SUGAR TRANSPORT IN NEUROSPORA CRASSA. III. AN INOSITOL REQUIREMENT FOR THE FUNCTION OF THE GLUCOSE ACTIVE TRANSPORT SYSTEM*

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SUMMARY

Germination of conidia of an inositol-requiring strain of Neurospora crassa on fructose plus inositol leads to the appearance of a previously described glucose active transport system. Removal of inositol from the growth medium leads to a decline in the activity of the system. Transport activity is rapidly restored upon addition of inositol to the culture. The inositol taken up by the cells during the restoration period is recovered largely as chloroform-methanol extractable material. It appears likely, therefore, that a lipid derivative of inositol is a necessary cofactor for the function of the glucose active transport system in cells of Neurospora crassa.

INTRODUCTION

The nature of the role of lipids in cellular processes is one of the oldest problems of modern biology. Recently, experimental systems have been developed to explore this problem (1-3) but information thus far is largely confined to bacteria. The eucaryote, Neurospora crassa, is a valuable experimental organism for studying this problem since, unlike bacteria, the phospholipid composition of Neurospora is quite similar to that of mammals, and since a variety of well-characterized lipid biosynthetic mutants of Neurospora are available. Previous communications from this laboratory have described two glucose permeases in germinated conidia of Neurospora (4,5). One, a facilitated diffusion system which is present when cells are grown on high levels of glucose; the other, a glucose active transport system which appears when cells are grown on

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fructose or limiting amounts of glucose. The experiments reported here demonstrate an inositol requirement for the function of the glucose active transport system. These studies are part of an overall plan to probe the roles of various classes of lipids in the structure and function of membrane systems in Neurospora using the biochemical-genetic approach.

MATERIALS

^{14}C-Sorbose was obtained from Amersham-Searle, ^{12}C-sorbose was from Pfanstiehl. ^{3}H-Inositol was purchased from New England Nuclear. Non-radioactive inositol and firefly lanterns came from Sigma. Neurospora crassa Strain 37102A, the inositol requiring mutant used in these studies, was obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire. This strain is allelic with strain 89601 (6) which has been shown to be lacking the enzyme which catalyzes the synthesis of myo-inositol phosphate from glucose-6-phosphate in the presence of oxidized NAD and Mg^{++} (7).

METHODS

Inositol Deprivation. Conidia were obtained from 8-10 day agar cultures (supplemented with 1.5% sucrose and 0.01% inositol) as previously described (4). Conidia thus obtained (2-4 x 10^6 conidia/ml) were shaken at 37° in Fries minimal medium (8) supplemented with fructose (50 mM) and inositol (10 µg/ml) until the optical density of the culture had just begun to increase (approximately 3.5 hours). At this point the cells were filtered, washed, and resuspended in minimal medium in order to remove the inositol from the culture. The culture was readjusted to 50 mM fructose and divided into two flasks. Part of the culture was supplemented with inositol (final concentration, 100 µg/ml) and the rest of the culture received no further additions. The cells were then incubated with shaking at 37° and aliquots were taken for the various assays at the indicated times during a 210 minute period.

Inositol Restoration. Cells which had been deprived of inositol for

the desired period of time (180-210 minutes) were supplemented with inositol (100 µg/ml), shaken for 5 minutes at 37°, and chilled. The cells were then extracted for ATP or K^+ determination or washed and resuspended in Tris-maleate-Mg^{++} buffer (0.1 M maleic acid adjusted to pH 6.5 with Tris and containing 0.01 M $MgSO_4$) and assayed for the activity of the glucose active transport system.

Assay of the Glucose Active Transport System. The uptake of sorbose, a nonmetabolizable sugar, by cells of Neurospora grown on fructose has previously been shown to be an assay for the glucose active transport system (5). In these studies, estimation of the activity of this system involved (a) harvesting cells at the desired time by filtration on Millipore HA filters; (b) washing the cells with Tris-maleate-Mg^{++} buffer; (c) resuspending the cells in Tris-maleate-Mg^{++} buffer to a density corresponding to 2.5 mg dry weight of cells per ml; and (d) assaying the cells for the ability to transport ^{14}C-sorbose under the conditions of the standard uptake assay. The standard uptake assay system contained Tris-maleate-Mg^{++} buffer at 20% of its original concentration, cycloheximide (1 µg/ml), ^{14}C-sorbose (S.A. approximately 15000 cpm/µmole) at a concentration of 10 mM, and cells at a concentration corresponding to 0.5 mg dry weight of cells per ml. Incubations were carried out with shaking at 37° in a final volume of 0.5 ml. After a 5 minute incubation period, the cells were filtered, washed, dried, and counted, as previously described (4).

Determination of Cellular ATP Levels. An aliquot of cells corresponding to 0.125 mg dry weight of cells was removed from the culture medium at the desired time and filtered on a Millipore HA filter. The cells were immediately resuspended in ice-cold 50% aqueous ethanol and allowed to stand in ice for 30 minutes. The cells were then removed by Millipore filtration and the ATP content of the extracts was estimated by comparison to standard solutions of ATP in 50% aqueous ethanol using a firefly lantern

extract method similar to that described by Forrest and Walker (9). Light output was measured in a Beckman LS-133 liquid scintillation counter.

Determination of Cellular Potassium Ion Concentrations. Aliquots of cells corresponding to 1.25 mg dry weight were removed from the cultures at the appropriate times, filtered on Millipore HA filters, and washed with 2 ml ice-cold water. The resulting pellets were treated with 2 drops of concentrated nitric acid for 30 minutes and resuspended in 5 ml of 10 mM LiCl. The potassium ion concentrations of the resulting extracts were estimated by comparison to KCl standards in 10 mM LiCl employing a Coleman Jr. flame photometer.

Lipid Extraction. Cells subjected to inositol deprivation and then to inositol reconstitution in the presence of ^{3}H-inositol were extracted by a method based on the methods of Bligh and Dyer, and Gibson, et al. (10,11).

RESULTS AND DISCUSSION

The data represented in Figure 1 demonstrate the important points of this communication. Cells deprived of inositol for 2 hours are almost normal with respect to growth and sorbose transport activity (i.e., the glucose active transport system). During the next 90 minutes there is a profound decline in the sorbose transport ability of cells deprived of inositol whereas control cells supplemented with inositol maintain a normal level of sorbose transport activity. During the 90 minutes when sorbose transport activity is declining, growth of the inositol deprived cells is the same as that of cells supplemented with inositol. Moreover, at any time during inositol deprivation, sorbose transport activity can be completely restored by a brief period of incubation with inositol.

In order to test the obvious possibility that the decline in activity of this active transport system is due to decreased energy levels in the cells deprived of inositol, the experiment described in Table I was carried out. Cellular ATP levels and sorbose transport capability were compared in cells which were (a) deprived of inositol for 210 minutes; (b) deprived

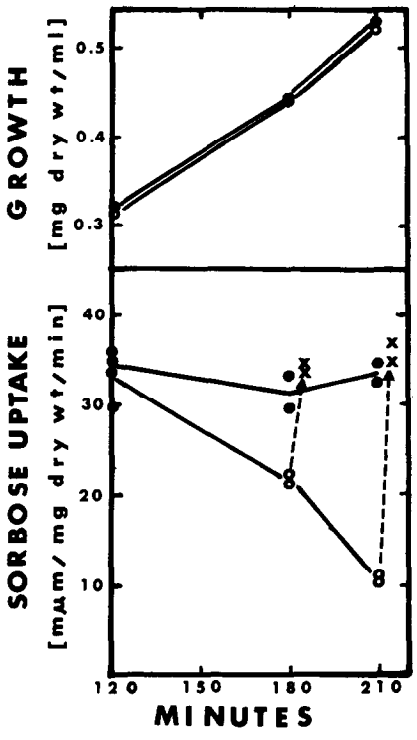

Figure 1. Growth of cells and sorbose transport activity during inositol deprivation.

See Methods for experimental procedure.

●——● cells supplemented with inositol.

○——○ cells deprived of inositol.

- - - -→ X inositol added to inositol deprived cells.

The abscissa represents minutes after removal of inositol.

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of inositol for 210 minutes and subjected to inositol restoration as described in Methods; or (c) supplemented with inositol throughout the experiment. Under conditions where inositol deprivation leads to a marked decrease in sorbose transport activity, cellular ATP levels are normal. A brief incubation with inositol restores the transport system and has little effect on the ATP levels. Thus, considering the normal growth rate and the normal ATP levels of inositol deprived cells, it is not likely that inositol deprivation leads to a decline in sorbose uptake activity by

TABLE I

Sorbose transport and ATP levels of inositol-deprived vs. inositol-supplemented cells

See Methods for experimental procedure.

Condition	Sorbose uptake	Cellular ATP concentration
	mμm/mg dry wt/min.	mM
Cells deprived of inositol for 210 min.	14.8	4.2
Cells deprived of inositol for 210 min. and then subjected to inositol restoration	35.1	4.5
Cells supplemented with inositol	33.6	4.6

affecting cellular energy levels.[1]

Another possible explanation for the decline in sorbose transport activity in inositol deprived cells is that as a result of inositol deprivation, the cell membrane becomes leaky for molecules the size of sorbose and smaller but not for molecules the size of ATP and larger (since cellular ATP levels remain normal in these experiments). In order to test this possibility, an experiment similar to that described in Table I was carried out but in addition to measuring sorbose uptake and ATP levels, cellular potassium ion concentrations were measured. The results indicated

[1]Since the mechanism of energy coupling in this active transport system is unknown, it cannot be stated with absolute certainty that the supply of energy for the system is normal in inositol deprived cells. However, the normal growth and normal ATP levels in such cells indicates that under the experimental conditions employed, inositol deprivation does not result in a gross disturbance of cellular processes.

that cellular potassium ion concentrations remain essentially normal throughout the inositol deprivation period and are not affected by inositol restoration. Thus, it is not likely that the decline in sorbose transport activity in inositol deprived cells is due to the development of a leaky cell membrane.

In order to determine the fate of the inositol taken up by inositol deprived cells during the restoration period, the following experiment was performed. Cells were deprived of inositol for 3.5 hours as described in Methods, incubated with ^{3}H-inositol for 5 minutes at 37°, and washed and resuspended in Tris-maleate-Mg^{++} buffer. An aliquot of the cell suspension was counted and the remainder extracted with chloroform-methanol as described in Methods. The chloroform phase in this experiment contained 63% of the radioactivity from the incorporated ^{3}H-inositol while the aqueous phase contained 36% of the radioactivity. Since free ^{3}H-inositol partitions greater than 99% in the aqueous phase of this system, the appearance of radioactivity from ^{3}H-inositol in the chloroform phase is evidence for conversion of ^{3}H-inositol to a lipid form during the inositol reconstitution period.

In summary, inositol, or more likely a lipid derivative of inositol, is responsible for the restoration of sorbose transport activity to cells of an inositol requiring mutant of Neurospora crassa which have been deprived of inositol. The biochemical nature of the role of inositol in the sorbose active transport system and the molecular events which take place during the inositol reconstitution period are currently under investigation in this laboratory.

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