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Carbon Balance of a Mannitol Fermentation and the Biosynthetic Pathway

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The carbon balance was determined for a fermentation in which mannitol is produced from glucose by an *Aspergillus* species. The products found were: cells (17% of carbon input), CO_2 (26%), mannitol (35%), glycerol (10%), erythritol (2.5%), glycogen (1%), and unidentified compounds (8%). Thus, 92% of the carbon input was accounted for. Cell-free enzyme studies showed that mannitol was synthesized via the reduction of fructose-6-phosphate and not by the direct reduction of fructose. If the cell yield from glucose was assumed to be 50% and the theoretical conversion efficiency from glucose to polyols was 90%, as calculated from the energy balance, then 34% of the glucose carbon was used for growth and 53% was used for polyol formation.

Mannitol is a common reserve product of fungi (14) and its accumulation by fungi and yeasts has been reported by a number of investigators. Coyne and Raistrick (6) reported in 1931 that a white *Aspergillus* strain produced 35% of mannitol from various sugars. Pruess et al. (15) obtained as much as 50% mannitol with a stationary culture of *Aspergillus* on Czapek-Dox (glucose) medium. Otani (13) studied mannitol production by *A. mannitosus* in shaken flasks and obtained a yield as high as 20%. The osmophilic yeast, *Zygosaccharomyces*, has been reported also to produce some mannitol, although the major polyols were arabitol and glycerol (17). Recently, K. L. Smiley et al. (Abstr. 152nd Meeting Am. Chem. Soc., p. Q15, 1966) described a submerged *A. candidus* fermentation process with a yield of 50% mannitol. In all the mannitol fermentations reported, no more than half the sugar was converted to mannitol. The only mannitol carbon balance reported was done in 1931 by Birkenshaw et al. (3) with stationary culture, and none of the products was specifically identified.

The fate of the sugar consumed but not converted to mannitol was of interest in this study. A strain of *Aspergillus* was chosen for its ability to produce mannitol in a simple defined medium. A complete carbon balance in shake flasks was carried out and is reported here.

The pathway of mannitol biosynthesis was also of interest. Other fungi have been reported to make mannitol either by the reduction of fructose with reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$; 7) or by the reduction of fructose-6-phosphate (F-6-P) with reduced nicotinamide adenine dinucleotide ($NADH_2$; 2). The possible presence of either or both pathways was examined in cell-free extracts of this *Aspergillus* species.

MATERIALS AND METHODS

Culture and medium. A strain of *Aspergillus* (Upjohn collection 4177) was used in these experiments. The culture was grown in a simple glucose and urea salts medium, which is similar to that described by Pruess et al. (15), in a New Brunswick reciprocal water bath shaker [2-inch (5.08-cm) stroke, 100 cycles per min] at 28 C. It was necessary to avoid the use of glass tubing within the flask of the fermentation train, to prevent plugging by mycelial growth (Fig. 1). Each shake flask was harvested and analyzed when a corresponding flask consumed all the glucose as determined by TesTape (Eli Lilly & Co., Indianapolis, Ind.).

Analyses. For convenience, all quantities are expressed in millimoles of carbon. The procedures of Neish (11) for collection of CO_2 and volatiles were followed. Carbon dioxide in the exit gas was determined gravimetrically by Ascarite (NaOH-impregnated asbestos, Arthur H. Thomas Co., Philadelphia, Pa.) absorption (11). The volatile compounds, acetic acid, acetaldehyde, and ethyl alcohol were assayed by Conway's diffusion methods (5). Acid-hydrolyzed glycogen and glucose were determined by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). The carbon content of the inoculum, dried filtrate solution, and mycelium were analyzed by combustion with oxygen. Three separate assay methods were used to determine the polyols:

(i) They were separated by descending paper chromatography on Whatman 3 MM paper with *n*-butanol-pyridine-water (6:4:3; 16). To detect the location of polyols, one paper was developed with

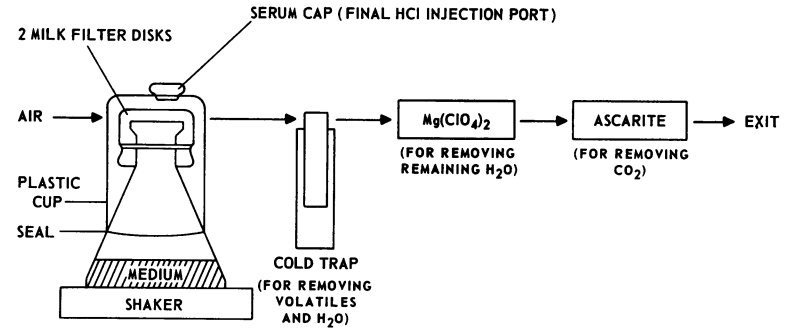

FIG. 1. *Schematic diagram of the mannitol fermentation train.*

TABLE 1. *Polyol analyses in 100 ml of fermentation broth*

Substance	Paper chromatography	Celite column	Gas chromatography
	g	g	g
Mannitol.....	4.85	—	4.75
Glycerol.....	0.96	0.825	Positive
Erythritol.....	0.30	0.258	Positive

$AgNO_3$ reagent (4). Then, the corresponding areas from another paper were cut out, and the polyols were eluted with water. Each fraction was analyzed quantitatively by periodate oxidation (11). Glucose and fructose also separated with the above system, and they were detected by *p*-anisidine-H_3PO_4 reagent (4).

(ii) Glycerol and erythritol were fractionated on a Celite column, and each fraction was assayed by periodate oxidation (11).

(iii) Mannitol was determined quantitatively by a modified gas chromatography method with inositol as internal standard (1). The results of the three different assay methods checked with one another (Table 1).

Enzyme assays. All materials were purchased from Calbiochem, Los Angeles, Calif., except mannose-6-phosphate which was purchased from Sigma Chemical Co., St. Louis, Mo. Mannitol-1-phosphate (M-1-P) was prepared according to the methods of Wolff and Kaplan (19). The cells for enzyme studies were grown as described, were harvested on the 3rd day by filtration, and were washed thoroughly. Cell-free extracts for mannitol dehydrogenase and M-1-P dehydrogenase were prepared in a chilled French pressure cell (10,000 to 15,000 psi) in 0.1 M potassium phosphate buffer (*p*H 7.0). A stable extract of mannitol phosphatase was prepared by sonic treatment in the cold in a buffer containing 0.1 M tris(hydroxymethyl)-aminomethane (*p*H 7.0), 0.005 M $MgCl_2$, 0.5 M KCl, and 0.1% mercaptoethanol (18). The method of Edmundowicz and Wriston (7) was used for the mannitol dehydrogenase assay. The method of Wolff and Kaplan (19) was used for the M-1-P dehydrogenase assay, but with 0.1 M $KHCO_3$ and 0.1% disodium ethylenediaminetetraacetic acid as buffer at *p*H 7.5 (2). The method of Yamada et al. (20) was used for mannitol-1-phosphatase assay, but with 0.1 M potassium acetate and 0.005 M $MgCl_2$ as buffer (*p*H 6.0). The inorganic phosphate liberated by mannitol-1-phosphatase was assayed by the method of Fiske and Subbarow (8). Protein in cell-free extracts was determined by the method of Lowry et al. (10).

RESULTS AND DISCUSSION

Carbon balance. The mannitol in the fermentation broths was crystallized by the addition of methanol, and its melting point, optical rotation, and infrared spectra were determined to be the same as for standard mannitol. It was still necessary to determine the other products of mannitol fermentation before running a carbon balance. Paper chromatography revealed that mannitol, glycerol, and erythritol were the fermentation products (Fig. 2). Their relative mobility in all three chromatographic systems was identical to standard mannitol, glycerol, and erythritol samples, as was their chemical reaction to periodate (11). Fructose was usually present early in the fermentation (Fig. 2), but it disappeared at the end and did not affect carbon balance. Only a trace of volatile substances was detected and was insignificant.

The carbon balance in Table 2 is the average of three single-flask experiments. An average of 92% of the carbon input was recovered. The total polyol yield of separate flasks was always about 50%, but the relative amounts of mannitol, glycerol, and erythritol varied from flask to flask. Variation of polyol composition was due to unavoidable drifts in fermentation conditions such as shaker speed and temperature. Polyols and "glycogen" accounted for 205 mmoles of the total 215 mmoles of carbon in the filtrate as determined independently by elemental analysis. The 10 μmoles of unidentified carbon compounds in the filtrate were undoubtedly soluble cellular substances.

Pathway of mannitol biosynthesis. For energy balance calculations, it is important to know

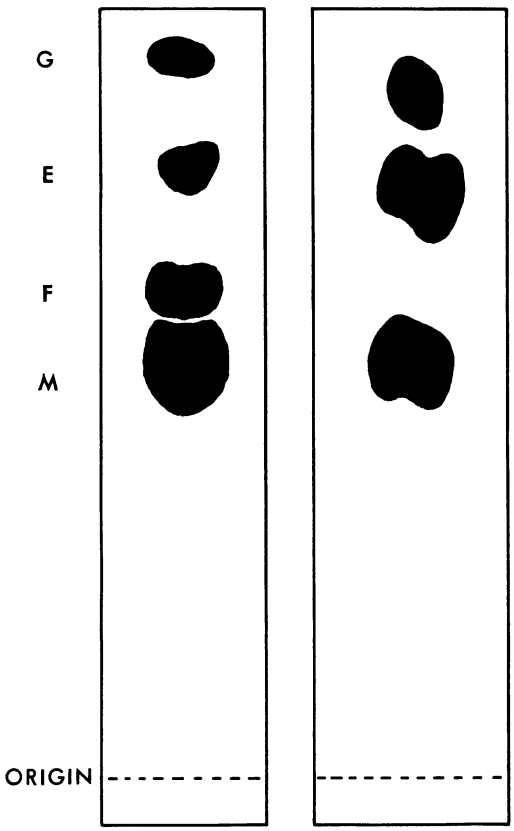

FIG. 2. *Paper chromatography of a 3-day-old fermentation broth showing fructose (F; left) and a mixture (right) of 3 μmoles of mannitol (M), erythritol (E), and glycerol (G).*

TABLE 2. *Mannitol fermentation balance*

Determination	Amt (mmoles) of "carbon" ± SD	% Carbon
Input		
Glucose	400	—
Urea	5	—
$CaCO_3$	3.3	—
Seed	8.6	—
Total input	417	100
Output		
CO_2	107.5 ± 5.7	26
Cells	71.7 ± 8.8	17
Mannitol	146.3 ± 21.8	35
Glycerol	43.5 ± 19.6	10
Erythritol	10.1 ± 4.4	2.5
Glycogen	4.7 ± 4.2	1.0
Total output	383.8	92

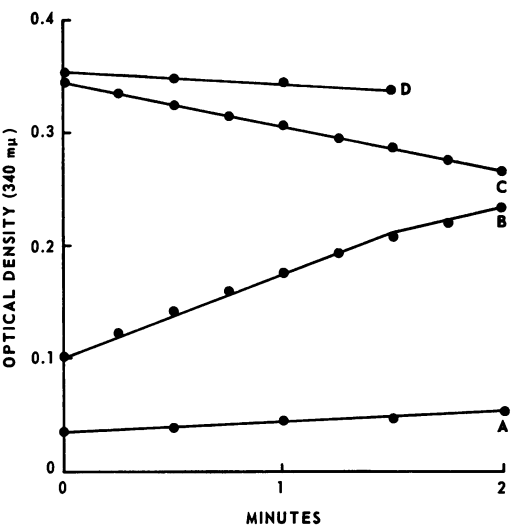

FIG. 3. *Mannitol-1-phosphate dehydrogenase forward and reverse reactions. Each tube contained (A) 0.5 μmole of NAD, (B) 0.5 μmole of NAD and 6.6 μmoles of M-1-P, (C) 0.5 μmole of $NADH_2$ and 10 μmoles of F-6-P, and (D) 0.5 μmole of $NADH_2$. Cell-free extract (0.175 mg of protein) was added, and the reaction mixture was made up to 3 ml with buffer. Room temperature.*

which pathway of mannitol biosynthesis is present in *Aspergillus* species. If mannitol were produced by the direct reduction of fructose by $NADPH_2$ (7), the reaction would be freely reversible, and no adenosine triphosphate (ATP) would be required. On the other hand, if mannitol biosynthesis proceeds via the reduction of F-6-P and hydrolysis (20), the synthesis would be an irreversible reaction, and, for each molecule of mannitol formed, one molecule each of ATP and $NADH_2$ would be utilized.

The forward and reverse reactions of both possible routes were tested. Cell-free extracts did not contain any mannitol dehydrogenase activity (reaction 1):

$$\begin{matrix}\text{fructose}\\ \text{glucose}\\ \text{mannose}\end{matrix} \underset{\text{NAD or NADP}}{\overset{\text{NADH}_2 \text{ or NADPH}_2}{\rightleftharpoons}} \text{mannitol} \quad (1)$$

On the other hand, active M-1-P dehydrogenase was always found in cell-free extracts, and both forward and reverse enzyme reactions were demonstrated successfully in Fig. 3 (reaction 2):

$$\text{F-6-P} \underset{\text{NAD}}{\overset{\text{NADH}_2}{\rightleftharpoons}} \text{M-1-P} \quad (2)$$

Finally, mannitol-1-phosphatase (Fig. 4) was identified in cell-free extracts (reaction 3). This reaction is essentially irreversible, and the K_m

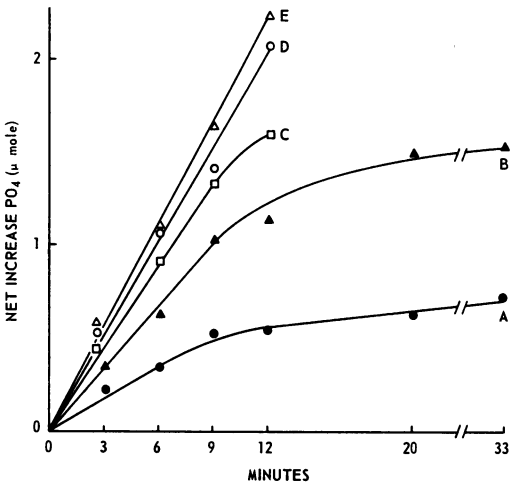

FIG. 4. *Activity of mannitol-1-phosphatase at various substrate concentrations. Each tube contained (A) 0.66 μmoles of M-1-P, (B) 1.65 μmoles of M-1-P, (C) 3.3 μmoles of M-1-P, (D) 6.6 μmoles of M-1-P, (E) 13.2 μmoles of M-1-P. Dialyzed cell-free extract (0.15 mg of protein) was added, and the mixture was made up to 2 ml with buffer. Temperature, 30 C.*

calculated from the rate data in Fig. 4 is 0.0013 M:

$$\text{M-1-P} \rightarrow \text{mannitol} + PO_4 \quad (3)$$

Mannitol is most likely synthesized by this *Aspergillus* species via enzyme reactions 2 and 3. The synthesis is essentially irreversible and should drive towards mannitol accumulation.

From known glucose oxidative pathways (9), it is estimated that upon oxidation to CO_2 each part glucose could yield about 9 parts $NADH_2$ or $NADPH_2$ and 9 ATP. Thus, the theoretical conversion efficiency of mannitol synthesis by this *Aspergillus* from glucose is 90% (reaction 4):

$$10 \text{ glucose} \rightarrow 9 \text{ mannitol} + 6\ CO_2 \quad (4)$$

We are currently studying some of the glucose catabolic enzymes as well as the mannitol catabolic enzyme, since this *Aspergillus* species also has the ability to degrade mannitol readily.

Distribution of glucose utilization. The carbon dioxide formed from glucose oxidation in mannitol fermentation is related to the energy used for cell growth and polyol synthesis. The fungal cell yield from glucose is reported to be 40 to 60% (14). If we could assume a 50% cell yield from glucose, then to form the 72 mmoles of cell carbon, 72 mmoles of CO_2 would be formed also. Thus, a total of 144 mmoles of glucose carbon would be used for cell growth. The theoretical conversion efficiency for mannitol synthesis as discussed above is about 90%. Strictly for convenience the theoretical conversion of glucose to glycerol, erythritol, and glycogen is also approximated at 90%. These products were not present in any quantity, and thus the error of the approximation is not large. The percentage of glucose used to form the various fermentation products based on the above assumptions is presented in Table 3. Out of 107 mmoles of the total CO_2 collected, 91 mmoles could be accounted for by cell growth and product formation. Thus, theoretical estimation and experimental data are in close agreement. Deviation from the assumed 50% cell conversion efficiency and the presence of minor unknown carbon compounds could explain the discrepancy.

TABLE 3. *Theoretical percentage of glucose used to form the various mannitol fermentation products*

Product	Glucose used (%)
Cells	34
Mannitol	37
Glycerol	11
Erythritol	3
Glycogen	1

The mannitol fermentation with this *Aspergillus* species has some resemblance to the polyol fermentation of osmophilic yeasts. They all produce about 50% polyols, although different in kind. One such yeast, *Pichia miso*, was reported to accumulate 50% glycerol from glucose (12). Another osmophilic yeast, a *Zygosaccharomyces* species, produced 8.8% arabitol, 8.4% glycerol, and a trace of mannitol from glucose (17). Since mannitol is a common reserve material in fungi, this *Aspergillus* species produces mannitol, glycerol, and erythritol instead of arabitol and glycerol like osmophilic yeast. It would be interesting to compare the mechanism of polyol production by *Aspergillus* and the osmophilic yeasts.

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