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Glucose-6-Phosphate Dehydrogenase and *Neurospora* Morphology*

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Abstract. In addition to the *Neurospora crassa* mutant *col*-2, mutations in two other unlinked genes, balloon and frost, lead to distinct morphological growth and abnormal glucose-6-phosphate dehydrogenases. The glucose-6-phosphate dehydrogenases from these strains have increased thermolabilities, altered kinetic parameters, and distinctive electrofocusing patterns. The altered enzyme segregates with each morphological phenotype in crosses to wild type, and there is a correlation between the severity of the enzymic defect and the degree of morphological abnormality in heterocaryons and double mutants.

These results are compatible with the hypotheses that the balloon, frost, and *col-2* genes are structural genes for glucose-6-phosphate dehydrogenase and that the pleiotropic effects of these altered dehydrogenases are responsible for the mutant morphologies.

Much of the network of biochemical pathways has been elucidated by the use of mutants in which one reaction is altered at a time. Events underlying morphological changes may be clarified in a like manner by employing single gene morphological mutants. The *Neurospora* morphological mutants offer several advantages for such a study, namely, well-defined genetics, characteristic morphological phenotypes,[1] and an ease of handling. These mutants are prototrophic, and the addition of metabolites to the growth medium does not usually alter their morphology, indicating that only those reactions concerned with morphological differentiation are affected. By tracing the primary biochemical lesions of these mutants, relationships between individual enzymes and morphology can be established and investigated.

Since the rigid cell wall of *Neurospora* appears to determine the external morphology of this organism, mutations that cause morphological changes should affect this structure. Many of the morphological mutants do, in fact, have altered cell wall compositions.[2, 3] Presumably, a large number of metabolic reactions influence *Neurospora* morphology, since the mutations involved map at about 80 independent loci in all seven linkage groups.[1] Such morphological changes may be caused by mutant enzymes concerned primarily with cell wall synthesis or by the secondary pleiotropic effects of altered metabolic reactions. Brody and Tatum have provided an example of the latter case.[4] They suggested that the *col-2* mutation affects the primary structure of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (D-glucose-6-phosphate:TPN oxidoreductase) and

causes a decreased stability of the enzyme plus a decreased affinity for G-6-P and TPN.

In this paper we present data to show that two additional mutations, balloon and frost, also alter the properties of G-6-P dehydrogenase and lead to distinctly different morphologies. It is suggested that the pleiotropic effects of these three unliked mutations result from a lowered TPNH level as a direct result of the defective G-6-P dehydrogenases.

Materials and Methods. Cultures: *col*-2 (Y5331-2-8A)[5] linkage group VII, balloon (R2409-2-48A)[1] linkage group II, frost (R2499-2(1-8)A and B110f$_3$A[1]) linkage group I, ragged (B53-A) linkage group I, and wild type RL-3-8A strains were obtained from stock cultures of the Rockefeller collection. Double mutants were constructed by the appropriate crosses of the single mutants. The identity of each double mutant was ascertained by segregation of the single mutants in a backcross to wild type. Heterocaryons were constructed on minimal medium from pairs of the single mutants, each containing a different biochemical marker.

Strains were maintained on slants of complete medium at 25°C and were grown in minimal medium at 30°C in 50-ml shake cultures or in aerated 5-gallon Pyrex carboys.

Purification of G-6-P dehydrogenase: All steps were performed at 4°C. Unless stated otherwise, the buffer used throughout this work was 0.1 *M* Tris, pH 7.4.

15 gm of lyophilized mycelia were ground with sand to a fine powder. The powder was extracted with 400 ml of buffer for 1 hr at 4°C and was centrifuged for 10 min at 3000 × *g*. The pellet was extracted once with buffer and the supernatant solutions were combined. To this crude extract was added solid $(NH_4)_2SO_4$ to 35% saturation. The pH was adjusted to 7.0 with 1 *N* NaOH (pH paper). After centrifugation the pellet was discarded and the $(NH_4)_2SO_4$ concentration of the supernatant fraction was raised to 60%. The resulting precipitate was centrifuged, resuspended in 40 ml of buffer, and dialyzed overnight against 2000 ml of the same. Any precipitate after dialysis was removed by centrifugation and discarded. To the supernatant solution were added 40 ml of calcium phosphate gel and the preparation was stirred for 16 hr. The mixture was centrifuged and the supernatant fraction was discarded. The enzyme was eluted from the calcium phosphate gel by three successive washes with 20% $(NH_4)_2SO_4$ in buffer. The washes were combined and the $(NH_4)_2SO_4$ concentration was brought to 70%. The precipitated enzyme was redissolved in 0.01 *M* buffer and was dialyzed overnight against the same. The dialyzed solution was chromatographed on a 2 × 22 cm DEAE-cellulose column with a linear gradient of 0–0.2 *M* NaCl in 0.01 *M* buffer (200 ml on each side). Fractions of 3 ml were collected at a flowrate of 36 ml/hr. The fractions containing enzymic activity were pooled and were concentrated by $(NH_4)_2SO_4$ precipitation. After passage through a 1.5 × 90 cm column of G-200, the enzyme had an average specific activity of 40–50 constituting a 200- to 250-fold purification.

Heat inactivation of G-6-P dehydrogenase: Fresh cultures were ground with $^1/_2$ part sand (by weight) and were extracted with buffer (2 ml/gm of tissue). The extracts were centrifuged at 2000 × *g* for 10 min and were dialyzed overnight against 100 vol of buffer. Protein was determined by the method of Lowry.[6] Extracts were diluted in buffer to 3–4 mg of protein/ml. Aliquots (0.1–0.2 ml) were heated in a 50°C water bath and were immediately placed in ice. Each time point represents duplicate determinations. The reported half lives of the G-6-P dehydrogenase from different strains are averages from at least two different inactivation curves.

Isoelectric focusing: Ampholine (pH 5–7) was mixed with a sucrose gradient (0–50%) in a 110-ml electrofocusing column.[7] The final ampholine concentration was 4%. The ampholine and electrofocusing column were obtained from LKB Productor AB, Stockholm, Sweden. Purified enzyme (8 mg) was introduced into the column by addition to the dense sucrose solution prior to gradient formation. Electrofocusing was performed at 700 V and ~3 mA for 60 hr at 4°C. Fractions of 1.0–1.2 ml were collected at

the end of the electrofocusing run with the aid of a peristaltic pump. Enzyme assays and pH determinations were done immediately.

G-6-P dehydrogenase assay: Assays were performed as previously described;[4] however, $MgCl_2$ was omitted from the buffers. Enzyme activity is expressed in international units (i.e., μmoles TPNH formed/min).

Results. Heat inactivation studies: It was previously shown that the G-6-P dehydrogenase from the *col-2* mutant is thermolabile.[4] Subsequently the heat stability of the enzyme was examined in dialyzed crude extracts of other *Neurospora* morphological mutants. Of these, the balloon and frost G-6-P dehydrogenase thermolabilities were significantly different from that of wild type, although the specific activities (0.2–0.3) in crude extracts of these strains were similar.

The kinetics of heat inactivation of the balloon and frost enzymes were compared with the decay of the *col-2* and wild type activities. At 50°C the half lives of the balloon, frost, and *col-2* enzymes are in the range of 2.8–3.5 min as compared to 9.0–11.0 min for the wild type G-6-P dehydrogenase (Table 1). A defective G-6-P dehydrogenase is not a symptom of all *Neurospora* morphological mutants. The heat stability of the enzyme from the ragged mutant, which is known to have a defective phosphoglucomutase,[8] is identical with that of wild type.

A detailed study of the conditions that influence the heat stability of the enzyme revealed that the pH and concentration of protein, TPN, and buffer are important. Of these variables, the TPN concentration has the greatest effect. Both the stability and aggregation state of the multimeric wild type G-6-P dehydrogenase are influenced by TPN.[9] High TPN concentrations increase the heat stability of the enzyme and convert the molecule from a dimer to a tetramer,[9] as also reported for the yeast enzyme.[10]

It was possible that altered endogenous TPN levels of the mutant extracts might account for the mutant enzyme thermolability. The effects of TPN levels on the mutant and wild type enzymes were therefore compared. A molar excess of TPN (10^{-3} *M*) increases the heat stability of the G-6-P dehydrogenase

TABLE 1. *Half-life of various G-6-P dehydrogenases at 50°C.*

Source of enzyme	$t_{1/2}$ (min)*
Wild type	9.0–11.0
Col-2	2.8–3.5
Balloon	2.8–3.1
Frost	2.8–3.1
Heterocaryons	6.0–8.0
Double mutants	1.5–2.5
Ragged	11.0

All cultures were grown at 30°C in minimal medium as shake cultures and supplemented when required with 20 μg inositol/ml. The cultures were harvested and treated as described in *Materials and Methods*. Time points of each inactivation curve were done in duplicate. The values given are the ranges found for at least three different extracts of each strain.

* Time for 50% inactivation.

TABLE 2. *Effect of TPN on the thermolability of the wild type and mutant G-6-P dehydrogenases at 50°C.*

Source of enzyme	$t_{1/2}$ (min)* (no TPN)	$t_{1/2}$ (min)* (10^{-3} *M* TPN)
Wild type	9.0	31.0
Col-2	3.0	12.5
Balloon	2.8	13.0
Frost	3.0	8.5

Values from a representative experiment. TPN was added to final concentration of 10^{-3} *M* where appropriate. See Table 1 for details.

* Time for 50% inactivation.

from all strains. However, the differences in thermolability between the mutant and wild type enzymes remained. No concentration of TPN (10^{-6} to 10^{-3} M) abolished the relative thermolability of the mutant G-6-P dehydrogenases (Table 2). Consequently, the instability of the mutant enzymes appears to be an inherent molecular property.

Based on the above observations, the genetics of the G-6-P dehydrogenase thermolability were examined. Both the morphological and enzymic defects are products of the defective balloon, frost, and *col-2* genes, since these properties segregate together in ordered tetrads isolated from crosses between the mutants and wild type. Complementation occurs in heterocaryons constructed with all pairs of the three mutants. The heterocaryons are nearly wild type in morphology in contrast to the tight colonial growth of balloon and *col-2* or the snowflake appearance of frost (Fig. 1). In addition, the enzyme thermolabilities of the heterocaryons are intermediate between those of the wild-type and the single mutants (Table 1), as would be expected for an *in vivo* recombination between the enzyme polypeptide chains. The correlation between morphology and G-6-P dehydrogenase thermolability also applies to the double mutants. These strains have more restricted growth patterns than the single mutants and also have a more thermolabile G-6-P dehydrogenase.

Properties of the partially purified mutant G-6-P dehydrogenases: To characterize further the apparent differences of the mutant G-6-P dehydrogenases, the enzymes were partially purified from the various strains (see *Materials and Methods*). These preparations had specific activities of 40–50 and contained no interfering activities that would reduce TPN or react with G-6-P. All kinetic and electrofocusing experiments were performed with G-6-P dehydrogenases of this purity.

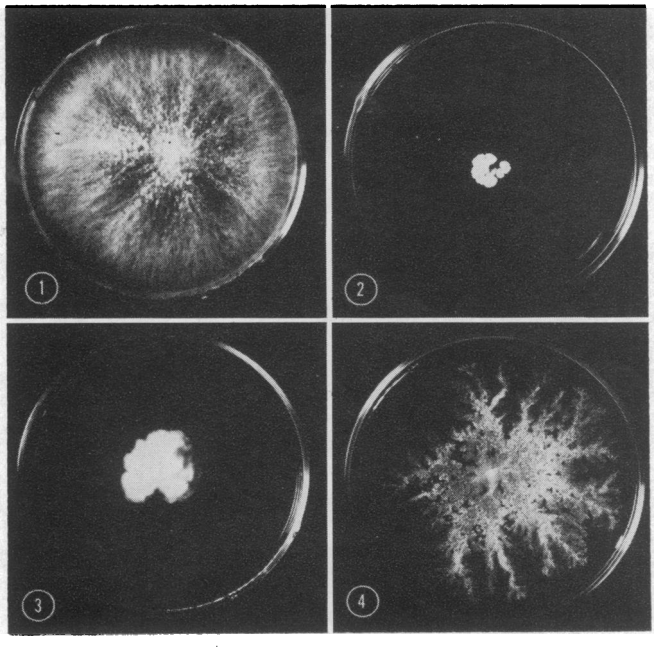

FIG. 1. — Typical growth of *Neurospora* on minimal agar medium at 30°C for 2 days, (*1*) wild type, (*2*) *col-2*, (*3*) balloon, and (*4*) frost.

The behavior of the balloon and *col-2* enzymes in the purification procedure was not significantly different from that of the wild type G-6-P dehydrogenase except that their over-all stability was less. Two peaks of enzyme activity were consistently observed in preparations from the frost mutant on DEAE-cellulose columns, although the enzyme from the other strains eluted as single peaks. Both peaks of G-6-P dehydrogenase activity from frost were pooled for subsequent experiments. In attempts to stabilize the mutant enzymes, various cations and anions were found to have little effect on the activity and stability of the mutant enzymes, except that phosphate inhibited the enzyme from all sources including wild type.

Kinetics: The kinetic properties of the mutant and wild type enzymes are different. The wild type G-6-P K_m is 2.9×10^{-5} M and that of the mutant enzymes is 1×10^{-4} M. The G-6-P K_m for the mutant enzymes is given as a single value since the observed differences are slight. The TPN K_m values for the balloon and frost strains are similar to the wild type value of 1.3×10^{-5} M. The *col-2* TPN K_m is higher (2.1×10^{-5} M), as previously reported.[4] These K_m values represent at least three separate determinations for each enzyme.

Electrofocusing: When the wild type G-6-P dehydrogenase was fractionated by electrofocusing (pH 5–7), three catalytically active components were found at pH values of 6.31, 6.42, and 6.51. A typical wild type pattern is shown in Figure 2*a*. The existence of multiple G-6-P dehydrogenase bands in *Neurospora* extracts was reported previously.[11] Typically, 60–70% of the initial activity was recovered in these peaks. From five separate determinations on different enzyme preparations the pI values of these peaks and those obtained from the other strains were reproducible to ± 0.02 pH units.

The balloon, frost, and *col-2* G-6-P dehydrogenase electrofocusing patterns are characteristic for each mutant. The mutant profiles differ from wild type either by shifted pI values of one or more components or by the absence of a peak. The frost profile more closely resembles that of the wild type than do those of the other two mutants (Fig. 2*b*). With the major component at pH 6.46, the three activity peaks are shifted to slightly higher pH values. In *col-2* preparations, two of the activity peaks are shifted to higher pH values (6.60 and 6.73) while the third component has a pI value (6.54) similar to that of the wild type peak at pH 6.51 (Fig. 2*c*). The relative proportions of these peaks differ from those of wild type. In contrast, the balloon profile is characterized by the absence of the pH 6.51 peak. The pH 6.41 peak constitutes the major component, with a minor peak at pH 6.33 (Fig. 2*d*). The electrofocusing pattern for the G-6-P dehydrogenase from the ragged mutant is identical with that of the wild type enzyme, which agrees with the heat inactivation data in suggesting that the ragged enzyme is normal.

The heterocaryon electrofocusing patterns are distinct from those of the wild type and the mutants. For example, five peaks of activity were observed in the balloon + *col-2* heterocaryon (Fig. 3). A major peak at pH 6.57 with a shoulder at pH 6.51 and a minor peak at pH 6.37, however, characterize the balloon + frost heterocaryon profile. Each heterocaryon profile is characteristic and intermediate between those of the two mutants constituting the heterocaryon.

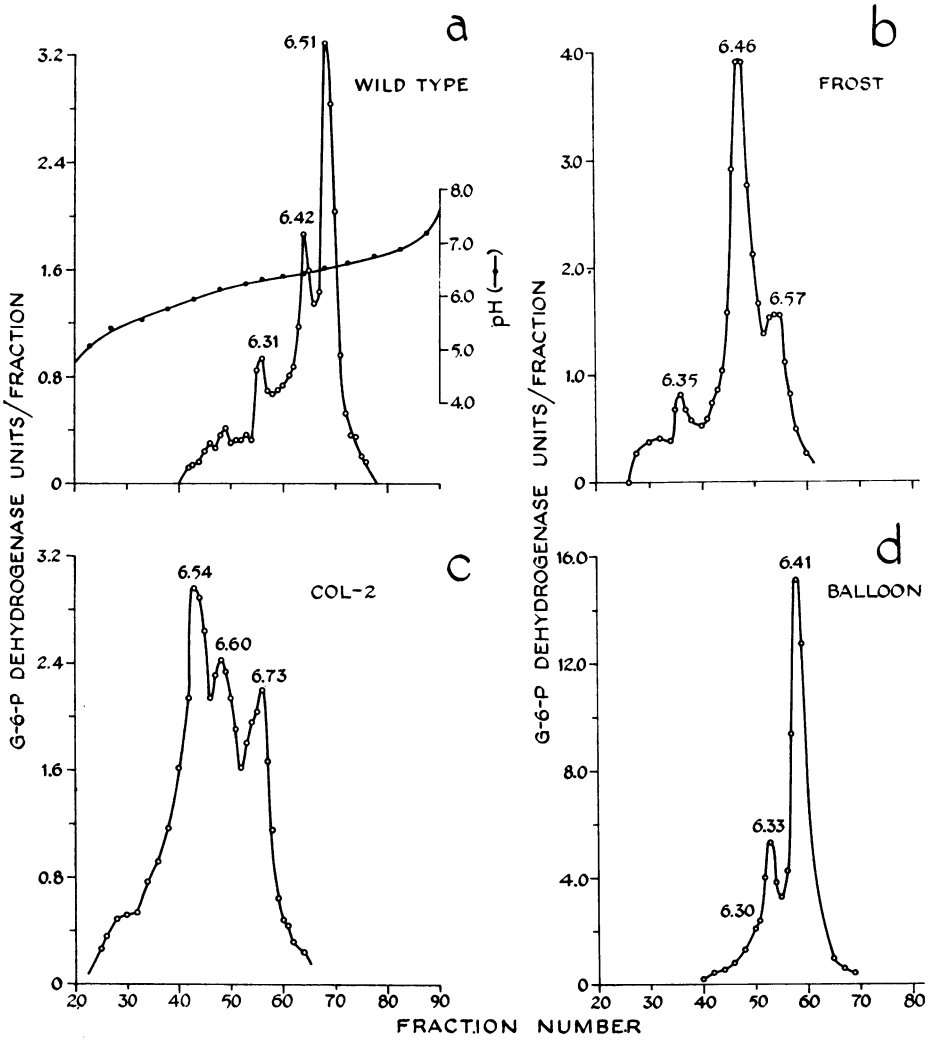

FIG. 2.—Electrofocusing pattern of *Neurospora* G-6-P dehydrogenase, (*a*) wild type, (*b*) frost, (*c*) *col-2*, and (*d*) balloon.

The intermediate thermolability and unique electrofocusing patterns suggest an *in vivo* recombination between the enzyme polypeptide chains in the heterocaryotic cell. These results indicate that the enzyme is composed of nonidentical polypeptide chains and therefore provide a molecular basis for the multi-genic control of *Neurospora* G-6-P dehydrogenase.

It is not known whether the different peaks of G-6-P dehydrogenase activity represent true isozymes. Both the dimeric and tetrameric forms of the enzyme are catalytically active.[9] One or more of the peaks may represent differences due to these forms, since a mixture of both dimers and tetramers is usually obtained in purified enzyme preparations. However, the addition of a molar excess of TPN to the wild type enzyme prior to electrofocusing does not alter the patterns.

Discussion. The balloon, frost, and *col*-2 G-6-P dehydrogenases differ from the wild type enzyme in thermolability, substrate affinities, electrofocusing patterns, and over-all stability. The observed changes of the mutant enzymes appear to be characteristic of these strains and not of all morphological mutants. Variables that affect the G-6-P dehydrogenase thermolability, such as the TPN concentration, do not alter the mutant and wild type differences. Since the enzyme from all strains was purified under identical conditions, the kinetic and electrofocusing differences would appear to be manifestations of structural changes rather than of extraneous variables such as ionic strength or possible contaminating molecules. These results suggest that the balloon, frost, and *col-2* genes control the primary structure of G-6-P dehydrogenase. A demonstration of amino acid substitutions in the mutant proteins is necessary, however, for an unequivocal proof. Of considerable interest is the similarity between the effects of these mutations in *Neurospora* and the enzyme changes found in nonspherocytic hemolytic anemia in man.[12] The G-6-P dehydrogenase in the abnormal erythrocytes also differs qualitatively from the normal enzyme (the K_m for G-6-P is about five times greater) and is considerably more labile.

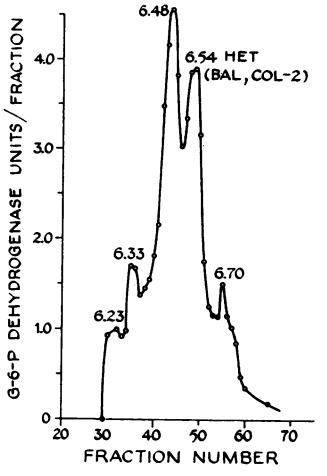

FIG. 3.—Electrofocusing pattern of the balloon + *col*-2 heterocaryon G-6-P dehydrogenase.

Each of the three *Neurospora* mutants has a distinctive morphological growth pattern (Fig. 1) although each has a common defective enzyme. The heterocaryons have a wild type-like morphology and intermediate enzymic properties while the double mutants are more extreme in both properties. This would indicate a relationship between the degree of morphological change and the severity of the enzymic defect. Such a correlation further strengthens the suggestion that the altered G-6-P dehydrogenases are indeed responsible for the abnormal morphology of the mutant strains.

The actual number of metabolic reactions that influence *Neurospora* morphology is probably smaller than indicated by the large number of mutant loci. If the enzymes affected by the morphological mutations consisted of two or more nonidentical polypeptide chains, the number of reactions would be reduced by a minimum factor of 2. The three enzymes known to affect *Neurospora* morphology, G-6-P dehydrogenase, phosphogulcomutase,[16] and 6-phosphogluconic acid dehydrogenase,[13] are all either multimeric molecules or exist in multiple forms.

Assuming that the cell wall of *Neurospora* is the target structure of the balloon, frost, and *col-2* mutations, it is not immediately obvious how an altered G-6-P dehydrogenase influences this structure. The role of the cofactor TPN may provide one explanation. A mutation in either of the first two enzymes of the pentose phosphate shunt, G-6-P dehydrogenase or 6-phosphogluconic acid dehydrogenase (reportedly altered in the *col-3* mutant[13]), would be expected to reduce the

TPNH content of the cell. Brody has, in fact, shown that the balloon, *col-2*, and *col-3* TPNH levels are reduced.[14] The pleiotropic effects of the defective G-6-P dehydrogenases may therefore be mediated by TPNH through its involvement in several diverse pathways such as the formation of lipids, glutamate, cell walls, and other cellular components.

It is impossible at the present to determine the relative importance of any of these pathways. However, fatty acid synthesis would seem to be a candidate for a significant role because of the correlation in many organisms between the rate of fatty acid synthesis and the production of TPNH by the pentose phosphate shunt, and also because of the involvement of fatty acids in membrane structures. Our preliminary results have indicated significant distortions in the relative ratios of unsaturated fatty acids in balloon and frost compared to wild type.[15]

In summary, mutations affecting enzymes such as G-6-P dehydrogenase that supply multifunctional cofactors would be expected to have drastic effects on the cell. The pleiotropic effects of such mutations as manifested by the abnormal morphologies emphasize the importance of the integration of metabolism. Studies, such as those reported here, can be expected to elucidate factors that influence morphology, and to provide further insight into the regulation of cellular metabolism.

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Abbreviations: G-6-P, glucose-6-phosphate; TPN, triphosphopyridine nucleotide; K_m, Michaelis constant; pI, isoelectric point.

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