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# Mutations affecting accumulation of glycogen

M. A. Morales
*Facultad de Farmacia y BioquÍmica*

A. J. Jobaggy
*Facultad de Farmacia y BioquÍmica*

H. F. Terenzi
*Facultad de Farmacia y BioquÍmica*



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## Abstract

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Recent findings suggest that the enzymatic mechanisms involved in the metabolism of glycogen in the mycelium of N. crassa are closely similar to those described for mammalian tissues (Téllez de Iñón, Terenzi and Torres 1969 Biochim. Biophys. Acta 191:765; Téllez de Iñón and Torres 1970 Proc. Nat. Acad. Sci. U. S. A. 66:459). It appears, therefore, that the basic pattern of control of glycogen synthesis and degradation mediated through enzyme conversions and chemical signals is a common feature among organisms at the eucaryotic level of organization. In view of that, we considered it of interest to investigate the genetic elements involved in the metabolism of glycogen in Neurospora. The preliminary part of this work consisted in the isolation of mutants with altered levels of glycogen in the mycelium.

Isolation of glycogen mutants. Two different N. crassa strains were used for mutagenic purposes: a microconidial (BAR 60-200, a; pe, fl; cot; inos), and a macroconidial one (BAR 46-68, cot). The microconidial strain was the sole source of mutants, since the mutagenic treatments of the macroconidial one have been so far fruitless. Conidia were irradiated using a UV germicidal lamp to allow survival of 1-10% of the population and plated on Vogel's medium supplemented with 2% glucose and inositol. The plates were incubated at 35°C for 3-4 days. After the colonies developed, 20 ml of iodine solution ($I_2$, 1.3 mg/ml; KI, 0.013 mg/ml) were added to the plates; the iodine solution was freshly prepared by diluting 10 ml of a concentrated stock solution ($I_2$, 26 mg/ml; KI, 0.26 mg/ml) in 190 ml of sterile distilled water. The iodine reagent was left in contact with the plates for about 1 minute; after that the solution was poured off and the surface of the plates was quickly rinsed with sterile distilled water. The stained colonies were observed immediately under a dissection microscope at low magnification. Any colony exhibiting a color different from the reddish-brown tonality shown by normal, glycogen-containing colonies was isolated and transferred to a small tube containing 1 ml of liquid complete medium. The tubes were incubated at 25°C to allow further growth and conidiation.

The plates were stained and scored one at a time, since the color given to the colonies by the iodine reagent faded away, resulting, after about ten minutes, in a bluish-grey uniform tonality for all colonies. No differences could be appreciated then. Although the iodine treatment proved to be quite toxic to the organism, since usually 40-50% of the isolates failed to grow further, a high proportion of those which grew were mutants with altered level of glycogen (9 mutants among 27 colonies isolated in one experiment.) Therefore, the procedure of staining colonies with iodine reagent can be regarded as a successful method for selecting glycogen mutants, as it has been shown to be for bacteria (Govons et al. 1969 J. Bacteriol. 97:970) and yeast (Chester and Byrne 1968 Arch. Biochem. Biophys. 127:556).

Extraction and quantification of glycogen. A conidial inoculum of the suspected mutants was seeded in 100 ml of Vogel's liquid medium supplemented with 2% glucose. Routinely, a series of flasks was inoculated with the same strain, in order to get information on the evolution of the glycogen content in the mycelium during the course of growth. The flasks were incubated at 27°C in a reciprocating shaker. At predetermined times the mycelium was harvested by filtration, rinsed with water, and dehydrated by several changes of ethanol and acetone. The acetone was finally evaporated under vacuum, and the remaining material was ground in a mortar to a fine powder.

Routinely, 100 mg of the acetonic powder were subjected to a KOH extraction (60% KOH, 15 minutes in a boiling water bath) and then centrifuged. The residue was washed three times with 33% KOH and finally discarded. The supernatant fractions were pooled and diluted to a 33% KOH final concentration. The extracted polysaccharides were precipitated by adding 1.3 vol. of 95% ethanol and a drop of 1 M ammonium acetate and were heated in a boiling water bath until the solution started to boil. The samples were then placed in a freezer for several hours to allow complete precipitation. The precipitated material was collected by centrifugation, dissolved in a small volume of water, and the precipitation procedure was repeated once more. The final precipitate was dissolved in 1 ml of saturated ammonium chloride solution and heated for 5 min in a boiling water bath. Glycogen content was determined in an aliquot of this solution according to the method described by Krisman (1962 Analyt. Biochem. 4:17). It has been demonstrated that this method, based on the colorimetric quantification of a glycogen-iodine complex, provides a reliable measurement of the glycogen present in the KOH-extracted material (Rothman and Cabib 1969 Biochemistry 8:3332).

Description of the mutant phenotypes. Three main phenotypic differences were observed among the colonies developed by the survivors after the mutagenic treatment as compared to wild-type, glycogen-containing colonies: blue or green colonies; yellowish (colorless) colonies; and very dark brown colonies. Any colony exhibiting these characteristics was isolated for further study. In liquid cultures at 27°C, the suspected mutants behaved in many instances as the wild type with regard to glycogen accumulation. However, a significant number of the isolates actually proved to be mutants that either failed to form the polysaccharide or were unable to degrade glycogen, as occurs in the wild type after reaching the stationary phase of growth. Such behavior was consistent with what was expected according to the color developed by the original colony in the presence of iodine.

KOH extraction and further quantification permitted us to distinguish two different glycogen-deficient phenotypes: First; the polysaccharide extracted with KOH formed a blue complex with iodine reagent. The absorption spectrum of such a complex exhibited a maximum at 580 nm (Figure 1) which resembled that of amylopectin. Colonies of these mutants stained blue or green with iodine. Second; the iodine complex of the KOH-extracted polysaccharide was identical to that of wild type glycogen (Figure 1); however, the amounts present in the mycelium were 6 - 12 times lower than in the wild type (Table 1). Colonies from these mutants did not stain with iodine. A third mutant phenotype was derived from colonies which stained in excess with iodine. It differed from the wild type in that a high level of glycogen was maintained during the stationary phase (Table 1). This is probably a result of alterations at the level of degradative processes.

Table 1. Glycogen content of N. crassa mycelia *

| Phenotype** | Hours of growth in liquid medium (27°C) | | |
|---|---|---|---|
| | 40 | 65 | 100 |
| Reddish-brown*** | 22.0 | 6.0 | 4.0 |
| Blue or green | -**** | -**** | -**** |
| Yellowish (colorless) | 2.7 | 0.5 | 0.7 |
| Dark brown | 21.6 | 20.8 | 31.6 |

* mg/100mg acetonic powder

**color developed by colonies in presence of iodine after 96 hrs. growth at 35°C

*** considered as wild type phenotype

**** if a large sample was used, a blue color developed. The absorption spectrum of the iodine complex of this material is shown in Figure 1, with spectrum of wild type glycogen.

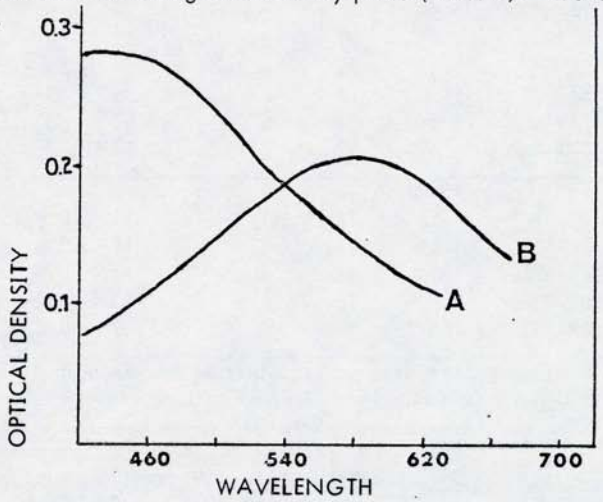


Figure 1. Absorption spectrum of iodine-stained Neurospora glycogen (A) and that of polysaccharide extracted with KOH from mutants of the first class (B).

The first and second mutant phenotypes were stable after repeated transfers, and grew in liquid medium as well as the original strain (BAR 60-200) did. In slants, the first class of mutants showed a tendency toward semi-colonial growth. Preliminary crosses performed using mutants of the first class and the STA4 strain gave a segregating pattern of glycogen-forming to glycogenless isolates of 1:1, indicating that the mutation affected a single nuclear gene. The third mutant phenotype (glycogen excess) turned out to develop rather poorly in solid or liquid medium and exhibited a marked tendency to revert to the wild type. Due to these inconveniences we still know little about their properties.

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