PHOSPHOGLUCOMUTASE MUTANTS AND MORPHOLOGICAL CHANGES IN NEUROSPORA CRASSA *

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The morphology of cultures of *Neurospora crassa* appears to be generally determined by the amount and frequency of branching from each filament, and by the actual shape and size of the individual filamentous hypha. Both of these structural features appear to be under genetic control, as indicated by the isolation of large numbers of different morphological mutants with different branching patterns and hyphal shapes. One of these morphological mutants has been described in a previous paper[1] as being the result of a mutation in the enzyme glucose-6-P dehydrogenase. With this example in mind, other mutants were screened for possible defects in carbohydrate metabolism. One particular mutant (B53), known as ragged (*rg*), accumulated much more glucose-1-P than the wild-type strain, and had much less phosphoglucomutase activity than the wild-type strain. In addition, two other independently isolated ragged strains, judged to be allelic to B53 and each other by genetic tests and complementation tests,[2] showed the same enzyme defect as the B53 strain. All three of these strains showed a dense compact colonial growth, due to an increase in the amount of hyphal branching, and exhibited a wavy, bumpy hyphal surface as opposed to the smooth tubular appearance of the wild-type hyphae. This paper describes the primary biochemical defect found in these mutants and provides additional evidence that changes in the structure of a single enzyme can bring about gross morphological changes in *Neurospora*.

Materials and Methods.—Purification of phosphoglucomutase: Eleven gm of a freshly lyophilized culture were mixed with an equivalent amount of acid-washed sand, and were ground with a cold mortar and pestle. Enzymes were extracted with 300 ml cold 0.05 *M* Tris-Cl pH 7.4–0.005 *M* $MgCl_2$ buffer, and residual material removed by centrifugation at 8000 × *g*, 15 min. To the supernatant solution, 5 ml 1 *M* $MnCl_2$ (neutralized) were added with rapid stirring for 10 min, followed by centrifugation. To this supernatant solution, solid $(NH_4)_2SO_4$ was added to 30% of saturation and adjusted to pH 7.0 by the addition of 1 *N* NaOH. After centrifugation, the $(NH_4)_2SO_4$ content of the supernatant fraction was raised to 55% and the pH adjusted to 6.4. The pellet formed upon centrifugation was dissolved in 10 ml 0.01 *M* Tris-Cl buffer, and the resulting solution heated in a 50° water bath for 6 min. After centrifugation at 8000 × *g*, 15 min, the supernatant solution was filtered through 2 gm of Sephadex G-25, and then adsorbed onto a 2 × 22-cm column of a Celite-$CaPO_4$ gel mixture. Approximately 100 ml of the 0.01 *M* Tris-Cl buffer, followed by 50 ml of a 16% $(NH_4)_2SO_4$ solution, in 0.1 *M* Tris-Cl buffer, were passed through this column. The active enzyme was then eluted with 50 ml of 25% $(NH_4)_2SO_4$ solution, in 0.1 *M* Tris-Cl buffer. Eluates of the highest specific activity were combined, and the $(NH_4)_2SO_4$ content was raised to 60%. After centrifugation, the precipitate was dissolved in 8 ml of 0.1 *M* Tris-Cl buffer and applied to the top of a BioGel P-150 column of dimensions 2 × 90 cm, equilibrated in 0.1 *M* Tris-Cl buffer. Solutions of 0.1 *M* Tris-Cl buffer were passed through this column, and fractions of highest specific activity were combined, concentrated by $(NH_4)_2SO_4$ precipitation, and reapplied to the same BioGel P-150 column. The highest specific activity fractions from this second column were used for kinetic analysis (wild-type).

The procedures for phosphoglucomutase preparation from the ragged mutants differed from the above only in that the 50° inactivation step was omitted, and an additional (final) step of *O*-(diethylaminoethyl)-cellulose (DEAE) column chromatography was employed. All operations were performed at 5°.

Determination of glucose-1-P (G-1-P) and glucose-6-P (G-6-P) level: Mycelia were rapidly harvested by suction filtration, continuously washed with distilled water, and were never completely dried on the filter paper. The washed cultures were then immediately immersed in boiling 80% ethyl alcohol, and were boiled for 5–10 min. The over-all time between removal of the flask from the shaker to immersion in the hot alcohol was approximately 30 sec. Assay of the ethanol extracts via chromatography and enzymatic analyses has been described previously.[1] After the assay of glucose-6-P was completed, 0.10 unit of phosphoglucomutase (Boehringer and Soehne), 0.2 μmoles glucose-1,6-di-P, and 0.02 μmoles ethylenediaminetetraacetate (EDTA) were added to all of the reaction mixtures, and the subsequent changes in the optical density at 340 mμ were recorded. These changes in $OD_{340\ m\mu}$ were taken to be indications of the amount of glucose-1-P present, and a molecular weight = 372 used for the calculations. The recoveries of glucose-6-P or glucose-1-P added to lyophilized material just before extraction were 85–90%. The phosphoglucomutase (PGM) preparation used for these assays contained $<0.1\%$ each of glucose-6-P dehydrogenase, hexose-6-P isomerase, or 6-phosphogluconic acid dehydrogenase.

Uridine diphosphoglucose (UDPG) determinations: Mycelia were extracted with hot 80% ethyl alcohol as described above. The extracts were concentrated to 1–2 ml in a vacuum rotary evaporator, and then centrifuged at 8000 $\times$ g, 15 min to remove insoluble material. To an aliquot of the supernatant solution, approximately 10 mg of extensively washed charcoal were added, and after 10 min, the suspension centrifuged at 8000 $\times$ g, 15 min. An additional 100 mg of charcoal was used to adsorb the resulting supernatant solution, and the two charcoal pellets were combined and washed twice with H_2O. Five ml of absolute ethyl alcohol–1 M NH_4OH (1:2) solution was twice used to elute the charcoal pellets, and the eluate concentrated to a very small volume in a vacuum rotary evaporator. This eluate was then chromatographed in an ascending 90% ethyl alcohol–1 M NH_4Ac pH 7.5 (7:3) system for approximately 40 hr. Ultraviolet-absorbing spots were located with the aid of an ultraviolet lamp, and the area corresponding to the marker UDPG spot was eluted. The UV-absorption spectrum was recorded for each eluate, and an $E_{OD262m\mu}$ = 10×10^6 was used for the calculations. The listed values have been corrected for the estimated 70–80% efficiency of extraction and assay.

Enzyme assay: The reaction mixture for phosphoglucomutase activity measurements was 2.5 ml 0.1 M Tris-Cl, pH 7.4, 0.01 M $MgCl_2$ buffer, 4.0 μmoles K_2 glucose-1-P·2 H_2O, 0.2 μmoles K_4 glucose-1,6-di-P·5 H_2O, 0.66 μmoles triphosphopyridine nucleotide (TPN), 0.02 μmoles EDTA, and 0.5 units glucose-6-P dehydrogenase (Boehringer and Soehne). Initial rates only were used in the kinetic analyses, i.e., at most a time interval comprising 10–15% of the time required for the completion of the reaction. The glucose-1-P (Sigma, grade I) contained less than 0.1% of glucose-1,6-di-P, as judged by the PGM reaction rate in the absence of any added glucose-1,6-di-P. The glucose-1,6-di-P (Calbiochem, grade A) contained less than 0.2% glucose-1-P and approximately 1.0% glucose-6-P, both assayed enzymatically. All enzyme assays were performed at 24–26°, the reactions followed at 340 mμ, and all values are given in international enzyme units.

Results.—Assays of ethanol extracts indicated that the steady-state level of glucose-1-P in the three ragged mutants was considerably higher than in the wild-type strains, and that the level of glucose-6-P was also somewhat higher (Table 1). Other colonial mutants,[1] such as *col-2* and *col-3*, as well as the wild-type strain grown on sorbose media (colonial morphology), did not show this 12$\times$ increase in glucose-1-P level, indicating that this increase was not a general symptom of the restricted colonial growth pattern, but was more specifically related to metabolic changes in the ragged mutants. It is interesting to note that the *in vivo* ratio of glucose-1-P: glucose-6-P, which is supposedly kept at an equilibrium ratio of approximately 1:20 by PGM appeared to prevail in the wild-type strain, but was 1:3 in the ragged mutants. This suggests that in the ragged mutants there is a deficiency in the PGM activity or in the physiological factors which regulate this enzyme activity *in vivo*. The levels of these intermediates are also affected by such factors as the breakdown of glycogen-like polymers. Enzymatic degradation of these polymers occurs normally in late log-phase mycelial cultures as a consequence of inadequate

TABLE 1

LEVEL* OF G-1-P, G-6-P, UDPG IN VARIOUS STRAINS

Strains	G-1-P	G-6-P	UDPG
Wild type (RL 3-8A)	0.02–0.03	0.30–0.60	6.0–7.0
Ragged mutants			
B53	0.25–0.40	0.70–1.20	6.0–7.0
$R2506b_1$	0.25–0.40	0.70–1.20	6.0–7.0
R2513	0.25–0.40	0.70–1.20	
col-2	0.04–0.08	3.0–4.0	8.0–9.0
col-3	0.02–0.03	0.30–0.60	
Wild type on sorbose	0.02–0.03	0.30–0.60	4.0–5.0

All cultures were grown as shake cultures at 30° on glucose-minimal media (Vogel and Bonner) supplemented with 20 μg inositol/ml. Extraction and assay procedures are given in *Materials and Methods*.

* All values are given in μmoles/gm residual dry weight and represent the range found from three or more separate determinations.

harvesting and extracting procedures of any culture.[4] Therefore, only liquid shake cultures which are actively growing log-phase cultures were used, as well as very rapid and careful extraction procedures (see *Materials and Methods.*)

Since glucose-1-P is the immediate precursor of uridine diphosphoglucose (UDPG) it was of interest to see if the increased levels of G-1-P caused an increase in the level of this compound (Table 1). The material that was eluted appeared to be only UDPG, based on identical chromatography in two different solvent systems versus marker UDPG, adsorption peak at 262 mμ, and mild acid hydrolysis yielding only glucose and uridine in approximately equal amounts. The steady-state range found for this intermediate appeared to be approximately the same in all of the strains, and agreed closely with that reported previously.[5] Therefore, the increase in over-all glucose-1-P content does not appear to have had any significant effect on the total amount of UDPG found throughout the mycelial mass.

Enzymatic studies: Freshly prepared crude, undialyzed extracts of wild-type and ragged strains were assayed for glucose-6-P dehydrogenase and phosphoglucomutase activity (PGM) as shown in Table 2. There was little difference between these strains with respect to the levels of the dehydrogenase or many other enzymes. The 12× lower activity in the PGM reaction in the ragged strains did not appear to be due to any inhibitor in these extracts, or activator in the wild-type extracts, as indicated by mixing and dialysis experiments. These low levels of activity were found only in the three ragged strains, whereas many other colonial strains (some

TABLE 2

ENZYME LEVELS IN CRUDE EXTRACTS

Strains	Phosphoglucomutase Specific Activity*	G-6-P dehydrogenase Specific Activity*
Wild type (RL 3-8A)	0.30–0.40	0.35–0.40
Ragged mutants		
B53	0.02–0.03	0.35–0.40
$R2506b_1$	0.02–0.03	0.35–0.40
R2513	0.02–0.03	0.35–0.40
col-2	0.30–0.40	0.35–0.40
col-3	0.30–0.40	0.35–0.40

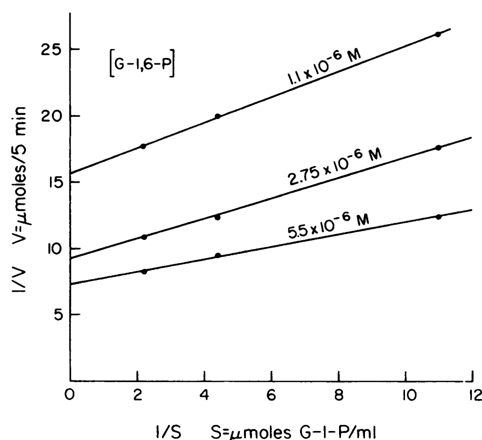
Cultures were grown in aerated carboys at 25° and after harvesting, were either lyophilized or immediately ground with sand to prepare extracts. Extracts were prepared and assayed as indicated in *Materials and Methods*.

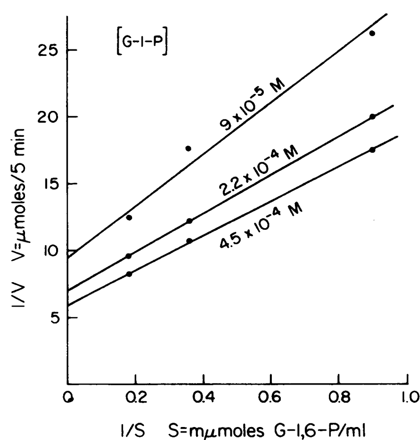
* Values given in IEU/mg protein, are the range found from duplicate determinations.

listed in Table 2) were found to have the wild-type activity level. The present assay conditions[6] consist of the optimum levels found for EDTA, glucose-1-P, glucose-1,6-di-P, Mg^{++}, pH, and substantial excesses of glucose-6-P dehydrogenase and TPN.

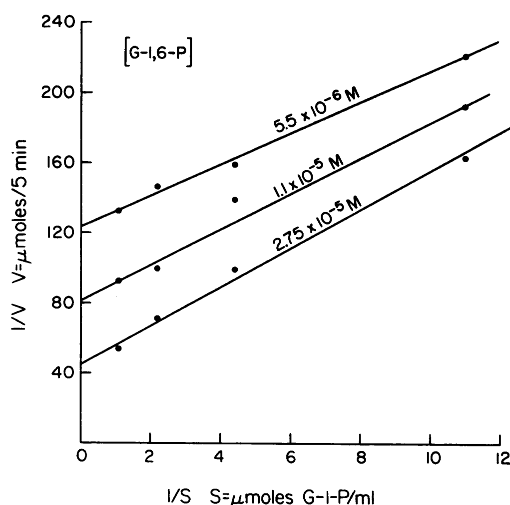
Crude extracts of ragged strains contained PGM activity of high lability, which we have not been able to stabilize appreciably by the addition of an excess of glucose-1-P, glucose-6-P, glucose-1,6-di-P, or a variety of other reagents. In contrast, the activity found in the wild-type strain was extremely stable to storage at 5°, freezing and thawing (100% recoveries), short periods of heat treatment, etc. Since the PGM activity in the ragged strains was so unstable, it would require immunological methods to determine the actual level of PGM protein in the crude extracts of these mutants.

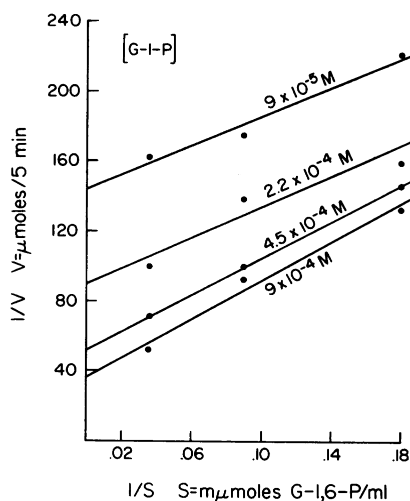
Extensive purification of the PGM activity from both mutant and wild-type strains was performed as outlined in the *Materials and Methods* section. Preparations from the wild-type strain could be obtained of specific activity 20–25. These preparations possessed no discernible activity (i.e., <0.1% of the PGM activity) for the following enzymes: glucose-6-P dehydrogenase, hexose-6-P isomerase, 6-phosphogluconic acid dehydrogenase, hexokinase, acid and alkaline phosphatase, glucose-1-P phosphate.[7] Preparations from two mutant strains (B53 and R2506b_1) were of specific activity 14–18, contained small amounts of glucose-6-P dehydrogenase and hexose-6-P isomerase, but none of the other enzymatic activities listed above. Although it is not known how much further both of these preparations can be purified, it is assumed that the values obtained from the kinetic studies on these preparations are due to PGM, and not to any unknown contaminants. In this regard, PGM preparations from the wild-type and mutant strains, with specific activities approximately tenfold lower than those reported above, had K_m values for glucose-1-P and glucose-1,6-di-P similar to the more purified preparations mentioned above.





FIGS. 1 (*left*) and 2 (*right*).—Lineweaver-Burke plot of kinetic data[9] on PGM preparations obtained from the wild-type strain. The assay conditions are listed in the *Materials and Methods* section except for the concentrations of glucose-1-P (G-1-P) and glucose-1,6-di-P (G-1,6-P). Each point is the average of two determinations, and the entire set of assays has been performed three times.



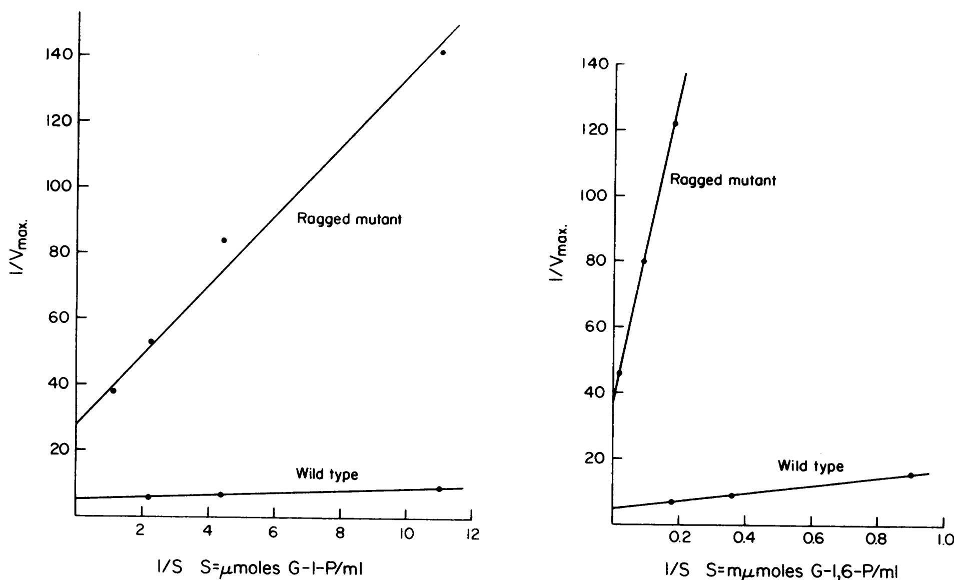


FIGS. 3 (*left*) and 4 (*right*).—Lineweaver-Burke plot of kinetic data on PGM preparations obtained from ragged mutant B53. Each point is the average of two determinations, and the entire set of assays has been repeated twice.

As shown in Figures 1–6, there were marked differences in the kinetic properties of the PGM preparations from the wild-type and ragged strains. These differences are indicated by the K_m(s) for glucose-1-P, $7.2 \times 10^{-5}M$ for the wild-type strain, $4.0 \times 10^{-4}M$ for the B53 and R2506b$_1$ strains. In addition, the K_m for glucose-1,6-di-P appeared to be altered too, being $2.5 \times 10^{-6}M$ for the wild-type strain, $1.8 \times 10^{-5}M$ for the B53 and R2506b$_1$ strains. All four of these K_m values were obtained by plotting the values of $1/V_{max}$ vs. $1/S$ (Figs. 5 and 6), although they also could have been obtained, in most cases, by the standard Lineweaver-Burke plotting method.

From these kinetic data, it appears that the PGM preparations from a wild-type *Neurospora* strain have a reaction mechanism similar to that of preparations obtained from *M. lysodeikticus* and *B. cereus*.[8] The converging lines in the kinetic plots (Figs. 1 and 2) for the wild-type preparations have been interpreted as indicating a metathetical reaction sequence for this enzyme, rather than a stepwise sequence involving a phosphorylated enzyme intermediate. At this time, no additional information pertinent to the reaction mechanism, such as the labeling of the enzyme with glucose-1,6-di-P^{32}, has been obtained for the preparations from either wild-type or ragged strains.

Heat-inactivation experiments have indicated that the PGM preparations from B53 and R2506b$_1$ were considerably more labile than the equivalent preparation from the wild-type strains. The wild-type preparations had a half-life at 50° of 180 ± 10 minutes, and the mutant preparations of 30 ± 5 min. First-order inactivation kinetics were obtained for both types of preparations under conditions similar to those given in the *Materials and Methods* section of a previous paper.[1] The observed kinetic and lability differences between these enzyme preparations seems not to be due to the aggregation state of the PGM, since the PGM activity from both strains behaved identically on BioGel columns.



FIGS. 5 (*left*) and 6 (*right*).—Plot of $1/V_{max}$ vs. $1/S$ for both ragged (B53) and wild-type PGM preparations. Data obtained from Figs. 1–4.

Two of the three ragged strains have had their PGM activity purified and examined, and the results reported for purified preparations were only for these two strains, B53, and R2506b$_1$. The R2506b$_1$ is the designation for an original ragged mutant isolate, after one backcross (b$_1$) to the wild-type strain. All of the ragged isolates from this backcross that were tested has low levels of PGM activity, indicating that the altered PGM activity was associated with the ragged mutant phenotype.

Discussion.—Mutations which severely diminish the PGM activity of an organism might be expected to have a serious, if not lethal, effect on the organism. It is interesting then that a strain apparently lacking 90 per cent of its PGM activity shows only morphological changes and is capable of fairly rapid growth under many different conditions. Although all of the metabolic aberrations due to this PGM defect may not be known, it appears that the twelvefold increase in glucose-1-P content has not in itself produced an increase in the level of an intermediate derived directly from glucose-1-P, such as UDPG. Of the many possible explanations, one is that the UDPG is not uniformly distributed throughout the mycelial mass, and that in the areas of glucose-1-P accumulation, little (if any) UDPG is formed. Spatial considerations may also be important to proper cell wall synthesis, which, in *Neurospora*, appears to occur primarily at the hyphal tips. In this regard, the ragged mutants all contain only 50 per cent of the over-all β 1–3 glucan content in their cell wall as compared to the wild-type strain.[10] Even though preliminary evidence has indicated that this polymer appears to be synthesized from UDPG,[10] the metabolic link between the decreased levels of the β 1–3 glucan and the glucose-1-P accumulation is not apparent at this time.

The level of PGM activity in the ragged strains appears to be approximately 6–8 per cent of that of the wild type, due to the effects of the mutation at the *rg*

locus. These levels were determined employing certain assay conditions, such as a saturating amount of glucose-1,6-di-P which gave the maximum level of PGM activity in crude extracts. The addition of saturating levels of glucose-1,6-di-P to the crude extracts increased the amount of discernable PGM activity by three- to fourfold for both the mutant and wild-type strains. This indicates that the level in crude extracts of glucose-1,6-di-P was not sufficient to maximally activate the PGM present, and that sufficient glucose-1,6-di-P was not formed from glucose-1-P during the assay. Therefore, the actual *in vivo* level of PGM activity in both strains may be considerably less than indicated by the assays, and may be more dependent upon physiological factors, such as the amount of glucose-1,6-di-P present, etc., than on the actual level of the enzyme itself. The mechanism of physiological regulation of PGM activity by activators or feedback inhibitors is not really known at the present time.

Phosphoglucomutase catalyzes the first reaction in a series of reactions resulting in the formation of part of the cell wall; and in the synthesis of a cytoplasmic glycogen-like polymer.[11] In this sense, it represents a "branch point" in metabolism and therefore might be required to adjust its activity so as to regulate these pathways. This need to respond to a variety of conditions, catabolic and synthetic, may account for the finding of two types of PGM in many organisms.[12–14] However, at the present time, there is no evidence of a second form or any unstable form of PGM in the wild-type *Neurospora* strains. Therefore, until a positive indication can be found for either isozymic forms or another type of PGM in *Neurospora*, the conclusion would be that the mutation at the *rg* locus specifically affects the primary structure of the PGM molecule, as evidenced by large changes in the kinetic properties, heat lability, and general stability of purified preparations.

Summary.—A particular morphological mutant of *Neurospora crassa*, called ragged (*rg*), has been found to be severely deficient in phosphoglucomutase (PGM) activity. Crude extracts of the mutant contained only 6–8 per cent of the wild-type PGM level, and this residual activity in the mutant was quite unstable. Purified enzyme preparations indicated that the PGM from the mutant strain had a K_m for glucose-1-P 6× higher, and a K_m for glucose-1,6-di-P 7× higher than the respective wild-type values. In the mutant strain, a direct effect of the PGM deficiency was a 12-fold increase in the *in vivo* level of glucose-1-P, a substrate for this enzyme. Two other independently isolated mutants, allelic by genetic tests, had the same phenotype, enzyme deficiency, and glucose-1-P accumulation. The apparent change in the structure of the PGM molecule appears to be the primary biochemical effect of the mutation at the *rg* locus and, therefore, is the cause of those pleiotropic effects which lead to the change in morphology.

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