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Salmonella typhimurium Mutants with Alternate Requirements for Vitamin B_6 or Isoleucine

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Several mutants of *Salmonella typhimurium* LT-2, isolated as auxotrophs for vitamin B_6, grew without the added vitamin when supplied with either isoleucine, α-ketobutyrate, or α-keto-β-methylvalerate, but not with threonine or with other α-keto acids. When grown on minimal medium supplemented with isoleucine, these mutants synthesized vitamin B_6 in amounts comparable to wild-type cells; they thus appeared to contain a modified L-threonine dehydratase and to belong to genotype *ilvA* (threonine dehydratase) instead of *pdx* (pyridoxine). Direct assays confirmed this hypothesis. Wild-type cells (toluene-treated) showed approximately the same threonine dehydratase activity whether grown in the presence or absence of added pyridoxal-P; mutant cells approached the activity of wild-type cells only when they were grown with added vitamin B_6 and were assayed in the presence of pyridoxal-P. In cell-free extracts, the threonine dehydratase from mutant cells was cold labile and more labile to oxidative inactivation than the wild-type enzyme; furthermore, activation of the mutant apoenzyme required a 10- to 20-fold higher concentration of pyridoxal-P than was required for the wild-type apoenzyme. These results show that cultures which appear auxotrophic for a given vitamin may synthesize that vitamin in normal amounts, the exogenous requirement arising from impaired binding of the vitamin-derived coenzyme to a genetically altered apoenzyme dependent on that coenzyme. Inadequate nutritional data to support the genetic findings can lead to erroneous genotype classification for such mutants.

The mutants of *Salmonella typhimurium* LT-2 described in this paper were obtained while attempting to isolate auxotrophs for use in studying vitamin B_6 biosynthesis. Mutants auxotrophic for vitamin B_6 were obtained with relative ease. However, since the selection had been made on minimal medium supplemented only with pyridoxine, the possibility existed that genetic alteration in a vitamin B_6-dependent enzyme necessary for synthesis of some essential metabolite other than vitamin B_6 had occurred, rather than in the biosynthetic pathway leading to vitamin B_6 per se. Therefore, these mutants were examined for alternate nutritional requirements and most of them were found also to respond to isoleucine. Similar groups of mutants have been isolated in this laboratory with alternate requirements for vitamin B_6 or histidine, and for pantothenic acid, thiamine, or related amino acids. The metabolic relationships involved are currently being studied; the present paper describes the vitamin B_6-isoleucine relationship.

While this study was in progress, a short communication (8) described a group of *Escherichia coli* mutants which require either vitamin B_6, α-ketobutyrate, or isoleucine for growth. The authors concluded that these alternate requirements probably resulted from a mutational change in the biosynthetic threonine dehydratase such that its dissociation constant for pyridoxal-P was increased. The work reported here establishes the validity of this explanation for nutritionally similar mutants induced in *S. typhimurium*. Several other mutants with alternate requirements for vitamin B_6 or amino acids (1, 5, 13) also have been reported. In the best documented of these reports, Bukhari and Taylor (1) studied mutants of *E. coli* that display an alternate requirement for pyridoxine or lysine. These mutants possessed a defective diaminopimelic decarboxylase which required higher concentrations of pyridoxal-P for activation than those required by the wild-type enzyme. They concluded that the mutation altered the binding site of the enzyme for pyridoxal-P. Since large numbers of enzymes require pyridoxal-P or other noncovalently bound coenzymes, binding mu-

tants of this general type may occur rather frequently.

MATERIALS AND METHODS

Isolation of mutants. Parent and mutant cultures of *S. typhimurium* LT-2 were grown on Nutrient Broth or Agar (Difco) supplemented with NaCl (14) or on the minimal medium of Vogel and Bonner (19) supplemented with the appropriate requirement. The parent strain was mutagenized with diethyl sulfate, enriched in mutants requiring vitamin B_6 by the penicillin technique (14), and then plated on minimal agar containing suboptimal (ca. 0.05 μg/ml) pyridoxine. Small colonies were picked and streaked onto solid media with and without pyridoxine, or streaked radially from edge to center of petri dishes containing minimal medium to which a small disc of filter paper impregnated with a suboptimal amount (1.25 to 3.0 μg) of pyridoxine was applied at the center (14). Vitamin B_6-requiring mutants showed growth along the streak only as far as pyridoxine had diffused.

Preparation and assay of cells and cell extracts. Cells were grown at 37 C from a 10% inoculum for 4 or 5 hr with moderate aeration on the minimal medium of Davis and Mingioli (4), modified by omitting citrate and increasing the glucose concentration to 0.5% (2). For vitamin B_6- or isoleucine-dependent mutants, this medium was supplemented either with pyridoxal-5-P or pyridoxine (0.33 μg/ml) or with the levels of L-isoleucine (8 μg/ml), L-leucine (50 μg/ml), and L-valine (100 μg/ml) recommended (6) for derepression of threonine deaminase in an auxotroph of *S. typhimurium*. After harvesting, the cells were washed with 0.05 M potassium phosphate buffer (*p*H 8.0). For preparation of cell extracts, cells were suspended in stabilizing buffer (2), subjected to sonic oscillation in an ice bath for 30 sec to 1 min by using the Sonic Disintegrator (Measuring & Scientific Equipment, Ltd.) or the Biosonik II (Bronwill) and then centrifuged at 27,000 × *g* for 20 min.

Cells and cell extracts were assayed for threonine dehydratase (L-threonine hydro-lyase, deaminating, EC 4.2.1.16) as described by Umbarger and Brown (17). Incubation mixtures contained: 80 μmoles of potassium phosphate buffer (*p*H 8.0), 50 μg of pyridoxal-P, appropriate amounts of cells or cell extracts, and water to 0.8 ml. When whole cells were used, toluene (0.02 ml) and sodium deoxycholate (0.02 mg) were added, and the mixture was incubated at 37 C for 10 min before addition of substrate (18). The reactions were started by addition of 0.2 ml (80 μmoles) of L-threonine in water solution and stopped after 20 min at 37 C by addition of 0.1 ml of 50% trichloracetic acid.

α-Keto acid was determined by the indirect method of Friedemann and Haugen (7) using toluene as extraction solvent, protein by the method of Lowry et al. (11), and total vitamin B_6 (after hydrolysis) by microbiological assay with *Saccharomyces carlsbergensis* (16).

RESULTS AND DISCUSSION

Fifty-eight mutants were isolated and purified by streaking. Most of the isolates responded equally on minimal agar to suboptimal amounts of pyridoxal, pyridoxine, or pyridoxal-P. A few grew slightly on pyridoxamine, and none responded to pyridoxamine-P. Reversion rates of the majority of the mutants were high.

Response of the mutants to amino acids. Growth studies with lactic acid bacteria and enzymatic studies both show that vitamin B_6 is required for synthesis of most of the amino acids (12). We therefore tested the ability of the mutants to grow on minimal medium supplemented with a vitamin B_6-free casein hydrolysate. All but four grew under these conditions; the majority of the isolates were therefore not mutants in a pathway leading specifically to vitamin B_6. Fifty-two of the mutants grew in the presence of isoleucine or either of its α-keto acid precursors, α-ketobutyrate and α-keto-β-methylvalerate (Table 1); these mutants are therefore blocked in the biosynthesis of isoleucine. Mutants 20 and 46 grew on pyridoxine or casein hydrolysate but not on isoleucine and therefore appear to be blocked in the biosynthesis of a different amino acid. Five of the mutants (no. 4 and 510 to 513) responded only to vitamin B_6 and appear to be true vitamin B_6 auxotrophs.

TABLE 1. *Growth response of various mutants of Salmonella typhimurium LT-2 to pyridoxine, α-amino acids, and α-keto acids*[a]

Mutant no.	Source	Supplements to minimal medium[b]							
		None	PN	Ile	Thr	α-KB	α-KMV	α-KIV	CH
2, 9, 22, 31, and *pdx* 502–509	This study	−	+	+	−	+	+	−	+
pdx 3 and *pdx* 6[c]	Sanderson	−	+	+	−	+	+	−	+
pdx 4	Sanderson	−	+	−	−	−	−	−	−
46 and 20	This study	−	+	−	−	−	−	−	+
pdx 510–513	This study	−	+	−	−	−	−	−	−

[a] Abbreviations: PN, pyridoxine; Ile, isoleucine; Thr, threonine; α-KB, α-ketobutyrate; α-KMV, α-keto-β-methylvalerate; α-KIV, α-ketoisovalerate; CH, casein hydrolysate.

[b] Growth response: −, no growth; +, good growth in 15 hr. Pyruvate, oxalacetate, and α-ketoadipate did not support growth.

[c] This mutant also requires histidine.

Growth response to isoleucine and vitamin B_6. The growth rate of mutants 22, 2, and 9 in the presence of pyridoxal-P is nearly equal to that of the parent strain on minimal medium, as shown for mutant 9 in Fig. 1; that of mutant 31 is slightly less. Growth with isoleucine is slightly slower. On prolonged incubation (>15 hr) growth occurs in minimal medium, probably because of proliferation of revertants.

Genotype of the mutants. Two pyridoxal-P enzymes, threonine dehydratase and transaminase B (leucine:2-oxoglutarate aminotransferase, EC 2.6.1.6), occur in the biosynthetic pathway leading to isoleucine; however, since α-ketobutyrate alone supports growth of all of these mutants, only threonine dehydratase must be defective. These mutants, therefore, represent alteration at the *ilvA* locus. Among the mutants labeled "*pdx*" in Table 1, no. 3, 6, and 502 to 509 also grow on either vitamin B_6, isoleucine, or its related α-keto acids, and are therefore *ilvA* mutants. The *pdx* locus which appears on the *S. typhimurium* chromosome map (15) was located in the region of the isoleucine-valine operon by time of entry experiments using strain HfrA as donor and one of these strains (*pdx* 3) as recipient; furthermore, the *pdx* 3 and *thrE* alleles were shown to be jointly transduced (K. E. Sanderson, *personal communication*). These results provide genetic confirmation of the nutritional evidence that these mutants are genotype *ilvA*. Mutants of this type, therefore, have been properly located on the chromosome map but improperly labeled.

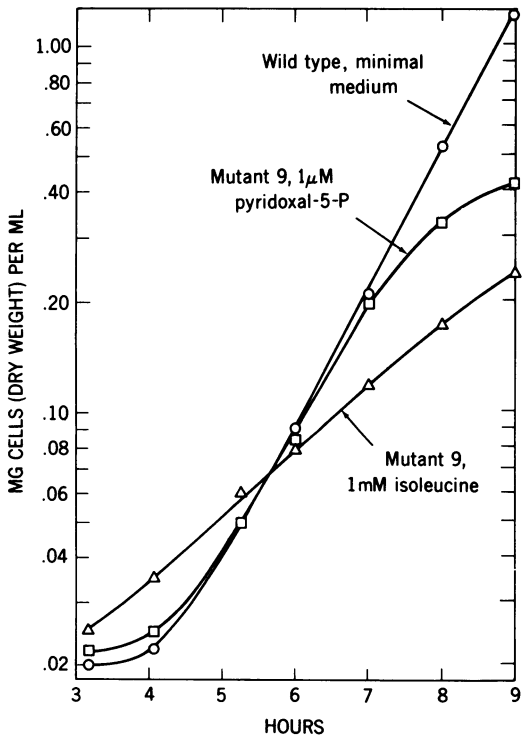

FIG. 1. *Comparative growth rates of S. typhimurium LT-2 (wild type) in minimal medium and of mutant 9 in the presence of pyridoxal-P or isoleucine. Growth of the mutant on minimal medium did not exceed 0.03 mg of cells per ml in 15 hr.*

Vitamin B_6 content of wild-type and mutant cells. The ability of these mutants to grow on minimal medium supplemented only with isoleucine (Table 1, Fig. 1) demonstrates their ability to synthesize vitamin B_6, since this vitamin is required for many other reactions necessary for growth. Direct analyses of hydrolyzed cells (Table 2) confirm this conclusion: mutant and wild-type cells grown under the same conditions in the absence of vitamin B_6 contain approximately equal amounts of the vitamin. Vitamin B_6 synthesis is not impaired, but amounts adequate for growth of the wild-type cell do not suffice to permit synthesis of α-ketobutyrate and hence growth of the mutant cells. The increased concentrations of vitamin B_6 in the mutant cells grown with external supplies of this vitamin (Table 2) are sufficient to activate this process and permit growth.

Properties of threonine dehydratase from parent and mutant cultures. Depending on the growth medium and the age of the cells at harvest, cell extracts from *S. typhimurium* LT-2 produced from 3 to 15.0 μmoles of α-keto acid from threonine per mg of protein per hr. Mutant cell extracts prepared under the same conditions possessed little or no measurable threonine dehydratase activity even when pyridoxal-P was added to the growth medium and to the cell-free extracts (Table 3).

Toluene-treated cells of the mutant cultures, however, showed readily detectable levels of threonine dehydratase which varied from mutant to mutant and, in some cases, were comparable to

TABLE 2. *Comparative vitamin B_6 content of wild-type and mutant cultures of Salmonella typhimurium under various growth conditions*

Supplement to minimal medium	Vitamin B_6 content (μg/mg)[a]			
	Wild type	*Pdx* 3	9	31
None	.08			
Ile, Val, Leu[b]	.05	.13	.06	.05
PN, 0.47 μM	.09	.14	.1	.11
PN, 1.4 μM	.13	.2	.13	.15

[a] Cultures (50 ml) were grown 15 to 20 hr, centrifuged, hydrolyzed for 5 hr at 120 C in 10 ml of 0.055 N H_2SO_4, neutralized with KOH, and diluted for assay.

[b] Ile, 8 μg; Val, 100 μg; Leu, 50 μg per ml of culture.

TABLE 3. *Comparison of threonine dehydratase activities of toluene-treated cells and cell-free extracts of Salmonella typhimurium LT-2 and several of the isoleucine auxotrophs*

Mutant no.	Supplement to growth medium	α-Keto acid formed from threonine per hr			
		Toluene-treated cells (μmoles/mg)		Cell-free extract (μmoles/mg of protein)	
		+PLP	-PLP	+PLP	-PLP
Wild type[a]	PLP	10.5	11.3	14.8	14.5
Wild type[a]	ile,leu,val	4.7	5.0	6.9	6.9
9[a]	PLP	2.4	1.8	0.08	0.03
9[a]	ile,leu,val	0.37	0.43	0.03	0
22	PLP	8.1	7.2	0.1	0.03
22	ile,leu,val	0.11	0	0.17	0.006
2	PLP	3.3	1.3	0.05	0.08
2	ile,leu,val	0.23	0.02	0	0
31	PLP	1.65	0.48	0.01	0
31	ile,leu,val	0.13	0	0.02	0.05
pdx 3	PLP	0.14	0.08	0.04	0.03
pdx 3	ile,leu,val	0.05	0.04		

[a] These cells were grown and assayed for threonine dehydratase activity on the same day.

the parent strain when the mutant cells were grown on medium supplemented with vitamin B_6 (Table 3). Incubation with pyridoxal-P did not increase activity of toluene-treated cells of the parent strain, but did increase that of the toluene-treated mutant cells to various degrees (Table 3). Cells and cell-free extracts of wild-type *S. typhimurium* were approximately equally active, and enzyme activity was partially repressed in extracts from cells grown with isoleucine, valine, and leucine.

Comparative affinities of wild-type and mutant enzymes for pyridoxal-P. It is clear from Table 3 that mutant cells contain appreciable levels of an altered threonine dehydratase which is more dependent on added pyridoxal-P than the enzyme in wild-type cells. Unlike the wild-type enzyme, the mutant enzyme is not stable to the procedures used in Table 3 for preparation of cell-free extracts. An extensive series of trials was undertaken to determine conditions under which active cell-free extracts of the mutant cells could be obtained. This was eventually achieved by suspending the cells [20 to 25 mg (dry weight) per ml] in stabilizing buffer (2) supplemented with pyridoxal-P (0.04 μmoles/ml), bovine serum albumin (0.3 mg/ml), and additional dithiothreitol (1 μmole/ml), subjecting them to sonic oscillation as described earlier, then [since the mutant threonine dehydratase proved to be cold labile (cf. 10)] immediately removing the broken cell suspension from the ice bath and centrifuging at 20 C. To remove pyridoxal-P, 3-ml portions of the cell-free preparations from both wild-type and mutant cells were dialyzed overnight at room temperature against 1 liter of the dialyzing buffer described by Hatfield and Burns (9). A representative mutant apoenzyme preparation obtained in this way requires much higher pyridoxal-P concentrations for maximal activation than the corresponding preparation from wild-type cells (Fig. 2A). Double-reciprocal plots of velocity versus substrate concentration were not linear under the conditions tested; maximal velocities (V_m) were obtained by extrapolating the curves (Fig. 2B) to the vertical axis and were 3 to 10 times higher for the wild type than for the mutant enzyme preparation. Comparative affinities for pyridoxal-P (K_{PLP}, analogous to K_m values) were calculated (6) from the corresponding Hill plots (Fig. 3). K_{PLP} for the wild-type enzyme was 2.5×10^{-6} M (other trials yielded values of 1.8×10^{-6} to 2.5×10^{-6} M) and agrees reasonably well with the value of 1.2×10^{-6} M reported by Burns and Zarlengo (2) for the purified enzyme. K_{PLP} for the mutant enzyme was 10 to 20 times higher.

These results demonstrate conclusively that the mutant cells contain an altered threonine dehydratase with a substantially lowered affinity for pyridoxal-P. In addition, the comparative V_m values show that the mutant enzyme is either present in lower concentration or, more probably, is catalytically less efficient than the wild-type enzyme. These altered properties together with the reduced stability of the enzyme in the

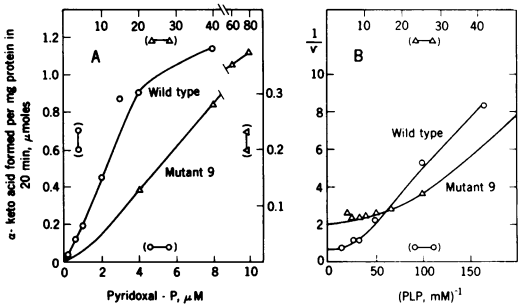

FIG. 2. *A, α-Keto acid formation by dialyzed cell extracts of parent strain (0.37 mg of protein per ml) and mutant 9 (0.85 mg of protein per ml) of S. typhimurium LT-2 as a function of the pyridoxal-P concentration. The apoenzyme preparations were incubated with pyridoxal-P for 30 min at 37 C before addition of substrate. B, Lineweaver-Burk plots of the data in A. V_m values calculated from these curves in micromoles of keto acid formed per milligram of protein in 20 min were: wild type, 1.3; mutant 9, 0.5; similar values obtained for other cultures were 0.14 (mutant 31) and 0.18 (mutant 22). Note change in scales.*

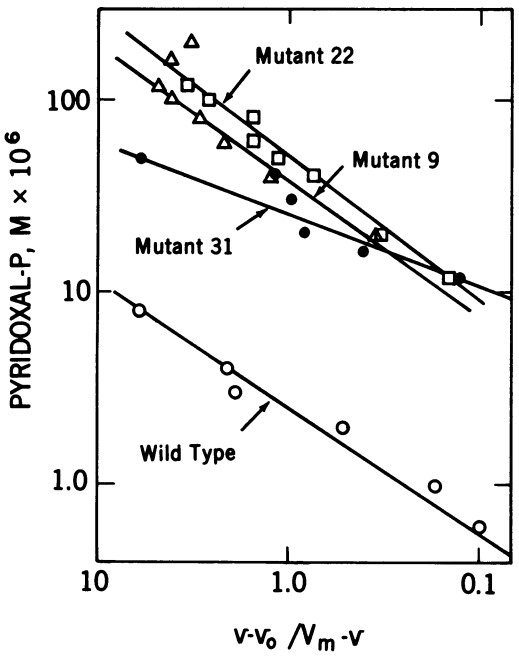

FIG. 3. *Estimation of dissociation constants of wild-type and mutant threonine apodehydratases from Hill plots; v = reaction rate at substrate concentration(s), v_0 = reaction rate at zero substrate concentration; V_m = maximum rate when saturated with substrate. Comparative dissociation constants for PLP were assumed to be numerically equal to the concentration of PLP at which $v = V_m/2$; they were: 2.5 μM for wild type; 37 μM for mutant 9, 49 μM for mutant 22, and 25 μM for mutant 31. These ilvA mutants have been assigned the following allele numbers: 9, ilvA 1452; 22, ilvA 1453; 31, ilvA 1454.*

absence of pyridoxal-P, appear to explain fully the alternative nutritional requirements for vitamin B_6, isoleucine, or α-ketobutyrate.

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LITERATURE CITED

1. Bukhari, A. I., and A. L. Taylor. 1971. Mutants of *Escherichia coli* with a growth requirement for either lysine or pyridoxine. J. Bacteriol. **105**:988-998.
2. Burns, R. O., and M. H. Zarlengo. 1968. Threonine deaminase from *Salmonella typhimurium*. I. Purification and properties. J. Biol. Chem. **243**:178-185.
3. Changeux, J.-P. 1963. Allosteric interactions on biosynthetic L-threonine deaminase from *E. coli* K_{12}. Cold Spring Harbor Symp. Quant. Biol. **28**:497-504.
4. Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17-28.
5. Dempsey, W. B., and H. Itoh. 1970. Characterization of pyridoxine auxotrophs of *Escherichia coli*, serine, and *pdx* F mutants. J. Bacteriol. **104**:658-667.
6. Freundlich, M., R. O. Burns, and H. E. Umbarger. 1962. Control of isoleucine, valine and leucine biosynthesis. I. Multivalent repression. Proc. Nat. Acad. Sci. U.S.A. **48**: 1804-1808.
7. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**:415-442.
8. Grimminger, H., and F. Lingens. 1969. Alternate requirement for pyridoxine or isoleucine in mutants of *Escherichia coli*. FEBS Lett. **5**:225-226.
9. Hatfield, G. W., and R. O. Burns. 1970. Threonine deaminase from *Salmonella typhimurium*. III. The intermediate structure. J. Biol. Chem. **245**:787-791.
10. Hatfield, G. W., and H. E. Umbarger. 1970. Threonine deaminase from *Bacillus subtilis*. I. Purification of the enzyme. J. Biol. Chem. **245**:1736-1741.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. Meister, A. 1965. The biochemistry of amino acids, vol. 1, p. 375-413. Academic Press Inc., New York.
13. Morris, J. G., and D. D. Woods. 1959. The synthesis of vitamin B_6 by some mutant strains of *Escherichia coli*. J. Gen. Microbiol. **20**:597-604.
14. Roth, J. R. 1970. General techniques in studies of bacterial metabolism, p. 3-35. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17. Academic Press Inc., New York.
15. Sanderson, K. E. 1967. Revised linkage map of *Salmonella typhimurium*. Bacteriol. Rev. **31**:354-372.
16. Snell, E. E. 1950. Microbiological methods in vitamin research, p. 327-505. *In* P. Gyorgy (ed.), Vitamin methods, vol. I. Academic Press Inc., New York.
17. Umbarger, H. E., and B. Braun. 1957. Threonine deamination in *Escherichia coli*. II. Evidence for two L-threonine deaminases. J. Bacteriol. **73**:105-112.
18. Umbarger, H. E., and B. Braun. 1958. Isoleucine and valine metabolism in *Escherichia coli*. VII. A negative feedback mechanism controlling isoleucine biosynthesis. J. Biol. Chem. **233**:415-420.
19. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97-106.