*THE PRIMARY BIOCHEMICAL EFFECT OF A MORPHOLOGICAL MUTATION IN NEUROSPORA CRASSA**

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Communicated August 17, 1966

Mutations affecting the external appearance of *Neurospora crassa* have been known since 1941.[1] In general, these mutations have the characteristic of point mutations, i.e., they can be located on a genetic map by recombination, they can revert back to their original state, and they are recessive in heterocaryons.[2] So far, morphological mutations have been located at about 80 different chromosomal sites,[3] thus explaining their ease of isolation in mutant hunts. These mutations do not lead to any nutritional requirements and, in general, the addition of supplements and growth factors does not alter the morphology of a given mutant in any obvious manner. Therefore, these mutations appear to affect only those biochemical reactions which ultimately determine the pattern of growth of this organism, and thereby its external morphology.

Many of these mutants differ from the wild type in that their cell wall has a quantitatively different carbohydrate composition.[4, 5] It is not known, however, whether these unique cell-wall changes in each mutant are the cause of the morphological changes, or just a symptom of an altered metabolism. In an attempt to resolve this problem, and to describe biochemically the primary enzymatic defects produced by morphological mutations, the carbohydrate metabolism of various mutants was studied.

This paper will describe one type of morphological mutant, known as a colonial, in which mutations at the *col-2* locus appear to alter the structure of the glucose-6-P dehydrogenase, leading to an *in vivo* accumulation of glucose-6-P, and ultimately to a striking change in the growth pattern of *Neurospora crassa*.

Materials and Methods.—Cultures: The strain *col-2* (Y5331) was backcrossed twice to the Rockefeller wild-type strain (RL 3-8A), and the resulting colonial isolates designated as *col-2b*$_2$. One of these strains was crossed to the *inos* strain (37401) yielding inositol-requiring colonial isolates *col-2b*$_2$, *inos*. Wild-type-like revertants were obtained from this strain either spontaneously (*S*) or after mutagenic treatment (*R*), and the wild-type-like segregants from backcrosses to another *col-2b*$_2$, *inos* strain were designated as *col-2b*$_2$, *inos* *S*1, or *R*1, *R*2, *R*3, etc. Y5296 (*col-3*) is a different colonial strain whose genetic defect is closely linked to the *col-2* marker.

All cultures were grown on a minimal medium supplemented with inositol, and were harvested during the actively growing phase of growth, i.e., a doubling of dry weight was still possible.

The *col-2* strain of *Neurospora* has a dense, compact, and highly branched colony structure as compared to the spreading, filamentous form of the wild-type strain (see Fig. 1).[6] Microscopic examination of the colony edge of liquid-shake cultures or agar-grown cultures of *col-2* indicated that each cell compartment was short and bulbous in shape, and that most of the cells were branch points for the outgrowth of two new cells.

Determination of glucose-6-P level: Mycelia were extracted with boiling 80% ethyl alcohol for 5–10 min; the extract was filtered and then concentrated in a vacuum rotary evaporator to 1–2 ml. After chilling and centrifuging 10 min at 8000 × *g*, an aliquot (0.4–0.5 ml) of the extract was chromatographed in an ascending n-butanol-acetic acid-water system (2:1:1) for approximately 40 hr. A marker strip from one edge was used to locate the glucose-6-P by $AgNO_3$ staining, and the area corresponding to glucose-6-P eluted in 2.0 ml H_2O. Different aliquots of the eluate were diluted to 3.0 ml with 0.1 *M* Tris-Cl buffer, 0.60 μmole TPN was added to each tube, and the OD_{340} then measured. The reaction was started with the addition of 0.2 unit of glucose-

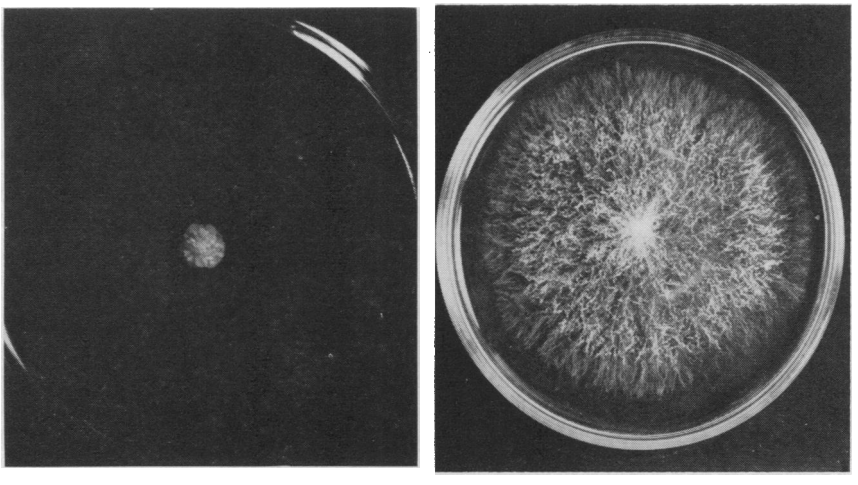

FIG. 1.—Growth on minimal agar after 48 hr at 30°. The wild-type strain is on the right, *col-2* strain on the left.

6-P dehydrogenase (obtained from Boehringer und Soehne), and after 30 min or more, the change in OD_{340} was recorded. The values of 1 μmole TPNH = 2.07 OD_{340} units, and glucose-6-P molecular weight = 340 were used for calculations. The recovery of glucose-6-P added to mycelia just before extraction was 85–90%.

Purification of glucose-6-P dehydrogenase: Eleven gm of a freshly lyophilized culture were briefly ground with 300 ml cold 0.10 *M* Tris-Cl pH 7.4–0.01 *M* $MgCl_2$ buffer in a Waring Blendor, and the mixture was centrifuged at 8000 × *g*, 15 min. The pellet was re-extracted in the same manner with 100 ml of buffer, and the second supernatant fraction combined with the first to form the crude extract. To this extract, 5 ml 1 *M* $MnCl_2$ (neutralized) were added with rapid stirring for 10 min, followed by centrifugation. To the resulting supernatant solution, solid $(NH_4)_2SO_4$ was added to 30% of saturation and adjusted by the addition of 1 *N* NaOH to pH 7.0 (pH paper). After centrifugation, the $(NH_4)_2SO_4$ content of the supernatant fraction was raised to 45% and adjusted to pH 6.4. The pellet formed upon centrifugation was dissolved in 15 ml 0.1 *M* Tris-Cl buffer and dialyzed overnight against 2 liters 0.01 *M* Tris-Cl buffer. After dialysis, insoluble material was removed by centrifugation, and the supernatant solution mixed with 40 ml of calcium phosphate gel. Adsorption of 95% of the enzyme took place after 10 min of stirring, and the mixture was then centrifuged. The gel was successively eluted with 50 ml of solutions of 8, 12, 12, and 16% saturated $(NH_4)_2SO_4$ all in 0.01 *M* Tris-Cl buffer. Eluates of the highest specific activity were combined and the $(NH_4)_2SO_4$ content raised to 50%. After centrifugation, the precipitate was dissolved in 8 ml of 0.1 *M* Tris-Cl buffer, and dialyzed overnight as before. The resulting solution was then chromatographed on a 2 × 22-cm DEAE-cellulose column (Cl^- form). The linear gradient was 0–0.25 *M* NaCl in 0.01 *M* Tris-Cl buffer (200 ml per bottle). All operations were performed at 5°.

Enzyme assays: The reaction mixture for the glucose-6-P dehydrogenase assays was 2.5 ml 0.1 *M* Tris-Cl, pH 7.4, 0.01 *M* $MgCl_2$ buffer, 3.0 μmoles Na_2 glucose-6-P·3 H_2O, and 0.66 μmole TPN. Initial rates only were used in the kinetic analysis, i.e., at most a time interval comprising 10–15% of the time required for the completion of the reaction. Heat inactivation experiments were performed on fresh enzyme preparations (sp. act. 25) in 0.1 *M* Tris-Cl, pH 7.4 buffer at a final protein concentration of 10 μg/ml. The reaction mixture for other assays was similar to the above, except that glucose-6-P was replaced by an excess of the pertinent substrate. All chemicals were Sigma grade, Sigma Chemical Co. All values are given in international enzyme units.

Results.—Comparable amounts of ethanol extracts of *col-2* and of the wild-type *Neurospora crassa* strains were subjected to ascending paper chromatography in an

n-butanol-acetic acid-water mixture. It was found that extracts of *col-2* contained more of a compound (located with $AgNO_3$ stain), subsequently identified as glucose-6-phosphate in both strains. The evidence for this identification is as follows: Paper chromatography of these extracts in three different solvent systems (butanol-acetic acid-water; phenol-water; and pyridine-ethyl acetate) all indicated that this compound had the R_f of authentic glucose-6-P. When C^{14}-labeled glucose-6-P was added to the extracts and the mixture subsequently chromatographed, 97 per cent of the radioactivity was located at the same position as the unknown compound. The unknown compound was eluted from chromatograms, precipitated as a barium salt with alcohol, and eventually rechromatographed. The eluate from the second chromatogram, when subjected to acid hydrolysis or to alkaline phosphatase action, yielded only glucose (chromatography and/or glucostat reagent[7]) and inorganic phosphate.[8] This eluate was also a substrate for highly purified glucose-6-P dehydrogenase,[9] whereas other phosphorylated sugars (fructose-6-P, glucose-1-P, 6-phosphogluconic acid, galactose-6-P, mannose-6-P, 2-deoxyglucose-6-P, and glucosamine-6-P) were not. The evidence above appeared to be sufficient for the purposes of this work to establish this compound as glucose-6-P, although further evidence could be gathered.

The glucose-6-P levels in ethanol extracts of various strains were estimated[10] as indicated in the *Materials and Methods* section, and are presented in Table 1. These normalized values are the ranges found for individual determinations on 6–8 separate cultures of these strains. They have been made comparable by using residual dry weight, since extractions[11] are done either on fresh mycelia or on lyophilized powder. It is clear that an elevated level of glucose-6-P is correlated with the presence of the *col-2* marker. In addition, eight ascospore cultures from a single ascus from a cross between *col-2* and wild type have been examined; the four colonial strains had an elevated glucose-6-P level while the four wild-type strains had the normal wild-type level. Since wild-type like revertants from the colonial mutant had the wild-type level of glucose-6-P, it would appear that the glucose-6-P level depends upon the state of the *col-2* gene. Although other genetic factors could also determine the steady-state level of this intermediate, it is interesting that of 11 other different colonial strains tested, none appears to have an elevated glucose-6-P level.

TABLE 1

LEVEL OF GLUCOSE-6-P IN VARIOUS STRAINS

Strains	Description	Glucose-6-P content (μmoles/gm residual dry weight)*
col-2	Colonial	3–4.0
col-2b₂	Colonial	3–4.0
col-2b₂, inos	Colonial	3–4.0
Y5296	Colonial	0.3–0.6
RL 3-8A	Wild type	0.3–0.6
RL 3-8A on sorbose	Colonial	0.3–0.6
col-2b₂, inos R2	Wild-type-like revertant	0.3–0.6
col-2b₂, inos R10	Wild-type-like revertant	0.3–0.6
col-2b₂, inos R24	Wild-type-like revertant	0.3–0.6
col-2b₂, inos S1 at 24°	Semicolonial revertant	1.0–1.5
col-2b₂, inos S1 at 34°	Colonial	3.0–4.0

Unless otherwise stated, all cultures were grown as shake cultures at 30° on glucose-minimal media supplemented with 20 μg inositol/ml. Extraction and assay procedures, and further descriptions of the strains listed above are given in *Materials and Methods*.

* Weight after ethanol extraction and drying.

The glucose-6-P levels in the wild-type and *col-2* strains were not altered by changes in the composition of the media, by addition of various compounds, or by various other conditions.[12] However, the growth temperature had a decided effect, as shown in Figure 2. Although the wild type showed little or no change with temperature, at 34° the *col-2* strain had almost twice the glucose-6-P present at 24°. A possible explanation for this increased glucose-6-P is discussed below.

In order to attempt to understand the glucose-6-P accumulation, various enzymes involved in the metabolism of glucose-6-P were assayed. Crude extracts of the wild-type and *col-2* strains grown at 30° had identical specific activity ranges for the following four enzymes: glucose-6-P dehydrogenase 0.22–0.27, 6-phosphogluconic acid dehydrogenase 0.06–0.08, phosphoglucomutase 0.04–0.06, and hexose-6-phosphate isomerase 0.12–0.14.

Heat inactivation studies of dialyzed crude extracts indicated that the glucose-6-P dehydrogenase from the colonial strain was more labile than the corresponding enzyme from the wild-type strain. This difference in heat stability also segregated with the colonial marker in all cases tested. On this basis, extensive purification of this enzyme from the two strains was undertaken in order to characterize further the apparent difference.

The enzyme preparations (see *Materials and Methods* section) from both strains which were used for all measurements had a specific activity of 25, i.e., approximately 100× purified over the crude extract. These glucose-6-P dehydrogenase preparations had no detectable activity[13] of those enzymes which might have interfered with the kinetic measurements, such as hexose-6-phosphate isomerase, phosphoglucomutase, 6-phosphogluconic acid dehydrogenase, and hexokinase. Also, no TPNase, glucose-6-phosphatase, or TPNH oxidase activity was found in either of the two purified glucose-6-P dehydrogenase preparations.[12]

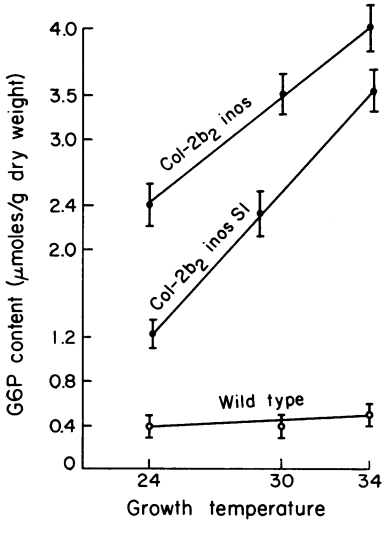

FIG. 2.—Temperature effect on glucose-6-P steady-state level. Experimental procedures listed in *Methods*. Dry weight refers to residual dry weight after ethanol extraction and drying.

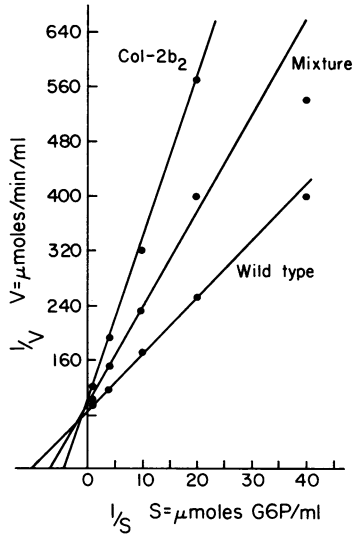

FIG. 3.—Lineweaver-Burk plot of kinetic data on two glucose-6-P dehydrogenase preparations. Conditions listed in text.

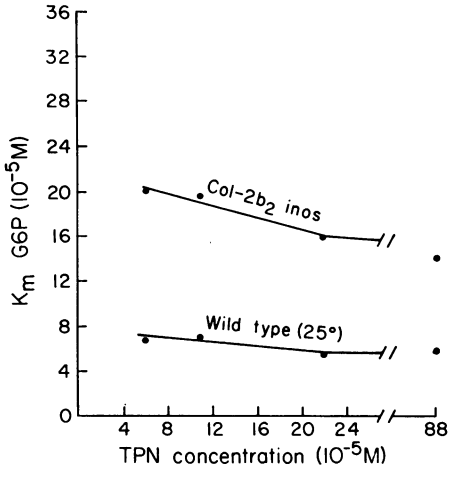

FIG. 4.—Effect of TPN concentration on the glucose-6-P K_m at 25°.

Figure 3 is a Lineweaver-Burk plot of kinetic data on the glucose-6-P dehydrogenase preparations from strains RL 3-8A (wild type) and *col-2b*$_2$ (colonial). The glucose-6-P K_m is 7.0×10^{-5} *M* (wild type) and 1.6×10^{-4} *M* (colonial) when assayed at 25° with excess TPN. A mixture of these two enzyme preparations had an intermediate glucose-6-P K_m. Various anions and cations, including the $(NH_4)_2SO_4$ used for stabilization of purified enzyme preparations, had no significant effects on these kinetic measurements, over a wide range of concentrations and conditions. Since the glucose-6-P dehydrogenase from these two strains was purified by an identical procedure, it would appear that the difference in affinity for glucose-6-P is due to a change in the structure of the enzyme, rather than to any possible contaminants, ionic strength effects, etc.

The affinities of both the colonial and wild-type enzymes for glucose-6-P are dependent upon the TPN concentration over a certain range, as shown in Figure 4. It is interesting that the enzyme from the colonial strain has an even poorer affinity for glucose-6-P under the assay conditions of less than saturating levels of TPN. Since the steady-state level of TPN in the cell appears to be quite low, relative to the amount of glucose-6-P dehydrogenase, this assay condition may correspond more closely to physiological conditions.

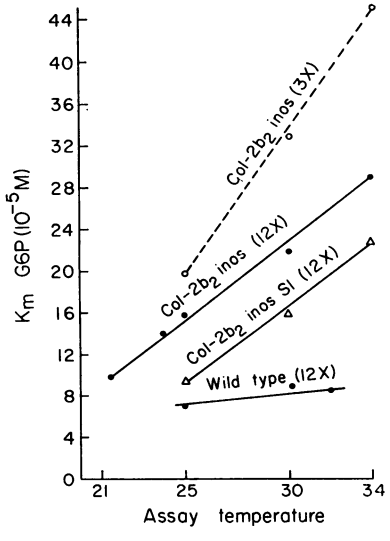

FIG. 5.—Variation of the glucose-6-P affinity with temperature. The figures in parentheses (3× or 12×) refer to the TPN concentration which is 3 or 12 times the respective TPN K_m value.

The K_m for glucose-6-P of both of the enzyme preparations varies with the assay temperature as shown in Figure 5. The wild-type enzyme varies only slightly, as might be expected. However, the enzyme from the colonial strain shows a considerable temperature effect, as might be expected for an altered enzyme. When assayed at low TPN levels, the temperature effect is even more pronounced, as indicated in Figure 5. There is a positive correlation between the temperature effect on the K_m of the enzyme for glucose-6-P and on the *in vivo* accumulation of glucose-6-P. At higher temperatures, the mutant enzyme has less affinity for glucose-6-P, and more glucose-6-P is found in the cell. Neither of these parameters varies much in the wild type. Therefore, it would appear that the *in vitro* K_m measurement and its variation with temperature may accu-

rately reflect the *in vivo* substrate affinity of this enzyme and its variation with temperature.

This last statement is supported by the properties of the temperature-sensitive revertant, *col-2b*$_2$, *inos* S1. This strain has a semicolonial morphology at 24°, and is colonial at 34°. The effect of temperature on the glucose-6-P level is given in Figure 2, and the effect of temperature on the glucose-6-P affinity of the glucose-6-P dehydrogenase is given in Figure 5. It is clear that for this strain also there is a correlation between lowered substrate affinity and increased substrate accumulation. This particular strain is interesting since the higher temperature simultaneously changes the enzyme affinity and the morphology of the culture. Also, it provides an additional genetically altered form of this enzyme due to mutation at the *col-2* locus,[14] thus strengthening the correlation between changes at the *col-2* locus and changes in the structure of the glucose-6-P dehydrogenase.

The TPN K_m for the wild-type enzyme is 6.6×10^{-5} M and for the colonial enzyme 1.3×10^{-4} M, when measured with excess glucose-6-P at 25°. These K_m values for each enzyme show little fluctuation with the glucose-6-P concentration. Other kinetic measurements on these two enzymes, such as the competitive inhibition with TPN shown by both phosphate and TPNH, indicated that the two enzyme preparations have similar K_I values, and that the Q_{10} (24–34°) is 1.5 for both enzymes.

Since the altered glucose-6-P dehydrogenase segregated with the *col-2* marker in eight strains tested, and the wild-type form was found in seven wild-type strains tested, wild-type-like revertants were examined. The glucose-6-P dehydrogenase from three separately isolated revertants, judged not to contain closely linked suppressor genes, was identical to the wild-type enzyme in all of the measurable kinetic parameters. Therefore, it would appear that the structural change in the glucose-6-P dehydrogenase, which restores it to a wild-type-like state, is sufficient to restore the wild-type morphology also. Also examined was the glucose-6-P dehydrogenase from other strains such as *col-3*, wild type grown on sorbose media (colonial phenotype), and a balanced heterocaryon on minimal medium between *arg* (wild-type morphology) and *col-2b*$_2$, *inos*. The *col-3* strain and the sorbose-induced colonial phenocopy both had the wild-type form of the enzyme, indicating that colonial growth alone does not cause a change in the glucose-6-P dehydrogenase. The heterocaryon, which had wild-type morphology, appeared to have both types of glucose-6-P dehydrogenase in the purified preparations, judging from the glucose-6-P K_m. A mixture of crude extracts from the two strains, grown separately, also yielded glucose-6-P dehydrogenase preparations with a glucose-6-P K_m intermediate between the value for the colonial and wild-type enzymes.

Heat-inactivation studies indicated first-order inactivation kinetics for both enzyme preparations, with a half life at 48° of 7.0 ± 0.8 min for the wild-type enzyme, and 3.5 ± 1.0 min for the colonial enzyme. Since the mixture of these preparations had a half life at 48° of 5.0 min, it would appear that the difference in heat stability is a significant one. Under conditions of substrate excess, both enzyme preparations are protected, as evidenced by a half life of 40 ± 2.0 min for the wild-type enzyme. and 12 ± 1.0 min for the colonial enzyme.

Enzyme preparations of 0.2–0.5 mg protein/ml lose approximately 20 per cent of their activity (wild type) and approximately 80 per cent of their activity (colonial strain) upon freezing and thawing. The addition of $(NH_4)_2SO_4$ to a final concentra-

tion of 2 per cent stabilizes both enzyme preparations considerably, although the enzyme from the colonial strain is still more labile. The differences in stability and heat sensitivity between these two purified enzyme preparations, under a variety of conditions, also suggests that the enzyme from the colonial strain is structurally different from the wild-type enzyme.

Discussion.—The higher steady-state level of glucose-6-P in the *col-2* strain appears to be characteristic of this particular mutant and not simply due to a general colonial growth pattern of *Neurospora* or to an artifact of extraction, etc. However, it is not known whether there is a higher average glucose-6-P concentration throughout the mycelia, or whether the additional glucose-6-P is localized in a particular area of the mycelial mass. Since *Neurospora* wild type shows localization of certain enzymes only in the growing tip region,[15] it is possible that the enzymes involved in glucose-6-P metabolism are not uniformly distributed throughout the mycelial cytoplasm. Because of these possible spatial considerations, it may not be meaningful to speak of a single pool of glucose-6-P or of an average intracellular glucose-6-P concentration in *Neurospora crassa*. It is clear, however, that the total level of glucose-6-P found in this organism is affected by the glucose-6-P dehydrogenase structure.

The glucose-6-P dehydrogenase from the *col-2* mutant differs from the wild-type enzyme in substrate affinities, temperature sensitivity, and general stability. Since these two enzyme preparations are identical with regard to pH optimum, acid lability, chromatographic behavior, and starch block electrophoretic patterns,[12] it would appear that the enzyme from the *col-2* strain is only slightly different from the wild-type enzyme. Although peptide patterns of mutant and wild-type enzymes are needed for unequivocal proof of a change in the primary structure of this enzyme, the altered properties of the mutant enzyme, by analogy to many similar instances, strongly suggests that these changes are due to an amino acid substitution in the structure of the protein. Furthermore, it has been experimentally shown that these changes are not due to the loss of enzyme-bound TPN, or to the state of the enzyme when purified, i.e., in a monomeric versus polymeric form.[12]

It seems unlikely that a mutation at the *col-2* locus changes a regulatory control system, leading eventually to the synthesis of a new type of glucose-6-P dehydrogenase and a repression of the wild-type form, since heterocaryons appear to have both forms of the enzyme. In addition, a model of the type on A form, off B form of the enzyme could not account for the finding of a third different type of enzyme, i.e., the temperature-sensitive glucose-6-P dehydrogenase in the temperature-sensitive revertant strain. These two different forms of the enzyme in mutant strains enhance the probability that the *col-2* gene is the structural gene for glucose-6-P dehydrogenase.

The pleiotropic effects produced by the slight change in the glucose-6-P dehydrogenase of the *col-2* mutant are not known at present. Employing specifically labeled C^{14}-glucose, it has been shown that the *in vivo* activity of the pentose-phosphate shunt appeared to be decreased by approximately 30 per cent in *col-2* as compared to wild type.[12] Although numerous supporting measurements are needed with this type of data, it is possible that this 30 per cent decrease effectively decreases the TPNH steady-state level in the *col-2* strain. A lowered TPNH content could easily have many effects on the metabolic regulation needed for the balance between

cellular synthesis and extension. In addition, the 6–8× accumulation of glucose-6-P might stimulate certain reactions in an allosteric fashion, or effectively inhibit other reactions. Whatever the cause of the pleiotropic effects, it is clear that the change in the glucose-6-P dehydrogenase is ultimately responsible for the change in the morphology. This type of finding supports the view that the synthesis and regulation of morphological structures can eventually be resolved into a series of steps, and some of these steps described in terms of enzymes and substrates.

Summary.—A mutation in a particular gene in *Neurospora crassa* which leads to a morphologically distinct colonial growth pattern causes a decrease in the affinity of the glucose-6-P dehydrogenase for both glucose-6-P and TPN. This decreased glucose-6-P affinity can be consistently correlated with an increased *in vivo* glucose-6-P level in the colonial strain under a variety of conditions. The altered enzyme and higher glucose-6-P level segregate with the *col-2* marker, and both are changed back to the normal wild-type state by mutations which restore a wild-type morphology. The evidence indicates that the *col-2* locus is the structural gene for the glucose-6-P dehydrogenase and that the primary effect of the *col-2* mutation in *Neurospora* is to change the structure of this enzyme, producing the pleiotropic effects which ultimately lead to the change in morphology.

The technical help of George Horn, George Sikorskyj, Mary Morgan, and especially Denny Meudt is gratefully acknowledged.

* This work was supported in part by an American Cancer Society postdoctoral fellowship to Stuart Brody, and by a research grant from the U. S. Public Health Service (CA 03610).

[1] Lindegren, C. C., and G. Lindgren, *J. Heredity*, **32**, 435 (1941).

[2] Barratt, R. W., and L. Garnjobst, *Genetics*, **34**, 351 (1949).

[3] Garnjobst, L., and E. L. Tatum, in preparation.

[4] de Terra, N., and E. L. Tatum, *Science*, **134**, 1066 (1961).

[5] Mahadevan, P. R., and E. L. Tatum, *J. Bacteriol.*, **90**, 1073 (1965).

[6] Courtesy of L. Garnjobst.

[7] Glucose oxidase from Worthington Biochemicals, Freehold, N.J.

[8] Assayed by the method of Fiske, C. H., and Y. Subba-Row, *J. Biol. Chem.*, **66**, 375 (1925).

[9] Sp. act. 140 units/mg, obtained from Boehringer und Soehne.

[10] Other methods of estimation, such as the anthrone method or diphenylamine method, gave values for the glucose-6-P present which were within 15% of the values obtained by the enzymatic assay.

[11] Other extraction methods, employing cold 1 *M* perchloric acid or cold 10% TCA, gave results comparable to the ethanol extractions.

[12] Brody, S., unpublished data.

[13] No activity was found for these enzymes, i.e., <0.1% present under the same assay conditions which gave appreciable activity of the four enzymes in crude extracts.

[14] A presumed mutation at the *col-2* locus, since a cross to the wild type yielded no *col-2* cultures among the 180 ascospore isolates tested.

[15] Zalokar, M., *Am. J. Botany*, **46**, 602 (1959).