CHROM. 6270

Isotope effect in the glucose oxidase reaction as determined by ion-exchange chromatography*

When D-arabinose, its enantiomer, and D-lyxose[2] were labeled on carbon-1 with ^{14}C, their mobility during countercurrent distribution, compared with that of their unlabeled counterparts, was different. This isotope effect was a positional one because it was not observed when the radioactivity resided on carbon-5 of, at least, arabinose. A possible site of this effect was the anomeric equilibrium because labeled glycosides had the same mobility as their unlabeled partners but, on hydrolysis, the isotope effect for labeled arabinose and labeled mannose again was observed[3]. These experiments stimulated our interest in the possibility that such isotope effects with ^{14}C might be discernible in an enzymatic reaction involving carbon-1 of a sugar. We selected glucose oxidase because of its known specificity for the beta-anomer of glucose and, thereby, the observations might provide insight into the influence of any effect on the anomeric equilibrium; besides, the substrate and product of this enzymatic reaction are conveniently separated by ion-exchange chromatography.

Experimental

Materials. Glucose oxidase was purchased from Schwarz BioResearch, Inc. [1-^{14}C] D-glucose (Lot No. 86-293-110 and Lot No. 86-293-109), [6-^{14}C] D-glucose (Lot No. 292-61-2), and [6-^{3}H] D-glucose (Lot No. 24-177) were purchased from New England Nuclear.

Procedure. Glucose oxidase was incubated with labeled glucose as indicated. A 0.5–1-ml aliquot of the final mixture was chromatographed on 17 × 1.8-cm columns containing a 3-cm height of Dowex 1 X8, 100–200 mesh, onto which was added 10 cm of Dowex 1 X8, 200–400 mesh. Glucose emerged in the effluent after the addition of 140 ml of water. Gluconic acid was eluted with an exponential 0.5 *N* formic acid gradient.

Samples were counted on a 6801 Automatic Scintillation Counter (Nuclear, Chicago) the windows of which were set so that tritium was counted with an efficiency of 26% in channel one while ^{14}C was measured with an efficiency of 40% in channel two. When an attempt was made to ascertain whether elaborated gluconic acid caused chemical quenching of radioactive glucose, progressively increasing amounts of the acid added to the doubly labeled sugar caused no change in channel one to channel two ratios. Glucose, after reaction with *ortho*-aminobiphenyl, was measured by the procedure of TIMELL *et al.*[5] on 1-ml aliquots of the 7-ml chromatographic fractions.

Results and discussion

The data from the incubation of carbon-1 labeled hexose for 0 and 10 min appear in Fig. 1 while the corresponding data for hexose labeled in the 6-positions appear in Fig. 2. The difference between the labeling of the residual glucose for the

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1-labeled and 6-labeled residual glucose indicate a positional isotope effect. At 20 min (Fig. 3) no difference appears. That the difference is greater at some time less than 10 min is evident from Fig. 4, where incubation at 1 min gives a greater difference between the areas for glucose than the incubation at 10 min. When the specific activity of the glucose remaining after incubation was compared to that of the original glucose, the observations (Table I) supported those adduced from the ^{14}C tritium data.

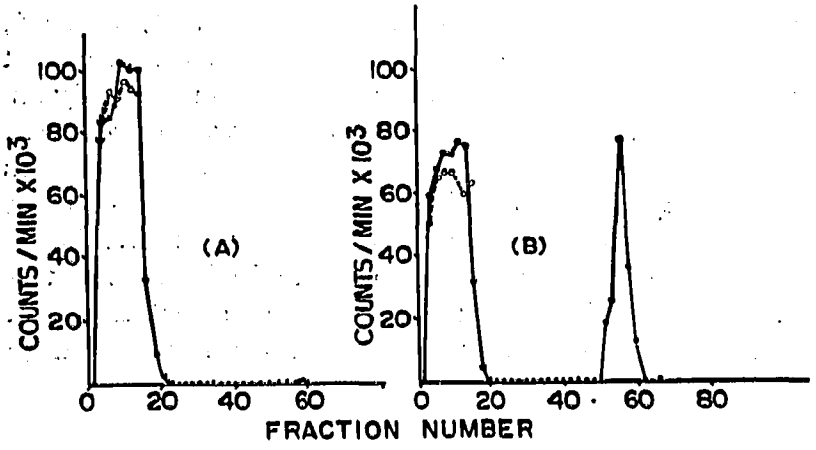

Fig. 1. Chromatographic separation of labeled glucose and gluconic acid at (A) 0 min and (B) 10 min. In a volume of 5 ml, 10 mg of glucose containing 12 μCi of 1[-^{14}C]glucose and 20 μCi of [6-^{3}H]glucose were incubated at 25° with 20 mg of glucose oxidase (200 units/mg) in 0.1 *M* acetate buffer pH 5.5. Counts of the peak fraction of the curve for gluconic acid, when the index was the measurement of tritium in channel one, were set to coincide with the counts for the peak fraction, when the index was the measurement of ^{14}C in channel two, and the values for glucose corrected correspondingly. ○, observed counts channel one; and ●, observed counts channel two.

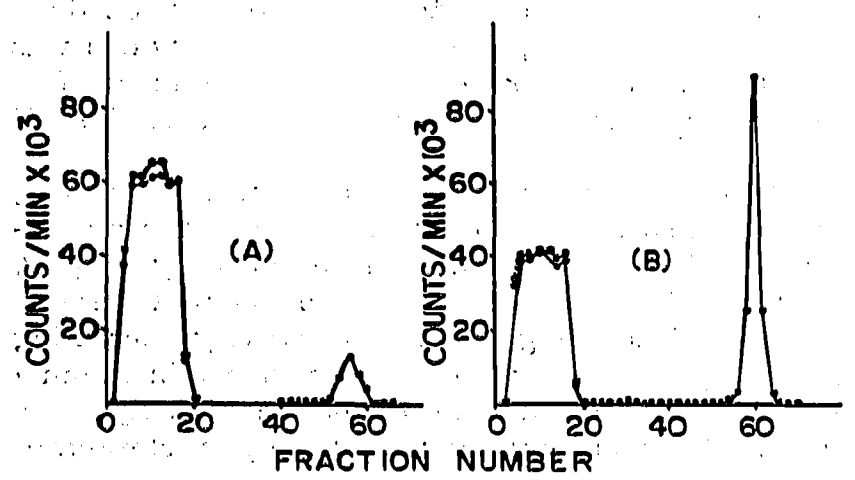

Fig. 2. Chromatographic separation of labeled glucose and gluconic acid at (A) 0 min and (B) 10 min. Conditions were the same as the ones for the results in Fig. 1 except that glucose contained [6-^{14}C]glucose and [6-^{3}H]glucose. ○, Observed counts channel one; and ●, observed counts channel two.

The results support a positional isotope effect in early times of incubation when glucose labeled on carbon-1 is converted to gluconic acid in the reaction catalyzed by glucose oxidase. The absence of the effect at 20 min, as evident from the

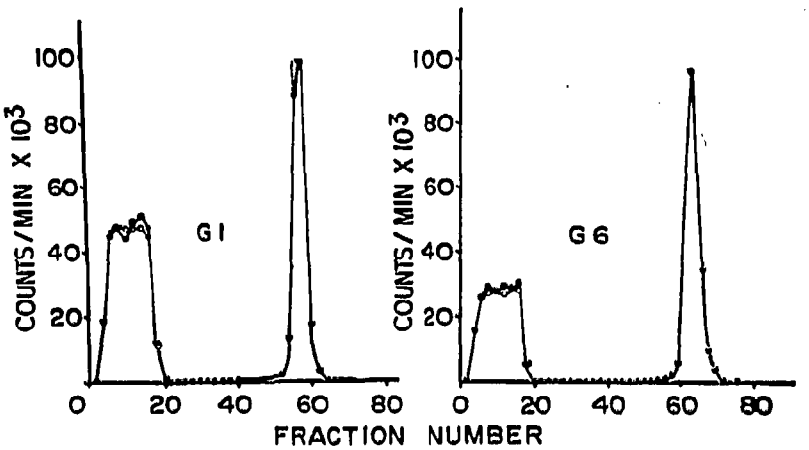

Fig. 3. Chromatographic separation of labeled glucose and gluconic acid at 20 min. Conditions were the same as those for Fig. 1 with the exception that the amount of radioactivity and tritium in the incubation of [1-^{14}C]glucose was increased. (G 1) Glucose containing 20 μCi [1-^{14}C]glucose and 112 μCi [6-^{3}H]glucose; (G 6) [6-^{14}C]glucose and 20 μCi [6-^{3}H]glucose. ○, Observed counts channel one; and ●, observed counts channel two, adjusted as in Fig. 1.

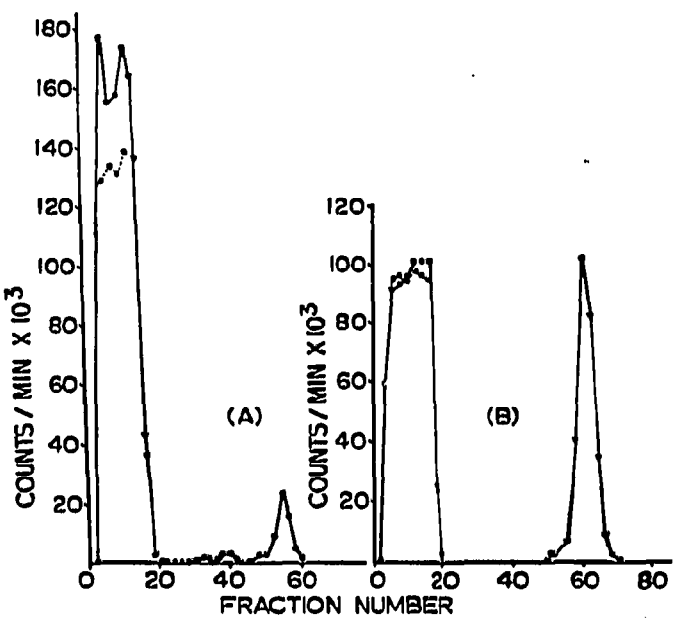

Fig. 4. Chromatographic separation of labeled [1-^{14}C]glucose and labeled gluconic acid at (A) 1 min and (B) 10 min. Conditions were the same as those for the results in Fig. 1 except that the quantity of tritium and ^{14}C-labeled glucose was approximately doubled. ○, Observed counts channel one; and ●, observed counts channel two.

similarity of the chromatographic areas for glucose (Fig. 3), would seem to rule out the possibility that radiochemical impurity could account for the data. Although, in so far as we know, this report is the first mention of a positional isotope effect

TABLE I

SPECIFIC ACTIVITIES OF LABELED GLUCOSE AFTER INCUBATION WITH GLUCOSE OXIDASE

Position of label	*Chromatographic fractions sampled*	*Time of incubation (min)*	*Counts/min/O.D. unit*[a]
C 1	4–16	0	4.13[b] ± 0.116
C 1	4–16	10	5.19[b] ± 0.197
C 6	6–16	0	3.80[c] ± 0.106
C 6	4–16	10	3.73[c] ± 0.065
C 1	4–16	1	9.01[d] ± 0.297
C 1	4–16	10	7.79[d] ± 0.226

[a] Each value, counts/min/O.D. unit, when multiplied by 10^4 is the mean of specific activities of 1-ml aliquots of designated fractions, expressed as counts/min divided by the optical density at 380 mμ of the *o*-aminobiphenyl derivative.

[b] 4.13 is significantly less than ($P < 0.01$) 5.19.

[c] 3.80 and 3.73 are not significantly different.

[d] 9.01 is significantly greater than 7.79 ($P < 0.01$).

involving an enzyme reaction that catalyzes a change at carbon-1 of a hexose, a ^{14}C-isotope effect in a biological system incorporating $^{14}CO_2$ has been previously reported[6]. If there is a difference between the electronegativity of ^{14}C and ^{12}C as PIEZ AND EAGLE[7] suggested, it would be reasonable to expect that the isotope effect could subtly influence the conformation of the sugar. Any effect on conformation thereby, would be expected to influence the velocity of the reaction because glucose oxidase, in spite of its apparent broad specificity, reacts with related substrates at vastly different rates. For example, the enzyme catalyzes the oxidation of D-xylose and D-allose at 10^{-2} and 2×10^{-4} times that of D-glucose. The isotope effect could reside in the anomeric equilibrium but the same rationalization concerning differences in electronegativity would be applicable because the enzyme catalyzes largely the oxidation of the beta-anomer only.

Setting the peaks of the curves of the chromatographic plots for the product to the same value and adjusting correspondingly the values for the substrate in such doubly labeled experiments (Figs. 1–4) can discerningly reveal isotope effects, should they occur in an enzymatic reaction. Essentially, the areas shown for glucose are a summation of the change in the area (an equivalent of the change in specific activity) of the curve for product and the change in the area of the curve for substrate, since a higher carbon/tritium ratio for residual glucose means that this ratio for the product would have been lower in phosphogluconic acid. The magnitude of the change in the specific activity of glucose alone can be found in Table I. The chromatographic plots, however, better reveal the isotope effects.

Summing up, following the rate of reaction by measuring tritium/^{14}C ratios as well as the specific activity of unreacted glucose, D-glucose was oxidized more rapidly than [1-^{14}C] D-glucose in the glucose oxidase reaction. [6-^{14}C] D-glucose was oxidized at

the same rate as unlabeled sugar. The chromatographic data are presented in a manner that shows, as a summation, the change in specific activity of substrate and product.

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