Carbohydrate Metabolism in Streptomycetes[1]

II. Isolation and Enzymatic Synthesis of Trehalose

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A number of streptomycetes were examined for their ability to synthesize trehalose phosphate as well as for the presence of α,α-trehalose. In each case, an enzyme system was demonstrated which catalyzed the transfer of glucose from guanosine diphosphate-glucose to glucose-6-phosphate to form trehalose phosphate. Thus, this group of organisms appears to synthesize trehalose phosphate by a different mechanism from that described in insects, yeast, and fungi. In addition, trehalose was isolated from each of these organisms. In several of these cases, crystallization of the sugar and determination of the physical properties showed that the sugar was α,α-trehalose.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is widespread in nature, being found in yeast, fungi, bacteria, plants, and invertebrates. In some organisms such as yeast, it is present in extremely high concentrations; Tanret (18) obtained up to 20 g of α,α-trehalose per kg of dried, pressed yeast.

In 1958, Cabib and Leloir (2) showed that the synthesis of trehalose phosphate in yeast occurred rom uridine diphosphate D-glucose (UDPG) and glucose-6-phosphate (glucose-6-P). Similar enzyme systems involving UDPG and glucose-6-P have been described in the insect fat body (3), silk moth (10), *Mycobacterium tuberculosis* (7), and the slime mold *Dictyostelium discoideum* (16).

Recently, we described the isolation of a new enzyme system from *Streptomyces hygroscopicus* which catalyzed the synthesis of trehalose phosphate from guanosine diphosphate D-glucose (GDPG) and glucose-6-P (5). This enzyme system (GDP glucose: D-glucose-6-phosphate 1-glucosyl transferase) appears to be widespread among the streptomycetes and has now been demonstrated in a number of these organisms, including *S. aureofaciens*, *S. bikiniensis*, *S. griseus*, and *S. rimosus*. Further, trehalose has been isolated from many organisms of this genus. Crystallization of the sugars in several of these cases followed by determination of the

physical properties proves that the sugar is α,α-trehalose.

In this communication, the isolation and characterization of α,α-trehalose from a number of different streptomycetes is described. In addition, the enzyme system catalyzing the synthesis of trehalose phosphate from GDPG and glucose-6-P (GDP glucose: D-glucose-6-phosphate 1-glucosyl transferase) is shown to be present in a number of streptomycetes. The purification and properties of this enzyme will be the subject of a future communication.

MATERIALS AND METHODS

Culture conditions. Cultures of the various streptomycetes were obtained from the American Type Culture Collection or were kindly supplied by G. Mallett, Eli Lilly & Co., Indianapolis, Ind. Oganisms were maintained and grown as previously described (6). After growth for 2 to 8 days on a rotary shaker, cells were harvested by filtration and were well washed with distilled water. The cell paste was then stored at −20 C until used. Cells were then extracted and the enzyme was isolated as previously described (5).

Chromatography. Descending paper chromatography was done with either Whatman no. 1 or Whatman 3 MM paper. Solvent systems were as follows: (1) propan-1-ol-ethyl acetate-water (7:1:2); (2) butan-1-ol-pyridine-water (6:4:3); (3) ethyl acetate-acetic acid-water (3:1:1); (4) butan-1-ol acetic acid-water (3:1:3); (5) butan-1-ol-pyridine-0.1 N hydrochloric acid (5:3:2); (6) methanol-formic acid-water (16:3:1) (7) methanol-ammonium hydroxide-water (6:1:3). Trehalose was detected on paper chromatograms by the alkaline silver nitrate reagent of Trevelyan et al. (18); after dipping through alkali, the papers were allowed to hang for 5 to 10 min before decolorizing, since the color given by trehalose develops very slowly.

Assay of the enzyme. The enzyme involved in the synthesis of trehalose phosphate could be determined either by a radioactive assay or by a colorimetric method (5). In the former case, 0.3 μmole of GDP-glucose-[14]C [or UDP-glucose-^{14}C, both prepared by a modification (4) of the method of Roseman et al. (15)] was incubated with 0.5 μmole of glucose-6-P and 0.5 μmole of MgCl₂ in the presence of 2 μmoles of phosphate buffer (*p*H 7.0) and the appropriate amount of enzyme. Incubations were at 37 C for 30 min. At the end of the incubation, HCl was added to a final concentration of 0.1 N, and tubes were heated at 100 C for 10 min to destroy any remaining sugar nucleotide. The complete reaction mixture was then streaked on Whatman 3 MM paper and was subjected to paper electrophoresis in 0.2 M ammonium formate buffer (*p*H 3.6). The area of the paper corresponding to trehalose phosphate was cut out and counted in a liquid scintillation spectrometer. In each case, the trehalose phosphate was further characterized as described in Results.

The formation of trehalose phosphate was also determined colorimetrically as described by Cabib and Leloir (2). Incubation mixtures contained 0.3 μmole of unlabeled GDPG, 0.5 μmole of glucose-6-P, 0.5 μmole of MgCl₂, 2 μmoles of phosphate buffer (*p*H 7.0), and the appropriate amount of enzyme (usually 0.1 ml of crude extract containing 500 to 700 μg of protein). After incubation at 37 C for 15 min, HCl was added to a final concentration of 0.1 N, and the tubes were heated at 100 C for 10 min to destroy any remaining sugar nucleotide. The tubes were cooled and NaOH was added to a final concentration of 0.1 N. Again the tubes were heated at 100 C for 10 min. This treatment destroyed sugars with free carbonyl groups, such as glucose and glucose-6-P. Trehalose phosphate could then be assayed by the anthrone procedure (9). This method will also determine free trehalose. Thus, organisms having phosphatases capable of cleaving trehalose phosphate to trehalose would also be determined.

Specific activity is reported as units per milligram of protein. One unit is that amount of enzyme which causes an optical density change of 0.1 at 620 mμ in 15 min (about 0.025 μmole of trehalose phosphate).

Isolation of trehalose. Trehalose was extracted from the cells with 70% ethyl alcohol (18). A 1-g amount of cell paste was suspended in 10 ml of ethyl alcohol, and the mixture was brought to a boil. The mixture was cooled and the precipitate was removed by centrifugation. The supernatant liquid was concentrated to remove ethyl alcohol, was treated with mixed-bed ion-exchange resin (equal parts of Dowex-50-H⁺ and Dowex-1-CO₃⁻²), and was again concentrated in vacuo. The extracts were then assayed for their content of trehalose as described below.

The extracts were then streaked on Whatman 3 MM paper and developed in solvent 1. The trehalose area of the papers was cut out, eluted, and rechromatographed on Whatman no. 1 paper in solvent 5. Again the trehalose area of the papers was cut out and eluted, and the amount of trehalose was determined.

Determination of trehalose. Trehalose was assayed as described by Cabib and Leloir (2). Appropriate samples (either before or after chromatography) were heated in 0.2 N NaOH at 100 C for 10 min. This treatment destroyed sugars with free carbonyl groups. After this procedure, trehalose was assayed by the anthrone method.

Crystallization of trehalose. In several cases (*S. aureofaciens, S. rimosus* and *S. hygroscopicus*), trehalose was isolated in amounts large enough to be crystallized. The sugar was crystallized in each case from aqueous ethyl alcohol and then was recrystallized several times from this solvent. Although trehalose crystallized as the dihydrate, the samples used for polarimetric studies were dried for 24 hr over P₂O₅ and probably represented the anhydrous sugars. Therefore, values were corrected for two molecules of water. In the case of trehalose isolated from *S. hygroscopicus*, the octaacetate derivative was also prepared (8).

Analytical methods. Total hexose was determined by the anthrone procedure (9), D-glucose with Glucostat (Worthington Biochemical Corp., Freehold, N.J.), protein by the method of Sutherland et al. (17) or by its absorption at 280 and 260 mμ, and reducing sugar by the method of Park and Johnson (14).

RESULTS AND DISCUSSION

The enzyme GDP glucose:D-glucose 6-phosphate 1-glucosyl transferase was demonstrated in extracts of a number of *Streptomyces* species, including two different strains of *S. hygroscopicus*, *S. aureofaciens*, *S. bikiniensis*, *S. griseus*, and *S. rimosus* (Table 1). In all of these cases, the reaction was specific for GDPG as the glucosyl donor; no formation of trehalose phosphate could be demonstrated when UDPG (^{14}C-UDPG) and glucose-6-P were incubated with these crude extracts. The slight activity found in the trehalose phosphate area of the electrophoretograms from ^{14}C-UDPG was due to contamination of this area by UDPG or its breakdown products, since this radioactivity did not correspond to trehalose phosphate when subjected to chromatography or to a second electrophoresis. However, the formation of trehalose phosphate from GDPG was easily demonstrated by use of either the radioactive or the colorimetric assay (Table 1). The specific activity of GDP glucose:D-glucose 6-phosphate 1-glucosyl transferase varied from 0.03 for *S. aureofaciens* to 2.2 for *S. hygroscopicus*. These results suggest that this enzyme system is fairly widespread among members of the genus *Streptomyces*, indicating that these organisms synthesize trehalose phosphate by a different mechanism than that previously described for other organisms. In all other cases examined thus far, the synthesis of trehalose phosphate occurred from UDPG and glucose-6-P. Thus, Cabib and Leloir (2) first demonstrated this reaction in yeast. Later, Candy and Kilby (3) and Murphy and Wyatt

TABLE 1. *Distribution of GDP-glucose-glucose-6-phosphate 1-glucosyl transferase*[a]

Crude extract	Counts/min in trehalose-P		Specific activity (colorimetric) from GDPG
	GDPG	UDPG	
Streptomyces hygroscopicus ATCC 10976......	8,470	225	1.0
S. hygroscopicus NRRLC 330......	11,127		2.2
S. rimosus NRRL 2234..............	5,322	14	1.1
S. griseus ATCC 10137.............	8,200	117	
S. bikiniensis........	8,000	117	0.8
S. aureofaciens NRRL 2209.......	734	80	0.03

[a] For the radioactive assay, incubation mixtures were as described in the text and contained either 15,000 counts of ¹⁴C-GDPG per min or 25,000 counts of ¹⁴C-UDPG per min and 0.1 ml of crude extract (500 to 700 μg of protein). For the colorimetric assay, incubations were as described in the text with unlabeled GDPG. After incubation, trehalose phosphate formation was determined as described. Specific activity is defined as an increase in optical density at 620 mμ (anthrone reagent) of 0.1 in 15 min (about 0.025 μmole of trehalose phosphate). Specific activity involving use of UDPG is not indicated, since this nucleotide was inactive as a glucosyl donor in this reaction.

(10) showed a similar reaction in insects, and recently Roth and Sussman (16) have shown its occurrence in the slime mold *D. discoideum* (17). The synthesis of trehalose phosphate has been shown in one other actinomycete, *M. tuberculosis*, and UDPG was also the glucosyl donor (7). Although the sugar nucleotide specificity was not examined in this case or in those others cited here, the fact that UDPG served as the glucosyl donor indicates a mechanism different from that described in the streptomycetes (5).

With each of the streptomycetes listed in Table 1, the product of the reaction of ¹⁴C-GDPG and glucose-6-P was further identified as trehalose phosphate as follows. The radioactive trehalose phosphate area of the electrophoretogram was eluted and further purified by chromatography in solvent 6 or 7. In both solvents, trehalose phosphate moves more slowly than either glucose-6-P or glucose-1-P ($R_{glucose-6-P}$ = 0.80 to 0.90). The radioactive material from the paper chromatograms having the same R_F as trehalose phosphate was eluted and treated with alkaline phosphatase. The neutral sugar resulting

from this treatment was shown to be trehalose by paper chromatography in solvents 1 and 5. The trehalose phosphate synthesized from GDPG and glucose-6-P by *S. hygroscopicus* was previously characterized by these as well as other means (5).

Trehalose was also isolated from a number of streptomycetes (Table 2). After extraction and deionization, the sugars were isolated by preparative paper chromatography. In each case, in addition to glucose, trehalose was either the only other sugar or the major one observed on the chromatograms. Trehalose was further characterized by paper chromatography (Table 2). Each of the sugars isolated from the various streptomycetes exhibited the same mobility as authentic trehalose in five different solvent systems. Further, the sugars reacted at the same slow rate with silver nitrate as did authentic trehalose. Each sugar was hydrolyzed in 3 N HCl at 100 C for 60 min, and the amount of glucose was determined by use of glucose oxidase. Approximately 2 moles of glucose was found for each mole of trehalose. In addition, it was possible to crystallize the sugar from several of these streptomycetes, and the crystalline samples exhibited the same physical properties as those of authentic trehalose (Table 3), indicating that in these cases the sugar was indeed α,α-trehalose.

Although trehalose has been isolated from a number of different organisms, including such microorganisms as yeast, fungi, and bacteria (18), this sugar has never been reported in streptomycetes. However, a glycolipid derivative of α,α-trehalose which was characterized as α,α-

TABLE 2. *Chromatographic identification of sugar from various streptomycetes*

Sugar isolated from	$R_{trehalose}$ in solvent[a]				
	1	2	3	4	5
Streptomyces antibioticus ATCC 8663...	1.03	1.07	1.03	1.02	1.03
S. aureofaciens NRRL 2209...	0.97	1.00	0.96	0.98	1.00
S. bikiniensis....	1.00	1.04	0.98	1.00	1.00
S. griseus ATCC 10137..	0.97	1.04	0.98	0.97	0.98
S. hygroscopicus ATCC 10976..	0.97	1.04	0.98	0.97	0.98
S. rimosus NRRL 2234...	0.97	1.00	0.95	0.97	1.01
Trehalose.......	1.00	1.00	1.00	1.00	1.00

[a] See Materials and Methods for composition of the solvents.

trehalose 6,6'dimycolate was found in mycobacteria (11, 13). Further, a phosphorylated form of this sugar, α,α-trehalose 6,6'diphosphate has been isolated from *M. tuberculosis*, in which it occurs as a phosphorylated polysaccharide (12). As already indicated, in the streptomycetes, trehalose is found as the free sugar, although small amounts are found as the monophosphate. However, Arcamone and Bizioli (1) isolated α,α-trehalosamine from the culture broth of a *Streptomyces* species. Interestingly enough, this compound acted as an antibiotic, probably by inhibiting mycobacterial trehalase.

Table 4 lists the relative amounts of trehalose found in each of the streptomycetes examined to date. Trehalose was determined in crude extracts as well as after isolation by paper chromatography. The results obtained by these two procedures are in fairly good agreement. The concentration of trehalose varied from 0:25 μmole per g of cell paste with *S. griseus* to 20.3 μmoles per g of cell paste in the case of *S. rimosus*. *S. hygroscopicus*, the organism used for the purification of GDP glucose:D-glucose 6-phosphate 1-glucosyl transferase, has a fairly high level of trehalose, about 10 μmoles per g of cell paste. Although *S. hygroscopicus* and *S. rimosus* have fairly high levels of GDP glucose:D-glucose 6-phosphate 1-glucosyl transferase and also contain large amounts of trehalose, very low levels of this enzyme were detected in *S. aureofaciens*, an organism which contains large amounts of endogenous trehalose. Thus, these results may reflect on the levels of several other enzymes involved in the synthesis and degradation of trehalose, namely GDP glucose pyro-

TABLE 4. *Quantity of trehalose in various streptomycetes*

Organism	Amt (μmoles) of trehalose/g of cell paste	
	Crude extract	After isolation
Streptomyces antibioticus ATCC 8663..............	0.55	0.36
S. aureofaciens NRRL 2209.....................	22.1	17.5
S. bikiniensis.............	0.91	0.56
S. griseus ATCC 10137.....	0.45	0.25
S. hygroscopicus ATCC 10976...................	10.7	10.0
S. lavendulae ATCC 8664...	22.5	17.8
S. rimosus NRRL 2234.....	22.9	20.3

phosphorylase, trehalose phosphatase, and trehalase. The last two enzymes have been detected in extracts of *S. hygroscopicus*, but their specificity is not known at the present time (A. Hey and A. D. Elbein, *unpublished data*). Experiments are now in progress to determine the levels of these enzymes at different times in the growth of *S. hygroscopicus*.

TABLE 3. *Physical properties of trehalose isolated from various Streptomyces species*

Organism	Melting point (C)	$[\alpha]_D$ (degrees)[a]	$[\alpha]_D$(degrees) for octaacetate
S. hygroscopicus ATCC 10976....	94–95	176.4 (194.8)	157
S. aureofaciens NRRL 2209....	93	173.8 (192.1)	
S. rimosus NRRL 2234....	97	175.3 (192.7)	
Authentic α,α-trehalose.......	95	178	163

[a] Values in parentheses are those actually observed for anhydrous samples (dried for 24 hr over P_2O_5). Values are corrected for trehalose dihydrate since reported rotations are for the dihydrate.

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