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SECONDARY TRITIUM AND CARBON-14 ISOTOPE-EFFECTS ON ION-EXCHANGE CHROMATOGRAPHY OF THE D-GLUCOSE–BORATE COMPLEX

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ABSTRACT

Secondary, hydrogen isotope-effects are observed when D-[1-^{3}H]glucose, D-[2-^{3}H]glucose, D-[3-^{3}H]glucose, D-[5-^{3}H]glucose, and D-[6-^{3}H]glucose are each co-chromatographed with nonlabeled D-glucose. A secondary, carbon isotope-effect is also observed when nonlabelled D-glucose is co-chromatographed with D-[U-^{14}C]-glucose; no isotope effect is seen with D-[6-^{14}C]glucose. These isotope effects have been quantitatively analyzed. The chromatographic separation of radiolabeled glucose from glucose is the result of a differential effect of isotopic substitution on complexation of the hydroxyl oxygen atoms with a proton and boron. Tritium in positions 2, 3, 5, and 6, but not in position 1, apparently increases complexation with a proton (a pK increase), but does not similarly increase complexation with boron. As a result, the tritiated glucose is eluted more rapidly on the ion-exchange resin. Based upon this effect, it is possible to obtain isotopically pure, tritiated glucose by ion-exchange fractionation.

INTRODUCTION

Isotope effects have been observed in various chromatographic systems. Gas–liquid partition chromatography has been used to effect separation of deuterated hexane from nonlabeled hexane[1], tritium-labeled from ^{14}C-labeled steroids[2], deuterated from nondeuterated ethanes[3], and trimethylsilyl ethers of deuterated D-glucose from nondeuterated D-glucose[4]. Multiple recycling by gas chromatography has resolved deuterated methane and butane from their respective, nondeuterated derivatives[5]. Isotope effects have also been observed in chromatography with paper[6], silica gel[7,8], silica–silver nitrate[9], Celite[10], and charcoal[11] columns.

Several isotope-effects have been observed for ion-exchange chromatography. In early work by Taylor and Urey[12], ^{6}Li was separated from ^{7}Li by using sodium zeolite. By a similar method, Brewer[13] fractionated ^{39}K from ^{41}K. Piez and Eagle[14] observed an isotope effect on Dowex-50 columns with several ^{14}C-labeled amino

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acids, and Gottschling and Freese[15] described an isotope effect with tritiated 2-aminopurine on Dowex 1-X8.

Previous studies in this laboratory[16] revealed partial resolution of D-[6-^{3}H]glucose from D-[U-^{14}C]glucose on a Dowex 1-X4 column; an isotope effect was presumed. In this work, we quantitate the isotope effect on chromatography of the D-glucose–borate complex on Dowex 1-X4, with tritiated D-glucose labeled in the 1, 2, 3, 5, and 6 positions and with D-[U-^{14}C]glucose and D-[6-^{14}C]glucose.

METHODS

Materials. — Intramolecularly labelled D-[6*R*,6*S*-^{3}H]glucose (33.99 Ci/mmol), D-[3-^{3}H]glucose (17.54 Ci/mmol), D-[1-^{3}H]glucose (18 Ci/mmol), intramolecularly labelled D-[U-^{14}C]glucose (360 mCi/mmol), D-[6-^{14}C]glucose (52.8 mCi/mmol) and Biofluor cocktail were obtained from New England Nuclear. D-[5-^{3}H]Glucose (12 Ci/mmol) and D-[2-^{3}H]glucose (19.3 Ci/mmol) were obtained from Amersham Corporation. 2,3-Butanediol was supplied by Aldrich Chemical Corporation. Chromatography was performed with Dowex 1-X4 (-400 mesh, Bio-Rad Laboratories) in a 0.6 × 100-cm, water-jacketed glass column (Glenco), eluted at 120–200 lb.in^{-2} by using a peristaltic pump (Milton Roy).

Column packing. — Buffers *A* and *B* and the Dowex 1-X4 resin slurry were prepared according to Walborg *et al.*[17], but with Brij-35 omitted. The 0.6 × 100-cm, water-jacketed column, maintained at 40°, was filled with the Dowex 1-X4 slurry in buffer *A* and packed at a 20 mL/h flow rate. After all but about 2.5 cm of the slurry height remained unpacked, the clear buffer was drawn off, and the column refilled with the slurry. This packing procedure was duplicated five times to effect a 97-cm column. The column was equilibrated by eluting at 40° with 500 mL of buffer *A* at 20 mL/h.

Sample addition. — Radioactive D-glucose (1.7–4.0 μCi) was evaporated to dryness and redissolved in 1.0 mL of 10mM D-glucose in buffer *A*. A 2-μL aliquot of this solution was counted, and the remainder added to the column by a 2-mL syringe fitted with a blunt, 15-gauge needle that was forced into the 1.58-mm (inner diameter) tefion inlet-tubing at the junction with the pump. After all of the sample had been forced out of the syringe, a second 2-mL syringe filled with 2 mL of buffer *A* was used to force 1 mL of this buffer behind the radioactive sample. The column inlet-tube was then connected to the pump while preventing air from entering the system. The elution rate was 20 mL/h, and 3-min fractions of 1.0 mL each were collected. The column was eluted at 40°, collecting 230 1-mL fractions, after which time the temperature-controlling water-bath was heated to 60°. After collecting 10 more fractions, by which time the column had equilibrated to 60°, stepwise elution with buffer *B* was begun. Elution was continued with buffer *B* until the D-glucose peak was completely eluted.

Assays. — Radioactivity was assayed by adding 5.0 mL of Biofluor cocktail to glass scintillation-vials containing 25 μL of the eluted fractions and 375 μL of

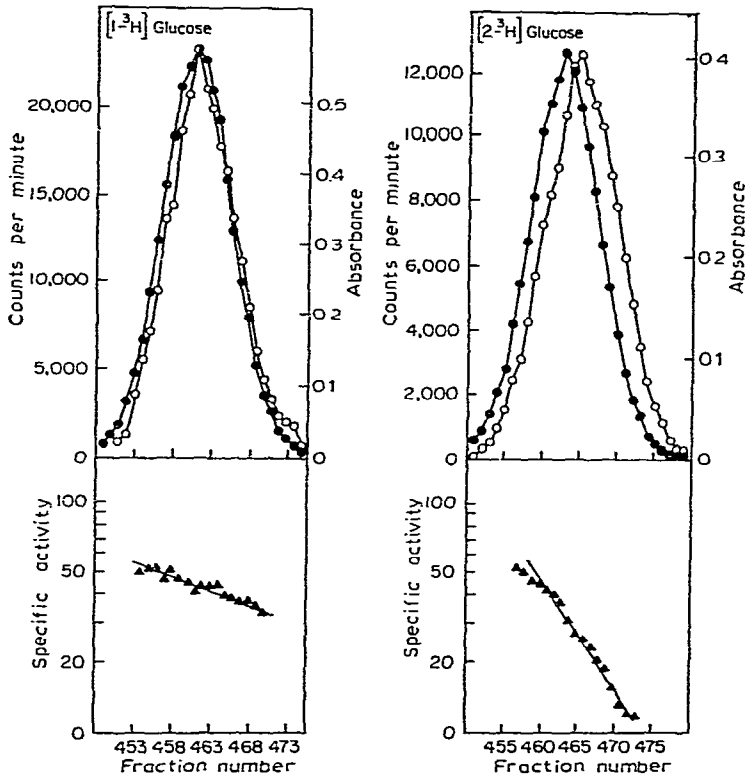

Fig. 1. Secondary tritium isotope-effect of D-[1-^{3}H]glucose and D-[2-^{3}H]glucose and comparison of fraction number to the specific radioactivity. Approximately 12 μCi of radioactive glucose in 1.0 mL of 10mM nonlabeled D-glucose was chromatographed. The 1.0-mL elution fractions were assayed for radioactivity (●—●) and for concentration of D-glucose (○—○). Fraction number is compared to specific radioactivity according to Eq. 2. (lower panel).

water. Colorimetric assays for D-glucose were performed according to the procedure of Walborg and Christensson[18] with heating of the marble-topped tubes in a water bath for 2 h at 100°.

RESULTS

Various degrees of fractionation were observed when D-[1-^{3}H]glucose, D-[2-^{3}H]glucose, D-[3-^{3}H]glucose, D-[5-^{3}H]glucose, D-[6-^{3}H]glucose, D-[6-^{14}C]glucose, and D-[U-^{14}C]glucose were separately chromatographed with nonlabeled D-glucose. Fig. 1 shows the chromatographic profiles for D-glucose-containing D-[1-^{3}H]glucose and D-[2-^{3}H]glucose. The fractionation of D-[2-^{3}H]glucose from nonlabeled D-glucose is greater than the fractionation observed when D-[1-^{3}H]glucose is chromatographed with nonlabeled D-glucose. When D-[3-^{3}H]glucose or D-[5-^{3}H]glucose was co-chromatographed with nonlabeled D-glucose (not shown), the fractionation of these labeled compounds from nonlabeled D-glucose was similar to that observed

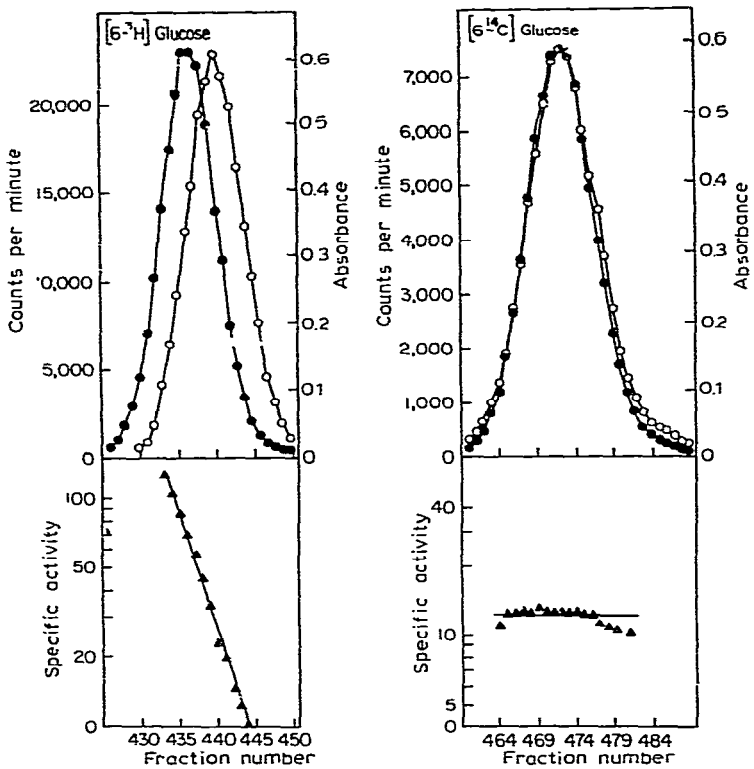

Fig. 2. Secondary tritium isotope-effect of D-[6-^{3}H]glucose and D-[6-^{14}C]glucose and comparison of fraction number to the specific radioactivity. Symbols are as in Fig. 1.

for D-[6-^{3}H]glucose (Fig. 1). D-[6-^{3}H]Glucose showed the largest separation from nonlabeled D-glucose (Fig. 2), while D-[6-^{14}C]glucose showed no fractionation (Fig. 2). D-[U-^{14}C]Glucose showed a small fractionation from nonlabeled D-glucose (Fig. 3).

The fractionation of radiolabelled and nonlabelled glucose results from an isotope effect; it is not the result of the presence of labelled contaminants that do not comigrate with glucose. We base this conclusion on the following: (*a*) A labelled contaminant would have to constitute an unreasonable fraction (30–70%) of the total radioactivity in order to account for the amount of radioactivity that does not fall under the nonradioactive-glucose peak. This 30–70% hypothetical contamination is a lower limit, calculated by assuming that the trailing edge of the radioactivity peak is constituted of pure radioactive glucose. From measurements of the amount of radioactivity and nonradioactive glucose in this region, the specific activity of the glucose is calculated. Based on this specific activity and assuming that radioactive and nonradioactive glucose cochromatograph (namely, that there is no isotope effect on chromatography), the shape of the radioactive-glucose peak may be constructed. The radioactive and nonradioactive glucose would peak at the same point,

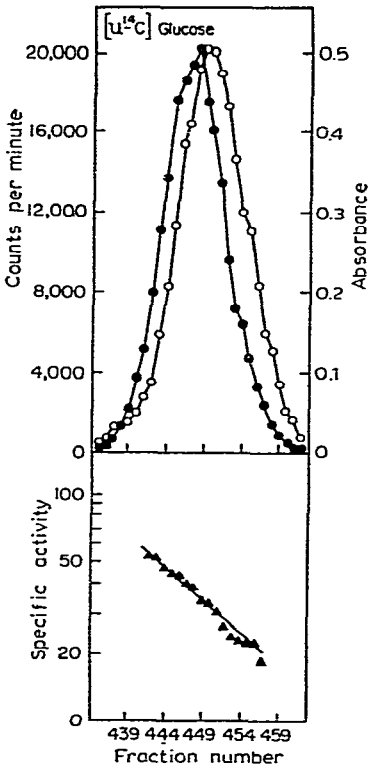

Fig. 3. Secondary carbon-14 isotope-effect of D-[U-^{14}C]glucose and comparison of fraction number to the specific radioactivity. *Symbols are as in Fig. 1.*

and the area outside this peak would consist of the hypothetical impurity. This area is 30–70% of the total radioactivity for the differently substituted, radioactive-glucose molecules. (*b*) The presence of a significant radioactive contaminant would not be expected to generate the symmetrical peaks observed in each of the experiments. (*c*) We have previously observed a similar separation of radioactive and nonradioactive maltose[16] and found that rechromatography of fractions from the leading and trailing edges of the radioactive peak gave identical chromatographic profiles.

Two methods were used to calculate the means of each chromatographic profile. (*a*) Probit least-squares analysis of the means[19] gave a significant displacement between the linear plots for the radioactivity and absorbance profiles, and allowed accurate determination of the two means. (*b*) The means ($\bar{N}$) of the elution profiles of each chromatographic fractionation were also determined by the following expression taken from Gottschling and Freese[15]:

$$\bar{N} = (1/R) \sum_{N=1} N \cdot r \qquad (1),$$

where R is the total radioactivity or optical density in the chromatographic profile;

TABLE I

ISOTOPE EFFECT ON ION-EXCHANGE CHROMATOGRAPHY OF GLUCOSE–BORATE COMPLEXES

Glucose Derivative	*Difference in means*		*Slope of specific radioactivity versus fraction number*[c]
	Probit Analysis[a]	$\bar{N} = 1/R \Sigma N \cdot r^b$ $N = 1$	
D-[1-^{3}H]Glucose	0.7	0.8	−0.19
D-[2-^{3}H]Glucose	2.3	2.3	−0.88
D-[3-^{3}H]Glucose	1.9	2.0	−0.84
D-[5-^{3}H]Glucose	2.0	1.9	−1.0
D-[6-^{3}H]Glucose	3.5	3.6	−1.8
D-[6-^{14}C]Glucose	0.1	0.2	0.0
D-[U-^{14}C]Glucose	1.3	1.2	−0.48

[a]Calculated as described in ref. 19. [b]Calculated as described in ref. 15. [c]Calculated as described in ref. 20.

N is the fraction number; and r is the distribution of radioactivity or optical density in each fraction. Means of all chromatographic peaks calculated by these methods are shown in Table I. The mean fractionation exhibited by D-[6-^{3}H]glucose (N = 3.6 mL), D-[5-^{3}H]glucose (N = 1.9 mL), D-[3-^{3}H]glucose (N = 2.0 mL), and D-[2-^{3}H]glucose (N = 2.3 mL) from labeled glucose is large. A small fractionation is observed with D-[2-^{3}H]glucose. D-[U-^{14}C]Glucose is also partially fractionated from D-glucose, whereas D-[6-^{14}C]glucose is almost coeluted with D-glucose.

The isotope effect may be quantitated by assuming that each pair of elution curves can be represented as a Gaussian distribution having the same standard deviations but different means. Based upon this, the specific activity (S) may be related to the fraction number (X) by the following equation[14]:

$$\ln S = \frac{\mu_1 - \mu_2}{\sigma}(X) + \frac{\mu_2^2 - \mu_1^2}{2\sigma^2} \qquad (2),$$

where μ_1 is the mean of the radioactivity curve, μ_2 is the mean of the absorbance curve, and σ is the standard deviation. When ln S is plotted versus the fraction number, a straight line is obtained (Figs. 1–3), and the slope of the line indicates the degree of fractionation; a zero slope indicates no fractionation. Slopes from these plots have the same relationships as the means of the peaks calculated by the method of Gottschling and Freese[15] and probit analysis[19] already discussed.

DISCUSSION

Complexation of borate with D-glucose occurs by a condensation reaction. Boric acid is unusual in that it ionizes in aqueous solution not by deprotonation, but by hydration followed by ionization. The negatively charged, tetrahedral borate ion

then complexes with D-glucose[20]. The equilibria for borate binding to D-glucose are complicated, and the existence of several D-glucose–borate complexes has been deduced from study of the relative ionophoresis of a series of D-glucopyranosides and D-glucofuranosides[21–23]: the *cis*-1,2-hydroxyl and 4,6-hydroxyl groups of the pyranose form and the *cis*-1,2-hydroxyl and 5,6-hydroxyl groups of the furanose form of D-glucose, complex with borate; a tridentate complexation of borate with the 3,5,6-hydroxyl groups of the furanose form of D-glucose has also been proposed; complexation of borate also apparently occurs with the open-chain aldehyde at the 2,4- or 4,6-hydroxyl groups. In alkaline borate solution, all of the hydroxyl groups of glucose probably take part in complexation to some extent[21]. Our observation that there are significant isotope-effects on chromatography of glucose–borate derivatives substituted with tritium at positions 1, 2, 3, 5, and 6 (Table I) is in accord with results[21–23] that indicate complexation with hydroxyl groups at these positions.

It should be noted that the secondary isotope-effects are normal and not inverse as might seem to be the case. The secondary isotope-effect is presumed to be on borate complexation with D-glucose and not on the binding of the D-glucose–borate complex to the ion-exchange resin. Thus, a normal isotope-effect that decreases complexation also decreases column binding and column retention-time, so that the isotopic glucose derivative is eluted ahead of the nonisotopic derivative.

The isotope effect on chromatography of various substituted glucose derivatives is expected to be a function of two parameters; (*a*) Isotopic substitution has an effect on the proton and boron affinity of the adjacent oxygen atom. The oxygen atom of a tritium-substituted derivative will have a higher affinity for a proton, as indicated by the higher pK for deuterated formic acid (pK 3.772), as compared with formic acid (pK 3.737)[24]; tritium substitution for hydrogen is expected to result in a larger increase[25] in pK. The increase in pK will decrease borate complexation, as it decreases the extent of the hydroxyl-group ionization that precedes complexation. This effect may, however, be offset by the isotopic substitution's increasing the strength of the oxygen atom's interaction with the boron Lewis acid. (*b*) The relative significance of a particular hydroxyl group on complexation of glucose is also important. If a hydroxyl group does not participate significantly, in formation of a borate complex, then the isotopic substitution is not expected to influence mobility in the ion-exchange chromatography.

A significant isotope-effect is seen for chromatography of glucose derivatives containing tritium at position 2, 3, 5, and 6. Apparently the isotope-induced increase in pK (which decreases the extent of ionization) is not offset by an equal increase in the strength of the interaction with boron. Less borate complexation occurs, and the isotopic compound is eluted more rapidly. This effect is greatest with [6-^{3}H]glucose, where the 6 position is more than single substituted with tritium. With D-[1-^{3}H]glucose, a very small isotope-effect is observed. It is suggested that this results from a near equivalence of the tritium's influence on the ionization of the relatively acidic 1-hydroxyl group and the strength of the boron complexation reaction.

Carbon isotope-effects are known to be quite small[26]. Thus, the absence of an

isotope effect with D-[6-^{14}C]glucose (Fig. 2) is expected. The isotope effect observed with D-[U-^{14}C]glucose (Fig. 3) is probably a result of the effect of the six ^{14}C atoms in each glucose molecule (the D-[U-^{14}C]glucose used as 100% isotopically substituted). This relatively large change in molecular weight may influence the elution pattern.

The large isotope-effect found with D-[6-^{3}H]glucose may be exploited to obtain isotopically pure, radiolabeled glucose. Approximately 48% of the tritium label and 16% of the nonlabeled glucose may be obtained by combining the fractions representing the left half of the radioactive peak. This constitutes a three-fold purification. Isotopic purification with decreased yield may be obtained by subsequent chromatography and fraction selections. It is calculated that multiple cycles through the same column with the effluent being pumped back into the resin, or use of a longer column, could give isotopically pure D-[6-^{3}H]glucose without decreased yield.

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REFERENCES

1 K. E. WILZBACH AND P. RIESZ, *Science*, 126 (1957) 748–749.
2 M. A. KIRSCHNER AND M. B. LIPSETT, *J. Lipid Res.*, 6 (1965) 7–9.
3 W. A. VAN HOOK AND M. E. KELLY, *Anal. Chem.*, 37 (1965) 508–511.
4 R. BENTLEY, N. C. SAHA, AND C. C. SWEELEY, *Anal. Chem.*, 37 (1965) 1118–1122.
5 J. W. ROOT, E. D. C. LEE, AND F. S. ROWLAND, *Science*, 143 (1964) 676–678.
6 E. V. JENSEN AND H. I. JACOBSON, *Recent Progr. Hormone*, 18 (1962) 387–414.
7 P. D. KLEIN, D. W. SIMBORG, AND P. A. SZCZEPANIK, *Pure Appl. Chem.*, 8 (1964) 357–370.
8 A. R. VAN DYKEN, *Abstr. Pap. Am. Chem. Soc. Meet.*, 128 (1955) 28.
9 D. S. SGOUTAS AND F. A. KUMMEROW, *J. Chromatogr.*, 16 (1964) 448–453.
10 V. CEJKA AND E. M. VENNEMAN, *Clin. Chim. Acta*, 11 (1965) 188–190.
11 P. L. GANT AND K. YANG, *J. Am. Chem. Soc.*, 86 (1964) 5063–5064.
12 I. W. TAYLOR AND H. G. UREY, *J. Chem. Phys.*, 6 (1938) 429–438.
13 K. BREWER, *J. Am. Chem. Soc.*, 61 (1957) 1597–1599.
14 K. A. PIEZ AND H. EAGLE, *Science*, 122 (1955) 968–969.
15 H. GOTTSCHLING AND E. FREESE, *Nature*, 196 (1962) 829–832.
16 C. R. SHOAF, M. CAPLOW, AND W. D. HEIZER, *Anal. Biochem.*, 96 (1979) 12–20.
17 E. F. WALBORG, JR., D. B. RAY, AND L. E. OHRBERG, *Anal. Biochem.*, 29 (1969) 433–440.
18 E. F. WALBORG, JR. AND L. CHRISTENSSON, *Anal. Biochem.*, 13 (1965) 186–193.
19 D. J. FINNEY, *Probit Analysis*, Cambridge University Press, London, 1952, pp. 20–30.
20 R. J. FERRIER, *Adv. Carbohydr. Chem. Biochem.*, 35 (1978) 31–80.
21 A. B. FOSTER AND M. STACEY, *J. Chem. Soc.*, (1955) 1778–1781.
22 A. B. FOSTER, *J. Chem. Soc.*, (1953) 982–986.
23 A. B. FOSTER AND M. STACEY, *J. Appl. Chem.*, 3 (1953) 19–21.
24 R. P. BELL AND W. B. T. MILLER, *Trans. Faraday Soc.*, 59 (1963) 1147–1148.
25 J. P. KLINMAN, *Adv. Enzymol.*, 46 (1978) 415–494.
26 H. SIMON AND D. PALM, *Angew. Chem. Int. Engl.*, 5 (1966) 920–933.