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Determination of Carbon 14 in the Terminal Positions of Sugars: Preparation of D-Arabinose-5-C^{14} From D-Fructose-1,6-C^{14} (1, 2)

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Methods are described for the determination of carbon 14 in the terminal positions of reducing sugars. The sugar in alkaline solution is oxidized by means of molecular oxygen to the next lower aldonic acid, and the acid is separated in the form of a convenient derivative. The loss in radioactivity coincident with the removal of carbon 1 is a measure of the activity located at this carbon. The activity of the carbon at the nonreducing end of the molecule is determined by periodate oxidation of the aldonic acid, followed by formation and radioactivity assay of the dimedone compound of the resulting formaldehyde.

The method was applied to the labeled D-fructose prepared from D-mannitol-1-C^{14} by the action of *Acetobacter suboxidans*, and the D-arabonic acid formed from the D-fructose was isolated as potassium D-arabonate. It was found that all of the activity of the labeled D-fructose was located equally at carbons 1 and 6.

Potassium D-arabonate-5-C^{14} was also prepared from D-mannitol-1-C^{14} in 52-percent yield (26-percent radiochemical yield) without the intermediate separation of crystalline D-fructose-1,6-C^{14}; it was then converted to D-arabinose-5-C^{14} in an over-all radiochemical yield of 18 percent of the original D-mannitol-1-C^{14}.

1. Introduction

Previous reports from this laboratory [1, 2] [3] have described the preparation of 1-C^{14}-labeled mannose, the reduction of this to D-mannitol, and the oxidation of the D-mannitol by *A. suboxidans* to labeled D-fructose. As the two halves of the mannitol molecule are stereomerically alike, oxidation by *A. suboxidans* can take place either at carbon 2 or at carbon 5. If oxidation took place without either an isotope effect or breakdown of the D-mannitol and resynthesis of the fragments, the derived D-fructose would be labeled equally and exclusively at carbons 1 and 6. The present report presents evidence that this fructose is, within experimental error, labeled equally and exclusively at carbons 1 and 6, and describes convenient methods for determining carbon 14 at these positions.

2. Discussion of Experimental Work

Ordinarily, considerable difficulty is experienced in the purification of D-fructose. In the course of the work on the labeled sugar, a convenient procedure was developed for the crystallization of D-fructose from crude sirups. This consists of dehydration of the sugar sirup by azeotropic distillation with absolute ethanol, followed by dissolution in about two parts of methanol, and addition of a higher alcohol such as butanol or isoamyl alcohol to the point of incipient turbidity. After nucleation, the solution slowly deposits large crystals of D-fructose.

For assay of carbon 1 of D-fructose, the method chosen was an alkaline degradation of the sugar in the presence of molecular oxygen [3, 4] adapted to a semimicro scale and so conducted as to avoid contact of the sugar and alkali except under oxygen pressure. The resulting D-arabonic acid was conveniently separated as crystalline potassium D-arabonate, and the assay of carbon 1 of the labeled D-fructose was obtained fron the difference in activity of the sugar and the potassium salt. The method is convenient, and can be used for the assay of carbon 1 of any simple aldose or 2-ketose by choice of a suitable derivative for separation of the lower sugar acid. For the assay of carbon 6 of D-fructose, the potassium D-arabonate described above was oxidized with sodium periodate, and the formaldehyde, which was derived only from the terminal carbinol carbon, was isolated directly from the oxidation mixture by means of the dimedone compound. The procedure is somewhat similar to that used by Reeves [5] for the quantitative determination of the primary carbinol groups of sugars; it does not require distillation of formaldehyde prior to formation of the dimedone compound. The method is suitable for the assay of the terminal carbinol group of any aldonic acid or simple sugar. Although aldoses can be assayed directly by the periodate oxidation method described here, direct assay of ketoses is not practicable because they contain two primary carbinol groups. Thus assay of ketoses by alkaline oxidation to the lower aldonic acid, and periodate oxidation of this, is particularly advantageous.

The C^{14}-labeled D-fructose, assayed as described above, yielded potassium D-arabonate with a molecular loss of 50 percent of the activity. The formaldehyde-dimedone derivative obtained after periodate oxidation accounted for all of the activity of

[1] Part of a project on the development of methods for the synthesis of radioactive carbohydrates, sponsored by the Atomic Energy Commission.

[2] The term "D-fructose-1,6-C^{14}" covers all materials labeled in positions 1 and 6, regardless of the proportion of the labels. As the fructose used in this work was prepared from D-mannitol-1-C^{14}, positions 1 and 6 are not labeled in the same molecule.

[3] Figures in brackets indicate the literature references at the end of this paper.

the potassium D-arabonate. Thus the original D-fructose was labeled equally and exclusively in positions 1 and 6.

The methods for the assay of carbon 1 of D-fructose can be used without change for the assay of glucose and mannose. By alkaline degradation in the presence of oxygen, it was shown that all of the activity of a sample of D-glucose-1-C^{14} prepared by the cyanohydrin synthesis from D-arabinose and radioactive cyanide is, unquestionably, located at carbon 1.

The semimicro procedure developed for the preparation of potassium D-arabonate-1-C^{14} is useful, also, for the preparation of D-arabonic-5-C^{14} acid and D-arabinose-5-C^{14}. In the preparation of these substances it is unnecessary to separate crystalline D-fructose after oxidation of D-mannitol by *A. suboxidans*. The fermentation mixture, after deproteinization, deionization, and alkaline oxidation, gave potassium D-arabonate in 52.4-percent yield, based on the D-mannitol fermented. However, because one-half of the activity was lost in removal of carbon 1, the carbon 14 recovered as potassium D-arabonate was 26.2 percent. The potassium D-arabonate-5-C^{14} was converted to D-arabonic-5-C^{14} lactone and this was reduced to D-arabinose-5-C^{14} in 69-percent yield by the procedure developed for the preparation of D-arabinose-1-C^{14} [6]. Obviously, the 5-labeled D-arabinose provides a starting material for the preparation of D-glucose-6-C^{14} and D-mannose-6-C^{14}.

3. Experimental Details

3.1. Purification of D-Fructose

For purification, 180 mg of crude, labeled D-fructose was dissolved in a few drops of water, and the solution was filtered through a microfilter, containing some decolorizing carbon, into a 50-ml round-bottomed flask. The funnel was carefully washed, and the combined solution and washings, after the addition of a drop of acetic acid, were concentrated under reduced pressure to a thick syrup at a temperature less than 35° C. The sirup was dissolved in absolute ethanol, and the solution was transferred to a heavy-walled test tube and reconcentrated under reduced pressure. The residue was dissolved in methanol, the solution was again evaporated under reduced pressure, and the thick sirup was diluted with 5 or 6 drops of methanol. By cautious addition of a methanol-isoamyl alcohol mixture (1:1), and finally of isoamyl alcohol alone, the volume was increased to approximately 1 ml, and the solution was brought to incipient turbidity. It was then lightly seeded and allowed to stand at room temperature. From time to time additional isoamyl alcohol was cautiously added. At the end of a few days the mother liquor was removed, and the crystals were washed in the tube with ethanol and dried. To recrystallize, the D-fructose was dissolved in methanol, the solution was concentrated under reduced pressure, and methanol and isoamyl alcohol were added as before.

3.2. Assay of Carbon 1 of D-Fructose-1,6-C^{14}

A 75-mg sample of labeled D-fructose having an activity of 2.62 μc per millimole was dissolved in 2 ml of water. The solution was transferred and frozen to a portion of the wall of a 200-ml pressure flask, previously cooled in a dry ice-acetone mixture. On a separate portion of the wall, 2 ml of 2 *N* potassium hydroxide was frozen. The flask was immediately placed on a shaking machine,[4] evacuated, and filled with oxygen under 10 lb of pressure; it was then shaken for 24 hr at room temperature. By this procedure, the solutions of alkali and sugar do not come into contact except in the presence of excess oxygen, and the deleterious effect of alkali upon the sugar in the absence of oxygen is avoided. After 24 hr, 50 ml of methanol was added and the solution was lightly seeded with dust of crystalline potassium D-arabonate and allowed to stand for 1 day. The mother liquor was decanted from the crystals, which adhered to the walls of the flask, and the crystals were carefully washed with methanol. The potassium D-arabonate was recrystallized once in the flask by dissolving it in a minimum quantity of water and adding methanol to the point of incipient turbidity. From time to time additional methanol was added to effect more complete crystallization. After 1 day, the mother liquor was removed with a capillary pipette. The crystals were washed with methanol, and then dissolved in water. The solution was removed from the flask, filtered through a microfilter containing some decolorizing carbon, and concentrated to approximately 1 ml in a small test tube under a stream of dry air; methanol was then added to the point of incipient turbidity. After crystallization had taken place, the mother liquor was removed by means of a capillary pipette, and the product was washed with 50-percent aqueous methanol. The potassium D-arabonate was recrystallized several times from water, by the addition of methanol, to give a product with an activity of 1.32 μc per millimole. This value was unchanged by further recrystallization. Thus the loss in activity accompanying removal of carbon 1 was 49.6 percent.

It was later found that the potassium D-arabonate could be purified to constant activity with 2 or 3 recrystallizations, if the final crystallization was conducted slowly by allowing an aqueous solution in a small test tube to stand undisturbed in a desiccator containing anhydrous calcium sulfate and also a beaker of methanol. In the course of 1 or 2 days large pure crystals formed. The activity of these was unchanged by an additional recrystallization.

3.3. Assay of Carbon 6 of D-Fructose-1,6-C^{14} (Carbon 5 of D-Arabonic Acid)

For the assay of carbon 6 of the labeled D-fructose, potassium D-arabonate was prepared and purified as described above, and then treated in the following manner:

[4] A Parr reduction apparatus (shaking device equipped with a low-pressure gas tank).

A 20-mg quantity of the labeled potassium D-arabonate having an activity of 1.18 μc per millimole was treated with 1.5 ml of 0.3 *N* sodium metaperiodate, and the oxidation mixture, in a glass-stoppered tube, was allowed to stand for 3 hr at room temperature. Aqueous sodium bisulfite was added dropwise in an amount sufficient to cause removal of the iodine that first appeared. The solution was neutralized to the end point of methyl orange by the dropwise addition of aqueous sodium bicarbonate. To the reaction mixture was added 2.5 ml of a freshly prepared solution containing 35 mg of dimedone and 35 mg of sodium bicarbonate. After the solution was carefully neutralized with aqueous hydrochloric acid to the end point of methyl orange, a voluminous crystallization took place. The product was separated and washed with water.

For recrystallization, the material on the filter was dissolved in the minimum quantity of methanol; the solution was passed through the filter and diluted with an equal volume of water. After one recrystallization, the dimedone compound had an activity of 1.20 μc per millimole, which was unchanged by further recrystallization. Thus, within the limits of error, the activity of the dimedone compound accounted for all of the activity of the potassium D-arabonate. Inasmuch as formaldehyde is derived, during the periodate oxidation, only from carbon 5 of the D-arabonic acid (carbon 6 of the parent D-fructose), 50 percent of the activity of the labeled D-fructose was found at carbon 6.

3.4. Assay of Carbon 1 of D-Glucose-1-C^{14}

The alkaline oxidation previously described was also carried out on D-glucose-1-C^{14} having an activity of 3.46 μc per millimole. In this case the potassium D-arabonate obtained was expected to be inactive. The salt was recrystallized once in the pressure flask and once in a small test tube by addition of methanol to an aqueous solution. Finally, the material in aqueous solution was filtered, and allowed to crystallize slowly, as described in section 3.2. The product thus obtained showed negligible activity (0.001 μc per millimole). Hence the parent sugar was labeled exclusively at carbon 1.

3.5. Preparation of Potassium D-Arabonate-5-C^{14} from d-Mannitol-1-C^{14}

A sterile solution consisting of 2 millimoles of D-mannitol-1-C^{14} (having 150 μc of radioactivity), 100 mg of yeast extract, 60 mg of potassium dihydrogen phosphate, and 20 ml of water was incubated at 30° C with a freshly prepared culture of *A. suboxidans*. (The surface to volume ratio was 1.6 cm^2/ml.) After 45 hr, 2 ml of a 20-percent solution of zinc sulfate was added, and then an equivalent quantity of aqueous barium hydroxide. The resulting precipitate was separated by filtration through a funnel heavily coated with diatomaceous earth and decolorizing carbon. The filtrate was concentrated to a volume of about 5 ml, and then frozen on the wall of a 200-ml pressure flask. (The volume with washings was about 8 ml.) Eight milliliters of 2 *N* potassium hydroxide was frozen on another portion of the wall, and the oxidation was conducted as described in section 3.2. The resulting solution was diluted with 5 volumes of ethanol and allowed to stand 24 hr for the crystallization of potassium D-arabonate. After separation and recrystallization, the product weighed 214 mg (52.4-percent yield).

3.6. Preparation of D-Arabinose-5-C^{14}

One millimole (204 mg) of potassium D-arabonate-5-C^{14} was dissolved in 10 ml of water and passed through a column containing 10 ml of cation exchange resin;[5] this was followed by 20 ml of wash water. The effluent was concentrated to a sirup under reduced pressure, and the sirup was transferred to a reduction tube such as that described in [7]. The sugar acid was lactonized by heating it in a current of air at 50° C for several days, with occasional addition of methanol. When the product had become entirely crystalline (about 1 week), the tube was immersed in an ice bath and connected with a stirrer, which reached to the bottom of the tube. After the addition of 3.2 g of crystalline sodium acid oxalate and 10 ml of water, vigorous stirring was begun, and 4.6 g of 5-percent sodium amalgam pellets was added through the sidearm of the reduction tube. Stirring was continued until the amalgam was spent, after which the mercury was separated. Five volumes of methanol was added; the crystalline sodium salts were separated by filtration, washed with methanol, and discarded. The alcoholic solution was concentrated under reduced pressure to about 5 ml, 25 ml of methanol was added, and the solution was filtered to remove a second crop of nonradioactive sodium salts. The solution was concentrated to remove the methanol, and was neutralized at ice temperature with sodium hydroxide to the end point of phenolphthalein. A small crop of nonradioactive salts was removed, and the solution was then passed through an ion-exchange column containing equal parts of cation and anion exchange resins.[6] The salt-free and neutral effluent was concentrated to 100 ml under reduced pressure, and finally was freeze-dried. The residue was dissolved in 0.5 ml of methanol, and isopropanol was added to the point of incipient turbidity. Crystallization of D-arabinose-5-C^{14} progressed readily, and ultimately 103 mg of crystalline D-arabinose was separated without the addition of carrier (yield=68.6 percent).[7] As potassium D-arabonate was obtained in 52.4-percent yield from D-mannitol, with the elimination of one-half of the carbon 14, the over-all radiochemical yield of D-arabinose-5-C^{14} was 18 percent.

[5] Amberlite IR 120, Rohm & Haas Co., Philadelphia, Pa.

[6] Amberlite IR 100, analytical grade, Rohm & Haas Co., Philadelphia, Pa., and Duolite A-4, Chemical Process Co., Redwood City, Calif.

[7] There has been considerable variation in yield in this preparation; a radiochemical yield of 60 to 70 percent may be expected.

4. References

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