ACTA CHEMICA SCANDINAVICA 12 (1958) 646—649

Formation of Trehalose during Dissimilation of Glucose by *Propionibacterium* *

RUNE STJERNHOLM**

Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland 6, Ohio, USA

A non-reducing carbohydrate has been isolated and purified from media and cells of three species of *Propionibacterium* fermenting glucose. This sugar was identified as trehalose after crystallization and preparation of its octaacetate derivative. Further criteria used were mixed melting points, paper chromatography and optical rotation.

A non-reducing carbohydrate has been observed in the media from fermentations of glucose by three species of *Propionibacterium* [1,2]. With proliferating cells 9 % of the fermented glucose was converted to a non-reducing sugar [1], and with resting cells [2] as much as 30 %. After the glucose had been utilized there was a subsequent conversion of the non-reducing carbohydrate to CO_2 and propionic, acetic and succinic acids [1]. The present report deals with the isolation and identification of this non-reducing sugar.

METHODS AND EXPERIMENTAL

The three species of *Propionibacterium* used in this investigation were *P. arabinosum* strain 34W, *P. shermanii* strain 52W and *P. pentosaceum* strain 49W. All fermentations were carried out anaerobically with washed cell suspensions. The bacteria had previously been carried through three transfers, grown for 4 to 5 days each, on the following medium which contained, per liter, glucose (5.0 g), yeast extract (5.0 g), K-phosphate buffer (0.5 M, pH 6.8), and 1 mg of each of the following vitamins: calcium-pantothenate, thiamine-HCl and biotine. The cells from the last transfer were collected by centrifugation and washed twice by resuspension in distilled water followed by centrifugation.

In a typical experiment 120 ml of a reaction mixture containing glucose (0.05 M), K-phosphate buffer (0.15 M, pH 7.2), and washed cells (2.5 % w/v) in a 300 ml round bottom flask was shaken and continuously gassed with nitrogen. After a variable period of time (6 to 22 h) the fermentation was stopped by the addition of sulfuric acid to pH 1, and the cells were then removed by centrifugation and washed twice with distilled water. The combined supernatant solutions (about 140 ml) were extracted with ether for 48 h

* This investigation was supported in part by a grant from the U.S. Public Health Service, No. E-1003 (C-2).

** DuPont Fellow, 1956—1958.

and the aqueous residue was then deionized by successive passage through a column containing Dowex-50 H^+ ($\times 12$, 200 mesh) and Duolite A4. The deionized solution was concentrated to 5 ml at 80° under an air jet, and placed on a celite-charcoal column [3] (30 mm × 500 mm if the total amount of carbohydrate did not exceed 300 mg). Water with an increasing concentration of ethanol was used for development of and elution from the column. The total carbohydrate in each fraction of eluate was determined by the anthrone method [4].

Table 1. Chromatography of fermentation residue on celite-charcoal.

Fraction	Eluant	Volume	Compound eluted
1	Water	100 ml	nil
2	1 % EtOH	100 »	»
3	2 » »	100 »	hexoses
4	3 » »	100 »	»
5	4 » »	100 »	nil
6	5 » »	100 »	»
7	6 » »	100 »	non-red. sugar
8	7 » »	100 »	» » »
9	8 » »	100 »	nil
10	9 » »	100 »	»
11	95 » »	500 »	»

A non-reducing sugar appeared in the fraction eluted with 6 and 7 % ethanol (Table 1). This elution behavior is typical of disaccharides [3], and when this substance was hydrolyzed with H_2SO_4 (1 M, 30 min, 100°) in a sealed tube glucose was the sole product. These preliminary data suggested that the non-reducing sugar might be trehalose [1-(α-D-glucopyranosido)-α-D-glucopyranoside].

Paper chromatography of the non-reducing material in four different solvent systems (Table 2) showed in each case a single spot, which could be detected when sprayed with reagents such as $AgNO_3$—NaOH [5] and HIO_4—KI-starch that react with non-reducing compounds [6]. The spot could not be detected when sprayed with aniline-phthalate [7] or *p*-anisidine-HCl [8] reagents which are specific for aldoses and reducing sugar in general. The R_F values of the non-reducing material were identical with that of known trehalose in the four solvent systems (Table 2).

Table 2. Paper chromatography of non-reducing compound and trehalose.

Solvent-system	R_F of non-red. compound	R_F of trehalose
Phenol-water	0.26	0.26
Collidine-water	0.28	0.28
n-BuOH-water	0.04	0.04
Ethylacetate-pyridine-water	0.09	0.09

When the non-reducing compound and known trehalose were mixed and chromatographed in each solvent system no separation took place.

Crystallization of the eluted non-reducing material from 80 % ethanol gave colorless rhombic crystals [m. p. 98°, $[\alpha]_D^{25}$ +179 (*c* 2, H_2O)]. The mixed melting point with a recrystallized commercial preparation of α,α′-trehalose-dihydrate [m. p. 98°, $[\alpha]_D^{20}$ +178 (*c* 2, H_2O)] showed no depression.

An acetate of the isolated material was prepared by reaction with excess acetic anhydride in pyridine [9] (12 h, 25°). Crystallization from 60 % ethanol gave colorless

needles [m. p. 100—102°, $[\alpha]_D^{25}$ +164.5 (*c.* 1.16, $CHCl_3$)]. The mixed melting point with a known sample of trehalose octa-acetate [9] (m. p. 100—102°, $[\alpha]_D^{20}$ +162.5, $CHCl_3$) showed no depression.

That trehalose is the only non-reducing sugar in the media was revealed from balance studies of several fermentations (Table 3). However, intracellularly a glycogen-like substance has been found in addition to trehalose.

Table 3. Balance studies on the aqueous residue after ether extraction of fermentation media.

Fermentation 1		
Total carbohydrate (anthrone method) [4]		141 mg *
Reducing sugar (Somogyi analysis) [10]		105 mg
Non-reducing sugar (calc.)		36 mg
Individual fractions from charcoal-celite column (anthrone method)	hexoses	118 mg
	trehalose	29 mg
Fermentation 2		
Total carbohydrate (anthrone method)		190 mg
Individual fractions from charcoal-celite column (anthrone method)	hexoses	129 mg
	trehalose	70 mg

* All values calculated as glucose.

Three species of *Propionibacterium* were analyzed for their cellular content of trehalose (Table 4). Cells of 34W, 52W and 49W, grown for two days on the above described glucose, yeast extract, phosphate and vitamin medium, were centrifuged, washed twice with water, lyophilized, and then treated according to the method given by Myrbäck [11] and Stewart *et al.*[12] for the isolation of trehalose from baker's yeast. By paper chromatographic assay (*cf.* Table 2), these preparations were found to contain trehalose and no other carbohydrates. Anthrone analysis of these fractions gave the trehalose content of the three species as shown in Table 4.

Table 4. Anthrone analysis of the trehalose content in the cells of *Propionibacterium*.

34W	5.3 %	trehalose (as glucose) *
52W	1.9 %	trehalose (as glucose) *
49W	0.7 %	trehalose (as glucose) *

* Calculated on dry weight of cells.

DISCUSSION

The findings of trehalose inside the cells of three species of *Propionibacterium*, and the production of the disaccharide in their media, may indicate that the presence of this sugar is characteristic of the entire genus.

Trehalose has earlier been identified or isolated from human tubercle bacilli [13], *Mycobacterium phlei* [14], mushrooms [15], brewer's yeast [16], lichens [17], algae [18] and insects [19]. The enzymatic synthesis of trehalose has been postulated to proceed *via* trehalose-6-phosphate, formed from UDPG and glucose-6-phosphate [20].

Trehalose samples, obtained from fermentation of glucose-1-^{14}C, -2-^{14}C, 3,4-^{14}C and -6-^{14}C by *P. arabinosum* and *P. shermanii*, after hydrolysis to glucose have been degraded by the method of Bernstein *et al.*[21], to determine the

isotopic distribution of the individual carbon atoms in order to study the mechanism of trehalose formation in these organisms. The results of these investigations will be published elsewhere.

Acknowledgement. The author wishes to express his sincere appreciation to Dr. H. G. Wood for suggesting this problem, and for helpful discussion during the progress of the work and the preparation of the manuscript.

REFERENCES

1. Wood, H. G. and Werkman, C. H. *J. Biol. Chem.* **105** (1934) 63.
2. Wood, H. G. and Werkman, C. H. *Biochem. J.* **34** (1940) 129.
3. Whistler, R. L. and Durso, D. F. *J. Am. Chem. Soc.* **72** (1950) 677.
4. Koehler, L. M. *Anal. Chem.* **24** (1952) 1576.
5. Trevelyan, W. E., Proctor, D. P. and Harrison, J. S. *Nature* **166** (1950) 444.
6. Metzenberg, R. L. and Mitchell, H. K. *J. Am. Chem. Soc.* **76** (1954) 4187.
7. Partridge, S. M. *Biochem. J.* **42** (1948) 238.
8. Hough, L., Jones, J. K. N. and Wadman, W. H. *J. Chem. Soc.* (1950) 1702.
9. Pangborn, M. C. and Anderson, R. J. *J. Am. Chem. Soc.* **58** (1936) 105.
10. Somogyi, M. *J. Biol. Chem.* **160** (1945) 61; Nelson, N. *Ibid.* **153** (1944) 375.
11. Myrbäck, K. *Ergeb. Enzymforsch.* **10** (1949) 68.
12. Stewart, L. C., Richtmyer, N. K. and Hudson, C. S. *J. Am. Chem. Soc.* **71** (1949) 2277.
13. Pangborn, M. C. and Anderson, R. J. *J. Biol. Chem.* **101** (1933) 105.
14. Anderson, R. J. and Newman, M. S. *Ibid.* **101** (1933) 499.
15. Inagaki, S. and Toki, M. *J. Pharm. Soc. Japan* **64** (1944) 132.
16. Elander, M. and Myrbäck, K. *Arch. Biochem.* **21** (1949) 249.
17. Lindberg, B. and Wickberg, B. *Acta Chem. Scand.* **7** (1953) 140.
18. Payen, J. *Compt. rend.* **236** (1953) 1811.
19. Wyatt, G. R. and Kalf, G. F. *J. Gen. Physiol.* **40** (1957) 833.
20. Leloir, L. F. and Cabib, E. *J. Am. Chem. Soc.* **75** (1953) 5445.
21. Bernstein, I. A., Lentz, K., Malm, M., Schambye, P. and Wood, H. G. *J. Biol. Chem.* **215** (1955) 137.

Received January 13, 1958.