of each species *generated* during chromatography, without consideration of any further reaction of either. $\beta 1$ and $\alpha 2$ are defined in an analogous fashion. The rate constants for conversion of α- and β-anomer into the opposite form are k_α and k_β, respectively. The elution times of the peak maxima for material eluted as unmutarotated α and β are t_α and t_β. For glucose in water, the ratio k_α:k_β has been shown to be constant from 0° to 45°C[26], with a value near 1.72[27]. From polarimetric studies, O'Connor *et al.*[28], have reported values of $k_\psi = k_\alpha + k_\beta$ for glucose in pH 5.0 water at temperatures from 275.0 K to 303.0 K. By means of a short extrapolation of the data of O'Connor *et al.* and using the k_α:k_β ratio of 1.72, values of $2.04 \cdot 10^{-5}$ s^{-1} for k_α and $1.19 \cdot 10^{-5}$ sec^{-1} for k_β can be predicted for glucose in water at 1.5°C. When these values are used in eqns. 1–5, with $t_\alpha = 25$ min, $t_\beta = 20$ min, $\alpha_0 = 0.369$, and $\beta_0 = 0.631$ (equilibrium ratios for a total sugar concentration of 1.0), we find that less than 1.42% of the injected β-anomer (β_0) will mutarotate during chromatography to form $\alpha 1$; only 1.52% of this $\alpha 1$ will in turn revert to β-anomer ($\beta 2$). Of the molecules injected as α-anomer, only 3.0% will mutarotate to form $\beta 1$; of these, only 0.71% will revert to α-anomer. We see, therefore, that a sugar with kinetic and chromatographic properties approximating those of glucose should meet the criterion that the vast majority of molecules that mutarotate on the calcium column should do so only once, provided (as will be confirmed later in this paper) the rate of mutarotation of glucose while on the column

does not differ greatly from that of glucose in pure water at the same temperature.

The expected distribution of mutarotated material along the elution profile has been determined by computer modeling of the chromatographic process (Fig. 4). The model employed is discrete, rather than continuous, in that in a two-step process it tracks each of a large number of discrete "molecules" the length of the column. In the first step, the column is divided into a number of generation zones (typically 100) of equal length. "First-generation mutarotated material" ($\alpha 1$ and $\beta 1$) is then generated in each of the zones by the first-order decay of a thin disk of injected sample (α_0 and β_0) as it passes through the column. The length of the generation zones is chosen so that, with the extrapolated mutarotation rate constants at 1.5°C, the change in the concentration of the generating species (α_0 or β_0) as it moves through the zone will be proportionately very small. The rate of generation of $\alpha 1$ or $\beta 1$, will then be very nearly constant as the generating material moves across the zone, and thus a model that assumes uniform distribution of product ($\alpha 1$ or $\beta 1$) along the zone is a useable approximation of reality. If the elution times for unmutarotated α and β are taken as 25 and 20 min, respectively, and the column is divided into 100 generation zones, then the injected α-anomer will spend 0.25 min crossing each zone; the β-anomer generating species will require only 0.20 min to cross each zone. In view of the long half-lives expected at 1.5°C (on the basis of the rate constants estimated above, 9.44 h for α, 16.2 h for β), there does not appear to be any difficulty in meeting the above criterion. In the second stage of the process, each "molecule" in each of the generation zones is individually tracked off the column. For each anomer, a standard time interval is chosen to equal the same specified fraction (usually 0.001 to 0.01) of the half-life of that anomer. At the beginning of each such interval, the

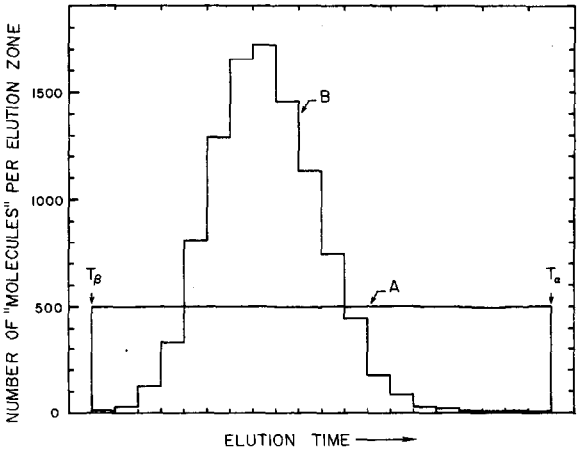

Fig. 4. Computer-generated models for the elution, at low and high temperatures, of glucose molecules that have mutarotated at least once during transit of the column. Curve A: distribution of mutarotated material assuming $k_\alpha = 0.00096\ \text{min}^{-1}$ and $k_\beta = 0.00066\ \text{min}^{-1}$, *i.e.*, rate constants approximating those expected in water in presence of packing material at 1.5°C. Curve B: distribution assuming rate constants on the order of those expected at 67–68°C ($k_\alpha = 0.96\ \text{min}^{-1}$, $k_\beta = 0.66\ \text{min}^{-1}$). The model does not take into account band-broadening effects other than those arising directly from mutarotation of the sample while on the column. In order to facilitate comparison of the curve shapes, the curves have been scaled in such a way that the areas under the curves will be equal.

random-number function provided by the computer is used to predict whether or not the molecule will mutarotate during the time interval. If mutarotation is predicted, the random-number function is used again to determine what portion of the interval will elapse before mutarotation occurs. At the time of mutarotation, the anomeric identity of the "molecule" is changed, along with the values for its standard time interval and velocity of migration. When the cumulative displacement of the "molecule" along the axis of the column shows that it is beyond the end of the column, a back-calculation is performed to determine the exact time of elution from the column. The "molecule" is then placed in the appropriate elution zone on the basis of its elution time, and the computer begins tracking the next molecule from its generation zone.

The two reaction-zone elution patterns shown in Fig. 4 represent two extreme cases of the value of the ratio of residence time on the column to the relaxation time for the mutarotational equilibrium. The elution profile labelled A was generated with values of the rate constants such that the average of the residence times of the two anomers is only 0.0365 times the relaxation time. Under these conditions, as shown earlier, the vast majority of those solute molecules that mutarotate at least once while on the column, mutarotate only once during transit, and the eluted material is spread evenly over the region between the elution times of the two unmutarotated anomers (T_α and T_β). For the other elution profile, labelled B, the rate constants for mutarotation are in the same ratio as for A, but have been increased by three orders of magnitude, so that the ratio of average residence time to relaxation time is now 36.5. Under these conditions, each sugar molecule will, on the average, mutarotate many times during residence on the column; the averaging effect of multiple successive mutarotations is seen in the gathering of the mutarotated material into a peak eluting at the weighted-average elution position for an equilibrium mixture of the anomers. The rate-constant determinations of O'Connor *et al.*[28] show that the rate constants for mutarotation of glucose in water increase by a factor of approximately 2.9 for each 10°C increase in temperature. The 100-fold increase in the magnitudes of the rate constants upon going from the values for curve A to curve B, therefore, is equivalent to increasing the temperature of the column by some 65°C, from 1.5°C to 66–67°C. The gathering of the eluted material into a single peak at high temperature is what one would expect for chromatography of a mutarotating sugar on ion-exchange columns[14].

It should be remembered that the curves shown in Fig. 4 are elution profiles of only that portion of the injected sample that has mutarotated while on the column. In the case of curve B, which represents the case with large rate constants, the reaction zone represents essentially all of the injected material, since almost all of the injected molecules (> 99.87%) will have mutarotated before exiting the column. On the other hand, the area under curve A, which illustrates the case of relaxation time much larger than chromatography time, represents only 1.7% of the injected material. For equal injections of sugar molecules, the area under curve A would therefore be much smaller than that under curve B. In order to facilitate comparison of the reaction-zone shapes, however, the two reaction zones have been scaled in such a way as to present equal areas in the figure.

The "discrete-molecule" computer simulation employed here requires substantial amounts of computer time, but has the advantage of being extremely simple and

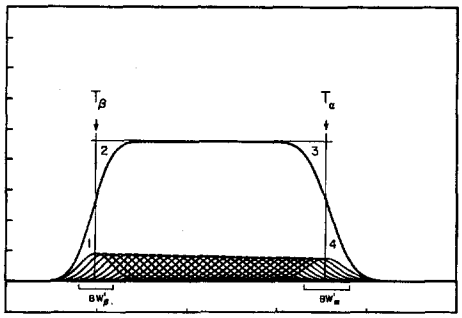

Fig. 5. Computer-generated elution curve showing effects of ordinary chromatographic band-broadening on the elution profile of material that has mutarotated only once while on the column (see curve A, Fig. 4). Details of curve-generating process given in text.

direct, and thus of involving a minimum of simplifying assumptions. As can be seen from curve A, Fig. 4, the model does not take into account any of the chromatographic band-broadening mechanisms (*e.g.*, non-uniform flow field, longitudinal diffusion, and mass-transfer resistances)[24] that operate in actual chromatography. Although the shapes of the reaction-zone elution profiles thus generated will differ substantially at each end of the zone from those of the actual profiles, the utility of such a model will be appreciated after consideration of the next two figures.

The important result obtained from the computer modeling demonstrated in Fig. 4 is that for small ratios of column residence time to relaxation time, and with band-broadening effects ignored, the mutarotated material will be distributed evenly over the region of the elution profile between the peak maxima for the two anomers. In Fig. 5, use is made of this finding in construction of a model for elution of the reaction zone that does take bandbroadening into account. The curve in Fig. 5 was constructed by the addition of 30 Gaussian peaks, evenly spaced over the region between T_β and T_α, the elution times for the β- and α-anomers. The band-width of the component Gaussians is increased linearly from BW'_β at T_β to BW'_α at T_α, with the area of the Gaussians held constant. The bandwidths BS'_β and BS'_α are related to the observed bandwidths for glucose β- and α-anomers, but since the peaks are slightly asymmetrical, the values used are obtained from the outer "halves" of the half-height bandwidths; *i.e.*, for the α peak the distance, at half-peak height, between the peak maximum position and the trailing edge of the peak is measured (see Fig. 6), and then doubled to obtain BW'_α. For BW'_β the distance measured at half-peak height is that between the peak maximum elution time and the leading edge of the peak. The rectangle shown in Fig. 5 is equal to the area under the predicted elution profile boundary, minus the areas marked 1 and 4, but with the addition of the areas marked 2 and 3. As the number of component Gaussians used to construct the elution profile becomes large, both the ratio between the size of area 1 and area 2 and the ratio between area 4 and area 3 approach a value of 1.0. The area of the rectangle shown in Figs. 5 and 6 is thus a good estimator of the area of the reaction-zone elution profile. As shown in Fig. 6, those regions of the elution time axis over which the height of the reaction zone is a strong function of elution time lie entirely beneath one peak or the other. It is thus immaterial whether the actual reaction-zone elution

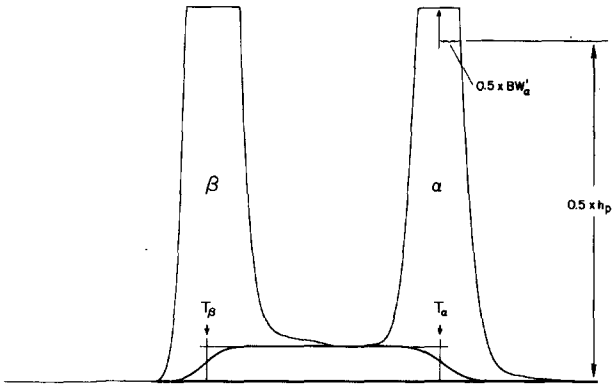

Fig. 6. Division of an actual elution profile into unmutarotated β peak, "reaction zone" (material mutarotated while on column), and unmutarotated α peak, according to the model developed in Figs. 4 and 5. The chromatogram shown is that of 0.1 mg D-glucose, injected in 0.020 ml and run at 1.5°C column temperature, 0.07 ml/min flow-rate. h_p = Half peak height; BW'_α = band-width.

profile or its rectangular approximation is used in applying baseline corrections to the peak areas.

Studies of the elution of the non-mutarotating 1-O-methylglycosides at 1.5°C on the calcium column reveal that, although there is noticeable tailing of peaks eluting in the region in which the sugar anomers elute, a profile composed of two non-interconverting peaks with retention times corresponding to those of the glucose anomers returns essentially to baseline between the peaks. For the other four sugars, the baseline region between the peaks will be wider than that for glucose (Fig. 1). For all five of the sugars, therefore, the height of the profile minimum between the peaks may be taken as the height of the reaction-zone elution profile. This value and the elution-time coordinates of the peak maxima provide all that is necessary for construction of a useable rectangular approximation of the reaction-zone elution profile.

Use of the peak quantitation method suggested in Fig. 6 permits quantitative expression (in Table I) of some of the trends seen qualitatively in Fig. 1. (With the exception of glucose, mutarotation of the sugars on the lead column proceeds to too great an extent for the assumptions underlying the method to hold. Area-based values, and some of the elution-time values, for these sugars on the lead column are shown in parentheses to indicate that they are merely rough estimates, and not based on a systematic method of analysis.) The selectivity ratios confirm that the failure of the lead column to provide analytically useful separations of four of the five sugars is not due to any lack of selectivity —for the five sugars the selectivity of the lead column is at least as good as that of the calcium column, with the selectivity of the lead column for mannose anomers being the highest for any sugar studied on either of the columns. The difference between the two columns is instead to be found in the percent mutarotation figures. The estimates for the lead column, rough as they are, clearly indicate in the case of mannose and xylose the destruction of well-separated anomeric peaks by rapid mutarotation. O'Connor *et al.*[28], have noted the differing effectiveness of various metal ions in catalyzing mutarotation of glucose, and have

TABLE I

COMPARISON OF THE SEPARATION OF SUGAR ANOMERS BY AQUEOUS CHROMATOGRAPHY AT 1.5°C ON CALCIUM-FORM AND LEAD-FORM ION-EXCHANGE COLUMNS

Values in parentheses were estimated from poorly-resolved to unresolved profiles and are thus less reliable than the other values shown.

Sugar	*Counter-ion**	*Retention times (min)***		*Selectivity* (t_2/t_1)	*Selectivity ratio (Ca/Pb)*	*Resolution factor****	*Percent mutarotation during chromatography*
		t_1	t_2				
D-Glucose	Ca^+	21.03	25.27	1.20	0.96	2.00	1.75
	Pb^+	26.45	33.07	1.25		2.04	15.0
D-Xylose	Ca^+	22.83	30.97	1.36	0.92	6.67	7.75
	Pb^+	28.77	42.7	1.48		(3.19)	(77)
D-Galactose	Ca^+	25.37	31.3	1.23	(1.03)	2.43	6.43
	Pb^+	(36)	(43)	(1.20)		–	(>90)
D-Mannose	Ca^+	26.03	31.17	1.20	0.63	2.08	8.98
	Pb^+	43.27	81.6	1.89		(6.17)	(78.0)
D-Arabinose	Ca^+	33.03	45.09	1.37	(1.08)	3.82	13.6
	Pb^+	(44)	(56)	(1.27)		–	(>90)

* Calcium-form column: BioRad HPX-87C; lead-form: BioRad HPX-87P.

** Column temperature 1.5°C, eluent deionized water at 0.3 ml/min.

*** Resolution factor = $(t_2 - t_1)/(1/2\ w_1 + 1/2\ w_2)$, where w_1 and w_2 are the base widths of the respective peaks as determined by extending to the baseline the tangents to the inflection points on either side of the peak[29]. Since these peaks are slightly asymmetrical, the "half-base-widths" used here are the distances between peak maximum and the trailing baseline intercept for the peak eluted first, and between peak maximum and the leading baseline intercept for the peak eluted second.

ascribed these differences to differences in the tightness of the metal–OH_2 bond in the aquated ions, which give rise to differences in the electrophilicity of the coordinated water molecules.

The resolution factors[29] shown for the calcium column (Table I) indicate that for at least three of the sugars (xylose, galactose, and arabinose) the 30-cm length of the column provides more than sufficient selectivity. For these sugars, the analysis time could be shortened, and the extent of perturbation of the anomeric ratio by on-column mutarotation thereby substantially reduced, by use of a column one-half or even one-third the length. The guard columns, Carbo-C and Carbo-P (Bio-Rad) used with these columns have packing materials that are chemically identical with those of the HPX-87C and HPX-87P, respectively, and are only 3.0 cm in length. Fig. 7 shows the performance of each of these pre-columns, used alone as a separation column, in comparison with the chromatography of the same sugar on the chemically-identical 30.0-cm analytical column. The Carbo-P, used as an analytical column in Fig. 7A, separates the mannose α- and β-anomers as two sharp peaks, whereas the 30-cm HPX-87P, with a much longer analysis time, shows only a tiny remnant of the second peak, with most of the injected material appearing in the reaction zone between the peaks. Ribose, which mutarotates extremely rapidly, elutes from the 30-cm HPX-87C as one peak near 86 min, with significant tailing beyond 230 min but with no clear evidence of any other peaks (Fig. 7D). In contrast, the 3-cm Carbo-C (Fig. 7C) resolves ribose into four distinct peaks, possibly reflecting pyranose–furanose equilibria as well as α–β equilibria. The examples chosen present the pre-columns in the best possible light, in that the respective packing materials have extremely high selectivities for these particular sugars; in the case of sugars with lower selectivity

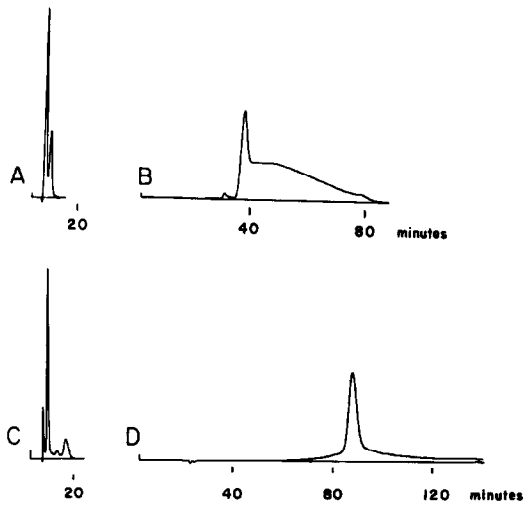

Fig. 7. The effect of residence time on the column upon resolution of the anomers of rapidly-mutarotating sugars. All columns maintained at 1.5°C. (A) D-Mannose (20 μg in 0.02 ml), chromatographed on 3.0-cm length Carbo-P (lead-form) guard column used as analytical column. Flow-rate 0.15 ml/min. (B) D-Mannose (100 μg in 0.02 ml), chromatographed on 30-cm HPX-87P (lead form), at 0.3 ml/min flow-rate. (C) D-ribose (20 μg in 0.02 ml), chromatographed on 3.0-cm length Carbo-C (calcium-form) guard column at a flow-rate of 0.15 ml/min. (D) D-ribose (100 μg in 0.02 ml), chromatographed on 30-cm length HPX-87C, at a flow-rate of 0.3 ml/min.

ratios, the relatively small separations provided by the pre-columns are insufficient in the face of extracolumn band-broadening (which is of the same magnitude for the 3-cm columns as for the 30-cm columns), to achieve resolution of the anomers. The examples shown are, nonetheless, striking evidence of the separations that should be possible with columns longer than the pre-columns, but shorter than the 30-cm columns. Increasing the flow-rate through the 30-cm columns is not a practical option for improving anomeric resolution, for two reasons: (1) at 1.5°C column temperature and 0.3 ml/min flow-rate, the systems already have a back-pressure near 900 p.s.i., so there is little room for further flow-rate increases in any event, and (2) at 1.5°C increases in flow-rate beyond 0.3 ml/min result in significant broadening of the peaks themselves, probably due to slow mass-transfer between phases at this temperature.

The relative importance of the different hydroxyl groups of the sugars is indicated by the results in Fig. 8, which shows the separation of anomers at 1.5°C by the HPX-87C, for a series of derivatives of each of the five principal sugars studied. Without exception, the more retained anomer is that which has the anomeric hydroxyl *cis* to the 2-hydroxyl. This finding is consistent with the early proposal by Goulding[2] that the formation of stable complexes between metal ions and axial–equatorial pairs of hydroxyls on adjacent carbon atoms was important in the reten-

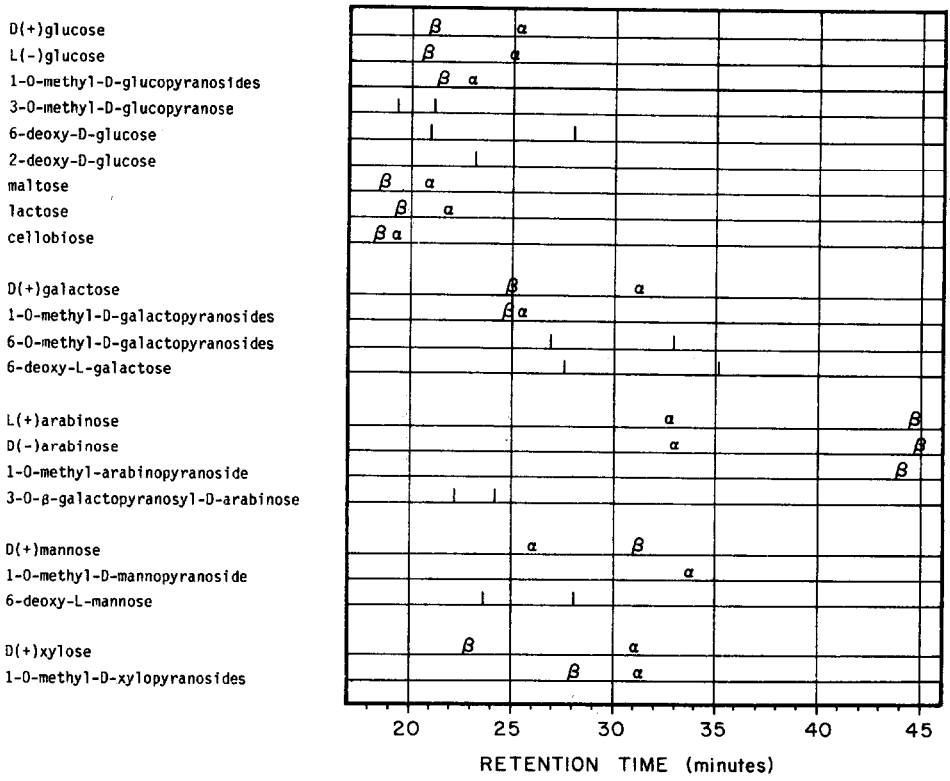

Fig. 8. Effect of hydroxyl-group derivatization upon retention times for sugars on calcium-form column. Chromatography at 1.5°C, 0.3 ml/min flow-rate on HPX-87C. Vertical lines indicate maxima positions of unassigned anomers.

tion of sugar molecules on metal-form ion-exchange columns. Replacement of the hydrogen of the anomeric hydroxyl with a methyl group does not appear to interfere seriously with the ability of the anomeric oxygen to form complexes with the packing material; where both α- and β-1-O-methyl glycosides were available, the glycosides were also separated by the column, and in the same order as were the anomers of the parent sugar. Methylation of the anomeric oxygen does tend to change significantly the retention of one or the other (or both) of the anomeric forms, but there does not seem to be any simple pattern. For glucose, the β-glycoside is retained slightly more than is the sugar β-anomer; the α-glycoside binds less tightly than does the α-sugar. In the case of galactose, the retention of the β-glycoside is essentially unperturbed (relative to that of β-glucose) by methylation of the anomeric hydroxyl, while the α-glycoside is retained much less strongly than the α-sugar, eluting quite near the position of the β-glycoside and the β-sugar. For xylose, it is the α-glycoside that closely mimicks the elution behaviour of the corresponding sugar anomer, and the β-glycoside that is bound considerably more tightly than is the β-sugar. Other factors, such as the effect of methylation on ring-conformation equilibria, will probably have to be considered along with the configurations about the 1- and 2-carbons, in order to account for the results.

The 2-hydroxyl appears to be absolutely essential for the separation of the anomers, at least on the basis of the one deoxy-sugar studied. 2-deoxyglucose shows no anomeric separation whatsoever, eluting as a single, narrow peak approximately centered between the elution positions of α- and β-glucose. Modification at the 3-hydroxyl does not destroy the ability of the calcium column to separate anomers of glucose and arabinose; even with the attachment of as bulky a group as a β-galactopyranosyl moiety, arabinose is still resolved into anomers, although both anomers are bound much less tightly than are the unencumbered parent-sugar anomers. Among the disaccharides tested, the interaction with the column appears sensitive to the stereochemistry of the 1,4-bond, with maltose (α-1,4) being better-resolved than cellobiose (β-1,4). The 6-hydroxyl appears to have little to do with the anomeric separation, since the three 6-deoxy-sugars tested were resolved almost as well as (6-deoxymannose) or slightly better than (6-deoxyglucose, 6-deoxygalactose) the parent sugars. The column shows no chiral selectivity; the elution patterns of L-glucose and L-arabinose were indistinguishable from those for the D-enantiomers.

When an LC column discriminates between two components of a sample that are reversibly interconvertible, determination of the way in which interaction of the sample with the column affects the apparent equilibrium between the two components can be instructive with regard to the mechanism of retention of the components[30]. The apparent effect of interaction with the HPX-87C packing material upon the equilibrium between α- and β-anomers of monosaccharides was studied in two different types of experiments. In the first type of experiment, the rate constants, k_α and k_β, for mutarotation of the α- and β-anomers of glucose were determined under "flow" conditions. Samples of glucose, pre-equilibrated in deionized water at the temperature of the column (1.5°C in this case), are chromatographed at a range of different flow-rates (0.3 ml/min down to 0.038 ml/min) in order to provide a range of different column residence times. As the residence time is increased, the area of the reaction zone increases at the expense of the two anomer peaks. Since those molecules that mutarotate while on the column are being transferred to the reaction

zone, rather than being transferred from one peak to the other, the mutarotation of the material injected as β and the mutarotation of the material injected as α can be treated as two separate first-order decay processes. The rate constants k_α and k_β can then be determined from the slopes of plots of the natural logarithms of the peak areas *versus* residence time on the column, the residence time for each anomer being taken as the elution time of the peak maximum. (The chromatogram used in Fig. 6 to illustrate peak area quantitation was taken from this series.)

The second type of experiment yields apparent equilibrium constants rather than rate constants. In these "stopped-pump" experiments, the sugar sample is run a short distance onto the column (2–3% of column length), the pump is then shut off, and the sample is allowed to equilibrate, at 1.5°C in contact with the packing material, for a period of time equal to 7–10 times the half-life for equilibration at this temperature. After this equilibration period, the pump is restarted, and the sample is eluted from the column at a flow-rate of 0.3 ml/min. Given the performance demonstrated for this column (Fig. 1, Table I), the ratios of the peak areas, as eluted, are reasonably good estimators of the overall equilibrium established on the column during the long equilibration period. The results of both types of experiments are shown in Table II. In all cases, interaction of the sugar with the column material was seen to shift the equilibrium in favor of the anomer more tightly bound to the column. This is not an especially surprising result, since preferential binding of one anomer would be expected to perturb the equilibrium, but the close agreement shown for glucose between results obtained by two different procedures, one in which the sample is allowed to reach equilibrium on the column, and another in which the sample remains far from equilibrium under column conditions, testifies to the ability of the peak-quantitation method of Figs. 4–6 to measure small changes in peak areas. Further, an examination of the purely kinetic results of the "flow" experiment suggests some possible features of the mechanism of anomer separation. The apparent perturbation of the glucose mutarotational rate constants appears to be primarily a perturbation of the value of k_α, with k_β as measured in the presence of the packing

TABLE II

EFFECT OF INTERACTION OF SUGARS WITH THE CALCIUM-FORM PACKING MATERIAL UPON THE APPARENT MUTAROTATIONAL EQUILIBRIUM CONSTANTS

Experiments carried out at 1.5°C on HPX-87C column. See text for details.

Sugar	*Expt. type*	$K_{col.}$*	K_{H_2O}**	$K_{col.}/K_{H_2O}$	t_β/t_α***
D-Glucose	Flow	1.45§	1.69§§	0.86	0.83
D-Glucose	Stopped-pump	1.43	1.74	0.82	0.83
D-Mannose	Stopped-pump	0.537	0.454	1.18	1.20
D-Xylose	Stopped-pump	1.55	2.12	0.73	0.74

* Apparent equilibrium constant (= [β]/[α]) at 1.5°C in the presence of column packing material.

** Measured equilibrium constant (= [β]/[α]) at 1.5°C in deionized water.

*** Ratio of retention times for the anomers at 1.5°C.

§ Calculated as k_β/k_α, with $k_\alpha = 1.60 \cdot 10^{-5}\ s^{-1}$ and $k_\beta = 1.10 \cdot 10^{-5}\ s^{-1}$. Compare with $k_\alpha = 2.04 \cdot 10^{-5}\ s^{-1}$ and $k_\beta = 1.19 \cdot 10^{-5}\ s^{-1}$, which are values calculated from the results of O'Connor *et al.*[28] for glucose in pure water at this temperature.

§§ Based on extrapolation of ln (peak area) to zero time to obtain the peak area ratio at injection.

material having approximately the same value it has in pure water at this temperature (Table II, 4th footnote). More than one set of assumptions might be invoked to explain this perturbation, but the following would seem to be the simplest: Suppose that the separation of the α- and β-anomers of glucose is due to the formation of a particular complex between α-glucose and resin-bound calcium (the "selectivity complex") for which no counterpart is formed by β-glucose. Assume further that α-glucose is not susceptible to ring-opening and mutarotation while a part of the "selectivity complex", but that both β-glucose, whatever the state in which it exists on the column, and all α-glucose not participating in the "selectivity complex", mutarotate with the same rate constants they would exhibit in the bulk mobile phase (water).

Given these assumptions, the "apparent" values of the rate and equilibrium constants as measured in the presence of the calcium-form resin will be related to the values in bulk water by eqns. 6–9.

$$k_{\alpha}^{(\text{app.})} = k_{\alpha}^{H_2O}\,(t_{\beta}/t_{\alpha}) \tag{6}$$

$$k_{\beta}^{(\text{app.})} = k_{\beta}^{H_2O} \tag{7}$$

$$K_{\text{eq.}}^{(\text{app.})} = [\beta]/[\alpha] = K_{\text{eq.}}^{H_2O}\,(t_{\beta}/t_{\alpha}) \tag{8}$$

$$K_{\text{eq.}}^{(\text{app.})}/K_{\text{eq.}}^{H_2O} = t_{\beta}/t_{\alpha} \tag{9}$$

Eqn. 8, and its rearrangement as eqn. 9, can readily be derived either by way of a kinetic approach corresponding to the "flow" experiments, or by way of an equilibrium approach corresponding to the "stopped-pump" experiments. As can be seen by reference to the last two columns of Table II, for either type of experiment the relationships between the experimentally measured values of $K_{\text{eq.}}^{(\text{app.})}/K_{\text{eq.}}^{H_2O}$ and t_{β}/t_{α} are in excellent agreement with eqn. 9.

The possible structure suggested in Fig. 9B for the "selectivity complex" illustrates two of the ideas discussed above. The direct interaction between calcium and the oxygen of the 2-hydroxyl is based on the findings that in all cases studied, the anomer most strongly retained is that with the 2-hydroxyl *cis* to the anomeric hydroxyl (Fig. 8) and that when the 2-hydroxyl is not present, as in 2-deoxy-D-glucose

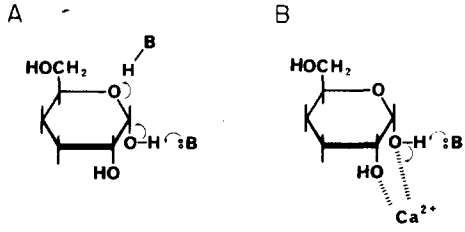

Fig. 9. Proposed effect of complexation with calcium ion upon the ring-opening step of mutarotation of α-glucose. (A) Ring-opening in the uncomplexed sugar. (B) Inhibition of ring-opening in the proposed "selectivity complex" between α-glucose and resin-bound calcium ion.

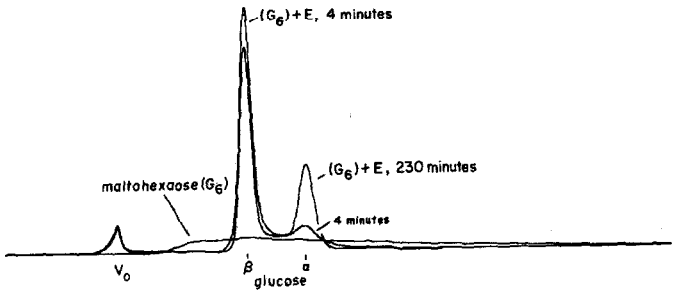

Fig. 10. Anomeric composition of glucose produced from the hydrolysis of maltohexaose by *A. niger* amyloglucosidase (E). Column temperature 1.8°C. Void volume elution (V_0) identifies enzyme elution and the lower-most plot shows the elution of substrate (G_6) and acetate buffer.

(Fig. 8), no anomeric separation is observed. The indication of direct interaction between the anomeric hydroxyl and the metal ion is based upon the proposed inability of "selectivity complex" α-glucose to mutarotate and upon the most widely accepted mechanism for ring-opening and mutarotation[27]. Whatever the differences in identity of catalysts and of rate-limiting steps between ring-opening mechanisms proposed for different conditions[31], one feature essential to all the mechanisms is displacement of the ring oxygen from its bond with the anomeric carbon through nucleophilic attack by an electron pair of the anomeric hydroxyl oxygen (Fig. 9A). If the glucose is bound to calcium as shown schematically in Fig. 9B, any excess electron density on the oxygen of the anomeric hydroxyl should shift toward the metal ion to form a tighter coordinate bond, rather than attacking the 1-carbon to displace the ring oxygen.

Figs. 10 and 11 illustrate the application of the aqueous chromatography on ion-exchange resins in determining the anomeric configuration of exo-glucanase and glucosidase enzyme reaction products. In Fig. 10 the lead-form column (HPX-87C) is used to analyze the products of total conversion of maltohexaose to glucose by the exo-glucanase type enzyme, *Aspergillus niger* amyloglycosidase. After 4 min. incu-

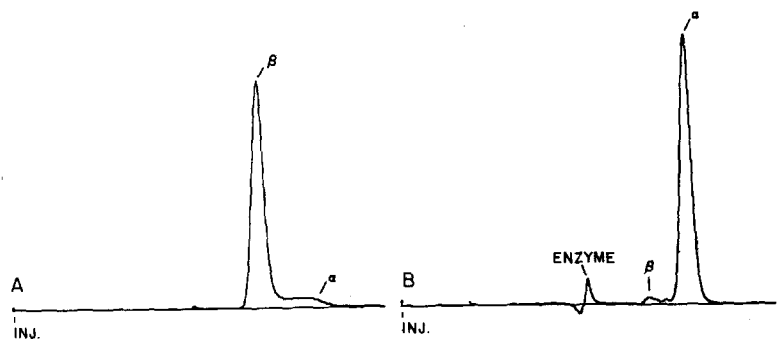

Fig. 11. Determination of anomeric product specificity for enzymic cleavage of synthetic substrates. (A) Chromatography on lead-form column (HPX-87P) of the products of a 2.5-min incubation at 22°C of 4.0 mg/ml salicin [2-(hydroxymethyl) phenyl-β-D-glucopyranoside] with 0.67 mg/ml almond β-glucosidase, at pH 5.0 in 3.33 m*M* acetate. (B) Chromatography on calcium-form column of the products of a 2.5-min incubation of *p*-nitrophenyl-α-D-glucopyranoside (2.5 mg/ml) with 1.25 mg/mnl brewer's yeast α-glucosidase at 22°C, pH 5.0 in 2.5 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) buffer.

bation, essentially all of the substrate has been hydrolyzed, and the preponderance of β-anomer in the product can be seen from the relative sizes of the α and β peaks, compared with the area ratio observed at 230 min., at which time the product has been in solution long enough to proceed most of the way to equilibrium. Hydrolysis of α-(1,4) glucosidic bonds by this enzyme is known, from other methods, to proceed with inversion of configuration at the anomeric carbon to produce β product from the α-linked substrate; the α-glucose observed at 4 min is due in small part to mutarotation of the product in solution after cleavage, but mostly can be traced to the reducing-end glucose unit, which in the intact substrate already consists of a mixture of α and β forms and is not affected in this regard by cleavage of the substrate.

The mixture analyzed in Fig. 11 represents products of enzyme action on very readily hydrolyzed p-nitrophenyl glucose esters. In these product mixtures the only glucose present is that liberated by cleavage of the glucosidic bond, and the reaction is sufficiently rapid that it is complete before the product has mutarotated to any great extent. In Fig. 11A, analysis on the lead column of the products of hydrolysis of 2-(hydroxymethyl) phenyl-β-D-glucopyranoside by almond β-glucosidase reveals that there is essentially no α-glucose in the reaction product (retention of configuration in the reaction); the elution profile consists almost entirely of a β-anomer peak with an appended "reaction zone" representing the mutarotation of β-glucose during chromatography. Analysis on the calcium-form column of the products of the hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside by brewer's yeast α-glucosidase (Fig. 11B) demonstrate the retention of configuration in this reaction also, in that the glucose product is seen to be at least 97% α-anomer.

A third column of this general type promises to be useful in determining the anomeric configuration of the products of enzymes that act on polysaccharides to liberate disaccharides rather than monosaccharides, such as β-amylase (1,4-α-D-glucan maltohydrolase; E.C. 3.2.1.2) and 1,4-β-D-glucan cellobiohydrolase; E.C. 3.2.1.91. The bead material of the calcium-form HPX-42C (Bio-Rad) is, like that of

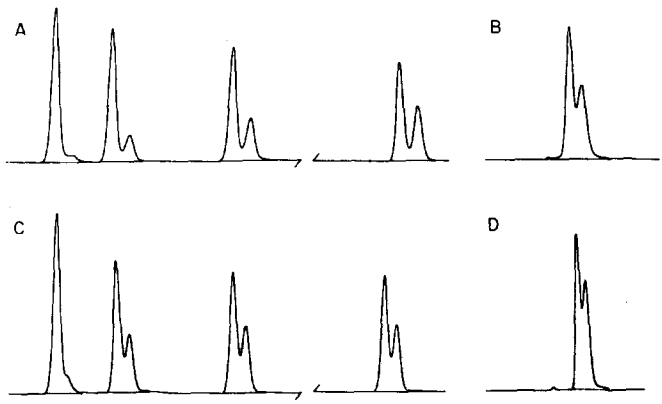

Fig. 12. Separation of the anomers of disaccharides on calcium-form ion-exchange columns. Injection volume 0.020 ml for all samples; column temperature 1.5°C, eluent deionized water at 0.15 ml/min. (A) Sequential injections of 5.0 mg/ml maltose at (from left to right) 70 s, 20 min, 63 min, and 29 h after time of solution in water at 22°. (B) *equil*-Maltose, 5.0 mg/ml, chromatographed on HPX-87C. (C) Cellobiose, 1.0 mg/ml, injected onto HPX-42C at (left to right) 2 min, 110 min, 4 h 10 min, and 31 h after time of solution in water. (D) *equil*-Cellobiose chromatographed on HPX-87C.

the HPX-87C and HPX-87P, a divinylbenzene-crosslinked polystyrene resin, but has a larger pore size (4% crosslinking compared with 8% for the other two columns) which allows the larger solute molecule greater access to the interaction sites. The successive injections of maltose put into solution as the β-anomer (Fig. 12A) show that the HPX-42C can be used to measure the preponderance of one anomer over the other in enzyme reaction products; comparison with Fig. 12B, a chromatogram of *equil*-maltose on the HPX-87C, shows the advantage of the more open resin matrix. For anomeric analysis of the disaccharides, a lower flow-rate (0.15 ml/min as opposed to 0.3 ml/min) was found to be optimal for both the HPX-42C and the HPX-87C, probably due to the increasing effect of mass-transfer resistance for the larger molecules at this low temperature. The anomers of cellobiose (Fig. 12C) are not as well separated on the HPX-42C as are those of maltose, but in this case also, the HPX-42C provides better resolution than does the more extensively crosslinked HPX-87C (Fig. 12D).

The method of anomeric analysis described here has a number of advantages with respect to other methods. The capability of injecting reaction mixture aliquots directly without any need for prior derivatization or extraction steps facilitates the examination of early stages of enzyme reactions. Since the aqueous metal-form ion-exchange method measures specific chemical species, rather than additive properties of solutions such as optical rotation, the LC method will be less susceptible to certain interferences. The sensitivity of the method, allowing determinations to be made on reaction mixtures of 100 μl or less, is certain to be welcomed by workers using limited and precious supplies of purified enzymes. Finally, of some practical advantage is the fact that many of the laboratories concerned with mechanistic studies of enzymes hydrolyzing glycosidic bonds will already have these columns, or their equivalents, on hand for use at high temperatures in routine assay of enzyme activity[32]. Implementation of the procedure described here will, therefore, involve only the provision of means for maintaining the columns at low temperatures.

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