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Glucose Transport-Deficient Mutant of *Neurospora crassa* with an Unusual Rhythmic Growth Pattern

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A new mutant of *Neurospora crassa* has been isolated from the *patch* strain. The phenotype of the new mutant includes the periodic production of sparse and dense aerial hyphae and the inability to utilize carbohydrates. The biochemical lesion was identified as a deficiency in the low-affinity glucose transport system. The high-affinity transport system appeared normal. External conditions such as medium composition, temperature change, and light-dark cycles affected the rhythm of hyphal production to a different extent in the mutant from that in the parental strain. The lesion in the mutant was mapped on the far left arm of linkage group IV.

One class of morphological mutants of *Neurospora crassa* exhibits a temporal change in their growth pattern that occurs periodically and is controlled by an unknown endogenous mechanism. For example, some strains of *Neurospora*, such as *patch* (19, 25) and *band* (20, 21), produce periodically, once in about 24 h (circadian), either conidia or sparse mycelia. The *clock* strain produces periodically either branched or monopodial mycelia in a noncircadian fashion (9, 26). However, there is no biochemical explanation for the control of morphological rhythms in these mutants. We recently isolated a new mutant of *N. crassa* that exhibits a morphological rhythm of periodic production aerial hyphae. The advantage of this mutant is that a particular biochemical lesion is associated with its phenotype which may help in future studies to elucidate the control mechanism for the oscillator associated with morphological rhythms. This paper describes the isolation and characterization of this mutant.

MATERIALS AND METHODS

Stocks. *N. crassa* strains *patch* (cm 125-5) and *band* and the wild type (65-20a and 65-1A) were obtained from J. F. Feldman. Strain cm 125-5 was a single spore isolate of *patch* which had been mutagenized with ethyl methane sulfonate and had the *patch* phenotype. Strain *band* was isolated from wild-type 74A and appears allelic to the *bd* gene originally isolated by Sargent et al. (20). Strains 65-20a and 65-1A are reisolates from wild-types 74-OR23-1A and 74-OR8-1a. The acetate-nonutilizing mutant (*acu-7A*) of Flavell and Fincham (11, 12) was obtained from David L. Edwards. Strains *patch a*, *cys 10*, *cot 1 cys 4*, and *lys 1* came from the Fungal Genetics Stock Center at California State University, Humboldt Foundation, Arcata, Calif.

Media and growth conditions. Stocks were maintained at 25 C with continuous light (fluorescent lamps, 20 ft-c [ca. 200 lx]) on slants of complete medium (8). Crosses were made at 25 C on Westergaard-Mitchell medium (28) supplemented with methionine (0.5 mg/ml) for the *cys 10* mutant. Rhythms were checked on Gray medium (4% glucose, 0.7% yeast extract, 1.5% agar, and 0.5% KH_2PO_4) prepared according to Brandt (4), or on Casamino Acids medium (Vogel salts [27]: 1.2% acetate, 0.5% Casamino Acids, and 1.5% agar). All percentage figures are weight per volume. Pyrex growth tubes (35 cm long) containing 8 ml of medium were used for phenotypic scoring, and tubes (63 cm long) containing 20 ml of medium were used for other experiments. The growth tubes were inoculated with a loopful of conidia at one end of the tube, incubated for about 20 h at 25 C in continuous light (fluorescent lamps, 20 ft-c [ca. 200 lx]), transferred to growth chambers (Sherer [model cel 4-4] or Environator Manufacturing Co.), and incubated in the dark at 25 C (unless otherwise specified) up to 2 weeks. A red safe light (General Electric BCJ, 60 W, transmittance about 635 nm) was used during daily marking of the growth front. Low-intensity red light has no effect on *Neurospora* circadian rhythm (20). Light-intensity measurements were made with a Weston illumination meter (model 756).

Genetic analysis. Crosses were analyzed by standard random spore and tetrad analysis methods (8). Progenies were scored for their nutritional markers on either glucose (2%) or acetate (1.2%) minimal liquid medium and on minimal medium supplemented with the required nutrient. Phenotypic scoring for rhythmicity was performed in growth tubes containing Gray complete or acetate minimal medium supple-

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mented with Casamino Acids as described above. Period length was determined by measuring the time elapsed between the formation of subsequent bands of dense mycelia.

Growth experiments. Growth experiments were performed by inoculating conidial suspensions (10^5 to 10^6 conidia) in 40 or 100 ml of liquid medium into 125-ml and 1-liter Erlenmeyer flasks, respectively, which were kept on a reciprocal shaker at 140 strokes per min (New Brunswick Scientific Co., model R-7) at 25 C. The concentration of the conidial suspension was determined with a Klett-Summerson colorimeter (no. 42 filter). Mycelia were harvested at different time intervals by filtration through a Büchner funnel. The mycelial pad was dried overnight in a 60 C oven.

Pyruvate dehydrogenase (EC 1.2.4.1). Large-scale cultures for enzyme assays were grown in low-form 2,500-ml culture flasks (Pyrex, no. 4422) containing 1,500 ml of Gray medium, and kept on a reciprocal shaker at 25 C. About 25 g (fresh weight) of mycelial pad was collected from each flask by filtration through a Büchner funnel.

Crude mitochondrial suspensions were prepared by the method of Harding et al. (14). The washed mitochondrial pellet was suspended in a solution of 0.1 M tris(hydroxymethyl)aminomethane (pH 8.0) and 0.1 M sucrose and treated for 20 s with a Branson Sonifier using a microtip horn at a setting of 3 (60-W output). The disrupted suspension was then centrifuged at 39,000 × *g* for 10 min in an IEC ultracentrifuge (model B-60, no. 495 rotor). Enzyme activity was lost with longer or more vigorous sonic treatment. The supernatant fluid was saved for spectrophotometric analysis. Enzyme activity was assayed by monitoring (Cary models 15 and 118-B) the formation of reduced nicotinamide adenine dinucleotide at 340 nm as described previously (14). Samples of the supernatant fluid were used for a Lowry protein determination (18).

Glucose determination. Glucose Fast-Pack (Calbiochem) was used for the estimation of glucose in media separated from the mycelia by filtration. A Cary model 15 spectrophotometer was used for spectrophotometric analysis.

Standard uptake assay. The standard assay of Scarborough (22, 23) was adopted. The uptake of [^{14}C]glucose and the ^{14}C-labeled analogue 3-*O*-methyl-D-glucose (New England Nuclear) was assayed in minimal medium containing 0.5 mg of cells (dry weight) per ml. Samples (1 ml each) of the assay mixture were removed at different intervals, filtered on a membrane filter (Millipore Corp., type HA), washed six times with 1 ml of ice-cold minimal medium, and dried in a 60 C oven for 10 min. The dried filters were then placed in a toluene-base liquid scintillation fluid containing 2,5-diphenyloxazole (5 g/liter) and 1,4 bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (0.3 g/liter), and radioactivity was determined in a Beckman LS-100 scintillation counter.

RESULTS

Isolation of the mutant and preliminary observations. The original morphological mutant I-20 (*LP car*$^-$, i.e., long period, carbohydrate negative) was isolated from a cross between the *patch* strain cm 125-5 and the acetate-nonutilizer strain *acu-7A*. In subsequent crosses, the *acu* mutation was not required for the appearance of the rhythmic morphological mutant.

The rhythm in the *patch* strain, described by Stadler (25), is expressed as alternating regions of dense and sparse mycelia, with heavy conidia formation in the dense region, and a period length of about 22 h on Gray medium in constant darkness at 25 C. However, the rhythm of the mutant is expressed, under the same conditions, only as bands of dense and sparse mycelia (Fig. 1), with little or no conidia and a mean period length of 53 ± 4.7 h. Daily variations in the periodicity of the mutant were high, with standard deviations ranging between 7.6 and 19 h.

In more detailed studies comparing the periodicity of mutant I-20 to its parent *patch*, we found the following differences.

(i) The periodicity of *patch* can be synchronized by light-dark cycles of 12 h of light and 12 h of darkness, with the resultant banding of conidia occurring at a specific and constant relationship to the light-dark cycles. However, regardless of the light-dark regimen (6, 8, or 12 h of light in a 24- or 48-h light-dark cycle) or light intensity, the period length was not significantly different from the dark controls in mutant I-20, and there was no establishment of a specific phase relationship to the external light-dark cycle. Similar results were obtained with 120-cm growth tubes, which allowed the rhythm to be followed for as long as 4 weeks at a time. Light, however, has an effect on the rhythm, since under constant illumination of about 20 ft-c (ca. 200 lx) at 25 C the rhythm disappeared, just as in the *patch* and *band* strains.

(ii) The rhythm of mutant I-20 was determined over a range of temperatures in constant darkness using Gray medium supplemented with 4% glucose (Table 1). Banding and growth were poor at 20 C. The data show that the periodicity is slightly temperature sensitive, with a Q_{10} of 1.6, compared to a Q_{10} of about 1 in the *patch* strain (19). The linear growth rate of this mutant is only slightly affected by temperature change (Table 1). On the other hand, the linear growth rate of *patch* is temperature sensitive, the value being 36 and 73.8 mm/24 h at 21 and 30 C, respectively.

(iii) A striking characteristic of the mutant is that its periodicity can be affected significantly by the composition of the medium, unlike the

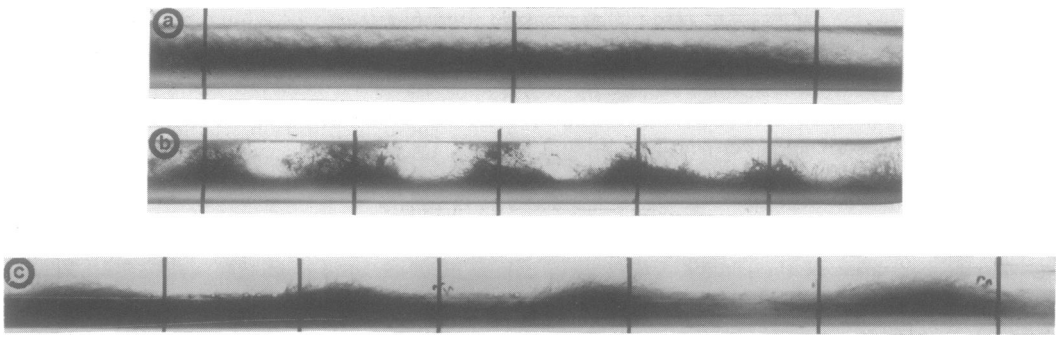

FIG. 1. *Pyrex growth tube cultures of wild type (a); patch, cm 125-5 (b); and mutant 1-20, LP car⁻ (c). Cultures were grown on Gray medium in constant darkness and at 25 C. Vertical lines delimit 24-h growth intervals.*

TABLE 1. *Period length of mutant LP car⁻ over a range of temperatures*[a]

Temperature (C)	Period length[b] (h)	Growth rate (mm/24 h)
22.5	44.6	35.9
25.0	45.3	39.5
28.0	38.4	35.5
30.0	33.4	44.2
	$S\bar{d}$ = 5.5	$S\bar{d}$ = 2.0

[a] Cultures were grown in Pyrex growth tubes on Gray medium supplemented with 4% glucose and kept in constant darkness for about 2 weeks. Values are means of six experiments. $S\bar{d}$, Standard error of a difference between treatment means, calculated by the analysis of variance.

[b] Q_{10} of period length = 1.6.

circadian rhythm observed in *patch* and other strains (29). Table 2 summarizes the periodicities of strain I-20 on Gray medium supplemented with various glucose concentrations (0.5 to 5%, wt/vol). The general trend is a longer period length with higher glucose concentrations. The rhythm could not be measured with a glucose-free medium, since there was no production of aerial hyphae. Linear growth rate was slightly reduced on 4 and 5% glucose; however, results from liquid medium experiments do not indicate that glucose acts as an inhibitor of growth. Growth was promoted without any delay when glucose was added to cultures growing on acetate minimal medium (Fig. 2), and the growth rate on Gray medium increased with increasing glucose concentrations (Fig. 3).

With Gray medium, in which acetate (1.2%) substituted for glucose as a carbon source, and acetate medium supplemented with Casamino Acids in constant darkness at 25 C, the period length was 25 ± 3 and 23.5 h, respectively. Light-dark cycles consisting of 12 h of light followed by 12 h of darkness synchronized this rhythm. It should be noted, however, that on a medium containing acetate the bands consisted mostly of conidia and very little aerial hyphae were produced. We are observing, therefore, two different morphological rhythms produced by the same strain, depending on the medium composition on which it was grown. The conidial-band rhythm is normal and was described in other strains. The aerial-hyphae rhythm is different and was not reported previously in *Neurospora*.

Liquid medium and growth rate experiments. In an effort to characterize the biochemical lesion of strain I-20, I assayed the growth of the mutant on Vogel minimal medium supplemented with different carbon sources (Table 3). The mutant did not grow on minimal medium supplemented with carbohydrates, but it did

TABLE 2. *Period length over a range of glucose concentrations*[a]

Glucose concn (%, wt/vol)	Mutant *LP car⁻*		*patch* strain	
	Period length (h)	Growth rate (mm/24 h)	Period length (h)	Growth rate (mm/24 h)
0.5	34.9	43.3	22.1	50.4
1.5	40.6	44.6	22.7	53.5
3.0	42.9	42.9	22.6	54.5
4.0	43.4	38.0		
5.0	53.5	36.6	23.0	54.2
	$S\bar{d}$ = 2.9	$S\bar{d}$ = 7.5	$S\bar{d}$ = 0.7	$S\bar{d}$ = 1.6

[a] Cultures were grown in Pyrex growth tubes on Gray medium supplemented with 0.5 to 5% glucose at 25 C in constant darkness for 2 weeks. Each value is a mean of three to four experiments.

[b] $S\bar{d}$, For explanation see Table 1.

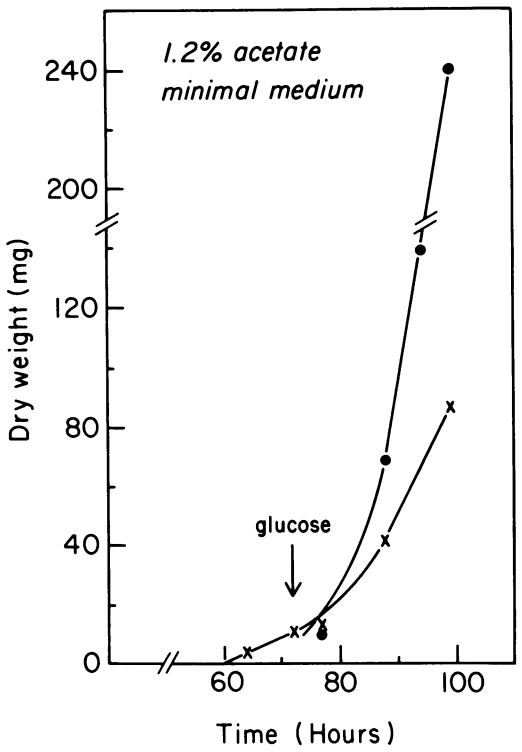

FIG. 2. *Growth rate enhancement by glucose. Cultures of mutant LPcar⁻ were grown in 100 ml of acetate minimal medium in 1-liter flasks on a reciprocal shaker at 25 C. After 72 h of growth (arrow), glucose was added to a final concentration of 2% (●). The controls were grown without addition of glucose (×). Samples were harvested at the indicated time and dried overnight in a 60 C oven.*

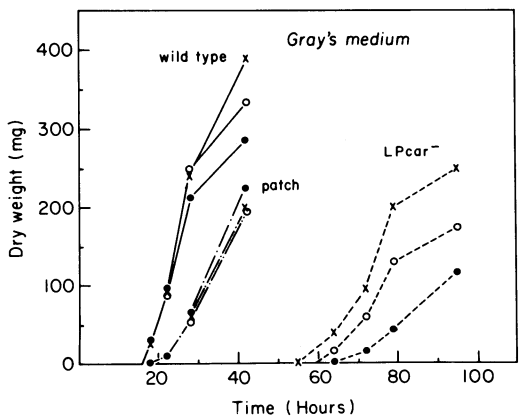

FIG. 3. *Growth rate on Gray medium supplemented with three concentrations of glucose. The three strains were grown in 40 ml of Gray medium in 125-ml Erlenmeyer flasks supplemented with 1% (●), 3% (○), and 5% (×) glucose, and harvested as described in the legend of Fig. 2.*

grow on acetate minimal medium and on acetate-glucose minimal medium with final yields similar to those of the wild type and *patch* strain. Direct measurements of glucose remaining in the medium after different periods of growth indicate that glucose is taken up in I-20 and the wild type at a rate proportional to the mass of hyphae produced. It should be noted, however, that the mutant required about 5 to 7 days to attain a mass of hyphae equal to that produced by wild type in 2 days.

Further information about the nature of the mutation in strain I-20 resulted from testing its growth rate on acetate minimal and Gray complete media (Fig. 3 and 4). Similar results were obtained with Horowitz complete and glucose-acetate minimal media (4 and 1%, respectively). In all cases the growth of strain I-20 lagged behind that of the wild type and *patch* strains. The differences were especially striking on rich media such as Gray complete medium, which enhanced the growth rate and shortened the lag phase of wild type and *patch* strains, but

TABLE 3. *Growth in liquid cultures*

Carbon source	Growth[a] of strain:		
	LP car⁻	*patch*	Wild type
Sodium acetate	14.0	12.0	11.5
Glucose	0.5	61.0	85.0
Glycerol	0.6	26.5	33.0
Arabinose	0	37.0	79.0
Glucose + sodium acetate	115.5	132.3	131.0

[a] Milligrams (dry weight) after 5 days in 20 ml of minimal medium shaken at 25 C.

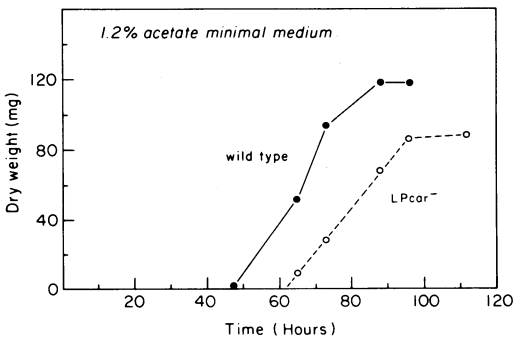

FIG. 4. *Growth rate on acetate minimal medium. Cultures were grown in 100 ml of acetate minimal medium in 1-liter Erlenmeyer flasks and harvested as described in the legend of Fig. 2.*

did not measurably affect the growth pattern of strain I-20.

Glucose transport. A deficiency in sugar transport in strain I-20 could explain the long lag period in growth and the inability of this mutant to utilize glucose as the sole carbon source. Scarborough (22, 23) and Schneider and Wiley (24) have shown that glucose uptake in *Neurospora* is mediated by two kinetically distinct systems, one with a high K_m for glucose (approximately 8 mM, operating at high glucose concentrations) and a second that operates maximally at extremely low glucose concentrations, with a K_m for glucose of approximately 10 μM. The low-K_m system was apparently absent in cells grown at high glucose concentrations (50 mM), implying a repression of the high-affinity active transport under conditions of adequate glucose levels in the medium (24). Similar studies were carried out on the I-20 mutant to determine whether one or both of these systems were defective. The uptake of glucose and the non-metabolizable analogue 3-*O*-methyl-D-glucose was followed in cells grown at high (50 mM) and low (1 mM) glucose concentrations. In these experiments, conidia of the wild-type strain were germinated in the medium containing glucose or glucose plus acetate at 30 C for 5 h; about 30 h were needed for the I-20 conidia to grow on medium supplemented with both glucose and acetate to achieve a similar physiological state (as determined by changes in optical density and microscope observations). To study the high-affinity transport system, conidia of both strains were germinated in 50 mM glucose supplemented with 20 mM acetate as before, transferred to salt solution for 90 min (glucose starvation), and then harvested for the assay. "Repressed" cells were assayed immediately after the germination period and were not subjected to glucose starvation. The conidia were harvested in a low-speed clinical centrifuge and suspended in the uptake medium. Cycloheximide (1 μg/ml) was added in one set of experiments to inhibit cell growth that could lead to inaccuracies in the calculation of intracellular sugar concentration. The data (Fig. 5a) for short-term uptake are similar, with or without the addition of cycloheximide. More important, these results indicate that the mutant I-20 is deficient in its low-affinity glucose transport system and exhibits rates of transport about eightfold lower than the wild type. The total capacity of the mutant to accumulate the sugar, as indicated by the uptake of 3-*O*-methyl-D-glucose (Fig. 5b), is about half that of the wild type. Supplementing the uptake medium with acetate did not restore the normal uptake (Fig. 5c). Furthermore, the lesion is specific for the low-affinity glucose transport, since at a 1 mM glucose concentration the uptake of glucose in the wild type and mutant was essentially the same (Fig. 6).

Enzyme assay. We examined the possibility that interference in glucose breakdown did not affect the ability of the mutant to use glucose. The acetate-requiring phenotype of *Escherichia coli* (15) and *Bacillus subtilis* (13), for example, is due to the inactivity of pyruvate dehydrogenase. The activities of this enzyme, however, in crude extracts of both mutant and wild-type *Neurospora* were similar, 5.14 $\pm$ 0.4 and 5.95 $\pm$ 0.8 μmol of reduced nicotinamide adenine dinucleotide per mg of protein per min, respectively.

Genetic analysis. The I-20 mutant was crossed to the wild type (65-20) and other strains, such as *band*, *patch*, *cys 10*, *cot 1 cys 4*, and *lys 1*, to characterize and map the genetic control of *LP car*$^-$ (long period, carbohydrate negative). In all crosses the progenies were tested for both phenotypes by growing the cultures on Gray complete medium in growth tubes and on glucose and acetate minimal media. In some crosses the progenies were also tested for conidial banding rhythm on acetate medium supplemented with Casamino Acids. In over 600 spores tested, progeny that exhibited long-periodicity rhythm also lacked the ability to grow in glucose minimal medium. However, some of the progeny that did not grow on glucose minimal medium did not exhibit the LP phenotype. The phenotypic expression of the aerial-hyphae rhythm was controlled by other modifying factors such as slow growth or sparse hyphae production. The lesion in the *LP car*$^-$ mutant was mapped on the far left arm of linkage group IV; it was tightly linked to *cys 10* (only 1% crossing-over) and less so to *band* (39% map units) and *cot 4* (57% map units).

The crosses to *band* and *patch* were of special interest with respect to the genetic control of period length. The double strain *bd LP car*$^-$ was glucose negative and had a normal circadian periodicity of conidial band formation on acetate Casamino Acids medium. On Gray medium, however, the two rhythms could not be resolved clearly and, in some cases, it appeared that both persisted in the same tube.

Strain I-20 was also crossed to strain *patch* cm 125-5. The cross did not give any viable spores, but a cross with the original *patch* strain was viable. Since the *patch* gene is linked to mating type *a* (25) it can be easily recognized in the progeny. The double mutant *patch LP car*$^-$

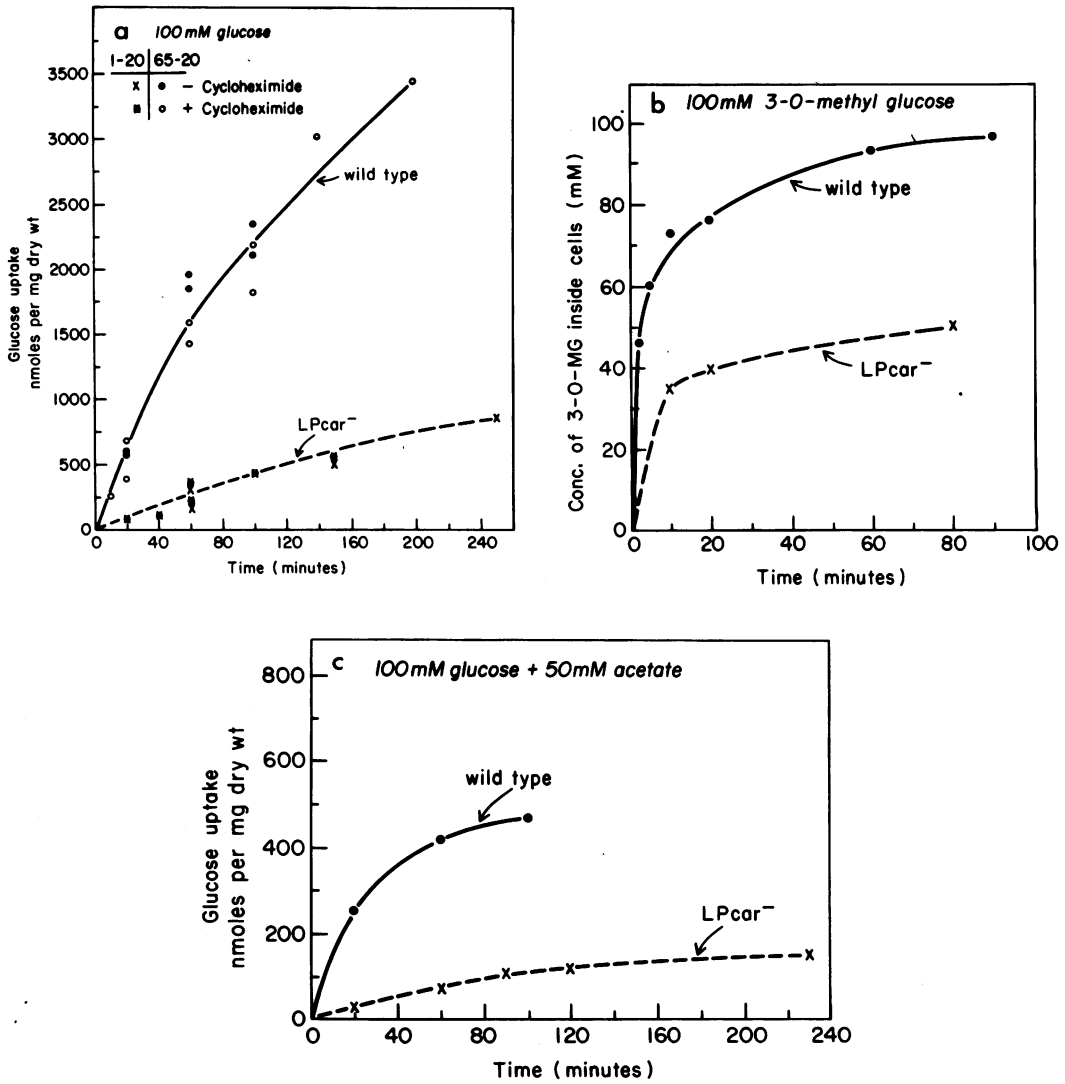

FIG. 5. *Time course of uptake of 100 mM glucose (a and c) and 100 mM 3-O-methyl-D-glucose (b). The cells were germinated in 50 mM glucose and 20 mM acetate. The incubation mixture contained minimal medium supplemented with (a) 100 mM glucose with or without 1 μg of cycloheximide per ml, (b) 100 mM 3-O-methyl-D-glucose, or (c) 100 mM glucose and 50 mM acetate. The concentrations of glucose and 3-O-methyl-D-glucose in the cells were calculated from the radioactivity inside the cells (specific activity, 0.5 μCi/mmol) and from the dry weight assuming a ratio of intracellular water to dry weight of 2.5.*

was of mating type *a* and glucose negative. Its rhythm, however, was either not expressed (sparse growth) or expressed in such a way that the long periodicity of the aerial-hyphae production was partially masked by conidia spread all over the growth tube. Under light-dark cycles, a synchronized conidial banding rhythm appeared with minimal aerial-hyphae production.

DISCUSSION

A new mutant of *Neurospora* was isolated (*LP car*$^-$) which exhibited an altered morphological rhythm associated with a deficiency in the glucose transport system. This morphological rhythm is expressed as alternating production of sparse and dense aerial hyphae, with a periodicity of about 50 h in a medium contain-

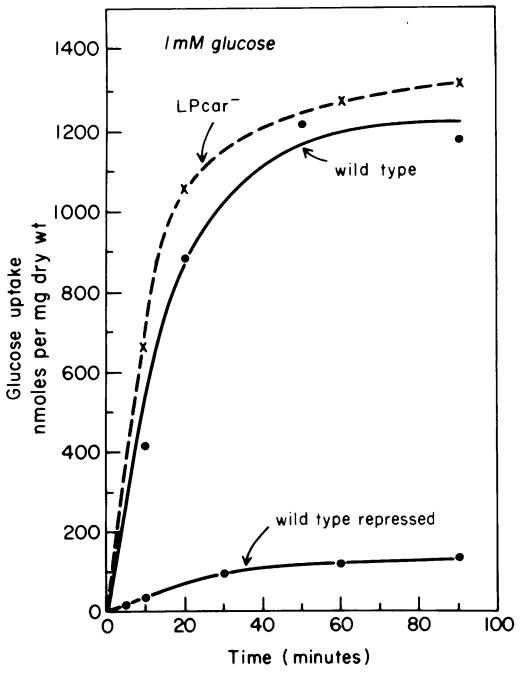

FIG. 6. *Time course of uptake of 1 mM glucose. The cells were germinated as before. Repressed cells were assayed immediately after germination, whereas derepressed cells were suspended in salt solution for 90 min before assaying. The assay medium contained 1 mM [^{14}C]glucose (specific activity, 0.05 μCi/μmol).*

ing 5% glucose. The period decreased with decreasing glucose concentrations. The rhythm of periodic aerial-hyphae production is not circadian, according to its dependence on the composition of the medium, its high Q_{10}, and its insensitivity to light-dark cycles. In this respect, it is similar to the rhythm of periodic production of sparse and aerial hyphae described by Bianchi (3) and to the rhythm of hyphal branching in *clock* described by Berliner and Neurath (1, 2).

These studies also demonstrated the coexistence of two different morphological rhythms controlled by two different mechanisms. In addition to a long-period rhythm of aerial-hyphae production, the mutant exhibits a normal circadian rhythm of conidial banding expressed on a medium containing acetate. This fact in itself is evidence that the deficiency in glucose transport does not affect the normal circadian periodicity. This conclusion is also supported by the fact that both double strains *bd LP car*$^-$ and *patch LP car*$^-$ exhibit a normal circadian rhythm of conidial banding. These observations are not surprising since we have concluded that the aerial-hyphae rhythm is not circadian and, therefore, the presence of the two is not mutually exclusive.

Mutants with altered periodicity have been previously isolated in *Neurospora* (10) and in other organisms such as *Drosophila* (16) and *Chlamydomonas* (6, 7), but in these reports the altered phenotype was not identified with a specific biochemical lesion. The most interesting aspect of this work is the identification, for the first time, of a specific biochemical lesion associated with an altered morphological rhythm. Some previously isolated morphological mutants of *Neurospora* appear to have a partial block in the pentose-phosphate-shunt pathway and contain, as a result, lower levels of reduced nicotinamide adenine dinucleotide phosphate (5). Those mutants exhibit their altered phenotype irrespective of changes in the external conditions. The mutant described here alternates between two physiological stages, sparse hyphae and dense aerial-hyphae production, the control of which is endogenous with the periodicity affected by the concentration of glucose. A deficient transport system for glucose was identified. The low-affinity transport system of glucose previously described in *Neurospora* (22–24) functions at a much lower level in the mutant than it does in the wild type. The enzymes responsible for the transport system are probably associated with the cell membrane, and it is possible that membrane alteration may affect the periodicity of the morphological rhythm. Speculation about the chain of events leading from the deficient transport system to the altered morphological rhythm must await further detailed biochemical descriptions of both.

The availability of this mutant facilitates the study of the transport mechanism and provides additional evidence that the low- and high-affinity transport systems are under different enzymatic controls since they can be genetically dissected. Strain I-20 does not grow on several other carbohydrates, such as arabinose and glycerol. In this respect, it is similar to the pleiotropic carbohydrate-negative mutants described in bacteria (17). These bacterial mutants were unable to grow on a variety of sugars and were also incapable of taking up these compounds. In I-20 we have not examined the uptake of any other sugar except glucose and its analogue 3-*O*-methyl-D-glucose, but the pleiotropic defect in *Neurospora* described here seems to be the manifestation of a single gene, as evidenced by genetic analysis. The mutant, therefore, is useful in determining whether the phosphoenolpyruvate-dependent carbohydrate uptake and utilization described by Rose-

man and co-workers (17) in bacteria also functions in *Neurospora*, and its availability facilitates the study of the links between biochemical mechanisms and morphological rhythms.

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