# Role of Vitamin $B_6$ Biosynthetic Rate in the Study of Vitamin $B_6$ Synthesis in *Escherichia coli*

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Nutritional auxotrophs of *Escherichia coli* synthesize vitamin $B_6$ compounds at a rate of $1 \times 10^{-10}$ to $2 \times 10^{-10}$ moles per hr per mg (dry weight) of cells when they are suspended in minimal medium lacking their required nutrients. A few auxotrophs have been found to stop or reduce vitamin $B_6$ synthesis during such an experiment. These include thiamineless, citrate synthaseless, and pyridoxineless mutants as well as mutants which require four carbon compounds for growth. Glycolaldehyde was found to restore vitamin $B_6$ synthesis in the last named of these mutants without restoring normal growth. A class of pyridoxineless mutants which responded with normal growth to 0.4 mM glycolaldehyde or $0.15 \times 10^{-3}$ mM pyridoxol was also found. The results suggest that a thiamine pyrophosphate-requiring step as well as glycolaldehyde may be involved in pyridoxal phosphate biosynthesis.

The sequence of reactions leading to the biosynthesis of pyridoxal phosphate in bacteria is largely unknown. Although several groups have recently presented preliminary or general data on this subject (6, 10, 16), no overall picture of the reactions involved has emerged, nor has any one, unique biosynthetic reaction been identified.

A previously unused approach to solving this synthetic pathway lies in the identification of those compounds (if any) in *Escherichia coli* that are required for continued synthesis of vitamin $B_6$. These compounds can be identified as necessary for vitamin $B_6$ biosynthesis if mutants lacking the capability to synthesize them are simultaneously unable to synthesize vitamin $B_6$. This type of measurement can be made by analyzing the vitamin $B_6$ content of cultures of various mutants during starvation for their required nutrients. Final identification of particular nutrients as necessary or direct precursors of vitamin $B_6$ as opposed to indirect or metabolically "coupled" compounds would be made by some other test, e.g., showing that the specific activity of vitamin $B_6$ was the same as that of the appropriately labeled radioactive test compound when tested in a mutant unable to make that test compound.

The purpose of this report is to communicate the results of one set of such tests made with *E. coli* B mutants in an attempt to identify metabolites required for vitamin $B_6$ synthesis. During these tests three unexpected findings were made

which appear to bear directly upon vitamin $B_6$ biosynthesis.

## MATERIALS AND METHODS

**Bacterial strains.** The several strains used in this study are listed in Table 1. Strains WG1, WG15, and WG139 have been described previously (4, 5, 8). WG1032 was identified as AroA phenotype by the nutritional test procedure of Pittard and Wallace (14). WG1390 was identified as citrate synthaseless (1) by assay of extracts of the organisms by the method of Srere et al. (15). All other strains were identified primarily by nutritional requirements.

**Media.** In all cases the basic medium used was the glucose minimal medium previously described (5). For growth of each different mutant this medium was supplemented as necessary with nutrients at the following final concentrations: pyridoxol, $6 \times 10^{-7}$ M; thiamine, $3 \times 10^{-7}$ M; L-glutamate, $3.4 \times 10^{-3}$ M; L-aspartate, $3.7 \times 10^{-3}$ M; L-lysine, $6.8 \times 10^{-4}$ M; L-leucine, $1.9 \times 10^{-4}$ M; uracil, $1.8 \times 10^{-4}$ M; and L-arginine, $5.8 \times 10^{-4}$ M. For strain WG1032 the following compounds were present during growth: L-tryptophan, $2.4 \times 10^{-4}$ M; L-phenylalanine, $3.0 \times 10^{-4}$ M; L-tyrosine, $2.8 \times 10^{-4}$ M; *p*-aminobenzoic acid, $1.5 \times 10^{-4}$ M; and *p*-hydroxybenzoic acid, $3.6 \times 10^{-5}$ M.

**Starvation procedure.** A 50-ml culture of the mutants in glucose minimal medium plus the required nutrients was inoculated with the appropriate mutants and grown overnight at 37 C, with vigorous shaking. In the morning, a portion of these cells containing 100 mg (dry weight) of cells was centrifuged, washed with saline, and used to inoculate 1 liter of identical medium. Shaking at 37 C was resumed until the mass at least doubled. Then the culture was centrifuged and washed

TABLE 1. *Strains used in this study*

| Strain 1 | Genotype | Requirements | Muta-gen |
|---|---|---|---|
| WG1 | wild type | None | None |
| WG15 | pdxD15 | Pyridoxine | UV[a] |
| WG139 | pdxC139 | Pyridoxine | UV |
| WG620 | pyrA | Arginine and uracil | NTG[b] |
| WG1002 | leu-2 | Leucine | NTG |
| WG1032 | aroA10 | Tyrosine and phenyla-lanine | NTG |
| WG1139 | lys-5 | Lysine | NTG |
| WG1225 | thi-22 | Thiamine | NTG |
| WG1256 | ppc-3 | 4-Carbon acid | NTG |
| WG1368 | ppc-4 | 4-Carbon acid | UV |
| WG1390 | glt-1 | Glutamate or $\alpha$-keto-glutarate | UV |

[a] UV, ultraviolet light.
[b] NTG, N-methyl-N'-nitro-N-nitroguanidine.

with saline, and a portion was used to inoculate unsupplemented glucose minimal medium to the cell densities indicated in each experiment. Duplicate 5-ml samples were withdrawn immediately and mixed at once with 5 ml of 0.11 N $H_2SO_4$. Sampling was repeated every half hour for at least 3 hr. The cell mass at each sampling time was determined from a standard curve relating apparent absorbancy at 420 or 650 nm to dry mass of cells. Samples were stored overnight at 2 C and hydrolyzed for 5 hr at 121 C, and portions then were assayed for total pyridoxine as described previously (3). In every case the entire experiment was repeated at least once with essentially the same results.

## RESULTS

The general procedure used in this study was based upon that of Wilson and Pardee (18). Cultures of mutants growing exponentially in a glucose minimal medium supplemented with whatever nutrients the particular mutants required were centrifuged, washed, and suspended in unsupplemented glucose minimal medium. Samples were withdrawn at different times, hydrolyzed with acid, and then assayed for total vitamin $B_6$ by measuring the growth response of *Saccharomyces carlsbergensis* to portions of the hydrolysate. The slope of the curve obtained by plotting the total vitamin $B_6$ content of the culture per milligram of cells at each time against time was equal to the rate of vitamin $B_6$ synthesis (18). The initial hypothesis upon which this work was based was that the vitamin $B_6$ biosynthetic rates would fall to a low, zero, or negative value if the particular nutrient required by the mutant was necessary for vitamin $B_6$ biosynthesis but would remain near some average value if there was no direct connection between vitamin $B_6$ synthesis and the mutation at hand.

Before assuming that such variations in this

vitamin $B_6$ biosynthetic rate could be interpreted as being related to vitamin $B_6$ biosynthesis, it was necessary to show that pyridoxineless mutants at least gave zero rates of vitamin $B_6$ synthesis. Figure 1 shows such data obtained when two pyridoxineless mutants were used in this experimental procedure. Similar data for other pyridoxineless genotypes has appeared (7). One can see that cell mass continued to increase during starvation, a behavior characteristic of vitamin mutants (18). In these particular experiments the medium was capable of supporting growth to 1.5 mg of dry cell mass per ml, so that the cells could be presumed to have finally stopped growth when they did because of their starvation for vitamin $B_6$. More importantly, the total vitamin $B_6$ content of the culture actually decreased while the cell mass increased. The result was a curve with negative slope or negative rate of $B_6$ synthesis. It was reasonable, therefore, to expect that either low or negative rates of $B_6$ synthesis might be found when mutants with alterations in pathways related to vitamin $B_6$ biosynthesis were tested in this manner.

Some of the mutants tested in this present series behaved in a manner similar to that previously reported for other mutants (3), that is, they continued to synthesize vitamin $B_6$ during starvation. Figure 2 shows results of four of these experiments. The data in this figure are tentatively interpreted as showing that arginine, pyrimidines, leucine, lysine, phenylalanine, and tyrosine are not directly involved in vitamin $B_6$ biosynthesis. (In each case except for WG1032, additional mutants of identical phenotype were also isolated and tested with results essentially identical to those shown here. No additional AroA mutants were found.)

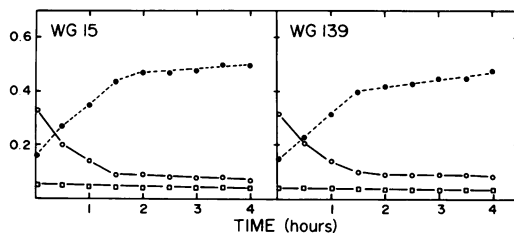Of more immediate interest however was the finding of a few mutant strains which were not



FIG. 1. *Effect of vitamin $B_6$ starvation upon two pdx mutants. WG15 (pdxD15) and WG139 (pdxC139) were shifted at time 0 to vitamin $B_6$-free glucose minimal medium. Samples were taken at various times to measure cell mass in mg of dry weight per ml (●), total vitamin $B_6$ per ml (□), and total vitamin $B_6$ per mg of cells (○).*
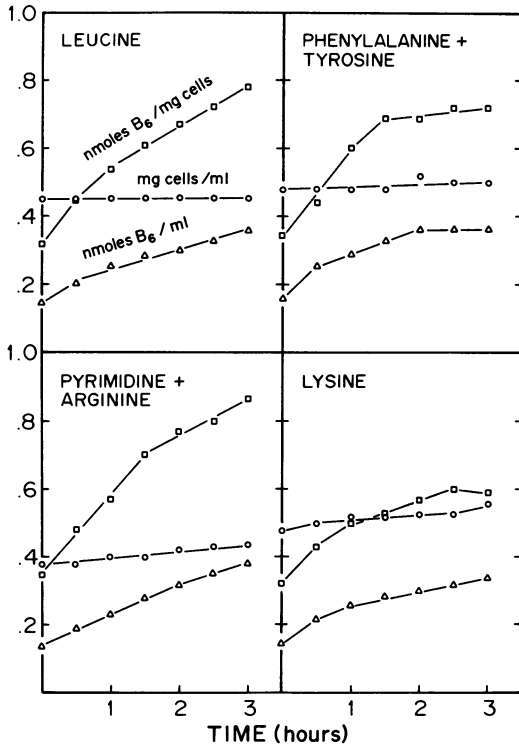
FIG. 2. *Vitamin B$_6$ content of cultures during starvation of mutants for their required compounds. WG1002 (upper left), WG1032 (upper right), WG620 (lower left), and WG1139 (lower right) were shifted at time 0 from minimal media containing the indicated required nutrients to medium lacking the nutrients. Samples were taken at various times to measure cell mass in mg of dry weight per ml (O), total vitamin B$_6$ per ml (△), and total vitamin B$_6$ per mg of cells (□).*

phenotypically pyridoxineless but which showed negative or very low vitamin B$_6$ biosynthetic rates in these tests. The first of these was WG1225, a mutant which required thiamine for growth.

Figure 3 shows that the vitamin B$_6$ biosynthetic rate was negative throughout the starvation of WG1225 for thiamine. This result suggests that vitamin B$_1$ may be required for vitamin B$_6$ synthesis. The data in Fig. 3 show that the cell mass of WG1225 increased sixfold during the period that vitamin B$_6$ increased barely twofold. Vitamin B$_6$ biosynthesis was found to resume in thiamine-starved WG1225 immediately upon the addition of thiamine to a final concentration of 100 μg/liter.

Niacinless and pantothenateless mutants of other strains of *E. coli* were also tested but were found to make vitamin B$_6$ at normal rates

throughout vitamin starvation. Nonleaky mutants for these and other vitamins were not found in *E. coli* B even after an extensive search using several types of mutagen.

The second mutant type to exhibit a negative vitamin B$_6$ biosynthetic rate was that group of mutants which have an absolute requirement for either glutamate or α-ketoglutarate. Mutants of this type have been shown by Ashworth et al. to lack citrate synthase (1). Two of the mutants of this type isolated in this laboratory, namely, WG1390 and WG1427, were found to have no citrate synthase activity when assayed by the method of Srere et al. (15). The results of a typical experiment with a citrate synthaseless mutant are shown in Fig. 4, which shows no growth during starvation for glutamate and some loss of total vitamin B$_6$ to give a net negative slope to the curve for vitamin B$_6$ per milligram of cells.
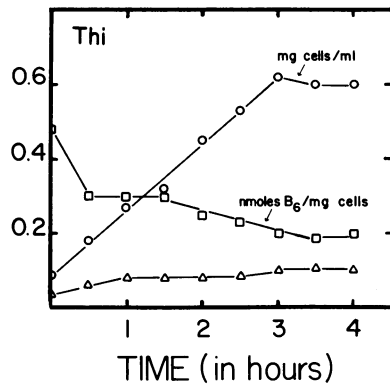


FIG. 3. *Vitamin B$_6$ content of a culture of a thiamineless mutant (WG1225) being starved for thiamine. Symbols are the same as in Fig. 2.*
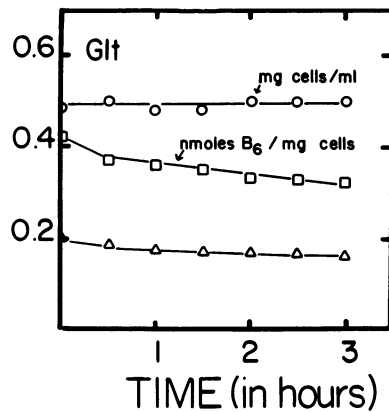


FIG. 4. *Vitamin B$_6$ content of a culture of a citrate synthaseless mutant (WG1390) being starved for glutamate. Symbols are the same as in Fig. 2.*

WG1390, a mutant of this type, made normal amounts of vitamin $B_6$ in media containing $\alpha$-ketoglutarate or glutamate but failed to incorporate any significant amount of radioactivity into pyridoxal phosphate when grown in glucose minimal medium with uniformly labeled $^{14}C$-glutamate (Dempsey, *in preparation*) as tested by the method described previously (6). This suggested that the carbon skeleton of glutamate itself was not necessary for vitamin $B_6$ synthesis and that, instead, a less direct role of glutamate or $\alpha$-ketoglutarate was involved.

The third mutant type to show a vitamin $B_6$ biosynthetic rate markedly lowered from the average value of $1.1 \times 10^{-10}$ moles per hr per mg of cells (3) was the type of mutant designated Ppc by geneticists (17). Mutants of this phenotype usually lack phosphoenolpyruvate carboxylase and require a compound such as succinate, fumarate, aspartate, glutamate, or $\alpha$-ketoglutarate for normal growth.

Results of experiments with two of these mutants are shown in Fig. 5. The rate of synthesis of vitamin $B_6$ in these mutants was $0.4 \times 10^{-10}$ mole hr per mg or less than half the average rate of $1.1 \times 10^{-10}$ moles per hr per mg (3). A large number of compounds was then tested for ability to restore vitamin $B_6$ biosynthesis to these mutants. The earlier finding of Morris and Woods that glycolaldehyde evidently played a role in vitamin $B_6$ biosynthesis (13) led to the inclusion of this compound in this test. Fortuitously, the result was that only glycolaldehyde had a positive effect. As can be seen in Fig. 5, addition of glycolaldehyde to a final concentration of $2 \times 10^{-4}$ M appeared to restore vitamin $B_6$ biosynthesis without affecting cell growth.
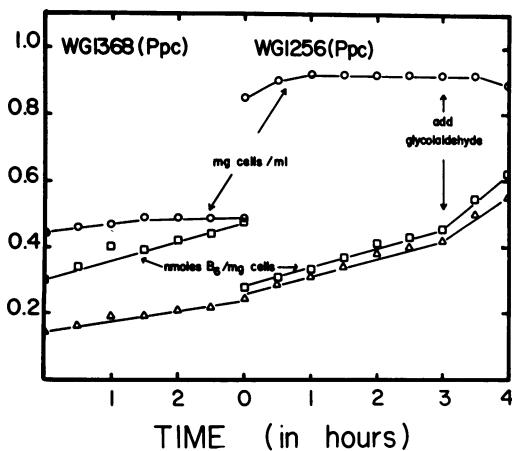


FIG. 5. *Vitamin $B_6$ content of cultures of two Ppc mutants being starved for aspartate. Symbols are the same as in Fig. 2.*

This experiment was repeated, but instead of adding glycolaldehyde after starvation, different 2-carbon compounds, each at $5 \times 10^{-4}$ M, were tested by having them present from the time starvation was initiated. Figure 6 shows that glycolaldehyde was the most effective of the compounds in restoring vitamin $B_6$ synthesis (middle panel) and that glyoxylate was most effective in restoring growth to the culture (left panel); rate of synthesis (right panel) was most effectively restored by glycolaldehyde. Glycolate was also tried in this series with results identical to those shown for acetaldehyde.

This finding suggested the interpretation that citrate synthaseless mutants might have failed to synthesize vitamin $B_6$ because they lacked isocitrate and, consequently, a source of 2-carbon units. If this were the case, then citrate synthaseless mutants suspended in media containing 2-carbon compounds ought to synthesize vitamin $B_6$. Table 2 shows that vitamin $B_6$ synthesis was not restored to normal in these mutants simply by the presence of 2-carbon compounds. In similar tests it was found that the presence of glycolaldehyde at $2 \times 10^{-4}$ M had no effect upon vitamin $B_6$ synthesis by WG1225 during starvation for thiamine.

The possibility that glycolaldehyde was a precursor of vitamin $B_6$ was first suggested by Morris (11). The above evidence added support to the argument that this 2-carbon compound was involved in vitamin $B_6$ biosynthesis. When glycolaldehyde was then tested by the replica plate method as a replacement for the vitamin $B_6$ requirement of over 100 Pdx mutants of all five Pdx groups, the finding was that $10^{-4}$ M glycolaldehyde could satisfy the growth requirement of some group I Pdx mutants as measured at 37 C after 24 hr. This group had previously been divided into three feeding classes or phenotypes on the basis of cross-feeding properties (8). A member of each phenotype was then tested to determine whether glycolaldehyde would support normal growth in terms of growth rate as well as growth extent. The growth rates of aerobic cultures of one mutant of each of PdxB, PdxC, and PdxD classes were tested. Figure 7 shows that $4.2 \times 10^{-4}$ M glycolaldehyde was equivalent in growth-supporting ability to $1.5 \times 10^{-7}$ M pyridoxol for two of the three classes. Samples of these cultures grown on glycolaldehyde had normal contents of vitamin $B_6$.

Figure 1 shows that PdxC and PdxD mutants do not synthesize vitamin $B_6$ when suspended in glucose minimal medium. These data for PdxB have already been published (7). Based on this type of test, these mutants clearly are true pyridoxineless mutants. The finding that they reach
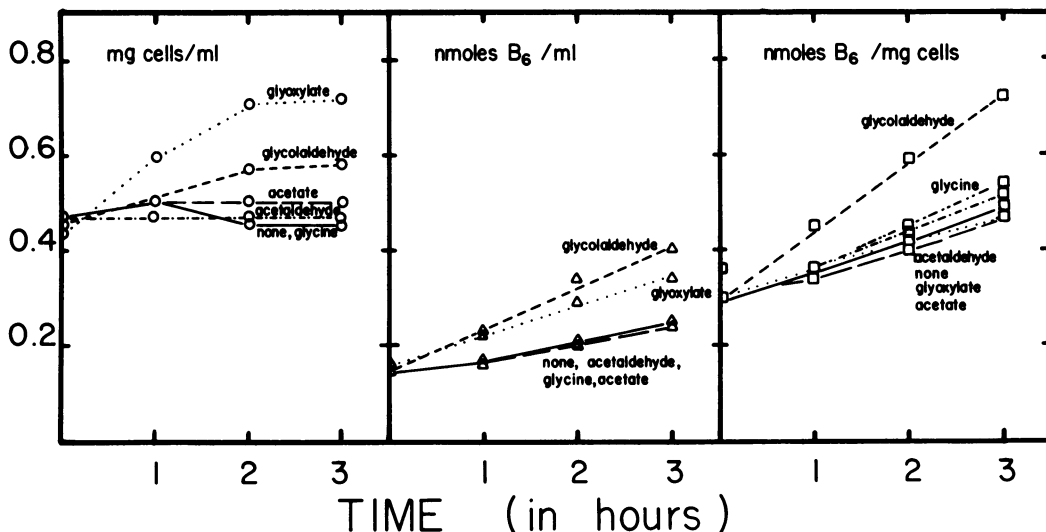
FIG. 6. *Vitamin $B_6$ content of cultures of WG1256 being starved for aspartate in the presence of 2-carbon compounds. The concentration of all compounds was $5 \times 10^{-4}$ M.*

TABLE 2. *Vitamin $B_6$ content in WG1390 cells during starvation for glutamate in different media*

| Medium | Rate of vitamin $B_6$ synthesis (nanomoles per milligram of cells) at: | | | |
|---|---|---|---|---|
| | 0 hr | 1 hr | 2 hr | 3 hr |
| Glycerol phosphate ...... | 0.36 | 0.37 | 0.39 | 0.42 |
| Glucose................ | 0.37 | 0.31 | 0.29 | 0.28 |
| Glucose and γ-aminobutyrate ............... | 0.36 | 0.32 | 0.30 | 0.28 |
| Glucose and glycolaldehyde ................ | 0.33 | 0.36 | 0.33 | 0.31 |
| Glucose and glyoxylate .. | 0.41 | 0.35 | 0.32 | 0.30 |
| Glucose and aspartate ... | 0.38 | 0.41 | 0.39 | 0.38 |

full growth in media containing no more vitamin $B_6$ than *E. coli* normally makes also indicates that they must be considered pyridoxineless mutants. Therefore, group I Pdx mutants are composed of pyridoxineless mutants which respond only to vitamin $B_6$ and pyridoxineless mutants which respond to either vitamin $B_6$ or glycolaldehyde.

## DISCUSSION

Mutants which lack citrate synthase do not grow or make vitamin $B_6$ in the absence of α-ketoglutarate or glutamate, but these mutants do grow normally and make normal amounts of vitamin $B_6$ when α-ketoglutarate or glutamate are present. The failure of these mutants to synthesize vitamin $B_6$ during starvation for glutamate might be due to the diminished adenosine tri-phosphate supply in the presence of a defective Krebs cycle, but this seems likely to be at most only a partial explanation because some adenosine triphosphate should be available for vitamin $B_6$ synthesis from fermentation of glucose via the Embden Meyerhof route. An equally likely explanation might derive from the fact that glutamate is a cosubstrate of 3-phosphohydroxypyruvate: glutamate aminotransferase, an enzyme which has been shown to be essential for vitamin $B_6$ biosynthesis (7); and in an absence of glutamate this essential reaction could not proceed.

Mutants which require thiamine for growth show greatly reduced vitamin $B_6$ synthesis during thiamine starvation. A relationship between the biosynthesis of these two compounds in yeast has been evident for some time (12), but it remains to be determined whether that relationship is as intimate as the sharing of a common precursor or as remote as the presence in the pyridoxal phosphate biosynthetic scheme of an enzyme which uses thiamine pyrophosphate as a coenzyme and vice versa. On the basis of numerous baffling observations with a large number of pyridoxineless mutants over the last several years, I favor the latter interpretation, but hard data in its support are not yet available. The existence of a class of pyridoxineless mutants which require either pyridoxine or glycolaldehyde for growth suggests that another type of pyridoxal phosphate "Km" mutant has been found. [This name applies to certain mutants of pyridoxal phosphate holoenzymes which respond with growth either to vitamin $B_6$ or to the product of the respective biosynthetic pathway such as iso-
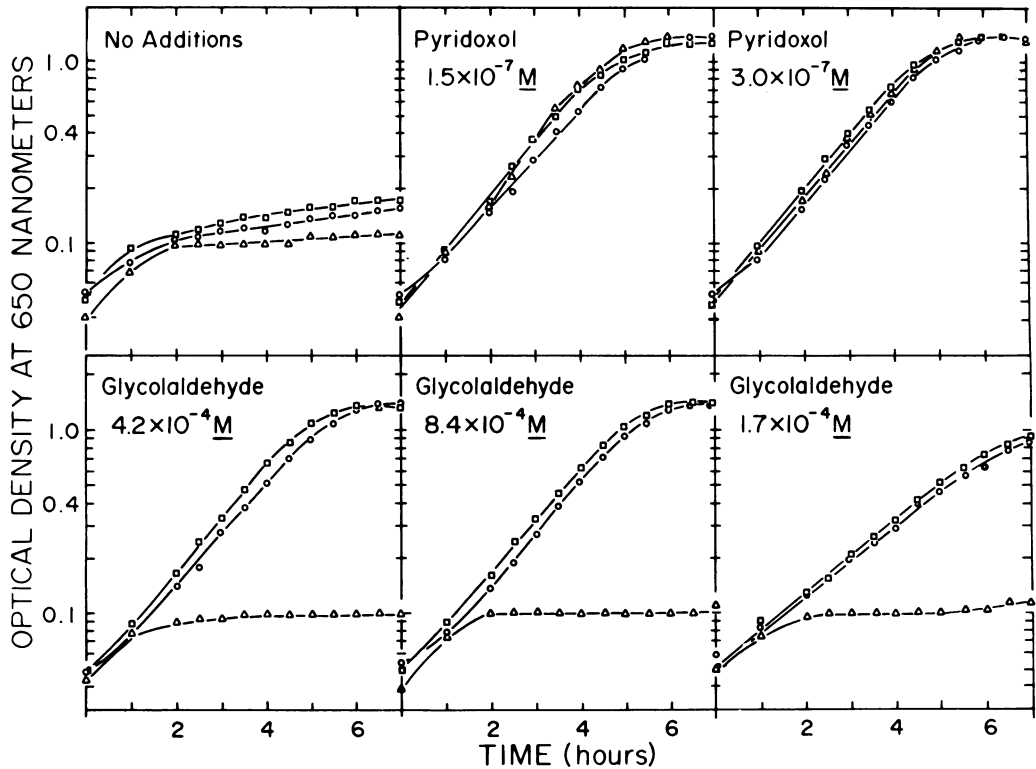
FIG. 7. *Effect of glycolaldehyde and pyridoxol upon the growth rates of group I Pdx mutants. Three strains, WG3 (pdxB3) (O), WG139 (pdxC139) (□) and WG15 (pdxD15) (△), were used.*

leucine (9), lysine (2), or serine (7).] But in this case, in contrast to the others, the mutants are unable to make any vitamin $B_6$ without glycolaldehyde being present. This finding implicates glycolaldehyde or a derivative directly in vitamin $B_6$ biosynthesis. In addition, the two phenotypes PdxC and PdxB which respond to glycolaldehyde or vitamin $B_6$ not only are very closely linked to each other genetically (4, 5), but also are linked to a nonglycolaldehyde-responsive pyridoxineless mutant phenotype PdxD to form a group of three separate *pdx* genes (5) supporting the interpretation that these are true pyridoxineless mutants. The data may presently be interpreted as suggesting that PdxB and PdxC mutants have blocks in enzyme(s) before the enzyme block in PdxD. This may be represented by: $X \xrightarrow{\text{PdxB}}$ $Y \xrightarrow{\text{PdxC}} Z \longrightarrow$ vitamin $B_6$. In this scheme metabolites X and Y are unknown and metabolite Z either is glycolaldehyde or is readily derived from it.

## ACKNOWLEDGMENTS

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