Trehalose Synthesis during Differentiation in
Dictyostelium discoideum

II: IN VIVO FLUX DETERMINATIONS*

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SUMMARY

The synthesis of [14C]trehalose has been studied in vivo at various stages of differentiation in Dictyostelium discoideum. The rate of synthesis was negligible from aggregation until late in the culmination process. At that time, a 100-fold burst of synthetic activity was observed, amounting to 0.04 μmole of trehalose per min per ml of packed cells. In the course of these studies radioactive maltose was also identified as a product of [14C]glucose metabolism.

Trehalose is known to accumulate during differentiation in Dictyostelium discoideum (1, 2). In the accompanying paper (3), kinetic models are presented which simulate the accumulation of this end product by various mechanisms. These models predict that trehalose β-phosphate synthetase could not increase in vivo during the period in which in vitro analyses indicate that this synthetic enzyme does accumulate (i.e. prior to 800 min), and that the rate of trehalose synthesis in vivo should be of the order of 0.03 μmole per min per ml of packed cells. In the present study, cells at various stages of differentiation were exposed to [14C]glucose. At 10-min intervals, cell samples were used for the isolation of trehalose, uridine diphosphate [14C]glucose, and [14C]glucose 6-phosphate. The rate of trehalose synthesis was determined by relating the increase with time in radioactivity of the trehalose pool to the specific radioactivity of UDP[14C]glucose and [14C]glucose-6-P. The data obtained substantiated predictions of the kinetic models.

EXPERIMENTAL PROCEDURE

Materials

Maltase was obtained from Koch-Light Laboratories, Ltd. (Colnbrook, Bucks, England); glucose 6-phosphate dehydrogenase and phosphoglucone isomerase were obtained from Calbiochem; 2-(N-morpholino)ethane sulfonic acid, alkaline phosphatase type III from Escherichia coli, and glucose 6-phosphate, disodium salt, were from Sigma. [14C]Glucose (uniformly labeled) was purchased from International Chemical and Nuclear Corporation, Kieselguhr G (according to Stahl) was from Brinkman Instruments, and No-Screen medical x-ray film was from New England X-ray and Electronic Equipment Company. Purified trehalase was the generous gift of Dr. Alan Elbein and Dr. Bertram Sacktor. Trehalose 6-phosphate, barium salt, was the kind gift of Dr. D. L. MacDonald. Prior to use it was converted to the sodium salt.

Methods

Preparation of Cells

D. discoideum strain NC-4 was grown according to the method of Liddel and Wright (4) except that the amoebeae were respread onto cookie sheets containing 2% agar, 0.01 M phosphate buffer (pH 6.5) and 0.001 M EDTA. The cells were allowed to recover at 23°C for 2 hours and then were put at 15°C overnight. Several hours before the desired stage of differentiation was reached, the cells were returned to 23°C, at which temperature the subsequent in vivo experiments were performed.

Labeling Conditions

The cells were harvested in Bonner’s salt solution in 10^{-3} M MES-NaOH, pH 6.5 (Bonner’s MES), such that 1 ml of cell suspension contained 0.1 ml of packed cells. Three milliliters of the suspension were placed into 40-ml plastic round bottom centrifuge tubes and incubated in the presence of 0.02 mM [14C]glucose (1.8 × 10^6 dpm per μmole) for 10 to 40 min on a Fisher rotator (setting of 205). Separate series of tubes were used for the isolation of UDP-glucose, trehalose, and glucose-6-P.

Thin Layer Plates

Kieselguhr G Plates for Trehalose Isolation—A slurry containing 20 g of Kieselguhr G plus 40 ml of 0.1 M phosphate, pH 6.0, was spread onto glass plates (20 × 20 cm) to a thickness of 250 μm. Samples containing trehalose were streaked across the plates 2 cm from the edge of the plate and run ascending in 1-butanol-acetone-0.1 M phosphate, pH 6.0 (4:5:1, v/v), to 14

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with 80% methanol and rinsed, the extract was evaporated to dryness, and the residue was dissolved in 1 ml of distilled water. The cell debris was centrifuged (14,500 \text{x} g, after which the supernatant solution was aspirated and the cells were extracted with 80% methanol for 30 to 60 min at room temperature (23°). It routinely took 6 min from dilution until the cells were extracted. Methanol was found to extract as much or more trehalose than treatment with 7% perchloric acid, chloroform-methanol (3:1, \text{v/v}) at 45°, acetone at 45°, or 80% ethanol. Heating the cells to 100° in the presence of 80% methanol did not improve the extraction. The cell debris was centrifuged (14,500 \text{x} g for 10 min) and washed once with 80% methanol, and the supernatant solution plus wash was evaporated to dryness. The dried extract was dissolved in 1 ml of distilled water and centrifuged to remove some of the lipid, and the supernatant solution was streaked across the origin on a Kieselguhr G thin layer plate. The lipid precipitate was washed once with 0.9 ml of distilled water and the wash was streaked over the origin. The plates were run three times in the solvent system described, and then put with x-ray film for 7 to 10 days. Following the development of the films the trehalose bands were scraped and either counted on x-ray film for 7 to 10 days, and the radioactive glucose-6-P area was cut out, eluted with distilled water, concentrated, and run two dimensions on unbound cellulose plates. (See "Thin Layer Plates"). The plates were then put with x-ray film for 10 to 12 days, and the radioactive glucose-6-P area was scraped off and eluted.

The specific radioactivity was obtained by comparing the radioactivity of the spot with the micromoles of glucose-6-P present as determined by assay with glucose-6-P dehydrogenase. Phosphogluconic dehydrogenase was found not to be present in the glucose-6-P dehydrogenase preparation. Contamination with fructose 6-phosphate, which runs close to glucose-6-P in these solvents, was excluded since addition of phosphogluconate isomerase to the completed reaction mixture gave no further increase in glucose 6-P conversion. Glucose 1-phosphate is present at one-sixth the concentration of glucose-6-P and would represent a minor contamination (with a comparable specific radioactivity).

**Preparation of Extracts for Trehalose 6-P Determination**

Cells at preculture were harvested in cold-distilled water and six 5 ml aliquots (packed cell volume of 0.50 ml/5 ml) were taken. The cells were either frozen and thawed before further treatment or were immediately treated as follows. Three aliquots were heated 10 min at 100°, after which 0.25 mg of trehalose-6-P or 0.18 mg of glucose-6-P was added to one tube to serve as an internal control. The precipitate was centrifuged and washed with distilled water, and the supernatant solution and wash were combined and evaporated to dryness. The remaining three aliquots were extracted for 30 min at 23° in the presence of 2 volumes of 95% ethanol (8). Again, trehalose-6-P or glucose-6-P was added to one tube as an internal control. Following extraction the cell debris was centrifuged, the precipitate was washed with 70% ethanol, and the supernatant solutions were evaporated to dryness.

**Alkaline Phosphatase Assay**

Both the extract alone and the extract plus glucose-6-P or trehalose-6-P were treated with alkaline phosphatase in the presence or absence of MgCl₂ as follows. To the dried extracts were added 0.008 mmole of Tris HCl (pH 8.0), 0.02 mmole of MgCl₂ when present, and 0.1 to 0.2 mg of alkaline phosphatase in a total volume of 1 ml. To determine the amount of trehalose present in the cells before alkaline phosphatase treatment, an identical tube containing extract was incubated at the same time in the absence of the enzyme. Following incubation at 25° for 4.5 hours the reaction was stopped by boiling briefly (approximately 2 min), the samples were either centrifuged and the supernatant solution chromatographed directly, or the samples were extracted with 2 volumes of 95% ethanol, the precipitate was washed, and the supernatant solution and wash were combined and evaporated to dryness. The sample was then dissolved in distilled water and equal aliquots were spotted with and without trehalose controls on Kieselguhr G plates and run in 1-butanol-acetone-6.1 M PO₄, pH 6 (see "Methods"). The intensity of the trehalose region was examined on plates following the detection of sugars by napthoresorcinol spray (9).

When MgCl₂ was present in the enzyme assay the samples were deasitized prior to chromatography by adsorbing the sugars on 1 g of a 1:2 (w/w) charcoal-Celite mixture and subsequently eluting the sugars with 5% 1-propanol. The eluates were then evaporated to dryness and treated as above.
Fig. 1 (upper left). Identification of trehalose in methanol extracts of young sorocarps (about 22 hours). X-ray film of thin layer plate over which is superimposed (hatched areas) the location of known sugars revealed by spraying the plate with naphthoresorcinol reagent. The same amount of extract was added at the origin of each strip. In addition, the following known sugars were added: 2, maltose; 3, trehalose; 4, extract alone; 5, cellotriose and cellotetrose (near origin). Glucose was added to each (1 to 6). See “Methods” for extraction and thin layer chromatography.

Fig. 2 (upper right). Thin layer chromatography of acetates prepared from an 80% ethanol extract of young sorocarp (~22 hours). Column 2 is extract alone; 3, extract plus maltose octaacetate. Acetate standards are in Columns 1 and 4. The area just above trehalose in Columns 2 and 3 did not give the characteristic sugar reaction with naphthoresorcinol spray and hence is not celllobiose. In fact, celllobiose has never been detected in extracts.

Fig. 3, A and B (lower). X-ray films showing the separation of trehalose from other sugars at two stages of differentiation. A, preceliumination (about 18 hours); B, young sorocarp (about 22 hours). The bands are as follows: 1, glucose; 2, maltose or celllobiose or both; 3, trehalose; 4 and 5, unknown.
Calculation of Rate of Trehalose Synthesis

The rate of \([^{14}\text{C}]\text{trehalose}\) synthesis was determined from the specific radioactivity of its precursors, \([^{14}\text{C}]\text{UDP-glucose}\) and \([^{14}\text{C}]\text{glucose-6-P}\). Since the glucose-6-P pool is relatively small and difficult to isolate, it was not feasible to determine the specific activity of glucose-6-P with every experiment. However, in two experiments performed at late culmination, the specific radioactivity of glucose-6-P was found to be 50% and 66% of the UDP-glucose specific radioactivity. Therefore, the specific radioactivity of the precursors was routinely taken as 100% of the UDP-glucose specific radioactivity. The rate of trehalose synthesis could then be expressed as an increase with time in micromoles of glucose incorporated per min per ml of packed cells. The per cent of the packed cell volume representing cells (6, 10) at late culmination (20 to 22 hours) was found to be about 50%.

Trehalase and Maltase Assays

Trehalase analyses were carried out in a volume of 0.5 ml containing 15 pmoles of potassium phosphate (pH 6.3), 7.5 pmoles of trehalose, and 0.02 ml of trehalase. One tube was boiled immediately as a control and the other was incubated for 18 hours at 23°C under 1 drop of toluene. Maltase analyses were carried out in a volume of 2 ml containing 1.25 pmoles of maltose, 8 pmoles of potassium phosphate (pH 6.9), and 4.0 mg of maltase. Again, one sample was boiled immediately and the other was incubated at 30°C for 3 hours. (A separate maltose control was included.) Glucose produced in these assays was separated from the disaccharides by paper chromatography (descending 24 hours in 1-butanol-ethanol-acetone-water, 5:4:3:2, v/v) and the counts per min and micromoles of sugar were determined. Cellobiose was not active as a substrate in these assays.

Other Methods

The phenol-sulfuric acid method of Dubois et al. (11) was used for sugar determinations. Octaacetates of the sugars were prepared according to the method of Wickberg (12), except that 1 ml each of acetic anhydride and pyridine was used. The acetates were spotted on silica gel plates (13) and run up to 10 cm (two or three times) in 5% ethanol in benzene. The acetates were detected by spraying the plates with naphthoresorcinol reagent.

RESULTS

Trehalose Identification—Fig. 1 shows the presence of radioactive trehalose and other sugars in an extract prepared after exposure of sorocarp cells to \([^{14}\text{C}]\text{glucose}\). Maximum trehalose accumulation occurs in the terminal stages of differentiation, as reported previously (2). Authentic glucose, trehalose, maltose, cellotriose, and cellotetraose were used as standards, and detected by spraying with the naphthoresorcinol reagent (hatched areas). Radioactive compounds were detected by x-ray film (dark areas). Since maltose and cellobiose have the same \(R_f\) value in this solvent system, the extract appears to contain trehalose, maltose.

![Graph](http://www.jbc.org/)
or cellobiose (or both), and some unidentified sugars. Further investigations including specific enzyme analyses revealed the presence of both radioactive trehalose and maltose. The presence of both disaccharides was further established by preparation of the octaacetate derivatives and cochromatography with authentic trehalose and maltose octaacetates (see Fig. 2).

**Trehalose 6-Phosphate**—There appeared to be no accumulation of trehalose-6-P in *vivo* at early or late prec culmination as evidenced by the lack of an increase in the amount of trehalose following alkaline phosphatase treatment of the extract. The enzyme was active on added glucose-6-P or trehalose-6-P in the presence of extract. It appears then that trehalose-6-P is a short lived intermediate in the synthesis of trehalose. Operti and Panek (14) were likewise unable to find any accumulation of trehalose-6-P in *vivo* in yeast cells under aerobic conditions, although trehalose readily accumulated.

**Rate of Trehalose Synthesis**—Fig. 3A shows that very little radioactive trehalose accumulates in 18-hour precultivating cells as compared to those in late culmination or early sorocarp 4 hours later (Fig. 3B). The rate of trehalose synthesis was determined from the increase with time in radioactivity, and from the specific radioactivity of cellular [14C]UDP-glucose and [14C]glucose-6-P (see “Methods”). Over the time period studied, the rate of incorporation of [14C]glucose into trehalose was linear, while UDP-glucose maintained a relatively constant specific radioactivity (Fig. 4). The specific radioactivity of UDP[14C]glucose was always one to two orders of magnitude higher than that of trehalose. The rate of trehalose synthesis was determined between aggregation and late culmination; determinations could not be made at later stages because of the drop in level of the two precursors. Although trehalose is present at all stages of differentiation, its rate of turnover is insignificant prior to late culmination. At that time, as indicated in Fig. 5, the rate of synthesis increases about 100-fold. This observation correlates well with the accumulation pattern of the trehalose and with the predictions of kinetic models simulating this system (see accompanying paper (3)).

**DISCUSSION**

This study emphasizes the potential pitfalls of extrapolating from *in vitro* data to changes in the specific activity of an enzyme to conditions in the living cell. In the case of trehalose synthesis, the maximum specific activity measured *in vitro* is about 0.01 of that which must be present *in vivo*; furthermore, the enzyme becomes active *in vivo* during differentiation only after *in vitro* analyses suggest that it begins to decline in activity. In the case of trehalase, the decrease *in vitro* in specific activity which is observed (15) cannot apply *in vivo* since, although trehalase is present, its rate of synthesis does not decrease but is extremely low and constant during this period. This lack of correlation in enzyme behavior *in vitro* and *in vivo* has been observed in many other cases (16). Changes in the specific activity of an enzyme during differentiation, with or without data concerning the effect of inhibitors of differentiation and enzyme activity, provide little information relevant to the control of metabolic activity *in vivo*. In other systems it has also been observed that enzyme profiles are a poor indication of the metabolic state or of the underlying control mechanism involved (17-19). As shown in the accompanying paper (3), kinetic models can serve effectively as a means of evaluating the applicability of *in vitro* data to metabolism in the differentiating cell.

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