JOURNAL OF BACTERIOLOGY, Oct. 1971, p. 415-421
Vol. 108, No. 1

Control of Vitamin B_6 Biosynthesis in *Escherichia coli*[1]

WALTER B. DEMPSEY

Basic Research Unit, Veterans Administration Hospital, and Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, Texas 75216

Received for publication 19 July 1971

Pyridoxineless mutants of *Escherichia coli* B which specifically require pyridoxal or pyridoxamine for growth can be divided into classes according to their growth responses in enriched media. Members of the slowest growing class synthesize vitamin B_6 at the fastest rates when starved for pyridoxal in glycerol minimal medium. After 80 min of synthesis at 4×10^{-10} moles of vitamin B_6 per mg of cells per hr, the rate increases four- to fivefold and continues at the new rate for several hours. The shift to the new rate is prevented by chloramphenicol, thus suggesting that a derepression mechanism exists to control vitamin B_6 synthesis in addition to the previously discovered feedback control.

Pyridoxal phosphate has such a strong affinity for certain amino groups of some proteins that it is used as a protein reagent (2, 7, 9, 10). Since these reactions occur readily in vitro, it is reasonable to expect that the living cell might have to protect itself rather rigorously against high intracellular pyridoxal phosphate concentrations to prevent these reactions in vivo. This might be accomplished in a number of ways, and it is the definition of the mechanisms which control pyridoxal phosphate concentration which is the general goal of this research.

The nomenclature of the compounds in this field can be extremely confusing, particularly since no single, totally unobjectionable name exists for the entire group of compounds metabolically and structurally related to pyridoxal phosphate. Because of this lack, this work adapts the recent suggestion (8) that "vitamin B_6" be used to refer to the group of compounds, pyridoxal, pyridoxamine, and pyridoxol to include the respective 5'-phosphate esters of these compounds. Pyridoxol is herein adopted as the least confusing name for the particular compound often referred to as pyridoxine. The terms pyridoxineless and Pdx are adopted in this present work as terms describing any mutant unable to grow normally because of an inability to synthesize adequate amounts of the coenzymatically active compounds in the vitamin B_6 family.

The mutants discussed in this work are a particular kind of pyridoxineless mutant which grows normally in minimal media supplemented with either pyridoxal or pyridoxamine. These mutants do not respond to pyridoxol. It has previously been shown that mutants of this type lack the enzyme required to oxidize pyridoxol phosphate to pyridoxal phosphate and thus, by their inability to make pyridoxal phosphate, they fall under the above definition of pyridoxineless mutants (4). Because of the position of the genetic block at the end of the biosynthetic pathway for pyridoxal phosphate, these mutants do accumulate pyridoxol and pyridoxol phosphate in the medium. Statements in the text about this particular property will appear confusing unless the above definitions are kept clearly in mind.

Finally, the bioassay organism used in these studies is one which is equally sensitive in growth response to pyridoxol, pyridoxal, pyridoxamine, and to mixtures of these compounds. The phosphate esters of these compounds must be hydrolyzed before they can be measured by this method. In all of the measurements herein reported, total vitamin B_6 (that is, the amount present in cells and medium after complete conversion of the phosphate esters to their corresponding alcohols) is measured and reported without regard to relative amounts of the different compounds.

Vitamin B_6 biosynthesis in *Escherichia coli* has already been shown to be under control of a mechanism which rapidly stops vitamin B_6 synthesis when pyridoxol is added to a growing culture (3), but no evidence has appeared to date to suggest that a repression mechanism operates in this control system. An effective inhibition of vitamin B_6 kinase by pyridoxal has also been shown (11)

[1] Presented at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

and could easily be part of a control system which could limit pyridoxal phosphate concentration.

Although several types of mutants are known which produce more vitamin B_6 per milligram of cell mass than wild-type *E. coli* (W. Dempsey, Bacteriol. Proc., p. 147, 1971), only one has been shown to synthesize vitamin B_6 at rates greater than three times the wild type (4). All of the others thus far examined synthesize vitamin B_6 at or near wild-type rates (W. Dempsey, *unpublished data*). The one exception, WG2 (formerly B-B_6-2) lacks pyridoxol phosphate oxidase and, as a result, requires pyridoxal or pyridoxamine for growth. During starvation for pyridoxal, this mutant strain begins to synthesize pyridoxol and pyridoxol phosphate at increasing rates and then rather abruptly stops (4). The particular research reported here was undertaken to answer two questions, namely, why the synthesis of vitamin B_6 by WG2 stopped so abruptly and whether the fourfold increase in the rate of vitamin B_6 synthesis seen with this strain was the maximal rate of synthesis in *E. coli*.

MATERIALS AND METHODS

Strains. Strain WG2 (formerly B-B_6-2) has been previously described (4, 5). All Pdx mutants reported here for the first time were derived from wild-type *E. coli* B strain WG1 which was originally obtained from A. L. Koch. WG1 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosomethylguanidine as described by Adelberg et al. (1), incubated overnight at 45 C, and treated with penicillin as described earlier (6). The strains isolated are listed with their allele numbers in Table 1. During the last few experiments reported here, the laboratory became contaminated with an unidentified virulent coliphage, which made it necessary to isolate phage resistant strains of WG1257 before doing the experiments shown in Fig. 4 and 5. Nine independent spontaneously arising phage-resistant colonies of WG1257 were isolated by consecutive single-colony isolations and then tested for physiological identity with strain WG1257 by repeating the experiment shown in Fig. 3. All nine strains were indistinguishable from WG1257 in this test. One of the nine resistant strains was then arbitrarily chosen, designated WG1439, and used for the remainder of the experiments.

Media. Enriched medium contained all of the components listed in Table 2 in a solution containing 0.15 M potassium phosphate (*p*H 7.8), 0.6% glucose, 15 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, and 0.01 mM $FeSO_4$. The minimal salts medium previously described (5) was used in all experiments except that using enriched medium. Carbon sources were either glucose or glycerol at 0.2% final concentration. Both of these were separately autoclaved before addition.

Pyridoxal when added was at a final concentration of 0.6×10^{-6} M. The amino acids in Table 2 were combined and autoclaved in a 100-fold stock concentrate. The purines and pyrimidines were likewise combined and autoclaved as a 100-fold concentrate. The vitamins were combined as a 1,000-fold concentrate and sterilized by filtration. The polyamines were combined and autoclaved as a 100-fold concentrate. The glycolaldehyde and choline were separately filter sterilized as 100-fold concentrates.

The techniques used to measure cell mass and vitamin B_6 synthetic rates were those previously described (3, 4). Vitamin B_6 content was measured by microbiological assay with *Saccharomyces carlsbergensis* by the methods previously described (3). The methods used for P1 transductions and phage stock preparations have been described (5). The pitfalls inherent in interpreting transduction data and in particular transduction frequency data have been pointed out previously (5). Chloramphenicol when present was at 0.1 mg/ml; it was present from zero time.

Growth response of the several mutants in enriched media at 37 C was measured by following optical density at 650 nm of duplicate 10-ml still cultures in culture tubes (18 by 150 mm). To inoculate these cultures, the mutants were first grown aerobically overnight at 37 C in glucose minimal medium with pyridoxal. All cultures were fully grown at this time. One-drop portions of these overnight cultures were then used to inoculate each 10-ml tube of enriched medium. After 6 hr of incubation at 37 C, 0.5 ml of these subcultures were transferred to 10 ml of fresh enriched medium at 37 C. Optical density readings were initiated at this time and continued for 6 hr.

Cell density was determined from a standard curve which related optical density of 650 nm to the dry

TABLE 1. *Full transductants per 10^8 phage*

Strain no.	Recipient alleles	Donor allele[a]				
		pdxH2	*pdxB3*	*pdxF5*	*pdxG25*	*pdxJ151*
WG2	*pdxH2*	0	850	540	383	405
WG1031	*pdxH154*	0.8	406	206	122	243
WG1114	*pdxH170*	15	196	146	58	201
WG1119	*pdxH172*	0	101	184	97	226
WG1132	*pdxH175*	0	68	44	72	70
WG1174	*pdxH176*	2.4	86	57	36	104
WG1224	*pdxH179*	6.7	68	222	149	279
WG1257	*pdxH181*	0	304	197	161	280

[a] Data are corrected for spontaneous revertants seen on control platings.

TABLE 2. *Components of enriched medium*

Component	Amt (mg/liter)	Component	Amt (mg/liter)	Component	Amt (mg/liter)
Choline chloride	100	Guanine	50	Riboflavine	0.1
Spermine·4HCl	15	Uracil	50	Thiamine·HCl	0.1
Putrescine·2HCl	15	Thymine	50	Calcium pantothenate	0.1
Spermidine·3HCl	15	Cytosine	50	Nicotinic acid	0.1
D-Glutamic acid	50	Adenine	100	Biotin	0.01
DL-Alanine	50	L-Alanine	44.6	*p*-Aminobenzoic acid	0.01
L-Arginine	43.7	L-Aspartic acid	52.2	*p*-Hydroxybenzoic acid	0.005
L-Cysteine·HCl	8.3	DL-Diaminopimelic acid	6.6	L-Glutamic acid	60.9
Glycine	23	L·Histidine	5.9	L-Isoleucine	23.7
L-Leucine	40.6	L-Lysine·HCl	50.4	L-Methionine	20.2
L-Phenylalanine	21.6	L-Proline	23.7	L-Serine	25.3
L-Threonine	22.2	L-Tryptophan	8.3	L-Tyrosine	15.0
L-Valine	25.3	Glycolaldehyde	10	Vitamin B-12	0.001

weight of cells. The parameter "nanomoles of vitamin B_6 per milligram of cells" was calculated by dividing the total vitamin B_6 content of the culture per milliliter (3) by the cell density.

All cultures from which samples were taken at various times for total vitamin B_6 analyses were 1-liter cultures in 2,800-ml Fernbach flasks shaken vigorously on a rotary shaker at 37 C. All centrifugations were performed at room temperature.

RESULTS

One possible reason why the synthesis of vitamin B_6 stopped a short time after the pyridoxol phosphate oxidase mutant WG2 was starved for pyridoxal (4) was that the mutation in the oxidase was a missense mutation which caused the enzyme to be "leaky" or subnormal but not totally inactive. If this were the case, pyridoxal phosphate could have been synthesized in small amounts; eventually, an amount sufficient to stop vitamin B_6 synthesis could have accumulated and stopped vitamin B_6 synthesis. Direct assay for trace amounts of pyridoxol phosphate oxidase activity in extracts of WG2 failed to reveal any detectable activity; at the same time, direct assay made it clear that the amount of activity present even in wild-type *E. coli* was so low that it would have been extremely difficult to gather the initial rate data necessary for valid comparison of enzyme activities. Accordingly, an alternative method of testing the existence of "leaky" oxidase mutants was sought.

If mutants with leaky oxidases existed, it seemed possible to find them without a satisfactory enzyme assay by comparing the growth rates of the different oxidase mutants. In a medium containing all of the amino acids, most transaminases would be repressed, thereby reducing the need for pyridoxal phosphate. Mutants with the ability to synthesize a little bit of pyridoxal phosphate should then be able to distribute what little they made over fewer apoenzymes and thus have a significant advantage over those mutants unable to synthesize any pyridoxal phosphate.

Several new Pdx mutants of the oxidase type were then isolated, and they and the previously isolated strains WG2 and WG1072 (5) were tested. These mutants were all characterized by a failure to grow in media supplemented only with pyridoxol, by a nutritional requirement which was satisfied by either pyridoxal or pyridoxamine and, where possible, by a transduction frequency test. In this last test, each mutant was transduced with P1bt phage preparations representing the five classes of pyridoxineless mutants in *E. coli*. The data in Table 1 show that the new mutants appeared to belong to the oxidase class by this test also. The first column of figures in Table 1 shows that phage grown on strain WG2 which contains the pyridoxol phosphate oxidase allele *pdxH2* either cannot transduce the other mutants in Table 1 or can do so only at a very low rate. Accordingly, by these several criteria, the mutants in this table were tentatively assumed to lack pyridoxol phosphate oxidase.

A medium containing all amino acids, purines, pyrimidines, polyamines, and vitamins except vitamin B_6 was then made; the rate and extent of growth of mutants in the medium was measured. The cultures were grown in still culture at 37 C as previously described. Figure 1 shows clearly that the mutants subjected to this test fell into two groups. One group grew without pyridoxal but with a continuously increasing doubling time. The other grew very poorly without pyridoxal; among this group was WG2. (WG1 at the top of Fig. 1 is wild-type *E. coli*.) After 24 hr in this medium, all members of the first group reached

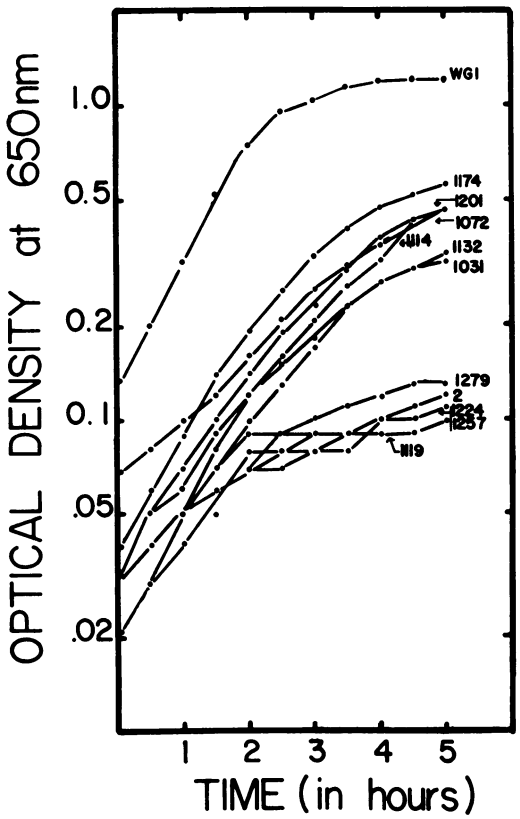

FIG. 1. *Growth of pyridoxol phosphate oxidaseless mutants in an enriched medium lacking pyridoxal. WG1 is wild-type E. coli. The other numbers are the WG strain numbers of the mutants tested.*

the same final optical density as that shown here for WG1. No members of the second group grew in this way; instead, after 24 hr, WG2 reached an optical density of 0.45 (equivalent to 0.25 mg of cells/ml), whereas the other four reached an optical density of 0.19 (equivalent to 0.075 mg of cells/ml).

The experiments were repeated in identical medium containing pyridoxal, and the mutants were again divisible into two groups (Fig. 2). One group grew like wild type (doubling time, 45 min), and the other grew at a slightly reduced rate (doubling time, 65 min). In all, three groups appeared to be distinguishable. Group A (WG2, 1119, 1224, 1257, 1279) grew normally with pyridoxal and only very slightly without it; group B (WG1031, 1114, 1132, 1174) grew normally with pyridoxal and moderately well without it; and group C (WG1072, 1201, 1224) grew more slowly than wild type with pyridoxal and moderately without it.

Although the true reason why these mutants do fall into groups with different growth responses remains to be demonstrated, we did find data that indicated that the three groups, especially group C, were more than an artifact of the inoculation procedure. Each group gave a distinctly different response when grown in glucose minimal medium supplemented with pyridoxal and then shifted in mid-exponential phase to pyridoxal-free glucose minimal medium. At least three members of each group were tested, and they all gave responses which were apparently characteristic of their group. The differences between the groups can be seen in Fig. 3. For these experiments, the cells were shifted at time zero from pyridoxal-containing medium to pyridoxal-free medium. After 80 min they were shifted again into fresh minimal medium. Samples were removed at various times, hydrolyzed, and then assayed for total vitamin B_6 with *S. carlsbergensis*.

Following the original line of reasoning, one would expect that those strains which grew least in the rich medium of Fig. 1, for example

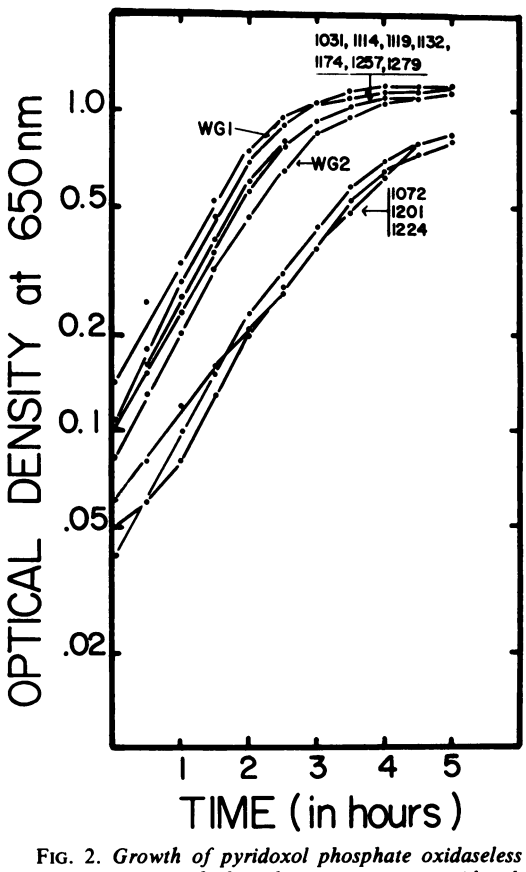

FIG. 2. *Growth of pyridoxol phosphate oxidaseless mutants in an enriched medium containing pyridoxal. WG1 is wild-type E. coli. WG2 and the other numbers are the WG strain numbers of the mutants tested.*

WG1257, would be most likely to contain totally inactive pyridoxol phosphate oxidase, whereas strains which grew moderately without pyridoxal would be likely to contain partially active pyridoxol phosphate oxidase, that is, they would be "leaky." Accordingly, if vitamin B_6 synthesis stopped in some pyridoxol phosphate oxidase mutants because the internal pool of pyridoxal phosphate is built up as a result of an incompletely blocked oxidase, one would reasonably expect that strains with completely blocked pyridoxol phosphate oxidase would synthesize the most pyridoxol and do it at the greatest rate. This expectation is consistent with the data found in Fig. 3. In this figure, the dotted line shows the response of a mutant from group C, the dashed line shows the response of a mutant from group B, and the solid line shows the response of a mutant from group A. Since both group B and C strains grow slowly without pyridoxal (Fig. 1 and 2), they may harbor leaky mutations. Group A strains appear to have the most absolute requirement for pyridoxal and consequently the most completely blocked pyridoxol phosphate oxidase.

The sum of the data to this point showed that classes of oxidase mutants existed which differed both in the amount of vitamin B_6 they synthesized and in their growth pattern in an enriched medium. These findings were consistent with the hypothesis that vitamin B_6 synthesis stopped at various times in at least some of these mutants because the oxidase was leaky and allowed accumulation of sufficient pyridoxal phosphate (or a metabolite derived from it) to activate the control mechanisms which *E. coli* has to stop vitamin B_6 biosynthesis. A more fundamental cause may have been operating on WG2 to cause it to stop vitamin B_6 synthesis in the original experiments (4), namely the exhaustion of energy supply or the accumulation of interfering metabolites. This mutant belongs to the same physiological class as WG1257 (Fig. 1 and 2). When WG1257 is centrifuged, washed, and suspended in fresh medium after 80 min of pyridoxal starvation, the synthesis of vitamin B_6 continues uninterruptedly for several hours (Fig. 3). If the WG1257 culture was not centrifuged at this time, however, this strain also stopped synthesis of vitamin B_6 after approximately 90 min of pyridoxal starvation in a manner quite identical to that previously reported for WG2. The same effect of several hours uninterrupted vitamin B_6 synthesis by WG1257 was seen if the cultures were inoculated to 0.1 mg of dry cell weight/ml, instead of to 0.4 mg/ml, at the time of the initial pyridoxal starvation. In this instance, a second shift was unnecessary. [WG1257 was used in these experiments for two reasons: (i) it is a significantly more stable mutant than WG2 and, as a result, reverts only rarely to wild phenotype; (ii) it grew less well in pyridoxal-free enriched medium than did WG2.]

The rate of B_6 synthesis (that is, the slope of the "nanomoles of B_6 per milligram" line) for strain WG1257 shifts from 4×10^{-10} moles per hr per mg during the first 80 min to 10×10^{-10} moles per hr per mg after the second shift. Similar rate shifts were not seen with mutants from

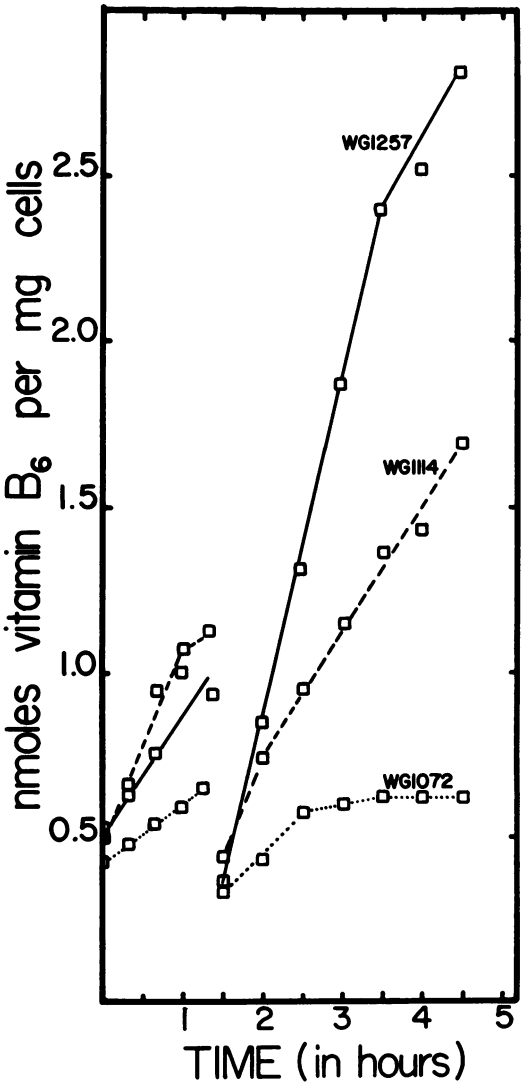

FIG. 3. *Total vitamin B_6 content of three pyridoxol phosphate oxidaseless mutants during starvation for pyridoxal. Each culture was centrifuged 80 min after starvation was initiated and suspended in fresh minimal medium. Initial inoculation was to approximately 0.4 mg of cells/ml.*

the other two groups. Thus, the second question, whether the rate of vitamin B_6 synthesis reported for WG2 previously (4) was the maximal rate for *E. coli*, namely, 4.5×10^{-10} moles per hr per mg, was answered negatively. A search for even better synthesis rates led to the discovery that the rate shifts are more clear cut and that the final rate is 17.5×10^{-10} moles per hr per mg if glycerol is used instead of glucose as the carbon energy source. Figure 4 shows a comparison of the two media when a phage resistant strain of WG1257, namely WG1439, is used.

The fourfold shift in rate after 80 min suggested that a repression mechanism might exist in vitamin B_6 control after all. If there were both feedback and repression, the increased rate after 80 min could arise from derepression, whereas the initial rate would be the result of relieving the feedback inhibition. If this were the case, then if the starvation for pyridoxal were performed with chloramphenicol present, the initial rate should be maintained but the quadrupling of the rate at 80 min should not occur.

This experiment was performed with WG1439, and the results are shown in Fig. 5. The results suggest that protein synthesis is essential for vitamin B_6 synthesis at the increased rate and, thus, that there is a repression-derepression mechanism operating as well as a feedback mechanism in the control of vitamin B_6 biosynthesis.

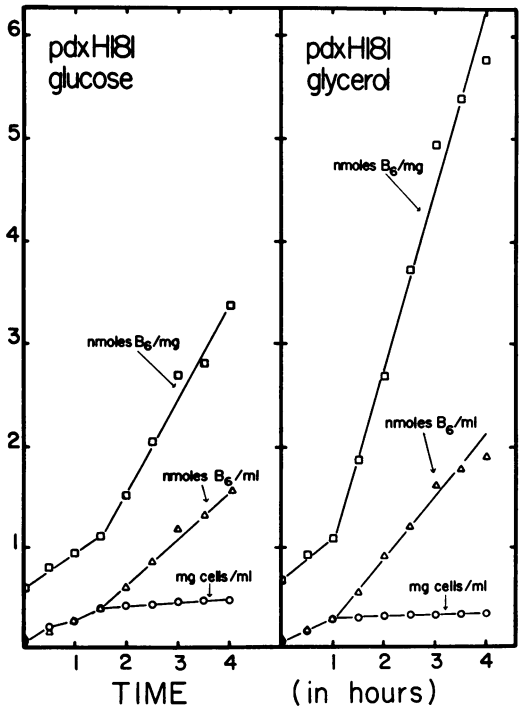

FIG. 4. *Total vitamin B_6 content of a pyridoxol phosphate oxidaseless mutant during starvation for pyridoxal in two different media. The cultures of WG1439 were inoculated initially to approximately 0.1 mg of cells/ml.*

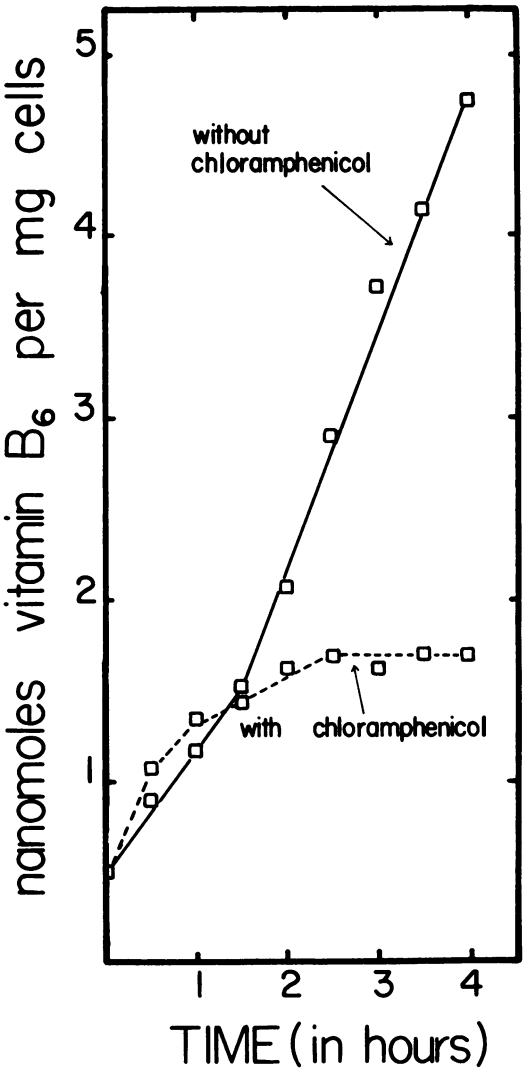

FIG. 5. *Effect of chloramphenicol on vitamin B_6 biosynthesis by strain WG1439 (pdxH181) while starving for pyridoxal in glycerol minimal medium. The cultures were inoculated initially to approximately 0.1 mg of cells/ml.*

DISCUSSION

The data presented show that several types of pyridoxol phosphate oxidase mutants can be isolated from *E. coli* which vary in their abilities to grow in well supplemented media. The inverse correlation between ability of the mutants to

grow without pyridoxal in rich medium and their ability to synthesize vitamin B_6 during starvation for pyridoxal in minimal medium suggests that the mutants may have mutant pyridoxol phosphate oxidases with various degrees of "leakiness."

The experiment with chloramphenicol indicates that vitamin B_6 biosynthesis is subject to a repression-derepression type of control in *E. coli* B. This finding, together with the previous findings that a rapidly acting mechanism is operative (3) and that an effective inhibition of pyridoxal kinase by pyridoxal (11), is reasonable evidence that the control systems which limit pyridoxal phosphate concentration in *E. coli* B are not unusual in concept or design. If the existence of unusually low biosynthetic enzyme concentrations or of very low K_m values is found, it may show eventually that the control of this cofactor is different than that of a metabolite which is used as a building block for macromolecules; however, from the data now available, it would appear that the mechanism of control of pyridoxal phosphate concentration is not unique.

The gradual cessation of all vitamin B_6 synthesis in the chloramphenicol-treated culture was an unexpected and presently unexplainable observation. It had been shown previously that vitamin B_6 synthesis in amino acid mutants of WG2, an oxidase mutant, continues for at least 3 hr during starvation for both pyridoxal and the required amino acid (4). The data of Fig. 5 may mean that one or more enzymes of vitamin B_6 biosynthesis is turned over or degraded under the conditions of this experiment or that chloramphenicol allows the accumulation of metabolites which interfere with vitamin B_6 synthesis.

If one extrapolates that portion of the line "nanomoles of B_6 per milligram" in Fig. 4 or 5 which has the greater slope to an abscissa drawn through the zero time point of that line, one finds that the extrapolated line intersects the abscissa at approximately 45 min. This suggests that the increased synthesis of vitamin B_6 by newly made enzymes begins at this time. Accordingly, the synthesis of those enzymes in response to a depletion in the pool of the controller form of vitamin B_6 begins some finite time before this. These data can mean then that it takes approximately 30 to 40 min to deplete the pool of the controller form of vitamin B_6 to the point where derepression occurs.

ACKNOWLEDGMENTS

This work was supported by research grant AM14157 from the National Institute for Arthritis and Metabolic Diseases.

The technical assistance of A. C. Kern and K. Sims is gratefully acknowledged.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. Churchich, J. E., and R. Irwin. 1970. Pyridoxal-5-phosphate lysozyme physical studies. Biochim. Biophys. Acta **214**:157–167.
3. Dempsey, W. B. 1965. Control of pyridoxine biosynthesis in *Escherichia coli*. J. Bacteriol. **90**:431–437.
4. Dempsey, W. B. 1966. Synthesis of pyridoxine by a pyridoxal auxotroph of *Escherichia coli*. J. Bacteriol. **92**:333–337.
5. Dempsey, W. B. 1969. Characterization of pyridoxine auxotrophs of *Escherichia coli:* P1 transduction. J. Bacteriol. **97**:1403–1410.
6. Dempsey, W. B. 1969. Characterization of pyridoxine auxotrophs of *Escherichia coli:* chromosomal position of Linkage Group I. J. Bacteriol. **100**:295–300.
7. Grillo, M. A. 1968. The effect of pyridoxal phosphate on yeast hexokinase. Enzymologia **34**:7–19.
8. Nomenclature for vitamin B_6 and related compounds. Tentative rules. 1970. Biochemistry **9**:4019–4021.
9. Rippa, M., and S. Pontremoli. 1969. Pyridoxal 5' phosphate as a specific photosensitizer for histidine residue at the active site of 6-phosphogluconate dehydrogenase. Arch. Biochem. Biophys. **103**:112–118.
10. Uyeda, K. 1969. Reaction of phosphofructokinase with maleic anhydride, succinic anhydride and pyridoxal 5'-phosphate. Biochemistry **8**:2366–2373.
11. White, R. S., and W. B. Dempsey. 1970. Purification and properties of vitamin B_6 kinase from *Escherichia coli* B. Biochemistry **9**:4057–4064.