

## Mutational Analysis of Dark Endogenous Metabolism in the Blue-Green Bacterium *Anacystis nidulans*

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Received for publication 29 April 1974

We describe a mutant (strain 704) of the obligate photoautotroph *Anacystis nidulans* which behaves like the wild type under continuous illumination but which in the dark rapidly loses viability, respire little, and incorporates label into ribonucleic acid and protein at rates considerably less than observed with the darkened wild type. Extracts of this mutant strain show no detectable 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activity. Spontaneous revertants of mutant 704 were selected as survivors of prolonged incubation in darkness. Of 10 such strains examined, none had regained 6-phosphogluconate dehydrogenase activity, and all had lost detectable glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity. Although dark survival of these revertants paralleled that of the wild type, rates of dark endogenous respiration and incorporation of labeled precursors into ribonucleic acid were still very low, comparable to those observed with strain 704. These results are consistent with the following hypotheses concerning dark endogenous metabolism in unicellular blue-green bacteria. (i) Although the oxidative pentose phosphate cycle (hexose monophosphate shunt) may play a major role in endogenous metabolism in *A. nidulans*, as proposed by others, it is not the only pathway capable of providing energy for maintenance of viability in darkness. (ii) Much of the endogenous metabolic activity (respiration and macromolecular synthesis) observed in darkened cultures of wild-type *A. nidulans* is not required for survival alone, and must therefore serve other functions.

One of the functions of endogenous metabolism in microorganisms is to supply maintenance energy for the support of metabolic activities essential for survival in the nongrowing state (3). Such activities include regulation of internal pH, retention of low-molecular-weight metabolites, and resynthesis of unstable macromolecules (2). For many blue-green bacteria (cyanophytes or "blue-green algae"), light is the only acceptable external energy source. In darkness, such organisms retain viability through endogenous metabolism of stored energy reserves. With the obligately photoautotrophic unicellular species *Anacystis nidulans*, this metabolic activity can be demonstrated in unilluminated cultures as respiration and continued incorporation of labeled precursors into ribonucleic acid (RNA) and protein (see below; 9, 11). The mutational analysis presented here is addressed to two questions concerning dark endogenous metabolism in this organism: what catabolic pathway(s) supports this metabolic activity, and are functions other than the minimal requirements for viability served by it?

In summarizing their own and earlier data, Pelroy et al. (16) proposed a unitary scheme for carbon and energy metabolism in blue-green bacteria. In light, CO<sub>2</sub> is assimilated via the reductive pentose phosphate (Calvin) cycle. A portion of the photosynthetically generated carbohydrate is stored as a polyglucose, presumably glycogen, which is assumed to be the substrate of endogenous metabolism during subsequent darkness. Dark catabolism of stored glycogen proceeds via the oxidative pentose phosphate cycle (hexose monophosphate shunt) and involves the following initial steps: (i) (glucosyl)<sub>n+1</sub> + P<sub>i</sub> (inorganic phosphate) → (glucosyl)<sub>n</sub> + glucose-1-phosphate, catalyzed by glycogen phosphorylase (EC 2.4.1.1); (ii) glucose-1-phosphate ⇌ glucose-6-phosphate, catalyzed by phosphoglucomutase (EC 2.7.5.1); (iii) glucose-6-phosphate + NADP<sup>+</sup> (nicotinamide adenine dinucleotide phosphate) → D-glucono-δ-lactone 6-phosphate + NADPH (NADP, reduced form) + H<sup>+</sup>, catalyzed by glucose-6-phosphate dehydrogenase (EC 1.1.1.49); (iv) D-glucono-δ-lactone 6-phosphate

→ 6-phosphogluconate, which can occur spontaneously (5) but may be catalyzed by gluconolactonase (EC 3.1.1.17); and (v) 6-phosphogluconate + NADP<sup>+</sup> → ribulose 5-phosphate + CO<sub>2</sub> + NADPH + H<sup>+</sup> catalyzed by 6-phosphogluconate dehydrogenase (EC 1.1.1.44). Subsequent steps complete the cycle by rearranging ribulose 5-phosphate to glucose 6-phosphate (% molar equivalent) and involve enzyme activities common also to the reductive pentose phosphate (Calvin) cycle. Only reactions i, iii, iv, and v, and the transfer of electrons from NADPH to O<sub>2</sub> are specifically required for dark carbon and energy metabolism, and flow through this pathway in the light is severely restricted by inhibition of glucose-6-phosphate dehydrogenase (16). This situation is presented schematically in Fig. 1.

The proposal of Pelroy et al. (16) is supported by measurements of metabolic pools in illuminated and darkened cells and by demonstration that unicellular blue-green bacteria have high levels of the enzymes catalyzing steps ii, iii, and v, but lack enzymes of the Entner-Doudoroff pathway and have very low levels of

phosphofructokinase (EC 2.7.1.11), a key enzyme in the Embden-Meyerhof-Parnas pathway. We here discuss the properties of an *A. nidulans* mutant which lacks 6-phosphogluconate dehydrogenase activity and of certain partial phenotypic revertants of this strain. Our results support the contention that the oxidative pentose phosphate cycle is a major pathway providing energy and metabolic intermediates for darkened cells but also indicate that it is not the only such pathway. They further show that much of the endogenous metabolic activities of darkened cells serve roles in addition to, and distinct from, those minimal functions required for maintenance of viability in the absence of growth.

## MATERIALS AND METHODS

**Organism and growth conditions.** Our wild-type strain of *A. nidulans* is that of J. Myers and presumably identical to the *Synechococcus* 6301 of Stanier et al. (18). Its routine maintenance and growth on liquid and solid media have been described (6). For preparation of crude extracts, 1,500-ml cultures were grown at 39 C in the medium of Allen (1), were continuously exposed to 5% CO<sub>2</sub> in air, and were stirred with a magnetic stirring bar in 2,500-ml capacity Fernbach flasks illuminated by four 40-W fluorescent tubes at an average distance of 30 to 50 cm.

**Mutant isolation.** A logarithmically growing culture was exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co.) at 15 μg/ml for 45 min while being vigorously shaken at 39 C in the light. The mutagen was removed by washing, and survivors were allowed to grow out in 10 separate subcultures vigorously shaken at 39 C in the light for 24 h. Light was then excluded for 15 h and, upon reillumination, penicillin (Eli Lilly & Co.) was added to each subculture to a final concentration of 10 U/ml. At cell densities employed during dark incubation, the mutants obtained remained viable, although at lower density at least one such mutant would not have survived this treatment (Fig. 2). After 24 h of penicillin treatment, the antibiotic was removed by centrifugation. Washed cells were suspended in fresh medium and incubated at room temperature (20 to 25 C) for 8 days with illumination. Cells were spread on agar to give approximately 100 per plate and, after growth at 39 C, colonies were replicated to a second series of plates which were subjected to 15 h of darkness at 39 C before illumination. Colonies which did not form replicas were picked from the original plates and cloned. They have since been maintained under continuous illumination.

**Measurement of dark survival.** Logarithmically growing cultures were spread on agar plates, as described by Allen (1), to give approximately 200 cells per plate. Plates were placed in the dark at 39 C and removed at intervals for subsequent illumination (four 40-W "cool-white" fluorescent tubes at an average distance of 25 cm). Colonies were counted after 4 days. At cell densities higher than those used

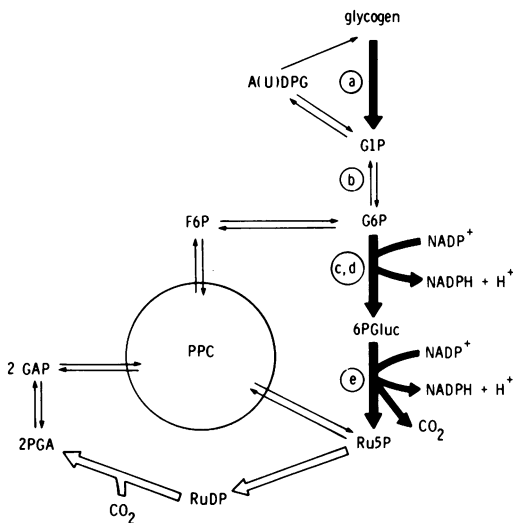


FIG. 1. Scheme for light and dark carbon metabolism in unicellular blue-green bacteria, adapted from Pelroy et al. (16). Heavy black arrows indicate reactions specific to respiratory or dark endogenous metabolism. Light arrows indicate reactions specific to photosynthetic CO<sub>2</sub> assimilation. Other reactions are essential both in light and darkness. Abbreviations: A(U)DPG, adenosine 5'-diphosphate (uridine 5'-diphosphate)-glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; 6PGluc, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; RuDP, ribulose-1,5-diphosphate; PGA, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; PPC, pentose phosphate cycle.

in this procedure, both wild-type and mutant strains remain viable for much longer periods of darkness, either in liquid medium or on agar. This phenomenon is still being investigated but undoubtedly accounts for the fact that mutant strain 704 survived a selection procedure including 15 h of dark incubation at high density but did not survive very much shorter periods of darkness as isolated cells on agar (Fig. 2).

**Photosynthesis and respiration.** For gas exchange measurements, logarithmically growing cells were pelleted quickly by centrifugation and resuspended at up to  $6 \times 10^8$  cells per ml in 5 ml of medium (1) buffered with Warburg no. 9 buffer (11). Gas exchange was measured at 39 C in a Gilson differential respirometer. Photosynthetic  $O_2$  evolution was monitored during illumination with two banks of eight 30-W incandescent bulbs 7 cm below the bottom of the flasks. Respiration was determined during incubation in the dark. Rates obtained were converted to microliters per milligram of cell dry weight using a value of  $1.1 \times 10^{-12}$  g (unpublished measurement) as the dry weight of a single cell.

**RNA synthesis.** RNA synthesis was monitored as incorporation of [ $^3H$ ]uracil (27 Ci/mmol, New Eng-

land Nuclear) into cold 5% trichloroacetic acid-insoluble material (4).

**Enzymatic activities and glycogen levels.** Harvested cells were resuspended in 2.0 ml of lysis buffer of Pelroy et al. (16) and disrupted in an Aminco French pressure cell at 16,000 lb/in<sup>2</sup> (ca.  $1.1 \times 10^7$  kg/m<sup>2</sup>). After clarification by centrifugation at 27,000  $\times g$  for 30 min, crude extracts were passed through a Sephadex G-25 column (1.4 by 21 cm) equilibrated with 2.2 mM glycylglycine (pH 7.4) and 4.4 mM  $MgCl_2$ , as described by Pelroy et al. (16). Glycogen phosphorylase was assayed as described by Shepherd et al. (17) at 40 C without added 5'-adenosine monophosphate, glucose-6-phosphate dehydrogenase as described by Kornberg and Horecker (13), 6-phosphogluconate dehydrogenase as described by Horecker and Smyrniotis (10), triose phosphate (glyceraldehyde-3-phosphate) dehydrogenase (EC 1.2.1.13) as described by Gibbs (7), and isocitrate dehydrogenase (EC 1.1.1.42) as described by Kornberg (12). In all dehydrogenase assays, reduction of NADP at 25 C was monitored. Glycogen was determined as free glucose after acid hydrolysis, as described by Govons et al. (8), which a glucose oxidase-peroxidase kit (Sigma Chemical Co.). Protein was measured by the method of Lowry et al. (14).

## RESULTS

**Properties of mutant 704.** Mutant 704 was obtained as described above. Its survival when held on plates in the dark at low density (200 cells per plate) is compared with that of wild type in Fig. 2. Wild-type cells survived as colony-forming units for at least 1 h in the dark and, indeed, remain viable for up to 20 h under these conditions (see Fig. 5). Cells of the mutant strain rapidly lost the ability to form colonies, with survival being less than 1% after only 30 min of darkness.

Figure 3 compares rates of photosynthetic  $O_2$  evolution and dark  $O_2$  consumption (respiration) in dense suspensions of mutant 704 and the wild type maintained at 39 C. Rates of  $O_2$  evolution in continuously illuminated cultures were equivalent for the wild type and mutant, consistent with the observation that the two strains show similar doubling times under usual growth conditions. When illumination was terminated, the wild type began to consume  $O_2$  at a rate of approximately 20  $\mu$ liters per h per mg of dry weight (similar to that reported by Kratz and Myers [11]). The respiratory rate of the mutant was less than 0.25 of that of the wild type and, upon reillumination, only the wild-type suspension resumed photosynthetic  $O_2$  evolution at significant levels.

RNA synthesis in mutant and wild-type cultures is compared in Fig. 4. Rates of incorporation of [ $^3H$ ]uracil into cold trichloroacetic acid-insoluble material were similar in continuously

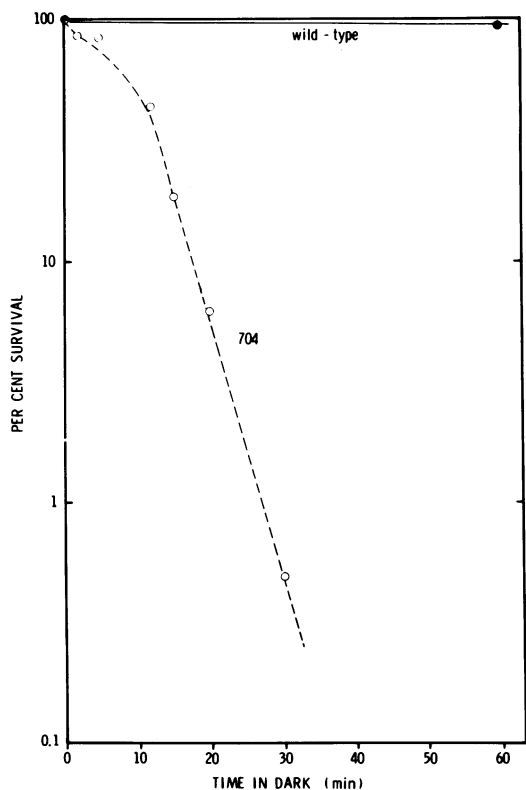


FIG. 2. Dark survival of isolated cells of wild type and mutant 704 on agar. Cells were spread on agar plates which were then held in darkness at 39 C for various intervals before removal to light at 39 C. Symbols: ●, percentage of wild-type cells forming colonies; ○, percentage of mutant 704 cells forming colonies.

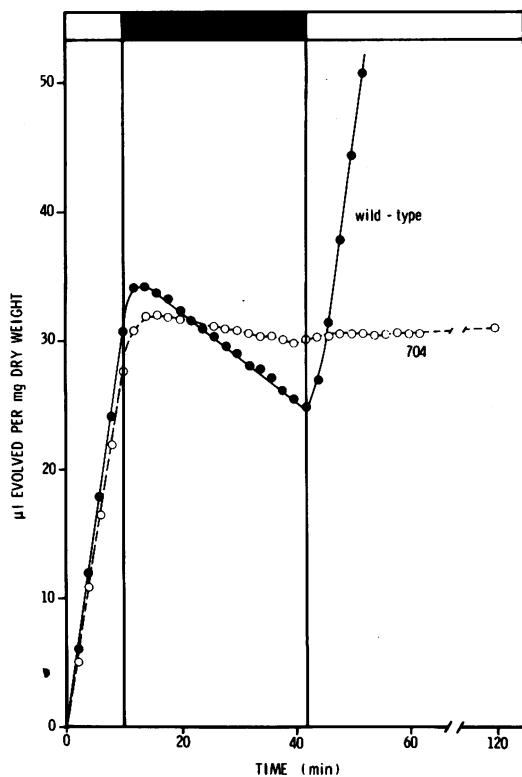


FIG. 3. Photosynthetic oxygen evolution and dark respiration in the wild type and mutant 704. Oxygen evolution was followed with illuminated cell suspensions in a Gilson differential respirometer. At 10 min, lights were extinguished, and  $O_2$  consumption (respiration) was monitored until 42 min, when the suspensions were again illuminated. Symbols: ●, wild type, ○, mutant 704.

illuminated cultures of either strain (not shown), but at least four times greater in the wild type than in the mutant when label was added in the dark (Fig. 4). Reillumination, after 60 min, stimulated label incorporation by both strains, although the rate of incorporation by the mutant remained substantially lower than that of the wild type. Protein synthesis, monitored as incorporation of  $[^3H]$ leucine into hot trichloroacetic acid-precipitable material, paralleled RNA synthesis: mutant and the wild type incorporated  $[^3H]$ leucine at comparable rates under continuous illumination, while only the wild type incorporated label (albeit at reduced rates) in the dark (data not shown).

**Enzymatic defect in mutant 704.** We interpreted the phenotype of mutant 704 to be the result of a lesion specifically affecting dark endogenous metabolism without impairing photosynthesis or other metabolic activities re-

quired for growth in the light (a type of conditional lethality). It seemed simplest to postulate that the lesion affected either the cell's ability to accumulate glycogen in the light or its ability to derive energy and required metabolic intermediates from glycogen in the dark. The first possibility was eliminated when it was determined that glycogen stores in continuously illuminated mutant cells were in fact somewhat higher than those in wild-type cells (260  $\mu$ g/mg of dry weight). The second possibility was tested by examining several of the enzymatic activities which, in the formulation of Pelroy et al. (16; Fig. 1), should be essential for dark endogenous metabolism of glycogen but dispen-

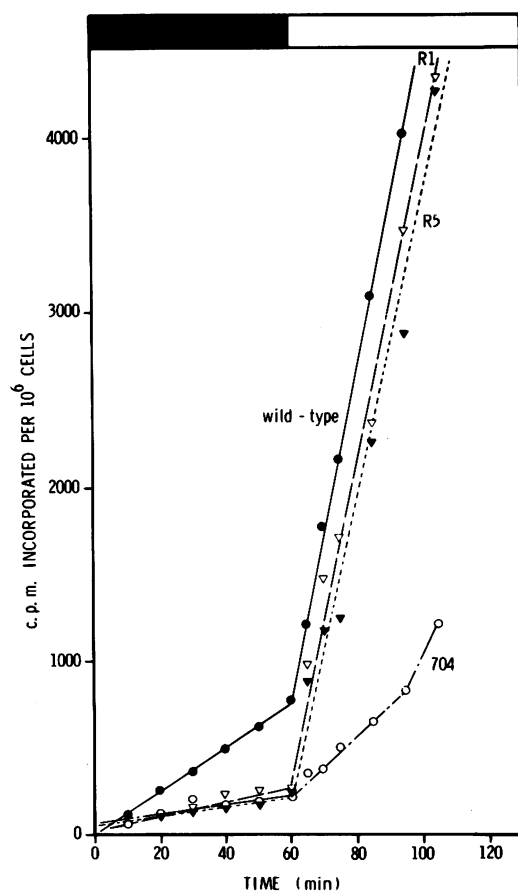


FIG. 4. Incorporation of  $[^3H]$ uracil by darkened cultures of the wild type, mutant 704, and two partial phenotypic revertants of 704 (R1 and R5). Lights were extinguished, label was added at zero time, and incorporation of  $[^3H]$ uracil into trichloroacetic acid-precipitable material was monitored. At 60 min, the cultures were reilluminated. Symbols: ●, wild type; ○, mutant 704; ▽, phenotypic revertant R1; ▾, phenotypic revertant R5.

sible during photoautotrophic growth. Specific activities of glycogen phosphorylase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and of two enzymes not specific to dark endogenous metabolism, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase and NADP-dependent isocitrate dehydrogenase, were determined with desalted crude extracts of the wild type and 704. (Glucanase and NADPH oxidase activities were not measured; respiration data [Fig. 2] indicated that mutant 704 retained at least some of the latter activity.) Results are displayed in the first two rows of Table 1. Strain 704 showed normal or near normal levels of all enzymes measured except 6-phosphogluconate dehydrogenase, which was not detectable (less than 0.5% of the wild-type levels) in this or other extracts of the mutant strain. Mixing experiments with the wild-type and 704 extracts showed that this absence of activity was not due to an inhibitor in the 704 extract.

The failure of strain 704 to survive and its relative inability to incorporate RNA and protein precursors in the dark appeared therefore consistent with one of two simple interpretations. (i) The oxidative pentose phosphate cycle is required for survival and endogenous metabolism, probably because it is the major or only source of energy (as proposed by Pelroy et al. [16]) or essential metabolic intermediates (e.g., pentose phosphates). (ii) Accumulation of 6-phosphogluconate in the unilluminated mutant blocks some activity essential for dark metabolism and survival. We found, for instance, that this compound strongly inhibits glucose phosphate isomerase (EC 5.3.1.9) activity in crude extracts of *A. nidulans* (60% inhibition when 6-phosphogluconate and the substrate, glucose-6-phosphate, are both present at 0.3 mM). Similar inhibition has been reported for this enzyme from a number of organisms (15), and a

possible role for it in endogenous metabolism is discussed below.

If the first of the above interpretations is correct, then all phenotypic revertants of 704 selected as survivors of darkness should have restored 6-phosphogluconate dehydrogenase activity, whereas if the second interpretation is correct, then at least some such revertants might survive because they no longer accumulate 6-phosphogluconate, as the result of a second genetic lesion. "Partial phenotypic revertants" of this latter class were in fact found, and their properties are discussed below.

**Enzymatic activities of partial phenotypic revertants.** Phenotypic revertants were obtained as surviving clones on plates of strain 704 incubated at approximately  $10^7$  to  $10^8$  cells per plate in darkness at 39 C for 48 h. After single-colony isolation, the abilities of two such clones (designated R1 and R5) to survive dark incubation on agar at low cell density were compared to those of their parent, 704, and its parent wild type, in the experiment illustrated in Fig. 5. Both phenotypic revertants maintained viability in the dark nearly as long as did the wild type, while 704 cells again rapidly died.

Specific activities of relevant enzymes in desalted extracts of continuously illuminated cultures of R1 and R5 are shown in Table 1. Neither partial phenotypic revertant strain had regained 6-phosphogluconate dehydrogenase activity, and both had acquired a second lesion resulting in loss of activity of glucose-6-phosphate dehydrogenase, the first enzyme specific to the oxidative pentose phosphate cycle. Eight other independently isolated revertants of strain 704, obtained by exposing freshly streaked 704 plates to 18 h of darkness before outgrowth, also showed this pattern of enzymatic activity. These glucose-6-phosphate dehydrogenase lesions are, in the classic sense, "indirect intergenic suppressors" of the original

TABLE 1. Enzymatic activities of desalted crude extracts<sup>a</sup>

Strain of <i>A. nidulans</i>	Activities <sup>b</sup>				
	Glycogen phosphorylase	Glucose-6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase	Triose phosphate dehydrogenase	Isocitrate dehydrogenase
Wild type . . . . .	2.5	14.3	7.8	48	1.7
Mutant 704 . . . . .	0.97	9.4	<0.04	35	1.8
Revertant R1 . . . . .	2.4	<0.01	<0.2	43	1.7
Revertant R5 . . . . .	2.0	<0.01	<0.1	35	1.4

<sup>a</sup> Specific activities of NADP-linked dehydrogenases and glycogen phosphorylase in Sephadex G-25 desalted crude extracts.

<sup>b</sup> Nanomoles of substrate used per minute per milligram of protein.

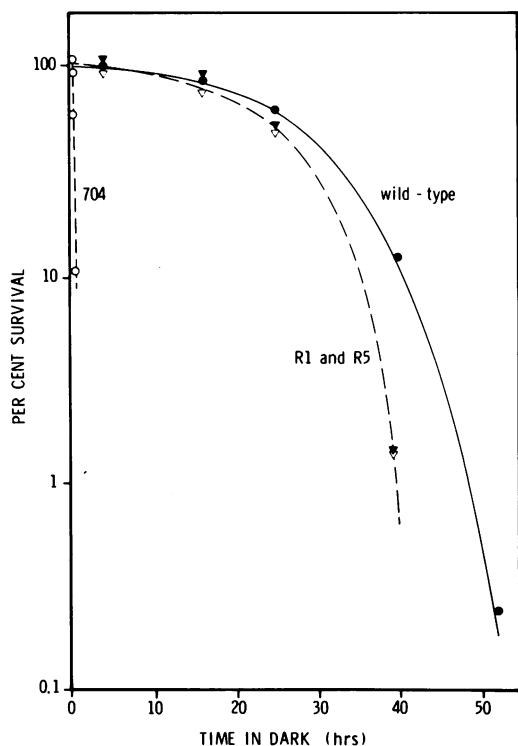


FIG. 5. Dark survival of isolated cells of the wild type, mutant 704, and two partial phenotypic revertants of 704. See Fig. 1, but note difference in abscissae. Symbols: ●, wild type; ○, mutant 704; ▽, phenotypic revertant R1; ▼, phenotypic revertant R5.

conditionally lethal 6-phosphogluconate dehydrogenase mutation.

The fact that R1 and R5 survive dark incubation while lacking two activities of the oxidative pentose phosphate cycle must mean that this pathway is not essential to maintenance of viability in the dark, although it undoubtedly plays a major role in endogenous metabolism in wild-type cells. These results further suggest that failure of mutant 704 to survive in darkness can be attributed to accumulation of toxic levels of 6-phosphogluconate which does not occur when glucose-6-phosphate dehydrogenase activity is also eliminated.

**Dark endogenous metabolism of phenotypic revertants.** Revertant strains R1 and R5, although regaining dark viability, did not show wild-type levels of dark respiration. Rates of endogenous  $O_2$  consumption were lower than those of 704, and less than 10% that of the wild type (Fig. 6). These strains did recover photosynthetic ability upon reillumination and thus differ from their parent, mutant 704, although their recoveries were much slower than observed

with the wild type. Rates of photosynthetic  $O_2$  evolution and growth under continuous illumination were, as expected, similar in all strains.

All strains incorporated [ $^3H$ ]uracil into trichloroacetic acid-precipitable material at similar rates under continuous illumination, but incorporation in the dark by strains R1 and R5 was much less than that of the wild type, and comparable to that of strain 704 (Fig. 4). Upon reillumination, the revertant strains regained the ability to accumulate labeled RNA nearly as rapidly as the wild type, and much more quickly than 704.

## DISCUSSION

The present work allows us to draw two general conclusions concerning dark endogenous metabolism in *A. nidulans*.

(i) Although the oxidative pentose phosphate cycle is probably the major energy-yielding pathway in darkened cells (since strains doubly blocked in the oxidative pentose phosphate cycle respire little and incorporate little label into RNA in the dark), functioning of this pathway is not essential for survival. Since,

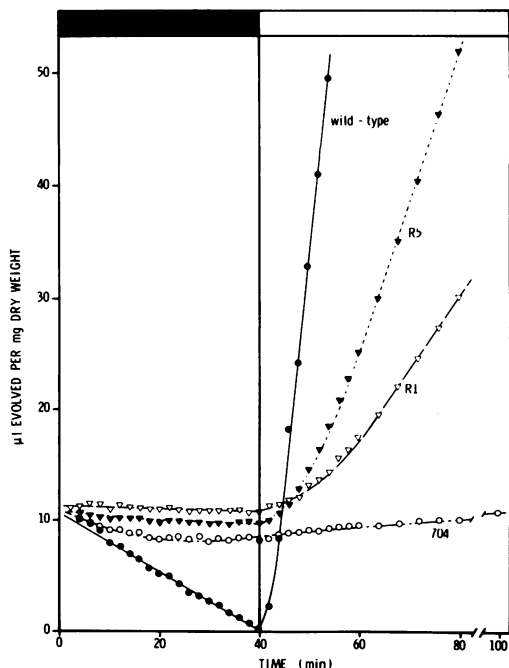


FIG. 6. Dark respiration in the wild type, mutant 704, and two partial phenotypic revertants of 704 (see Fig. 3). Oxygen consumption was followed for 40 min in darkness, at which time cell suspensions were reilluminated and photosynthetic  $O_2$  evolution was monitored. Symbols: ●, wild type; ○, mutant 704; ▽, phenotypic revertant R1; ▼, phenotypic revertant R5.

however, it is likely that some energy must be generated for maintenance of viability in the dark (2), we suggest that an alternate (perhaps normally minor) energy-yielding pathway operates in the doubly mutant revertants R1 and R5. The nature of this alternate pathway is unknown. Dark respiration was not observed in strains R1 and R5, and their metabolism might thus be fermentative, although levels of O<sub>2</sub> consumption less than 2  $\mu$ liters per h per mg of dry weight would not have been detected. Involvement of the Embden-Meyerhof-Parnas pathway is suggested by our finding that 6-phosphogluconate, which inhibits glucose phosphate isomerase, is toxic to strains which cannot further metabolize it in the dark. However, Pelroy et al. (16) report that levels of phosphofructokinase, a key enzyme in this pathway, are very low in *A. nidulans*. Our data cannot, of course, exclude a role for a more unusual energy-yielding biochemical process, such as hydrolysis of stored polyphosphate (3), in providing adenosine 5'-triphosphate for energy of maintenance.

(ii) The relatively high rates of O<sub>2</sub> consumption observed in darkened cultures of wild-type *A. nidulans* are not essential for survival of this organism, since the partial phenotypic revertants R1 and R5 remain viable while respiring very little if at all. Dark survival of these latter strains also appears not to require RNA synthesis at the level observed in unilluminated wild-type cells (although here we stress that the experiments performed cannot distinguish between decreased transcription and decreased uptake of exogenous uracil). In the wild type, respiration and synthesis of macromolecules presumably serve functions separate from and additional to those involved in maintenance of viability. We suggest that these additional functions help prepare the cell for rapid resumption of growth upon reillumination. At least one such preparatory function could be maintenance of pools of intermediates of the pentose phosphate cycle at levels which do not restrict photosynthetic CO<sub>2</sub> fixation during the first few minutes after reillumination. Failure to replenish these pools may account for the tardiness with which strains R1 and R5 resume photosynthetic O<sub>2</sub> evolution after a short period of darkness (Fig. 6). We cannot say if the RNA and protein synthesis seen in the darkened wild type serve specific functions related to recovery from darkness, but have shown elsewhere that RNA (R. A. Singer and W. F. Doolittle, manuscript in preparation) and protein (Singer and Doolittle, manuscript in preparation) species accumulated in the dark do differ from those

which accumulate under continuous illumination.

#### ACKNOWLEDGMENT

This work was supported by grant MA-4467 from the Medical Research Council of Canada.

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