

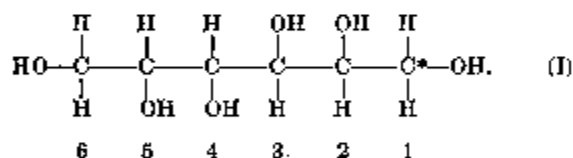
Preparation of D-Mannitol-C¹⁴ and Its Conversion to D-Fructose-1-(and 6)-C¹⁴ by Acetobacter Suboxydans¹

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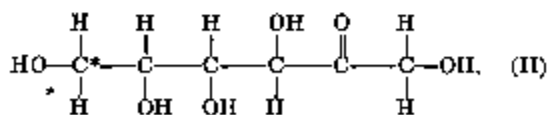
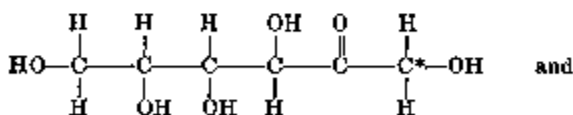
Methods are described for the production of D-mannitol-1-C¹⁴ and D-fructose-1-(and 6)-C¹⁴. These materials are powerful new tools for research in biology and chemistry. D-Mannitol-1-C¹⁴ was prepared in 80-percent yield from D-mannono-γ-lactone. D-Fructose-1-(and 6)-C¹⁴ was produced in 54-percent yield by growth of Acetobacter suboxydans on a D-Mannitol-1-C¹⁴ substrate.

1. Introduction

The Bureau recently undertook a program for the development of methods for the production of a variety of position-labeled sugars [1].² The preparation of D-glucose-1-C¹⁴ and D-mannose-1-C¹⁴ in high yield was described in a previous report [2]. This paper deals with the production of D-mannitol-1-C¹⁴ and its conversion to D-fructose-1-(and 6)-C¹⁴ by oxidation with Acetobacter suboxydans.³ Prior to the present investigation, randomly labeled fructose had been prepared by photosynthesis [3], but position-labeled fructose had not been reported. It was known that D-mannitol could be obtained by reduction of D-mannose, and that it could be converted to D-fructose by oxidation with Acetobacter suboxydans [4]. D-Mannitol differs from D-mannose in that the ends of the molecule are alike. For this reason a 1-labeled mannitol (I) can be considered as either 1- or 6-labeled, and any unsymmetrical derivative prepared from this substance would be 1,6-labeled, provided the carbon chain remains intact. Thus, by oxidation of D-mannitol-1-C¹⁴ one would obtain D-fructose-1-(and 6)-C¹⁴ (II).



D-Mannitol-1-C¹⁴



D-fructose-1-(and 6)-C¹⁴

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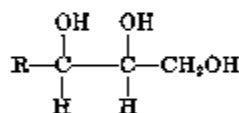
² Figures in brackets indicate the literature references at the end of the paper.
³ A proof by chemical degradation for the 1- and 6-structure ascribed to the fructose formed will be presented in a later report.

As both D-mannitol-1-C¹⁴ and D-fructose-1-(and 6)-C¹⁴ would provide powerful tools for the elucidation of many problems in biology and chemistry, the preparation of these compounds was undertaken.

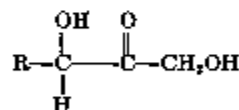
2. Discussion of the Processes

The production of D-mannitol-1-C¹⁴ includes: (1) preparation of D-mannono-γ-lactone-1-C¹⁴ by the method already described [2], (2) conversion of the lactone to D-mannose-1-C¹⁴ by sodium amalgam reduction, and (3) catalytic reduction of the sugar with hydrogen. Each step in the process was studied in detail, and procedures were developed to obtain D-mannitol-1-C¹⁴ from D-mannono-γ-lactone-1-C¹⁴ in an over-all radiochemical yield of about 80 percent.

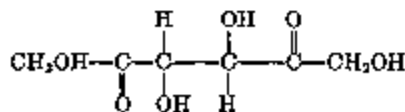
The production of D-fructose-1-(and 6)-C¹⁴ involves oxidation of D-mannitol-1-C¹⁴ with Acetobacter suboxydans. It has been established [5] that Acetobacter suboxydans preferentially oxidizes the



group to the



group. In agreement with this property, D-fructose-1-(and 6)-C¹⁴ is obtained by oxidation of D-mannitol with Acetobacter suboxydans.⁴ As pointed out in section 1, the ends of the molecule are alike. For this reason, oxidation could proceed equally well from either end of the molecule, and, in the event that oxidation took place at both ends, a diketo derivative would be formed. Thus, prolonged action of Acetobacter suboxydans on mannitol should yield the diketo derivative



(D-threo-5-oxo-2-ketohexose).

⁴ See footnote 3.

To ascertain whether the oxidation did, in fact, proceed beyond the fructose stage, a series of mannitol *Acetobacter suboxydans* cultures were set up and, as given in table 1, the course of the reaction was followed by determinations of fructose and total copper-reducing substance. The fructose concentration was determined from the polarization at two temperatures [6]. Total reducing substance was determined by the modified Scales method [7]. Curve 1 of figure 1 gives the yield of D-fructose determined from the difference in optical rotation at two temperatures. Curve 2 gives the copper-reducing power of the solution expressed as the percentage of the reducing power of a solution containing the amount of D-fructose molecularly equivalent to the mannitol originally present. The copper-reducing value represents all reducing substances present and is expressed arbitrarily on the fructose scale. The yield of D-fructose reached a maximum in about 2 days, and then decreased for about 5 days when active growth of the organism had slowed down. In every sample, after the first day the copper-reducing value exceeded that of the D-fructose determined from the polarization at two temperatures. Hence, the solution contained a constituent having copper-reducing power other than D-fructose. Presumably, this substance was the anticipated diketone compound or a substance derived therefrom. The results clearly show that it is necessary to control the reaction time to obtain maximum yields of D-fructose. The experiment of figure 1 was conducted with a surface-volume ratio of 1.28. In agreement with the work of Fulmer, Dunning, and Underkofler, a somewhat higher yield of fructose was obtained with a higher surface-volume ratio. In the preparation of D-fructose-1-(and 6-) C^{14} on a tracer level, a surface-volume ratio of 1.59 was used, and a radiochemical yield of 54 percent was obtained.

3. Experimental Procedures

3.1. D-Mannono- γ -lactone- $1-C^{14}$

This substance was prepared in 67-percent yield by the sodium-bicarbonate-carbon-dioxide method previously described [2].

3.2. Conversion of D-Mannono- γ -lactone- $1-C^{14}$ to D-Mannitol- $1-C^{14}$

One millimole of D-mannono- γ -lactone- $1-C^{14}$ having an activity of 10 μ c was dissolved in 20 ml of water; 1.5 g of benzoic acid was added, and the mixture was stirred in an ice bath until cold. Sodium amalgam pellets (4.6 g, containing 5 percent of sodium) were added and stirring was continued. After 2 hours the mercury was removed, and another addition of 1.2 g of benzoic acid and 4.6 g of amalgam was made. Stirring was continued at 0° to 5° C for 2 hours, after which the mercury was removed, and 1.26 g of oxalic acid was added. Then the mixture was extracted with chloroform to remove the benzoic acid. The sodium oxalate in the aqueous solution was precipi-

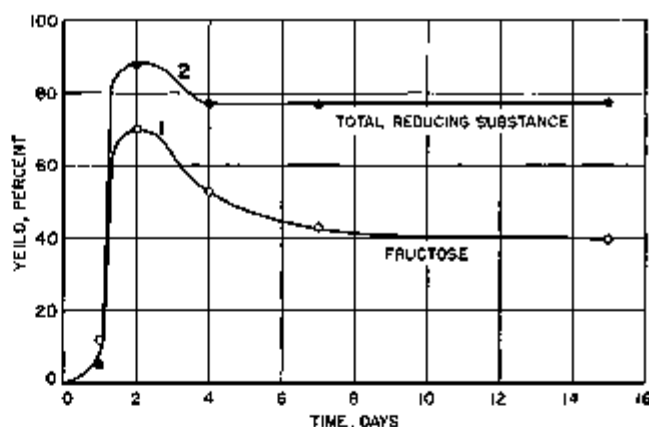


FIGURE 1. Fructose formed from mannitol by *Acetobacter suboxydans*.

tated by the addition of approximately 2 volumes of methanol and 2 of ethanol. The insoluble salts were separated by filtration and washed with methanol. The resultant alcoholic liquor was concentrated under reduced pressure to about 5 ml and was diluted again with 2 volumes of methanol and 2 of ethanol. The resultant precipitate was separated and washed with methanol. The alcoholic liquor was evaporated to approximately 2 ml and was then transferred with 3 ml of water to the tube of a high-pressure hydrogenator containing 0.1 g of calcium carbonate and 0.5 g of Raney nickel catalyst. The mixture was hydrogenated for 2 hours at 125° C and 1,000 lb/in². Then the mixture was cooled to room temperature, filtered, and passed through a column (1.4 by 25 cm) containing equal portions of Amberlite IR 100-H and Duolite A4. The effluent and wash liquor were combined and lyophilized. The residue was dissolved in the minimum quantity of hot methanol. After the addition of an equal volume of isopropanol, and seeding, the solution was set aside for crystallization of D-mannitol. In the course of 3 days 117 mg of D-mannitol- $1-C^{14}$ (6.4 μ c) was obtained. It melted at 165° C, in substantial agreement with the accepted value of 166° to 168° C. "Carrier" D-mannitol (100 mg) was dissolved in the mother liquor and, after crystallization, 98 mg of D-mannitol- $1-C^{14}$ was obtained with a radioactive content of 1.6 μ c. The total radioactive recovery was 80 percent.

3.3. Preliminary Study of the Oxidation of D-Mannitol to D-Fructose with *Acetobacter Suboxydans*

Aliquots (25 ml) of an aqueous solution containing 9.1 g of D-mannitol, 2.5 g of yeast extract, and 1.5 g of potassium dihydrogen phosphate in 500 ml were placed in 125-ml Erlenmeyer flasks and sterilized for 15 minutes at a steam pressure of 15 lb. Each flask was then inoculated with five drops of a previously prepared inoculum of *Acetobacter suboxydans* and allowed to stand at 30° C in an incubator. The inoculum was prepared by growth, at 30° C for 45 hours, of a fresh culture of *Acetobacter suboxydans* on a solution containing 2 g of mannitol, 0.2 g of yeast extract, and 40 ml of water.

At regular intervals following the inoculation, one of the flasks was removed from the incubator. The liquid was transferred to a 25-ml volumetric flask and diluted to volume. One-half gram of neutral lead acetate trihydrate was then added. The mixture was transferred to a centrifuge tube, and the precipitate was removed by centrifugation. To the clear solution was added 0.25 g of potassium oxalate monohydrate, and the resulting precipitate was again removed by centrifugation. The clear solution was then used directly for measurements of optical rotation and copper-reducing power.

TABLE 1. *Acetobacter suboxydans* oxidation of mannitol
(Series—volume 7510, 1, 2)

Incuba- tion time	Polarization at two temperatures				Fructose yield		Reducing sugar yield	
	°C	°S	°C	°S	g/100ml	Percent	g/100ml	Percent
1.0	20	-0.47	57.3	-0.20	0.21	11.7	0.09	5.0
2.0	20	-0.52	53.5	-4.67	1.17	55.0	1.58	37.8
4.0	20	-3.34	54.5	-4.01	0.95	52.8	1.39	77.2
7.0	20	-3.10	53.2	-3.87	.77	42.8	1.39	77.2
15.0	20	-4.86	56.0	-3.92	.71	39.4	1.40	77.8
28.0	20	-4.50	53.0	-3.45	.66	36.7	1.46	81.1

The optical rotations given in table 1 were made at two temperatures with a Bates saccharimeter and a 2-dm tube. The rotations at the higher temperatures were corrected [6] for expansion by use of the coefficient 0.00044 for each degree rise in temperature. The difference between the corrected rotation and that at 20°, divided by the temperature difference and by 0.03441, equals the number of grams of D-fructose per 100 ml of solution. Complete conversion to D-fructose of the mannitol originally in the solution would have given 1.8 g of D-fructose per 100 ml.

Copper-reducing values, also given in table 1, were determined on the solutions by the modified Scales method [7] standardized against D-fructose. The values so obtained represent all substances that reduce the alkaline copper reagent.

3.4. Conversion of D-Mannitol-1-C¹⁴ to D-Fructose-1- (and 6)-C¹⁴

One millimole (182 mg) of D-mannitol-1-C¹⁴, with a radioactive content of 8.8 µc/millimole, was dissolved in 10 ml of aqueous solution containing 50 mg of yeast extract and 30 mg of potassium dihydrogen phosphate in a 50-ml Erlenmeyer flask (surface/volume=1.59 cm²/ml). The mixture was allowed to incubate at 30° C with three drops of a previously prepared inoculum of *Acetobacter suboxydans* to the point of maximum rotation and reducing power (45 hours). To this solution was then added 1 ml of a 20-percent solution of zinc sulfate and an equivalent quantity (previously determined with bromothymol blue indicator) of a saturated solution of barium

hydroxide. After removal of the precipitate by centrifugation, the solution and washings were passed slowly through a (1.2 by 22 cm) column containing a mixture of Amberlite IR 100-H and Duolite A4. The effluent and aqueous wash liquor were concentrated by lyophilization to an amorphous residue weighing 145 mg. This residue was dissolved in several drops of methanol, isopropanol was added to the point of incipient turbidity, and the mixture was seeded with D-fructose. Crystallization was allowed to proceed for several days, after which 56 mg of crystalline product was obtained. The specific rotation ($[\alpha]_D^{20} = -82^\circ$) as well as the radioactivity (0.0428 µc/mg) of the material was about 10 percent too low. Hence, the yield of C¹⁴-labeled fructose was actually 49 mg (27%) instead of 56 mg. An additional quantity of labeled fructose was separated by the use of 200 mg of carrier D-fructose. An aqueous solution of the carrier was added to the mother liquor, and the solution was evaporated to substantial dryness. The residue was taken up in 1 ml of methanol, and crystallization was induced by the addition of 2 ml of isopropanol and seeding with crystalline fructose. The crystals that separated weighed 210 mg and had an activity of 0.0114 µc/mg. The crop of fructose undiluted with carrier contained 2.40 µc, and the carrier-diluted sugar contained 2.39 µc; thus the radiochemical yield was 4.79 µc, or 54.4 percent.^a

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4. References

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^a A limited quantity of D-fructose-1-(and 6)-C¹⁴ with an activity of 0.5 µc/mg made by this process, is now available from this Bureau at a cost of \$1.00 per microcurie.