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Phosphoglucomutase Mutants of *Neurospora sitophila* and Their Relation to Morphology*

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Abstract. Biochemical and genetic evidence is presented that the *rg-1* and *rg-2* genes control the structure of the two isozymes of phosphoglucomutase (phosphoglucomutase I and II) in *Neurospora sitophila*. Results of kinetic and gel filtration studies show that the two phosphoglucomutases may exist *in vitro* either as separate or as a single but complex molecular species depending on the ionic concentration. The complex phosphoglucomutase molecule is suggested to be the physiologically active form of the enzyme. The change in the enzyme structure is discussed in relation to the morphological change in the ragged mutant strains.

The study of morphological mutants can provide an insight into the architecture and function of the molecules which directly or indirectly determine the cell structure. In a previous communication[1] from this laboratory it was reported that the morphology of the ragged (*rg*) mutant of *Neurospora crassa* is related to low specific activity of the enzyme phosphoglucomutase (E.C.2.7.5.1) and to significant accumulation of the substrate. It was concluded that the *rg* locus controls the structure of phosphoglucomutase in *N. crassa*.

The work described here was undertaken to study the biochemical basis of ragged morphology in *N. sitophila*, where this morphological change is known to occur as a result of mutation in either the *rg-1*$^+$ or the *rg-2*$^+$ gene.[2] A specific suppressor (*su-2*) is known to restore wild-type growth in strains (carrying both *rg-2* and *su-2* genes) previously designated as "apparent wild type" (AWT). In this paper we present evidence that the *rg-1* and *rg-2* loci are the structural genes for two isozymes of phosphoglucomutase (PGM) in *Neurospora*.

Materials and Methods. Strains: Strains of *N. sitophila* used in the present investigation were wild type (Wa, WA), ragged-1 (M-17, M-17Wa-1), ragged-2 (SFT-5, SFT-9), AWT(15-6). The genes *rg-1* and *rg-2* are nonallelic in *sitophila*[2] and *crassa*[3] genetic background. These strains have been described elsewhere.[2]

Chemicals: Glucose-1,6-diphosphate (G-1,6-P) was obtained from Calbiochem, glucose-6-phosphate dehydrogenase from Boehringer & Schone Co., Sephadex G-100 and G-25 from Pharmacia & Co., and human γ-globulin from Pentax Co. Barbitol buffer, ampholine (pH 3–10, 3–6), and equipment for electrofocusing were obtained from LKB Products Co. Glucose-1-phosphate (G-1-P) and all other reagents used were purchased from Sigma Chemical Co. A 0.1 M Tris-HCl buffer containing 0.01 M $MgCl_2$, pH 7.5, was used throughout the work except where specified.

Enzyme preparation and assay: Phosphoglucomutase was extracted and purified by a modification of the method described earlier.[1] Its activity was estimated by the coupled assay method as described earlier.[1]

Electrophoresis and location of PGM on gel: Agar gel slides were prepared by a method described elsewhere.[4] Agar gel electrophoresis was run in 0.05 M barbitol buffer (pH 8.7) at 4°C. Starch gel electrophoresis was run in Tris-malate buffer (pH 7.6) as described elsewhere.[5] After electrophoresis, gels were immersed in 100 ml of the reaction mixture (containing 5 μmole G-1-P, 0.5 μmole G-1,6-P, 0.6 μmole TPN, 0.5 IEU glucose-6-phosphate (G-6-P) dehydrogenase, 0.1 mg nitroblue tetrazolium, and 0.05 mg phenazine methosulfate/ml of buffer) and incubated in the dark at 37°C in a moist chamber to localize the PGM activity. The purple color indicating PGM activity was maximal after 40 min. No color developed when G-1-P was omitted from the reaction mixture.

DEAE-cellulose chromatography: A partially purified PGM preparation was placed on the DEAE-cellulose column (1.5 × 30 cm) and eluted by a gradient of 0–0.15 M NaCl in buffer. Each fraction was assayed for PGM activity, and for protein by absorption at 280 mμ. PGM I and PGM II, separated by DEAE-cellulose chromatography, were concentrated on Sephadex G-25 and were then further purified by gel filtration on Sephadex G-100.

Electrofocusing: Electrofocusing was performed as described by Vesterberg and Svensson.[6] PGM (5 mg of protein) was introduced into the column (LKB 8100, 110-ml capacity) containing a 4% solution of the carrier ampholytes (pH 3–6) in a sucrose gradient (0–50%). A final potential of 700 V was applied by a current of 5 mA. Each experiment was run for about 70 hr at 4°C. At the end of the experiment, 1-ml fractions were collected with a peristaltic pump and then assayed for PGM activity. The isoelectric point of PGM was determined by measuring the pH of the fraction of highest enzyme activity.

Gel filtration: The standard method of gel filtration was employed.[7] For the routine purification of PGM, the enzyme preparations were applied on the Sephadex G-100 column and eluted with buffer. Each fraction was assayed for PGM activity, and for protein by absorption at 280 mμ. During the study of the effect of ionic concentration on PGM, the wild-type PGM sample and the marker proteins were applied in a 2-ml volume to the top of the Sephadex G-100 column (1.8 × 100 cm) and then eluted with buffer at low ionic (0.01 M Tris HCl and 0.005 M $MgCl_2$, pH 7.5) or high ionic concentration (0.05 M–0.25 M $MgCl_2$). The flow rate was adjusted to 4–5 drops/min and 2-ml fractions were collected. Each fraction was assayed for PGM activity and for the marker proteins: catalase (mol wt 250,000) was assayed by measuring the decrease in ultraviolet absorption of a standard H_2O_2 solution as described elsewhere;[8] human γ-globulin (mol wt 160,000), hemoglobin (mol wt 64,000), and cytochrome *c* (mol wt 12,500) were determined by absorption at 280, 408, and 412 mμ respectively.

Kinetic studies: The Michaelis constant (K_m) studies were performed at 25°C with purified or semipurified PGM preparations. The K_m values were determined by standard Lineweaver and Burke plots. Heat inactivation studies were done with the total PGM or with PGM I and PGM II separately at low (0.001–0.005 M $MgCl_2$) or high (0.05–0.2 M $MgCl_2$) ionic concentrations.

Results. Wild-type PGM isozymes: The purified wild-type PGM had a specific activity of about 70 and contained very little protein other than PGM.[3] The wild-type PGM was found to be quite stable during purification and storage. Subsequent study by electrophoresis, DEAE-cellulose chromatography, and electrofocusing revealed two PGM isozymes in the enzyme preparations.

Agar gel electrophoresis of the wild-type PGM showed two bands of enzyme activity, both migrating towards the anode (Fig. 1). These are designated as PGM I (slow band) and PGM II (fast band). No change was found in the relative activity or mobility of the two bands when PGM preparations were incubated with G-1-P, G-6-P, or G-1,6-P before electrophoresis. On starch gel electrophoresis, similar slow (PGM I) and fast (PGM II) bands of activity were seen migrating towards the anode at distances of 6 and 11 cm respectively under

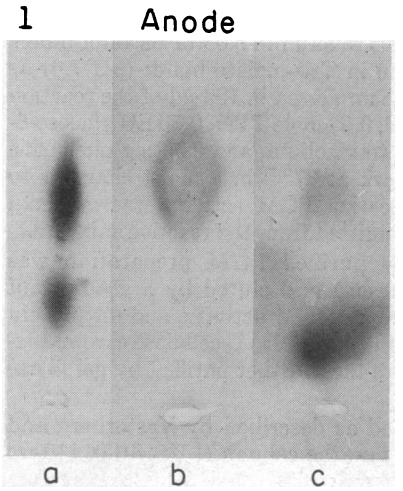

FIG. 1.—Agar gel electrophoresis of PGM. (*a*) Wild type, (*b*) ragged-1, (*c*) ragged-2.

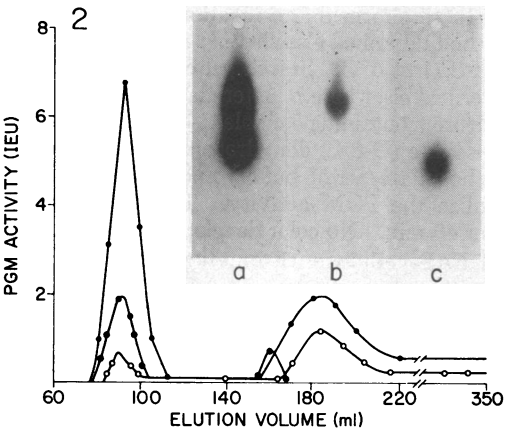

FIG. 2.—DEAE-cellulose chromatography of PGM. Wild type (——), ragged-1 (—○—) ragged 2 (—●—).
Inset: agar gel electrophoresis. (*a*) Input on DEAE-cellulose column, (*b*) peak-1, (*c*) peak-2 obtained after chromatography.

conditions described in *Material and Methods*. The wild-type PGM preparations were found to give two peaks of enzyme activity when chromatographed on DEAE-cellulose column (Fig. 2). The PGM I was first to elute; the PGM II was eluted only on increase in ionic concentration of the gradient. The activities of these two peaks (PGM I and PGM II) were approximately 60 and 40% respectively. On subsequent agar gel electrophoresis, peak I gave only the slow band (PGM I) of enzyme activity, whereas peak II gave only the fast band (PGM II) (Fig. 2). On electrofocusing, the wild-type PGM was found to have two distinct peaks of enzyme activity. The isoelectric points of these two peaks were 4.23 ± 0.02 and 4.43 ± 0.02 respectively. Essentially the same isoelectric point values were obtained for the wild-type PGM I and PGM II when these were subjected separately to electrofocusing.

Ragged PGM isozymes: The specific activities of the partially purified preparations from the ragged-1 and ragged-2 strains were 8–10. The ragged PGM's were relatively unstable during purification and on storage. Two isozymes of PGM were also found in the ragged strains, but differed markedly in their activity and kinetic properties. The ragged PGM gave two bands of enzyme activity on agar gel electrophoresis. In ragged-1 most of the PGM activity was found in PGM II, while very little activity was found in PGM I (Fig. 1). In ragged-2 most of the enzyme activity was found in PGM I with little in PGM II (Fig. 1). The ragged PGM also showed two peaks of PGM activity on DEAE-cellulose chromatography (Fig. 2).

Comparison of the wild-type and ragged PGM's: The level of PGM in the crude extracts of the mutant was found to be only about 10% of the wild type (Table 1). The results of DEAE-cellulose chromatography showed that the levels of the isozymes were significantly reduced in the mutants (Fig. 2). The

TABLE 1. *A comparison of the level and specificity of PGM in different strains of N. sitophila.*

Strains	Specific activity (IEU/mg protein)	Michaelis Constant G-1-P (M)	Michaelis Constant G-1,6-P (M)
Wild type	0.35–0.40	7.8×10^{-5}	1.8×10^{-6}
Ragged-1	0.03–0.04	3.3×10^{-4}	1.1×10^{-5}
Ragged-2	0.025–0.035	3.5×10^{-4}	1.3×10^{-5}
AWT	0.20–0.30	1.6×10^{-4}	3.7×10^{-6}

wild-type and the ragged PGM's were found to show no stimulation or inhibition of enzyme activity in mixtures. These results make unlikely the presence of an activator of PGM in the wild type or an inhibitor in the mutants.

A marked difference in the heat stability of the wild-type and mutant PGM was observed. The wild-type PGM's were relatively heat-stable (Table 2). The wild-type PGM I showed an initial increase (20%) in the activity of the enzyme following heat treatment. In ragged-1, PGM I was found to be heat-labile while PGM II was as heat-stable as wild-type PGM II (Table 2). In contrast, in ragged-2, PGM I was found to be relatively heat-stable but PGM II was heat-labile (Table 2). When the ragged PGM preparations were briefly heat-inactivated and then subjected to agar gel electrophoresis, the gel showed only PGM II activity from ragged-1 or PGM I activity from ragged-2; the second band of PGM activity in each mutant strain was lost in such experiments. The heat treatment had no effect on the two electrophoretic bands of the wild-type PGM. The heat inactivation studies clearly demonstrated that only PGM I in ragged-1 and PGM II in ragged-2 were altered.

Significant differences were found between the affinity of the wild-type and ragged PGM's for G-1-P and G-1,6-P as measured by the Michaelis constant. The G-1-P K_m and G-1,6-P K_m of the total ragged PGM's were found to be at least 6–8 times greater than those of the wild type (Table 1). The G-1-P K_m and G-1,6-P K_m of the PGM II in ragged-1 and PGM I in ragged-2 were found to be similar to the wild-type K_m values. The K_m values of the wild-type PGM I or PGM II were found to be similar to those of the total wild-type PGM. The K_m values obtained with PGM samples of different specific activities were essentially the same, as reported earlier.[1]

The wild-type PGM showed a three to fourfold increase in activity when preincubated with imidazole (0.1–0.5 M) and Mg^{2+} (0.01 M). The ragged PGM, however, showed no increase in activity when preincubated similarly with imidazole and Mg^{2+}.

The effect of ionic concentration on the behavior of PGM: The specific activities of the ragged PGM preparation when dialyzed to low (Mg^{2+}) were found to increase by 70–100%. The wild-type PGM instead showed a slight decrease in specific activity on dialysis to low (Mg^{2+}).

TABLE 2. *Heat stability of Neurospora PGM isozymes.*

	Half Life at 50°C (min) PGM I	Half Life at 50°C (min) PGM II
Wild type	150 ± 10	210 ± 10
Ragged-1	10 ± 1	210 ± 10
Ragged-2	150 ± 10	10 ± 1

The change in ionic concentration was found to have a marked influence on the course of heat inactivation of the ragged PGM but not of the wild-type PGM. The wild-type PGM was found to be relatively heat-stable at both high and low (Mg^{2+}); the half life of the enzyme was 180 ± 10 min. The ragged PGM at high (Mg^{2+}) was found to be quite heat-labile (half life of 30 ± 5 min). The ragged PGM at low (Mg^{2+}) was, however, relatively heat-stable (half life of 150 ± 10 min) and showed a marked change in the rate of heat inactivation. About 30% of the total PGM activity was lost within the first 20 min. Afterwards, the heat inactivation proceeded at a rate comparable to that of the wild-type PGM.

Similar effects of the ionic concentration were observed on the heat inactivation curves of an artificial mixture of the mutant PGM I (from ragged-1) and the wild-type PGM II (from wild type) (Fig. 3). At low (Mg^{2+}), the heat

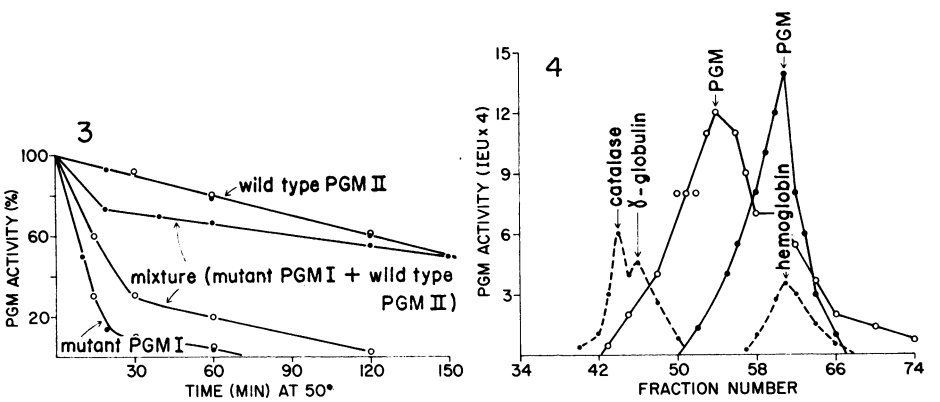

FIG. 3.—Effect of (Mg^{2+}) on the heat stability of mutant PGM I and wild-type PGM II in an artificial mixture. High (Mg^{2+}) (—○—) or low (Mg^{2+}) (—●—) buffer.

FIG. 4.—Effect of (Mg^{2+}) on the molecular weight of PGM; gel filtration on Sephadex G-100; elution with high (Mg^{2+}) (—○—) or low (Mg^{2+}) (—●—) buffer.

inactivation curve of the mixture was found to compare with that of the wild-type component of the mixture. The same mixture at high (Mg^{2+}) was found to be almost as heat-labile as the mutant component of the mixture. In addition, the K_m of the mixture was found to be strikingly affected by (Mg^{2+}). At high (Mg^{2+}) the G-1-P and G-1,6-P K_m's of the mixture were found to be 3.2×10^{-4} M and 1.2×10^{-5} M whereas the corresponding values at low (Mg^{2+}) were 1.3×10^{-4} M and 3.7×10^{-6} M. Thus in respect both to heat stability and K_m values, the mixture of mutant PGM I and wild-type PGM II behaved like the mutant component at high (Mg^{2+}).

The total PGM from AWT strains showed similar changes in heat stability with changes in the ionic concentration. The total PGM from the two ragged strains when mixed at high (Mg^{2+}) was found to show relatively greater heat lability (half life 10 ± 1 min) than either ragged-1 or ragged-2 PGM alone (half life 30 ± 5 min). This suggested that the heat lability of the PGM was further increased when both PGM I and PGM II were present in the mutant forms.

The effect of ionic concentration on the heat stability of enzyme was detected only when both PGM isozymes were present. When PGM I and PGM II were heat-inactivated separately, the curves remained essentially unchanged under different (Mg^{2+}) (also see Fig. 3). In another experiment the total ragged-1 PGM at low (Mg^{2+}) was briefly heated at 50°C to destroy the heat-labile PGM I. The enzyme preparation (containing the wild-typelike PGM II and also denatured PGM I) was then brought to high (Mg^{2+}) and subjected to heat inactivation. The enzyme was found to be quite heat-stable, like the wild-type enzyme (half life 150 ± 10 min). These results also suggested that denatured mutant PGM I had no effect on the heat stability of wild-typelike PGM II.

The effect of (Mg^{2+}) on the molecular weight of PGM: Whether or not changes in the behavior of the enzyme at different ionic concentrations were accompanied by changes in its molecular weight was investigated by gel filtration. A significant change in the elution pattern of PGM was observed depending on the ionic concentration of the elution buffer. When the enzyme was eluted with buffer of low (Mg^{2+}), the PGM came out as a single peak corresponding to the peak of hemoglobin. Thus the molecular weight of PGM eluting at low (Mg^{2+}) was calculated to be approximately 64,000. However, on elution with buffer of high (Mg^{2+}), the PGM activity was found to shift towards the peak of human γ-globulin. The elution curve of the PGM still showed a shoulder corresponding to the peak of hemoglobin (Fig. 4). This shoulder disappeared and all PGM activity was found in the peak shifted towards the human γ-globulin when the elution was performed with buffer of higher (Mg^{2+}) (0.2 M Tris and 0.25 M $MgCl_2$, pH 7.5). The majority of the PGM eluting under high (Mg^{2+}) was calculated to have a molecular weight between 120,000 and 140,000.

Genetic studies: The progeny of the cross between mutant and wild-type strains always showed 1:1 segregation of the altered and the wild-type PGM characteristics; the altered PGM was always found in ragged progeny.

Discussion. Results of our genetic, electrophoretic, chromatographic, and electrofocusing studies demonstrate that there are two isozymes of phosphoglucomutase in *N. sitophila*. These two isozymes, designated as PGM I and PGM II, correspond respectively to the slow and fast anodally migrating bands of PGM activity in agar gel. The marked differences in the G-1-P and G-1,6-P K_m values between the mutant and wild-type enzymes and the increased thermolability of PGM in mutant strains suggest a change in the primary structure of the enzyme in the mutant strains. The heat-inactivation studies of PGM I and PGM II (Table 2) demonstrate that only one isozyme of PGM is altered in each mutant strain. The results of agar gel electrophoresis and DEAE-cellulose chromatography lead to the same conclusion. Thus these studies demonstrate that mutation of the *rg-1*$^+$ or *rg-2*$^+$ gene specifically changes either PGM I or PGM II in the mutants and establish that the *rg-1* and *rg-2* loci in *Neurospora* are the structural genes form PGM I and PGM II. The existence of the two forms of PGM in *Neurospora* is in agreement with the occurrence of multiple forms of PGM in other organisms including bacteria, yeast, and man.[4,5,9,10]

The behavior of the enzyme at different ionic concentrations clearly suggests that PGM I and PGM II exist as two distinct molecular species at low (Mg^{2+}) or interact to form a single complex molecular species at high (Mg^{2+}). Such interaction should cause a change in the molecular weight and conformation of the enzyme. The results of gel filtration, showing a twofold increase in the molecular weight of PGM at high (Mg^{2+}), indeed provides evidence for such interaction, and the behavior of PGM's at different (Mg^{2+}) suggests a change in the conformation of the enzyme molecule. The final conformation of the complex PGM molecule appears to be much more like the mutant component and thus leads to mutantlike behavior of the enzyme. An interaction between mutant and wild-type polypeptides which renders an enzyme unstable or stable has been shown to occur in multimeric proteins.[11,12] No such interaction between two active isozymes as seen in *Neurospora* PGM's has been reported previously.

If such interaction occurs *in vivo*, the physiologically active form of the enzyme may be the complex PGM molecule. This may provide a regulatory mechanism for control of the production of the two isozymes *in vivo*. A common regulation of the two isozymes is indicated by the fact that in ragged strains, the level of the wild-typelike isozymes is also significantly reduced. The fact that both PGM's are required for the expression of wild-type morphology is consistent with the idea that the complex PGM molecule is the physiologically active form of the enzyme. This would also provide the basis for the typical mutant morphologies of ragged strains despite the presence of at least one wild-typelike PGM isozyme. The nature of the agent promoting such complex formation, if it occurs *in vivo*, remains to be determined. The role of Mg^{2+} *in vivo* is uncertain because of the high concentration required for *in vitro* interaction. Cations like Mg^{2+}, spermine, spermidine, and Tris-HCl are known to cause aggregation of yeast UDP-galactose-4-epimerase[13] and also an increase in the activity of this enzyme. Polyamine cations may also be involved *in vivo* with PGM; the presence of spermidine has been reported in *Neurospora*.[14]

The defects in PGM seem to be instrumental in causing a change in the series of biochemical events which ultimately determine the expression of the ragged morphology. This is concluded from the facts that ragged morphology is always associated with the altered PGM and that in the AWT strains, the presence of the suppressor gene restores not only the wild-type morphology but also the wild-type characteristics of the PGM.

The morphology of *Neurospora* is apparently determined by the rigid cell wall, and many morphological mutants show alterations in cell wall composition.[15,16] Preliminary findings[3,17] show that ragged mutants have only approximately 50% of β-glucanlike polymers as compared to wild type. Since glucan is a major component of the cell wall, any change in its level would be expected to affect the morphology. Thus a decrease in glucan level appears to be a secondary effect of mutation of the *rg-1*$^+$ or the *rg-2*$^+$ gene. The manner in which such a secondary effect is produced remains to be elucidated. However, modification of glucan biosynthesis may arise, in part, from the allosteric effects[18] of the phosphorylated sugars which accumulate in significant excess in the

mutants due to altered PGM.[1,3] Interactions of metabolic pathways have long been known to produce multiple effects.[19] Recently, Kirk[20] and others[21] have demonstrated decreases in the specific activity of PGM in fatty or fibrous atherosclerotic lesions of human aorta or during experimental arteriosclerosis in rabbits on high-cholesterol diets.

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Abbreviations: *rg*, ragged; AWT, apparent wild type; PGM, phosphoglucomutase; G-6-P, glucose-6-phosphate; G-1,6-P, glucose-1,6-diphosphate; K_m, Michaelis constant.

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[1] Brody, S., and E. L. Tatum, these PROCEEDINGS, **58**, 923 (1967).

[2] Mishra, N. C., and S. F. H. Threlkeld, *Genetics*, **55**, 113 (1967).

[3] Mishra, N. C., unpublished results.

[4] Tsoi, A., and H. C. Douglas, *Biophys. Biochim. Acta*, **92**, 513 (1964).

[5] Spencer, N., D. A. Hopkins, and H. Harris, *Nature*, **204**, 742 (1964).

[6] Vesterberg, O., and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).

[7] Andrews, P., *Biochem. J.*, **91**, 222 (1961).

[8] Bergmeyer, H. U., in *Methods of Enzymatic Analysis* (New York: Verlag Chemie A.P., 1963), p. 886.

[9] Joshi, J. G., H. Hooper, T. Kuwaki, T. Sakarada, J. R. Swanson, and P. Handler, these PROCEEDINGS, **57**, 1482 (1967).

[10] Dawson, D. M., and A. Mitchell, *Biochemistry*, **8**, 609 (1969).

[11] Fincham, J. R. S., in *Molecular Genetics*, ed. Wittman and Schuster (New York: Springer Verlag, 1967), vol. 1.

[12] Schwartz, D., and W. J. Laughner, *Science*, **166**, 626 (1969).

[13] Darrow, R. A., and R. Rodstrom, these PROCEEDINGS, **55**, 205 (1966).

[14] Bachrach, U., and I. Cohen, *J. Gen. Microbiol.*, **26**, 1 (1961).

[15] de Terra, N., and E. L. Tatum, *Amer. J. Bot.*, **50**, 669 (1963).

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

[17] Mahadevan, P. R., personal communication.

[18] Wakil, S. J., J. K. Goldman, I. P. Williamson, and R. E. Toomey, these PROCEEDINGS, **55**, 880 (1966).

[19] Chaikoff, I. L., *Harvey Lectures*, **47**, 99 (1953).

[20] Kirk, J. E., *J. Geront.*, **21**, 420 (1966).

[21] Killinger, A., *Enzyme Biochemistry of Arterial Wall*, ed. T. Zemplenyi (London: Lloyd Luke Ltd., 1968).