

# Studies on the Mechanism of the Interconversion of D-Glucose, D-Mannose, and D-Fructose in Acid Solution<sup>1</sup>

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**Abstract:** Hydrogen transfer reactions, apparently intramolecular, were found to occur during the acid-catalyzed isomerization of aldoses to ketoses and ketoses to aldoses. D-Glucose-2-<sup>3</sup>H, in acid, gave D-fructose-1-<sup>3</sup>H with the tritium nearly evenly distributed between the pro-1-*R* and pro-1-*S* positions (by enzymic assay). (1*R*)-D-Fructose-1-<sup>3</sup>H in acid gave both D-glucose-<sup>3</sup>H and D-mannose-<sup>3</sup>H with specific radioactivities 18 and 85% of the starting compound, respectively. The tritium in the glucose (by enzymic analysis) was at C-1 (16%) and at C-2 (79%); in the mannose (by both chemical and enzymic analysis) it was at C-1 (11%), at C-2 (66%), and elsewhere (23%, by difference). The data are consistent with a mechanism involving, in part, a 1 → 2 hydride shift for the isomerization and are thus quite different from the reaction in base where no transfer reactions are observed and substantial solvent isotope exchange occurs. Except for the random stereochemistry of the reaction, it is similar to enzymatic transformations catalyzed by isomerases.

The interconversion of 2-ketoses and the appropriate C-2 epimeric aldoses constitutes well-known reactions in the carbohydrate series which are subject to general acid-base catalysis in chemical systems<sup>2</sup> (the Lobry de Bruyn-Alberda van Eckenstein transformation) and enzymatic catalysis in biological systems.<sup>3</sup> The mechanism for the transformation in basic solution has been extensively studied, largely by use of isotope-exchange techniques, and it has been concluded that the reaction probably involves a common 1,2-enediol intermediate which gives rise to an aldose by protonation at C-2 and the ketose by protonation at C-1. The observed labeling patterns on sugars produced, which have been observed to contain carbon-bound deuterium<sup>4</sup> when the reactions were performed in alkaline deuterium oxide solution, are consistent with such a mechanism.

In biological systems, a number of similar reactions catalyzed by isomerase enzymes occur which are quite different from the base-catalyzed reactions, in that the reactions are much more stereospecific; only one aldose is produced from a ketose,<sup>5</sup> only one of the two possible hydrogens at C-1 of the ketose appears to be acted upon,<sup>5,6</sup> only one anomer appears to react,<sup>7,8</sup> and the transformation involves the intramolecular transfer of hydrogen from C-2 of the aldose to C-1 of the ketose and *vice versa*. For a number of enzymes such as D-xylose isomerase and L-arabinose isomerase<sup>7,8</sup> the intramolecular hydrogen transfer is complete; that is, solvent exchange never appears to occur during the reaction and for others, such as phosphoglucose isomerase and phosphomannose isomerase, a combination of intramolecular transfer and solvent proton exchange occurs.<sup>9</sup> In spite of the striking differences observed with respect to stereochemistry and isotope exchange data between base and enzyme catalysis, the enzyme mechanisms are nevertheless currently discussed in terms of 1,2-enediol intermediates,<sup>3,10</sup> as are proposed for the base-catalyzed reaction, even though some very high energy ring forms of sugars<sup>3</sup> must be postulated as substrates in order to provide a plausible explanation for the mechanism.

Although similar transformations appear to proceed in acid media, as evidenced by Ward and Ohno's<sup>11</sup> isolation of D-fructose from D-glucose, until recently no mechanism studies have been performed on the transformation in acid solution.

In a recent study in this laboratory concerning some dehydration reactions, it was found that, during the conversion of D-glucose to 2-(2-hydroxyacetyl)furan, a C-2 → C-1 intramolecular transfer of hydrogen occurs.<sup>12</sup> The transfer was found to be complete in strong acid and involved an iso-

tope effect ( $K_h/K_t$ ) of 4.3. The exact step for the transfer was found to be in the conversion of D-glucose to D-fructose.<sup>13</sup> This information suggests that, in acid solution, sugars are interconverted and the products are similar to those expected during a Lobry de Bruyn-Alberda van Eckenstein transformation, but the mechanism may be more similar to that which prevails in enzyme reactions.

In this work, the importance of such transfer reactions was further investigated using isotope tracer techniques. The studies involved the conversion of D-fructose-1-<sup>3</sup>H into D-glucose-<sup>3</sup>H and D-mannose-<sup>3</sup>H followed by measurements of the distribution of tritium on the aldose molecules. In addition, D-fructose-1-<sup>3</sup>H was prepared from D-glucose-2-<sup>3</sup>H by treatment with acid and the stereochemistry of the labeling at C-1 of the ketose was determined. The purpose of the present study was to determine the extent of the analogy between the acid-catalyzed process and the enzyme reactions.

## Results

For studies on the conversion of D-fructose to D-mannose and D-glucose, a sample of commercially obtainable D-fructose-1-<sup>3</sup>H was used. This sugar was labeled using the phosphoglucose isomerase reaction<sup>9</sup> and thus was stereospecifically labeled in the pro-*R* position<sup>14</sup> at C-1. The D-fructose-1-<sup>3</sup>H was treated with acid and the resulting D-mannose-<sup>3</sup>H and D-glucose-<sup>3</sup>H were isolated in pure form. The chromatographically pure D-glucose-<sup>3</sup>H contained 18.4% the specific activity of the starting D-fructose-1-<sup>3</sup>H and the D-mannose contained 84.6% the activity of the D-fructose-1-<sup>3</sup>H.

To determine the distribution of tritium on the D-glucose-<sup>3</sup>H, it was allowed to react with D-glucose oxidase,<sup>15</sup> a reaction which quantitatively converts β-D-glucopyranose into D-glucono-δ-lactone, thus removing all C-1 bound tritium and hydrogen atoms from the molecule. The tritium released was measured by determining the radiochemical activity of the water which contained 16% the activity of the starting D-glucose-<sup>3</sup>H, indicating the per cent of total activity at C-1. To determine the amount of tritium at C-2, a further sample of D-glucose-<sup>3</sup>H was quantitatively converted to D-glucose-<sup>3</sup>H 6-phosphate using hexokinase and ATP and the resulting product equilibrated with phosphoglucose isomerase. This process releases all the tritium and hydrogen atoms at C-2 into the solvent<sup>9</sup> at the conditions employed. The activity of the water showed that 79% of the activity was located at C-2, thus showing that 95% of the total activity of the original D-glucose-<sup>3</sup>H was located at C-1 and

C-2.

To examine the distribution of tritium on D-mannose-<sup>3</sup>H, several procedures were used. Thus, the D-mannose-<sup>3</sup>H was converted to the crystalline D-manno benzimidazole derivative,<sup>16</sup> a reaction which removes the tritium and hydrogen atoms from C-1. This derivative contained 89% the activity of the original sugar, indicating that 11% of the total activity was located at C-1. Periodate oxidation of this derivative to give 2-formylbenzimidazole, a derivative which contains only the C-2 bound hydrogen and tritium atoms,<sup>17</sup> showed that this compound contained 66% the activity of the starting sugar. Thus 77% of the total activity can be accounted for being distributed 11% at C-1 and 66% at C-2.

In order to further verify the latter measurement, a sample of D-mannose-<sup>3</sup>H was converted to D-mannose-<sup>3</sup>H 6-phosphate with hexokinase and ATP and then allowed to react with phosphomannose isomerase, an enzyme which ultimately exchanges all the hydrogen and tritium at C-2 with solvent. A determination of the radioactivity of the water, at equilibrium, indicated, again, that 66% of the total isotope on the sugar molecule was located at C-2.

A prior study<sup>13</sup> has shown that the D-fructose-<sup>3</sup>H derived from D-glucose-2-<sup>3</sup>H is exclusively labeled at C-1, but the stereochemistry of the labeling at this carbon was not investigated. Thus, in this work D-glucose-2-<sup>3</sup>H was converted to D-fructose-1-<sup>3</sup>H at conditions where the C-2 → C-1 intramolecular hydrogen transfer is quantitative, and the resulting D-fructose-1-<sup>3</sup>H was isolated in chromatographically pure form. The resulting material was converted to D-fructose-1-<sup>3</sup>H 6-phosphate using hexokinase and ATP and was then treated with phosphoglucose isomerase in order to exchange the tritium and hydrogen atoms located at the pro-*R* position of C-1 with solvent. The results showed that 48% of the isotope at C-1 was released into the solvent.

## Discussion

The data obtained show that D-fructose, on acid treatment, gives rise to detectable amounts of both D-glucose and D-mannose and that D-glucose, on acid treatment, produces both D-fructose and D-mannose. The reaction is thus consistent with products expected during a Lobry de Bruyn-Alberda van Eckenstein transformation at conditions of acid catalysis.

The labeling results show that C-1 → C-2 intramolecular hydrogen transfer reactions appear to be an important feature of the isomerization of ketoses to aldoses and that C-2 → C-1 intramolecular transfers are also important for the reverse reaction. The data are most consistent with a portion of the reaction pathway involving a hydride shift mechanism where the function of the acid catalyst is to protonate the carbonyl oxygen atom of the sugars to facilitate the shift. Such a mechanism, which involves a low-energy, symmetrical intermediate, would be consistent with the relatively small tritium isotope effect measured for the conversion of aldose to ketose. It is noteworthy that the only other report of an intramolecular hydrogen shift reaction in the carbohydrate series is found in Gleason and Barker's<sup>18</sup> report of a partial transfer during the interconversion of D-ribose-2-<sup>3</sup>H and D-arabinose in alkaline solution. These authors have also observed a relatively low isotope effect for the transfer reaction. Also noteworthy is the fact that these authors found that label was retained within the sugars in the alkaline system at positions other than C-1 or C-2 as is reported here in the case of D-mannose-<sup>3</sup>H derived from D-fructose-1-<sup>3</sup>H.

Also noteworthy is the fact that Isbell has recently reported<sup>19</sup> that when D-glucose-2-<sup>3</sup>H is converted to D-fructose in alkali, the latter is not radioactive, thus indicating that C-2 → C-1 transfers do not appear operative in this

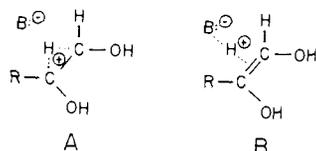
system.

Experimentally verifiable explanations are not available for the difference observed in the specific activities of the D-glucose-<sup>3</sup>H and D-mannose-<sup>3</sup>H obtained from the same sample of D-fructose-1-<sup>3</sup>H, for the unequal distribution of label between C-1 and C-2 of the aldoses, or for the fact that the D-mannose does not contain all the tritium at C-1 and C-2. With respect to the latter case, it does not seem likely that the distribution measurements on D-mannose-<sup>3</sup>H are an anomaly or due to a contaminant, since 66% of the activity was found at C-2 using two independent methods, one chemical and the other enzymatic. The reaction mixtures obtained are exceedingly complex and, when the ketose to aldose transformations were made, advantage was taken of the fact that D-fructose undergoes acid-catalyzed dehydration and subsequent degradation at a much faster rate than D-mannose or D-glucose, and, as a result of this, a large portion of the sugars was destroyed during the reaction. The complex reaction mixture contained, aside from the three sugars, a number of degradation products such as "Humic materials," furans such as 5-(hydroxymethyl)-2-furaldehyde and 2-(hydroxyacetyl)furan, levulinic acid (as evidenced by thin-layer chromatography), and a number of unidentified compounds, all of which could arise from any of the three sugars. It is apparent that the complexity of the reaction precludes complete kinetic knowledge of all aspects, but isotope effects involved in side degradative reactions may explain some of the data. For example, a side degradation reaction involving the breaking of the C-2 bound hydrogen (or tritium) atom as an important step would permit the protiated material to react much faster than the tritiated compound, thus leaving unreacted aldose-<sup>3</sup>H having a substantially greater amount of 2-<sup>3</sup>H than 1-<sup>3</sup>H. Since it is also known that D-glucose and D-mannose undergo dehydration and degradation at different rates, similar arguments can be used to explain differences in specific activities observed in these two sugars which arose from the same source. For the above reasons, the data obtained should be considered as qualitative in nature but, nevertheless, serve to demonstrate that intramolecular hydrogen transfer is a feature of acid-catalyzed aldose-ketose isomerizations.

The stereochemistry of the acid-catalyzed reaction appears to be, in all respects, of a random nature since D-fructose, stereospecifically labeled at C-1, gave rise to D-glucose and D-mannose, both of which contained substantial carbon-bound isotope at C-2. Likewise, the D-fructose-1-<sup>3</sup>H obtained from D-glucose-2-<sup>3</sup>H was randomly labeled as evidenced by the fact that only 48% of the activity at C-1 was located at the pro-*R* position. If stereospecific labeling had occurred the enzyme assay used would have released either 0 or 100% of the label. The random stereochemical features of the reaction indicate that the transformation in acid solution probably does not proceed *via* a cyclic form of the sugar, since these forms, with stereochemically fixed substituents, would be expected to give rise to products which are labeled in a more stereochemically precise manner. The data are more consistent with an acyclic form as the reactive species and further point to a heretofore undiscovered mechanism operative for this type of interconversion.

Details of the mechanism of isomerization as catalyzed by either acid, base, or enzymes are not yet known. Enzyme mechanisms for isomerization reactions are usually discussed in terms of base catalysis,<sup>3</sup> presumably because of the large body of information available on such transformations in alkaline solution, and the similarities of isotope incorporation observed. Hydride shifts have usually not been considered as important in such mechanisms. The kinetics of incomplete transfer are usually taken to be due to an ex-

change of the abstracted proton with water while it is associated with a basic group on the enzyme. In support of this theory evidence has been accumulated to show that a carboxylate anion<sup>20</sup> (in the case of triosephosphate isomerase) and an imidazole group<sup>21</sup> (in the case of phosphoglucose isomerase) are required for activity. The stereochemistry of labeling observed has led to the further suggestion that the intermediate enediol is of the *cis* configuration, and the finding that glycidol phosphate (1,2-epoxypropanol 3-phosphate),<sup>22</sup> a compound having a planar configuration similar to a *cis*-1,2-enediol, inactivates both triosephosphate isomerase and enolase has been given as supportive evidence for the mechanism. It is noteworthy that these same facts are consistent with the transition state structure for a hydride shift mechanism shown in A or for a concerted four mechanism intermediate as shown in B in which the abstracted



proton remains associated with the developing  $\pi$ -electron cloud and is stabilized by a negative counterion. In both cases the oxygen atoms on C-1 and C-2 would be *cis*, a negatively charged counterion would stabilize the transition state, and the intermediate would have a planar configuration, similar to a *cis*-1,2-enediol.

### Experimental Section

**Materials and Methods.** Ultraviolet spectra were obtained with a recording Coleman Model 124 double beam grating spectrophotometer. Radiochemical determinations were performed with a Packard Model 3003 Tri-Carb liquid scintillation counter using 15 ml of Brays scintillation cocktail. Efficiencies were determined using a toluene-<sup>3</sup>H internal standard. Paper chromatograms were developed by the descending method using Whatman No. 1 paper for analytical purposes and Whatman 3 mm paper for preparative work. In most cases, the developing solution consisted of ethyl acetate, acetic acid, formic acid, and water (18:3:1:4). Sugars were visualized using aniline hydrogen phthalate<sup>23</sup> followed by heating at 110° for 5 min. Quantitative estimation of sugars was performed using the Somogyi alkaline copper method<sup>24</sup> and the Nelson arsenomolybdate chromogenic reagent.<sup>25</sup> Standard curves were prepared using reagent grade sugars. D-Fructose-1-<sup>3</sup>H was purchased from New England Nuclear where it was prepared by an enzymatic method using phosphoglucose isomerase. This material was examined for radiochemical purity by examining paper chromatograms, developed in the above irrigant as well as 1-butanol-ethanol-water (104:66:30) and 1-butanol-pyridine-water (1:1:1), using a Packard paper radioscaner. In all three cases, no contamination by either D-glucose or D-mannose was detected, and 99% of the total activity was located at the zone corresponding to D-fructose. D-Glucose-1-<sup>3</sup>H was purchased from Amersham-Searle, Inc., where it was prepared by reduction of methyl D-*arabino*-hexopyranosid-2-uloside with NaB<sup>3</sup>H<sub>4</sub> followed by hydrolysis and separation of the D-glucose-2-<sup>3</sup>H from the D-mannose-2-<sup>3</sup>H by paper chromatography. An assay of this material, using the same procedures as for D-fructose showed no contamination by either D-fructose or D-mannose and indicated that 96% of the activity was located at the D-glucose zone with the remainder of the activity being evenly distributed on the paper.

**Conversion of D-Fructose-1-<sup>3</sup>H to D-Glucose-<sup>3</sup>H and D-Mannose-<sup>3</sup>H.** D-Fructose-1-<sup>3</sup>H (1 mCi) was diluted to 25 g with inert D-fructose, placed in 500 ml of 2 N sulfuric acid, and refluxed 16 hr. The resulting mixture, which contained suspended insoluble material and accompanying degradation products, was neutralized with barium carbonate and filtered, and the filtrate was concentrated to a syrup. The syrup, which amounted to approximately 1 g, was streaked on four Whatman 3 mm papers 9 in. wide and was developed for 18 hr. The bands corresponding to D-glucose and D-mannose were located on thin strips cut from both sides of each

chromatogram using aniline hydrogen phthalate for visualization. The region containing each sugar was marked and cut from each of the four chromatograms and eluted from the paper with methanol. Both solutions were separately rechromatographed using the same procedure. These eluates were evaporated to dryness, taken up in 1 ml of water, and qualitatively examined by paper chromatography. No contamination by D-fructose or the corresponding 2-epimer was observed in either case. Each aldose was made up to a volume of 10 ml and 0.2 ml was taken for quantitative estimation of sugar content. Radiochemical analysis was performed by counting 0.5 ml of each sugar solution. In the case of D-glucose-<sup>3</sup>H, 0.5 ml of solution contained 1.79 mg of sugar having a specific radiochemical activity of 1.32  $\mu$ Ci/mmol. For the D-mannose-<sup>3</sup>H, 0.5 ml of solution contained 0.55 mg of sugar having a specific radiochemical activity of 6.08  $\mu$ Ci/mmol. To the remaining sugar solution was added 5 g of the respective inert sugar and each sample was recrystallized from water-methanol to constant radiochemical activity. In each case three recrystallizations were done; 4.1 g of D-glucose-2-<sup>3</sup>H was obtained having a specific activity of  $9.08 \times 10^{-3}$   $\mu$ Ci/mmol and 3.8 g of D-mannose-2-<sup>3</sup>H was obtained having a specific activity of  $1.01 \times 10^{-2}$   $\mu$ Ci/mmol. These crystalline sugars, after drying *in vacuo* over anhydrous calcium chloride, were used for determining the distribution of tritium labeling.

**Determination of Tritium Label on C-1 of D-Glucose-<sup>3</sup>H Derived from D-Fructose-1-<sup>3</sup>H.** To 6.0 ml of water was added 180 mg (1 mmol) of D-glucose-<sup>3</sup>H ( $9.08 \times 10^{-3}$   $\mu$ Ci/mmol), 930 mg (1.5 mmol) of adenosine 5'-triphosphate disodium salt (ATP), and 9 mg of magnesium chloride. The pH was adjusted to 7.3 with solid tris(hydroxymethyl)aminomethane (Tris) and 1000 units of hexokinase (Sigma Type F-300) was added. The solution was allowed to stand at 25° for 1 hr at which time paper chromatograms showed that all of the D-glucose was converted to the 6-phosphate ester. Following this, 1000 units (2 mg) of phosphoglucose isomerase (Sigma Type III) was added. Samples of 1 ml were removed at timed intervals and placed in a test tube containing 2 drops of concentrated sulfuric acid. Each aliquot was distilled at 40° under reduced pressure and 0.5-ml samples of the distillate were used for radiochemical analysis. After 2.3 hr of reaction the radiochemical activity of the water reached a constant value of  $2.16 \times 10^{-5}$   $\mu$ Ci/mmol.

**Determination of the Position of Tritium Label on D-Mannose-<sup>3</sup>H by Conversion to the Benzimidazole Derivative.** The procedure outlined by Moore and Link<sup>16</sup> was used. To a 250-ml three-necked flask equipped with a thermometer and addition funnel were added 1 g of D-mannose-<sup>3</sup>H (specific activity of  $1.01 \times 10^{-2}$   $\mu$ Ci/mmol), 2 ml of water, 13 ml of methanol, 2.5 g of barium iodide, and 2.8 g of iodine in 40 ml of methanol. The solution was warmed to 40° and the heating mantle removed. Over a period of 15 min, 22 ml of 4% methanolic potassium hydroxide was added. The solution was stirred 19 min and cooled, and the precipitated barium D-mannonic acid was filtered and dried in a vacuum desiccator over calcium chloride. The barium salt (2 g) was then suspended in 10 ml of water and the solution neutralized with dilute hydrochloric acid. To this solution was added 0.8 ml of concentrated hydrochloric acid. To precipitate the barium ions, 0.5 ml of 1:1 concentrated sulfuric acid-water was added. After centrifugation the filtrate was concentrated to a volume of 4 ml and 0.7 g of *o*-phenylenediamine and 0.5 ml of 85% phosphoric acid were added. After boiling for 2 hr at 135° the solution was decolorized with charcoal and filtered. The filtrate was made alkaline with ammonium hydroxide and the benzimidazole precipitated. The product which had mp 229° (lit. mp 228°) was recrystallized from ethanol and water to a constant specific activity ( $8.96 \times 10^{-3}$   $\mu$ Ci/mmol). The resulting benzimidazole was converted, by periodate oxidation,<sup>17</sup> to 2-formylbenzimidazole, which had mp 242° and a specific activity of  $6.67 \times 10^{-3}$   $\mu$ Ci/mmol.

**Determination of the Amount of Tritium at C-2 of D-Mannose by Enzymatic Techniques.** To 6 ml of water were added 90 mg of D-mannose-<sup>3</sup>H ( $1.01 \times 10^{-2}$   $\mu$ Ci/mmol), 465 mg (0.75 mmol) of adenosine 5'-triphosphate disodium salt (ATP), and 4.5 mg of magnesium chloride. The pH was adjusted to 7.6 with solid tris(hydroxymethyl)aminomethane (Tris) and 500 units of hexokinase (Sigma Type F-300) was added. The solution was allowed to stand at 25° for 1 hr. After this time had elapsed, 375 units of phosphomannose isomerase (Sigma) was added and 1-ml samples were removed at timed intervals and placed in a test tube contain-

ing 2 drops of concentrated sulfuric acid. Each sample was distilled at 40° *in vacuo* and 0.6-ml aliquots of the distillate were taken for radiochemical analysis. The maximum radiochemical activity obtained was  $9.89 \times 10^{-4}$   $\mu\text{Ci}/\text{mmol}$  which corresponds to the release of 66% of the tritium label on D-mannose- $^3\text{H}$ .

**Conversion of D-Glucose-2- $^3\text{H}$  to D-Fructose-1- $^3\text{H}$  in 2 N Sulfuric Acid Solution.** Into a 4-l. erlenmeyer flask were added 100 g of D-glucose-2- $^3\text{H}$  (1.5 mCi) and 3500 ml of 2 N sulfuric acid solution. The solution was autoclaved at 15 lb/in.<sup>2</sup> for 10 hr. It was then neutralized with barium carbonate and filtered, and the resulting solution was concentrated to a syrup. The syrup was seeded with crystals of  $\alpha$ -D-glucose and allowed to stand at room temperature overnight. After removal of the crystals by filtration, the resulting syrup was again concentrated and seeded. This process was repeated until no more D-glucose would crystallize, leaving a solution containing D-glucose, D-mannose, and D-fructose as evidenced by paper chromatography. The remaining D-glucose was removed by treatment with glucose oxidase as described by Ohno and Ward,<sup>11</sup> followed by treatment with Dowex-50 (hydrogen form) and Dowex-1 (carbonate form) to remove contaminating ions and proteins. The resulting solution was concentrated and then streaked on Whatman 3 mm paper and chromatographed. The region on the paper corresponding to D-fructose, as evidenced by aniline hydrogen phthalate visualization of strips cut from either side of each paper, was excised. The D-fructose was eluted from the paper with methanol and concentrated and the chromatographic procedure repeated. To the resulting chromatographically pure D-fructose-1- $^3\text{H}$  was added 100 mg of inert D-fructose. The solution was then seeded and allowed to crystallize. The crystalline D-fructose-1- $^3\text{H}$  was dried over calcium chloride in a vacuum desiccator and used for determining the stereochemistry of labeling at C-1.

**Determination of Stereochemistry of Tritium Labeling at C-1 of D-Fructose Produced from D-Glucose-2- $^3\text{H}$ .** Into a test tube was placed about 10 mg of D-fructose-1- $^3\text{H}$  to be assayed. To this, 5.0 ml of water was added followed by the addition of 1.0 ml of a solution containing 3 mg of magnesium chloride. To this solution was added 300 mg of adenosine 5'-triphosphate disodium salt (ATP) and the pH adjusted to 7.3 with about 76 mg of tris(hydroxymethyl)aminomethane (Tris). About 1400 units (3 mg) of hexokinase (Sigma Type F-300) was added and the solution allowed to stand at 25° for 1 hr. Following this reaction, 0.5 ml of the solution was removed, counted, and found to contain 5700 dpm, indicating

that the original 10-mg sample of D-fructose-1- $^3\text{H}$  had a specific activity of  $5.55 \times 10^{-1}$   $\mu\text{Ci}/\text{mmol}$ .

To the remaining 5.5 ml of solution was added 0.5 ml of a solution containing 84 units (0.4 mg) of phosphoglucose isomerase (Sigma Grade III). At timed intervals 1-ml samples were removed and placed in labeled test tubes containing 2 drops of concentrated sulfuric acid. Samples were distilled at 40° under reduced pressure. The distillate was warmed to 25° and a 0.5-ml sample of each was taken for radiochemical determination. After a 20-min reaction time, the radiochemical activity of the water samples reached a constant value of  $4.21 \times 10^{-5}$   $\mu\text{Ci}/\text{mmol}$ .

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