Enzymic preparation of L-fructose*

L-Fructose, a sugar which has not yet been detected in Nature, was first synthesized by Fischer[1], who treated α-acrose with phenylhydrazine. The resulting DL-glucose phenylosazone was isolated, and hydrolyzed to the glycosulose (osone), which was then reduced to DL-fructose. The D-fructose was then fermented with yeast, to yield a solution of L-fructose which was identified by formation from it of L-*arabino*-hexose phenylosazone, but it was not, itself, isolated in crystalline form.

L-Fructose was later synthesized by Wolfrom and Thompson[2] by the following sequence of reactions: L-arabinonic acid tetraacetate→L-arabinonoyl chloride tetraacetate→1-deoxy-1-diazo-*keto*-L-fructose tetraacetate→*keto*-L-fructose pentaacetate →L-fructose. The L-fructose was crystallized, and identified by elemental analysis, polarimetry, and derivatives. To date, this procedure is the only satisfactory method available for the synthesis of L-fructose.

The present report describes an alternative and more simplified procedure for the preparation of L-fructose. It involves the isomerization of L-mannose to L-fructose catalyzed by an enzyme present in cell-free extracts of *Aerobacter aerogenes* grown on L-rhamnose. L-Fructose has been isolated in crystalline form, and identified by paper chromatography, polarimetry, and derivatives.

The enzymic method for the preparation of L-fructose described here is considerably shorter than the chemical method previously described[2]. It involves only the isomerization of L-mannose, a compound which can be readily prepared or purchased; the enzyme does not need to be purified, but can be used directly as it occurs in cell-free extracts. Unreacted L-mannose may be recovered from the equilibrium mixture by regeneration from its phenylhydrazone. The yield (28–32%) of L-fructose is equal to or better than that obtained with the chemical method starting from L-arabinonic acid tetraacetate[2].

Although the isomerization of L-mannose to L-fructose represents a new enzymic reaction, the isolation of L-fructose is an adaptation of a standard procedure. Mannose (D or L) phenylhydrazone is well known for its low solubility in water; thus, it has been used to separate mannose quantitatively from other hexoses. Sowden and Fischer[3] succeeded in separating L-mannose from L-glucose through their corresponding phenylhydrazones, and we have succeeded in separating L-mannose from L-fructose by the same means. Two precautions may be specifically noted. First, a high excess of phenylhydrazine should be avoided, because L-fructose and L-fructose phenylhydrazone readily react with the excess reagent to produce insoluble L-*arabino*-hexulose phenylosazone[4], thus decreasing the yield of L-fructose. Second, mixed-bed resins possessing strong anionic groups should not be used for deionizing solutions of fructose because they cause partial isomerization to glucose.

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Since the extent of isomerization is based solely on establishing equilibrium, the amount of L-fructose that can be obtained is limited only by the amount of L-mannose available; the time required to reach equilibrium is inversely proportional to the proportion of enzyme. Thus, larger quantities of L-fructose could be prepared, simply by increasing the initial amounts of the reaction components.

EXPERIMENTAL

Materials. — L-Mannose was prepared by the method of Sowden and Fischer[3] and possessed the following properties: m.p. 129–135°, $[\alpha]_{578}^{24}$ −14.8° (*c* 5, water). (It is commercially available from Calbiochem, Los Angeles, California.) L-Fructose for use as seed crystals was a generous gift from Professor M. L. Wolfrom, The Ohio State University. Duolite resins C-25 (Na^+) and A-6 (Cl^-) were purchased from the Diamond Alkali Company, Redwood City, California; they were converted into the H^+ and OH^- forms, respectively, and, prior to preparation of the column, were mixed in the ratio of cation:anion of 3:5 (wet weight).

Analytical. — L-Fructose was determined by the Roe method[5]. Polarimetric measurements were made with a Zeiss photoelectric polarimeter (courtesy of Dr. J. C. Speck, Jr.). Protein was measured[6] at 210 nm. L-*arabino*-Hexulose phenylosazone was prepared by the procedure of Garard and Sherman[7], and *arabino*-hexulose phenylosotriazole by the procedure of Hann and Hudson[8]. Chromatography was performed on Whatman No. 1 paper with (1) water-saturated phenol, and (2) 15:5:2 (v/v) butanone–acetic acid–water; the sugars on the chromatograms were located with a bath of silver nitrate[9], or sprays of orcinol[10] and *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride[11].

Preparation of cell-free extracts. — *A. aerogenes* PRL-R3 was grown aerobically at 30° in 500 ml of a medium consisting of 0.71% of Na_2HPO_4, 0.15% of KH_2PO_4, 0.3% of $(NH_4)_2SO_4$, 0.01% of $MgSO_4$, 0.0005% of $FeSO_4 \cdot 7H_2O$, and 0.4% of L-rhamnose (autoclaved separately). The cells were harvested by centrifugation after growing for 10–12 h in a Fernbach flask on a rotary shaker. They were washed with cold water, recentrifuged, and suspended in 15 ml of 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer (pH 7.6). They were disrupted by sonication for 4 min in a Raytheon 10-kHz sonic oscillator equipped with an ice-water cooling-jacket. The cellular debris was removed by centrifugation at 31,000 *g* for 10 min. The supernatant solution was the cell-free extract.

Enzymic preparation of L-*fructose.* — The reaction mixture (75 ml) consisted of the following: 28 mmoles of L-mannose, 5 mmoles of cobalt chloride, 12 mmoles of 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer (pH 7.6), and cell-free extract (300–350 mg of protein); it was incubated for 2–3 h at 30°, or until equilibrium was established. At equilibrium, the ratio of L-mannose:L-fructose was 19:31. The reaction mixture was then heated in a boiling-water bath for several minutes, and the denatured protein was removed by centrifugation at 31,000 *g* for 15 min.

L-Mannose was separated from L-fructose by adding a solution containing 3.1 g of phenylhydrazine in 7.0 ml of glacial acetic acid, and keeping the resulting mixture overnight at 4°. The L-mannose phenylhydrazone which crystallized was removed by suction filtration. The filtrate was concentrated under vacuum to about 50–60 ml, and stored an additional 2–3 h at 4°. The precipitate that formed was removed by suction filtration.

Free L-fructose was regenerated from the dissolved phenylhydrazone by refluxing the filtrate for 2–3 h with 13 ml of ethanol, 8 ml of benzaldehyde, and 0.8 g of benzoic acid. After the suspension had been cooled to room temperature, the liquid phase was decanted from the insoluble 2-benzyl-2-phenylhydrazone, washed with three 100-ml portions of chloroform, and decolorized with Darco G-60 carbon. This solution (about 60 ml) was deionized by passage through a column (2.7 × 30 cm) of mixed-bed resins consisting of Duolite C-25 (H^+) and A-6 (OH^-). About 500 ml of effluent was collected, which provided a 96% recovery of L-fructose. The neutral effluent was evaporated under diminished pressure to a syrup, which was dissolved in warm absolute alcohol; the solution was nucleated with crystals of L-fructose, and kept for 24 h at room temperature. The fine, needle-like crystals of L-fructose were collected by gravity filtration, and the mother liquor was reprocessed to give additional product. The overall yield of L-fructose was 1.4–1.6 g (28–32%), m.p. 93–95°, $[\alpha]_{578}^{24}$ +91.0° (*c* 2.5, water)[12]. Heating the crystals for 2 h in an Abderhalden vacuum dryer at 83° or 110° resulted in a weight loss of no more than 1.5%; thus, the crystals were considered to be essentially anhydrous.

When chromatographed on paper, with two different solvent-systems, L-fructose migrated with the authentic D-enantiomorph, and was free from detectable L-mannose. L-Fructose was converted into L-*arabino*-hexulose phenylosazone, m.p. 205–208°, a value in good agreement with that recorded by Garard and Sherman[7]. The osazone was subsequently converted into L-arabino-hexulose phenylosotriazole, m.p. 193–197°, $[\alpha]_{578}^{24}$ +80.0° (*c* 4, pyridine); these values are in good agreement with those given by Hann and Hudson[8].

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REFERENCES

1 E. FISCHER, *Ber.*, 23 (1890) 389.
2 M. L. WOLFROM AND A. THOMPSON, *J. Amer. Chem. Soc.*, 68 (1946) 791.
3 J. C. SOWDEN AND H. O. L. FISCHER, *J. Amer. Chem. Soc.*, 69 (1947) 1963.
4 J. ASHMORE AND A. E. RENOLD, *J. Amer. Chem. Soc.*, 76 (1954) 6189.
5 J. H. ROE, *J. Biol. Chem.*, 107 (1934) 15.
6 M. P. TOMBS, F. SOUTER, AND N. F. MACLAGAN, *Biochem. J.*, 73 (1959) 167.
7 D. D. GARARD AND H. C. SHERMAN, *J. Amer. Chem. Soc.*, 40 (1918) 955.
8 R. M. HANN AND C. S. HUDSON, *J. Amer. Chem. Soc.*, 66 (1944) 735.
9 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444.

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10 A. BEVENUE AND K. T. WILLIAMS, *Arch. Biochem. Biophys.*, 34 (1951) 225.
11 R. B. KOCH, W. F. GEDDES, AND F. SMITH, *Cereal Chem.*, 28 (1951) 424.
12 W. C. VOSBURGH, *J. Amer. Chem. Soc.*, 42 (1920) 1696.

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Recovery of useful carbohydrates in nucleoside syntheses: 2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranose*

The mercury derivative of 2-acetamido-6-chloropurine[1] was first employed in nucleoside synthesis for the preparation of the anomers of 2′-deoxy-6-thioguanosine and related compounds. The method has been extended[2] to the preparation of 3′-deoxy-6-thioguanosine, L-thioguanosine, and some related nucleosides. The proposed[2] generality of this synthetic route and the interesting biological properties attributed to various nucleosides derived from thioguanine[3], which necessitates their preparation in amounts sufficient for chemotherapeutic evaluation, prompt us to record our further observations on this synthetic method.

In previous work[4] on the synthesis of moderately large quantities of the anomers of 2′-deoxy-6-thioguanosine[1], we have examined the coupling mixture after removal of the nucleosides and the unreacted purine starting-materials, and have found the protected, (1→1)-linked, disaccharide of 2-deoxy-D-*erythro*-pentofuranose to be a major artifact in this reaction. This compound has proved valuable from a practical standpoint in that it is readily reconvertible into a glycosyl halide that could be re-used in the nucleoside synthesis, thus decreasing the cost and effort necessary to prepare quantities of these compounds sufficient for biological evaluation. The formation of the disaccharide was attributed to the presence, or generation, of water during the nucleoside synthesis or to the formation of a labile purine–mercury–sugar complex, which was decomposed during the isolation and purification process. The exact nature of this mechanism has remained in doubt largely because of the obscure nature of the mercury complex of 2-acetamido-6-chloropurine[1], wherein the elements Hg and O_2 are added to give a complex of unknown structure. In the preparation of 2-acetamido-6-chloro-9-(2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranosyl)-9*H*-purine[2] by the coupling of the mercury complex of 2-acetamido-6-chloropurine and 2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranosyl chloride[2], it was found[2] that the addition of molecular sieves** is useful. It has further been observed that the mode of addition of the molecular sieves is important. The overall yield in the coupling is materially increased when the azeotropically dried mercury

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**Linde, Type 4A, 1/16″ pellets.