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Evidence for a New, Oxygen-Regulated Biosynthetic Pathway for the Pyrimidine Moiety of Thiamine in *Salmonella typhimurium*

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The synthesis of the pyrimidine moiety of thiamine (vitamin B_1) shares five reactions with the de novo purine biosynthetic pathway. Aminoimidazole ribotide (AIR) is the last common intermediate before the two pathways diverge. Evidence for the existence of a new pathway to the pyrimidine which bypasses the de novo purine biosynthetic pathway is reported here. This pathway is only expressed under anaerobic growth conditions and is denoted alternative pyrimidine biosynthesis or APB. Labeling studies are consistent with pantothenate being a precursor to the pyrimidine moiety of thiamine that is synthesized by the APB pathway. The APB pathway is independent of the alternative *purF* function which was proposed previously (D. M. Downs and J. R. Roth, J. Bacteriol. 173:6597–6604, 1991). The alternative *purF* function is shown here to be affected by temperature and exogenous pantothenate. Although the evidence suggests that the APB pathway is separate from the alternative *purF* function, the relationship between this function and the APB pathway is not yet clear.

Thiamine (vitamin B_1) is a required nutrient for the cell and is a coenzyme for two classes of reactions in metabolism in which aldehydes are removed and/or transferred between substrates (14). Thiamine consists of a pyrimidine moiety and a thiazole moiety, which are synthesized separately and later joined. Much work has been focused on how these two components are synthesized prior to being joined (7, 8, 10, 15–17, 25–27).

Early studies indicated that in *Salmonella typhimurium*, the first five reactions in de novo purine synthesis are common to the synthesis of the pyrimidine portion of thiamine. The connection of these two pathways was first observed in physiological studies and through the subsequent isolation of mutants which required both purine and thiamine for growth (2, 30). Newell and Tucker demonstrated that the dual nutritional requirement was attributable to a common intermediate in the two pathways, aminoimidazole ribotide (AIR) (16). The common steps of the two pathways are illustrated in Fig. 1. Radiotracing studies determined that carbon from AIR is incorporated into thiamine and, furthermore, that all the carbons in the pyrimidine moiety of thiamine are derived from AIR (9, 28, 29). In addition, [^{14}C]formate and [^{14}C]glycine (precursors of the purines) were incorporated into the pyrimidine moiety, indicating that the source of AIR was de novo purine biosynthesis (10). The biochemical steps involved in the conversion of AIR to the pyrimidine moiety of thiamine have not been fully characterized, although a partial pathway has been proposed by Brown and Williamson (3).

In spite of the fact that the pyrimidine moiety of thiamine is derived from AIR, there is no evidence to suggest feedback or transcriptional control over AIR biosynthesis by thiamine. The genes of the purine biosynthetic pathway are regulated by a common repressor protein (PurR) (13, 20). This protein responds to a variety of purines (19), but no regulation by thiamine has been noticed (22a).

Recent studies of *S. typhimurium* suggest that cells can make the pyrimidine of thiamine independent of the first gene in de novo purine biosynthesis (*purF*). All *purF* mutants could have their thiamine requirement satisfied by either exogenous pantothenate or a mutation in the *panR* locus (6). Yura (30) first observed the ability of exogenous pantothenate to satisfy the thiamine requirement of certain *pur* mutants. This was prior to the assignment of the purine genes to biochemical reactions in the pathway, but presumably these were *purF* mutants (30). The function responsible for growth in the presence of pantothenate is designated the *purF* alternative to indicate its ability to bypass only the PurF enzyme. PurD, -G, and -I are still required for thiamine synthesis from exogenous pantothenate. Data presented here show that the alternative *purF* function is affected by temperature and pantothenate.

I also report here evidence for the existence of an additional pathway for the synthesis of the pyrimidine moiety of thiamine in *S. typhimurium*, which has been designated alternative pyrimidine biosynthesis (APB). Genetic evidence indicates that this pathway is distinct from the alternative *purF* function because it is independent of all purine biosynthetic genes prior to the formation of AIR. The APB pathway shows negative regulation by oxygen. The data suggest that this APB pathway uses pantothenate as a precursor for the pyrimidine moiety, since the pantothenate precursor aspartate appears to be directly incorporated into thiamine. Although pantothenate appears to be involved in thiamine biosynthesis by both the alternative *purF* function and the APB pathway, the relationship between these two biosynthetic routes is not yet clear.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. All strains used in this study are derived from *S. typhimurium* LT2 and are listed with their sources in Table 1. Mu dJ is used throughout the manuscript to refer to the Mud dl1734 transposon, which has been described previously (5).

The E medium of Vogel and Bonner (24), supplemented with 11 mM glucose, was used as minimal medium. Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) added was used as rich medium. Difco Bacto-Agar was added to a final concentration of 1.5% for solid medium. When present in the culture media, and unless otherwise stated, the final concentrations of the following compounds were as follows: adenine, 5 mM; thiamine, 50 μM; aspartate, 50 μM; and pantothenate, 0.1 mM. The final concentrations of the anti-

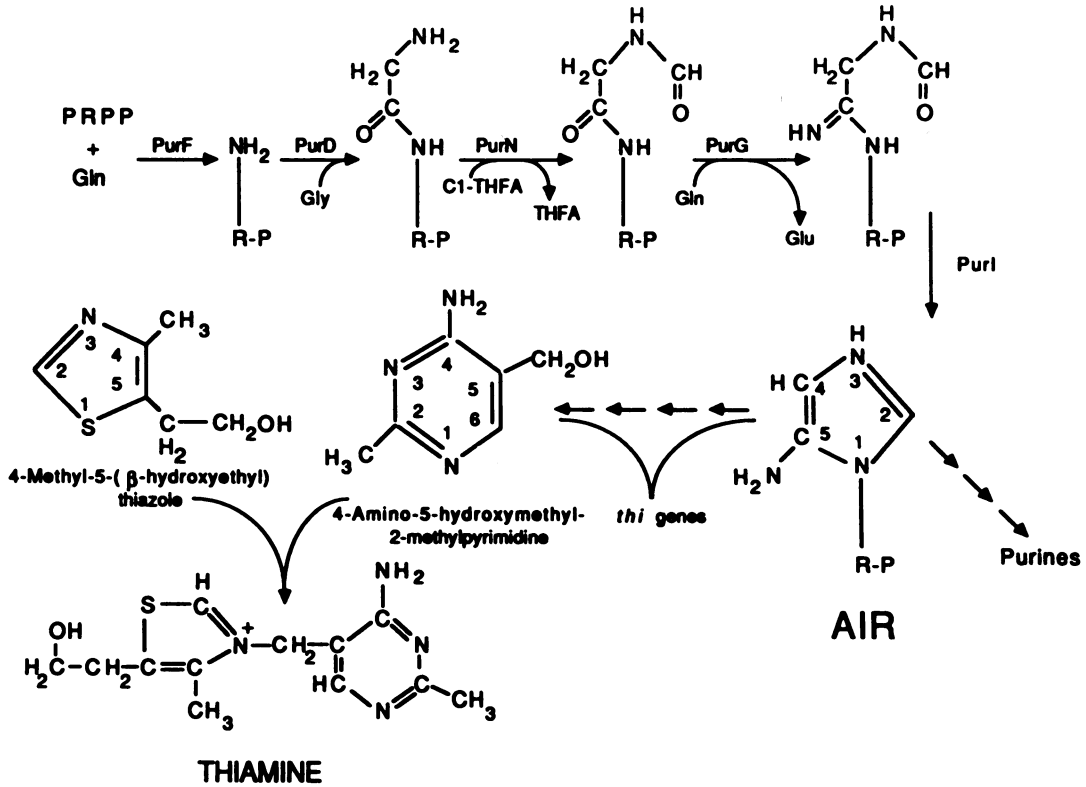

FIG. 1. Biosynthetic pathway for thiamine. The purine gene product required for each reaction is indicated above the arrows. The number of steps involved in the formation of the 4-amino-5-hydroxymethyl-2-methylpyrimidine from AIR is not known (3). AIR also serves as an intermediate in the synthesis of complete purines. Abbreviations: R-P, ribose phosphate; PRPP, phosphoribosyl pyrophosphate.

biotics tetracycline and kanamycin in rich medium were 15 and 50 μg/ml, respectively.

Sources for supplies were as follows: silica thin-layer chromatography (TLC) plates (13179), Kodak Chemicals (Rochester, N.Y.); Amberlite CG-50, Aldrich (Milwaukee, Wis.); L-[U-^{14}C]aspartate (specific radioactivity, 125 mCi/mmol), ICN Biomedicals (Costa Mesa, Calif.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

TABLE 1. *S. typhimurium* strains

Strain	Genotype	Source[a]
TT176	*asp-544*::Tn*10*	J. Roth
TT315	*purG1739*::Tn*10*	J. Roth
TT501	*thiA541*::Tn*10*	J. Roth
SA2696	*thiI195*	M. Demerec
DM4	*purF2100*	
DM40	*purG2324*::Mu dl1734[b]	
DM41	*purG2324*::Mu dJ *purF2100*	
DM42	*purI2152*::Mu dJ	
DM43	*purI2152*::Mu dJ *purF2100*	
DM44	*purD1874*::Mu dJ	
DM45	*purD1874*::Mu dJ *purF2100*	
DM129	*purG1739*::Tn*10 purF2100*	
DM132	*purF2085 asp-544*::Tn*10 panR561*	
DM142	*purI2152*::Tn*10 purF2085 asp-544*::Tn*10 panR561*	

[a] Unless otherwise stated, strains came from the laboratory collection or were constructed for this work.

[b] All Mu dl1734 insertions are referred to as Mu dJ throughout. This defective transposon has been described previously (5).

Growth conditions. Growth curves were determined as follows. Nutrient broth cultures of the relevant strains were grown to stationary phase. The cells were pelleted and suspended in an equal volume of 85 mM NaCl. A 0.2-ml aliquot of this cell suspension was used to inoculate 5 ml of culture medium in an 18- by 150-mm tube. Tubes were shaken (if required) on a rotary shaker at 250 rpm. All tubes were incubated in a water bath at 30 or 37°C. For experiments testing the effects of variation in oxygen concentration, anoxic culture tubes were prepared as previously described (1). Sterile oxygen equivalent to the desired percentage in the headspace of the tube was injected by syringe. For more than 1% oxygen, the correct amount of atmospheric headspace gas was removed prior to the addition of oxygen. In all cases, cell turbidity was monitored with a Bausch and Lomb Spectronic 20D spectrophotometer at 650 nm. Specific growth rate was determined as $\mu = \ln(X/X_o)/T$, where $X = A_{650}$ during the linear portion of the growth curve and T = time. Routinely, the A_{650} at time zero was between 0.02 and 0.04.

Genetic techniques and strain constructions. Strains were constructed by transduction using a derivative of bacteriophage P22 containing HT-105/1 and *int-201* (22). The isolation of *panR* mutations has been described previously (6).

Thiamine purification. The purification of thiamine was accomplished by modifying a previously described method (26). The appropriate strain was inoculated into 1 liter of minimal medium containing 22 mM glucose, 5 mM adenine,

and 50 μM aspartate. Cultures were grown without shaking at 30°C in 4-liter Erlenmeyer flasks. Cells at late log phase (1 to 2 g) were separated from the medium by centrifugation (10,000 × *g* for 20 min), resuspended in 8 ml of 0.1 N HCl, placed in a boiling-water bath for 20 min, and cooled to room temperature. One milliliter of 1 M sodium acetate (pH 4.7) was added, and the pH was adjusted to 4.7 with 4 M KOH. Centrifugation followed at 14,000 × *g* for 15 min to remove cell debris. Acid phosphatase (20 mg) and chloroform (100 μl) were added to the supernatant. This mixture was incubated for 18 h at 37°C; the pH was adjusted to 7 and the volume was adjusted to 10 ml before the mixture was loaded on an Amberlite CG-50 (H^+) column (1 by 13 cm; bed volume, ~10 ml). After the column was washed with 20 ml of glass-distilled water, thiamine was eluted with 15 ml of 0.1 N HCl. This fraction was evaporated to dryness in a Savant Speed-Vac evaporator and resuspended in either water (for bioassay) or 100% methanol (for TLC).

Quantitation of thiamine. Quantitation of thiamine in purified fractions was accomplished by a thiochrome assay. A modification of the method of Kawasaki was used (12). To a 1.25-ml aqueous sample, 0.75 ml of 0.0265% $K_3Fe(CN)_6$ in 30% NaOH was added. The solution was mixed, and fluorescence was measured on a PCI photon-counting spectrofluorometer (SLM Instruments, Urbana, Ill.). Excitation was set at 375 nm, and emission was measured at 432 nm. A standard curve with thiamine HCl was generated, and unknown samples fell within the linear portion of the curve, allowing quantitation of thiamine.

[^{14}C]aspartate labeling of thiamine. Cultures (50 ml) of both strain DM132 and DM142 were grown to full density in minimal medium containing 22 mM glucose, 5 mM adenine, and 5 μM aspartate. The cells were pelleted and suspended in saline to remove excess aspartate. The suspended cultures were used to inoculate 1 liter of E medium containing 22 mM glucose, 5 mM adenine, 0.5 μM aspartate, and 10 μCi of L-[U-^{14}C]aspartate. This concentration of carrier aspartate was found to be the minimal concentration which allowed optimal growth of these aspartate auxotrophs. The cultures (in 2-liter Erlenmeyer flasks) were incubated without shaking in a 30°C water bath for 24 h. Cells were harvested by centrifugation, and thiamine was purified as described above.

Cleavage of thiamine into its pyrimidine and thiazole moieties. The method for bisulfite cleavage of thiamine has been described previously (23). Carrier, nonradioactive thiamine HCl (1 mg) was added to the fraction containing ^{14}C-labeled thiamine (30,000 dpm), and the mixture was suspended in 120 μl of 2.5 M $NaSO_3$ (pH ~4.7). This mixture was placed in a boiling-water bath for 30 min and cooled to room temperature. The solution was kept undisturbed at room temperature for 48 h and evaporated to dryness in vacuo with a Brinkman REIII rotovapor (Buchi Institute, Switzerland). The pellet was extracted twice with methanol (120 μl) to remove most of the $NaSO_3$. The hydrolysate was dried in a Speed-Vac concentrator, suspended in a final volume of 50 μl of methanol, and used in subsequent experiments.

TLC. TLC separations were done on silica gel plates (Kodak 13179) without fluorescent indicator, developed in solvent system a (acetonitrile-water [40:10, pH 2.54 with formic acid]), solvent system b (pyridine-acetic acid-water [29:2:79]), and solvent system c (pyridine-acetic acid-water [10:2:88]). Thiamine was detected by autoradiography and/or bioassay. Thiazole was easily visualized by iodine staining.

Bioassay for thiamine. The bioassay for thiamine was based on the thiamine-dependent growth of TT501 (*thiA541*::Tn*10*). This mutant requires thiamine (or the pyrimidine moiety of thiamine) for growth. Another strain, SA2696 (*thiI95*), was used to assay the thiazole moiety, since this strain is satisfied only by thiazole. The correct tester strain was combined with 0.7% agar and overlaid on E minimal medium. The sample to be tested for biological activity was then spotted on a sterile disk (r = 1 cm) on this plate. This assay, using TT501 as a tester strain, can detect 10 pmol of thiamine as a 2-cm growth zone around the disk.

RESULTS

The PurF, -D, -G, and -I enzymes are needed for the synthesis of AIR, the last common intermediate in the biosynthetic pathways for purine and the pyrimidine moiety of thiamine. In the experiments described below, adenine was routinely included in the medium to satisfy the purine requirement of the strains that carried *pur* mutations. Thus, the only phenotype assessed was the ability of the cells to synthesize thiamine.

Thiamine synthesis in the absence of *purF* and *purG*. The experiments shown in Fig. 2 defined conditions that demonstrate the existence of an APB pathway for the synthesis of thiamine. Figure 2 shows the growth behaviors of *purF*, *purG*, and *purF purG* mutants grown either with or without vigorous shaking at 37°C. Figure 2A, C, and E show growth of cultures without shaking, while panels 2B, D, and F show growth of cultures with shaking on a rotary shaker at 250 rpm. All strains in shaking tubes showed a requirement for exogenous thiamine, as predicted by the known biochemical pathways.

In contrast, all strains in standing culture tubes showed significant growth in the absence of exogenous thiamine. In the case of the *purF* mutant (Fig. 2A), growth in standing tubes was identical, in both specific rate and final cell density, with or without exogenously added thiamine. Thus, the *purF* mutant can completely bypass the need for exogenous thiamine under conditions of low aeration. In the cases of the *purG* mutant (Fig. 2C) and the *purF purG* double mutant (Fig. 2E), only partial suppression of the thiamine requirement occurred. The specific growth rates of these mutants in standing cultures were increased about twofold by the addition of exogenous thiamine.

The difference in specific growth rate (μ) between the *purF* mutant and the *purG* mutant in standing cultures without added thiamine was significant (μ = 0.4 and 0.24, respectively; Fig. 2A and C). This suggested that a block in *purF* had a less severe effect on thiamine biosynthesis than did a block further down the purine pathway.

The data in Table 2 demonstrate that the APB pathway bypasses the requirement for all *pur* genes which are necessary for the synthesis of AIR through the de novo purine pathway. As expected, the *purG*, *purI*, and *purD* mutants behave similarly with or without a *purF* deletion. This result suggests that no metabolite flow through the purine pathway is required for the synthesis of thiamine via the APB pathway. The simple interpretation of these results is that the APB pathway results in the formation of AIR, which is then converted by the standard thiamine pathway (3) to the pyrimidine moiety of thiamine.

In experiments using standing cultures, the maximum A_{650} was significantly lower (~55%) than that reached in shaken cultures. This probably reflects the reduced efficiency of glucose utilization by fermentation compared with that by respiration. Since cultures in medium containing exogenous

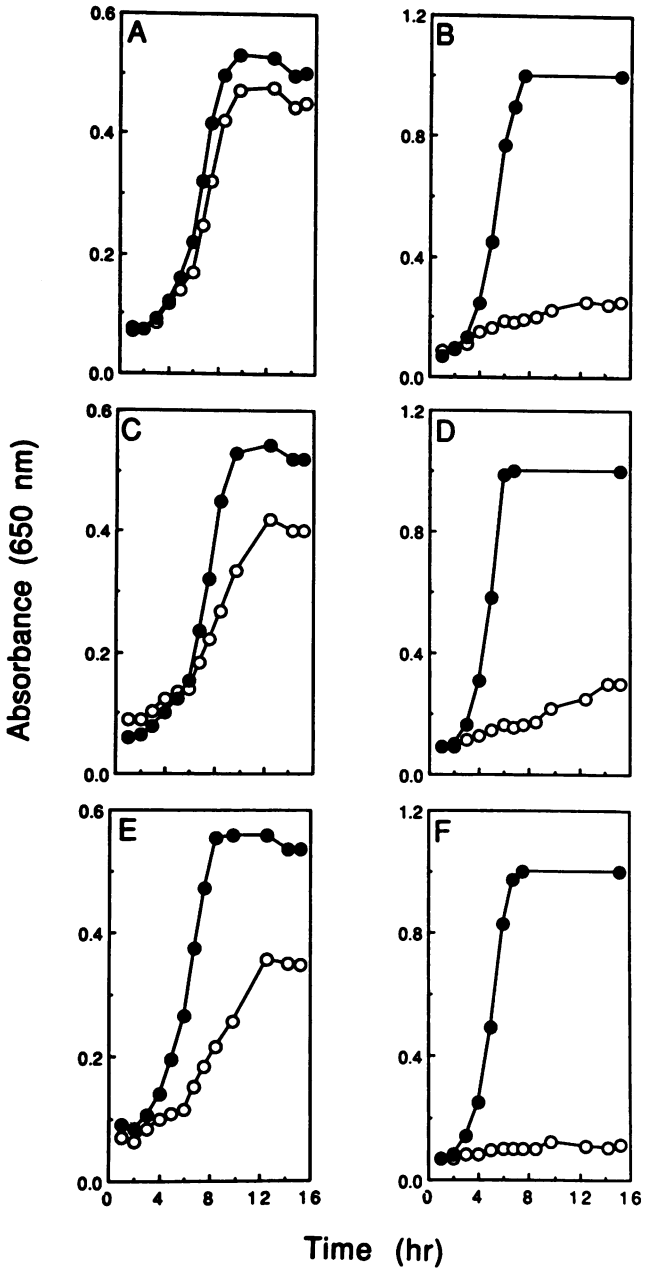

FIG. 2. Standing versus shaking growth tubes. Cultures were grown in either standing or shaking (250 rpm) tubes as described in Materials and Methods. Growth was carried out in a 37°C water bath. Panels A, C, and E show growth in standing tubes, and panels B, D, and F show growth in shaking tubes. The strains were as follows: A and B, DM4 (*purF2100*); C and D, TT315 (*purG*); and E and F, DM129 (*purF purG*). Medium supplements were adenine (○) and adenine + thiamine (●).

thiamine reached only this lower cell density, it was clear that this limitation was due to differences in growth conditions and not to strain differences.

Oxygen inhibits thiamine synthesis by the *apb* pathway. One obvious difference between shaking and standing tubes is the amount of dissolved oxygen available to the cultures. To address the issue of whether oxygen concentration was causing the growth differences described above, the initial oxygen concentration in the growth medium of standing cultures was varied. For these studies, growth of strain DM4 (*purF2100*) was tested in medium either with or without exogenous thiamine at 37°C.

TABLE 2. Effect of pre-AIR *pur* blocks on the APB route[a]

Strain	Genotype	Specific growth rate (μ) (doublings h^{-1}) in:			
		Standing culture with:		Shaking culture with:	
		Adenine	Adenine + thiamine	Adenine	Adenine + thiamine
DM40	*purG2324*::Mu dJ	0.21	0.40	<0.02	0.59
DM41	*purG2324*::Mu dJ *purF2100*	0.24	0.38	<0.02	0.57
DM42	*purI2152*::Mu dJ	0.23	0.44	<0.02	0.56
DM43	*purI2152*::Mu dJ *purF2100*	0.24	0.31	<0.02	0.62
DM44	*purD1874*::Mu dJ	0.24	0.44	<0.02	0.47
DM45	*purD1874*::Mu dJ *purF2100*	0.31	0.35	<0.02	0.48
DM4	*purF 2100*	0.41	0.43	<0.02	0.46

[a] Strains were grown in minimal medium with the indicated supplement(s) in a 37°C water bath. Shaking cultures were grown on a rotary shaker at 250 rpm. Final A_{650} of standing cultures at stationary phase was between 0.42 and 0.53. Final A_{650} of shaking cultures with added thiamine was between 1.0 and 1.2; without added thiamine, final A_{650} was between 0.06 and 0.17.

The data in Fig. 3 show that oxygen inhibited thiamine synthesis through the APB pathway. In Fig. 3A, for which thiamine was present in the growth medium, the specific growth rates for DM4 at all oxygen concentrations were essentially the same (0.4 to 0.5). The culture without any oxygen had a slightly lower specific growth rate (0.33), which again may reflect the difference between the efficiency of glucose utilization by fermentation and that by respiration. This difference was also reflected in the increase in yield of the cultures with increasing oxygen concentrations. Figure 3B shows growth of DM4 in medium without added thiamine. The specific growth rate of cultures with no added oxygen was essentially the same as that for cultures grown in

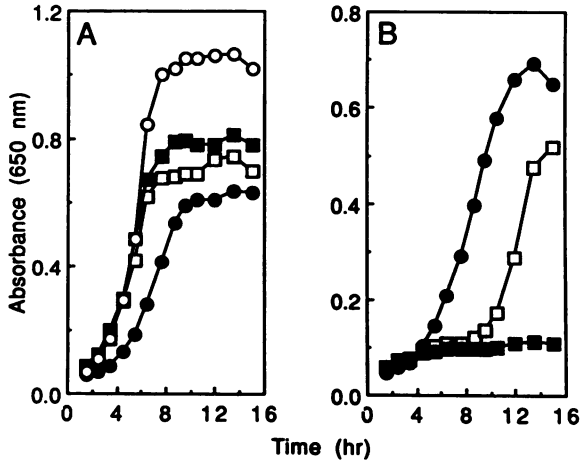

FIG. 3. Repression of the APB pathway by oxygen. Strain DM4 was grown in sealed tubes at 37°C as described in Materials and Methods. The medium was minimal medium containing adenine + thiamine (A) or adenine (B). In both panels, the initial P_{O_2} was as follows: ●, none (0 kPa); □, 3.75 kPa; ■, 7.5 kPa; ○, 26.25 kPa (atmospheric pressure).

TABLE 3. Effect of temperature and exogenous pantothenate on growth of *pur* mutants[a]

Strain	Genotype	Specific growth rate (μ) (doubling h^{-1}) and final A_{650} of cultures in minimal medium at:											
		37°C with:						30°C with:					
		Adenine		Adenine + thiamine		Adenine + pantothenate		Adenine		Adenine + thiamine		Adenine + pantothenate	
		μ	A_{650}	μ	A_{650}	μ	A_{650}	μ	A_{650}	μ	A_{650}	μ	A_{650}
DM129	*purG1739*::Tn*10 purF2100*	0.03	0.10	0.44	1.0	0.06	0.10	0.04	0.10	0.29	1.1	0.03	0.13
TT315	*purG1739*::Tn*10*	0.04	0.13	0.42	1.0	0.02	0.10	0.06	0.15	0.34	1.1	0.05	0.10
DM4	*purF2100*	0.05	0.15	0.46	1.2	0.07	0.42	0.14	0.57	0.30	1.2	0.17	0.86

[a] Strains were grown in minimal medium with the indicated supplement(s) in a water bath. All cultures were shaken in a rotary shaker at 250 rpm. A_{650} at time zero was between 0.02 and 0.05.

thiamine-supplemented medium (μ = 0.3). With an initial partial pressure of oxygen (P_{O_2}) of more than 7.5 kPa (6%), no significant growth of DM4 was seen, indicating that there was no synthesis of thiamine. With a P_{O_2} of 3.75 kPa (3%), the specific growth rate was 0.34, similar to that seen with no added oxygen, but the lag prior to growth increased significantly (from 3 to 9 h). This lag may reflect the time needed for the inoculum to consume the oxygen present in the headspace to a level which allows APB function. Since only initial P_{O_2} was measured, no control for consumed oxygen was done. It is clear that oxygen negatively affects the growth of *pur* mutants in the absence of thiamine. This suggests that oxygen negatively affects the function of the APB pathway. In similar experiments with *purG*, *-D*, and *-I*, the same pattern was seen, although the specific growth rates in the absence of thiamine were ~50% of those measured for *purF* mutants.

The *purF* alternative is distinct from the APB pathway. *purD*, *-G*, and *-I* mutants are completely dependent on the APB pathway for the synthesis of thiamine. This is demonstrated by their inability to grow aerobically in the absence of thiamine. *purF* mutants are capable of growing aerobically in the absence of thiamine due to the alternative *purF* function. As reported above (Fig. 2), this growth does not occur at 37°C in medium containing only adenine. The data in Table 3 show that pantothenate and/or low temperature are required for *purF*-independent aerobic synthesis of thiamine. The data in Table 3 indicate the following. (i) Temperature had a dramatic effect on the growth rate of *purF* mutants in unsupplemented medium (adenine alone). The growth rate of *purF2100* increased from 0.056 at 37°C to 0.14 at 30°C. To compensate for the fact that the optimal growth temperature for *S. typhimurium* is 37°C, one should compare the specific growth rate in medium without thiamine to that in medium with thiamine at each temperature. By this criterion, the relative growth rate increases fourfold when *purF2100* is grown at 30 instead of at 37°C (relative growth rates are 0.47 and 0.12, respectively). (ii) At each temperature, the presence of pantothenate increased the specific growth rate of the *purF* mutant slightly (growth rate at 30°C, 0.17 versus 0.14; growth rate at 37°C, 0.073 versus 0.056). However, exogenous pantothenate exerted a large effect on the final yields of the cultures (i.e., final A_{650}). At 37°C, the yield was increased almost threefold (0.42 versus 0.15) by the addition of pantothenate.

Neither temperature nor exogenous pantothenate had any affect on the growth rate or yield (final A_{650}) of the *purG* mutant, the *purF purG* double mutant (Table 3, lines 1 and 2), the *purD* mutant, or the *purI* mutant (data not shown). In standing cultures, neither pantothenate nor temperature affected the growth of any strain (data not shown).

[^{14}C]aspartate is a precursor to thiamine in *pur* mutants. Observations made throughout this work suggested that pantothenate may be involved in the APB pathway as an intermediate. My working hypothesis (Fig. 4) predicted that pantothenate was a precursor of the pyrimidine moiety of thiamine via the APB pathway. Here, the assumption (the simplest from the genetic data) is made that the *pur* and APB pathways share AIR as a common intermediate. Since appropriately radiolabeled derivatives of pantothenate were not commercially available to directly test this hypothesis, aspartate, which is a direct precursor of pantothenate, was utilized. The model in Fig. 4 predicts that two carbons (C-2 and -3) from aspartate will be incorporated into the pyrimidine of thiamine via the APB pathway. For in vivo labeling studies, uniformly labeled [^{14}C]aspartate was used.

Internal levels of pantothenate are closely regulated, and a significant fraction of exogenous aspartate would not be expected to enter the pantothenate pool in a wild-type strain (11). To increase the incorporation of aspartate into pantothenate, I used a strain containing a *panR* mutation (6). Since this mutation causes strains to excrete large amounts of pantothenate, these cells probably convert more aspartate into pantothenate than does the wild type. This idea was

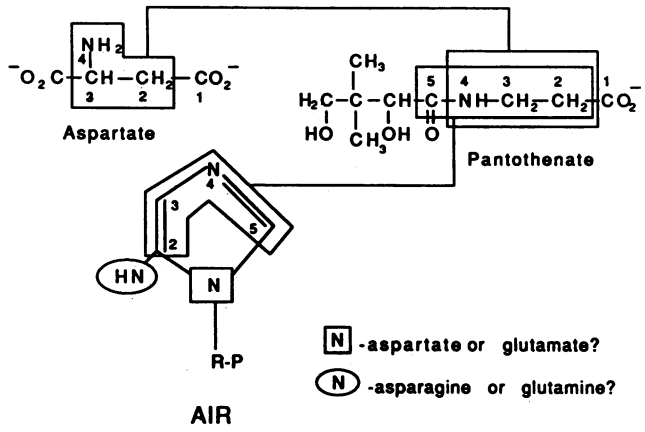

FIG. 4. Proposed metabolic origin of AIR through the APB pathway. The schematic illustrates proposed precursors of AIR via the APB pathway. AIR is assumed to be an intermediate in the APB pathway. What is known about the conversion of AIR to 4-amino-5-hydroxymethyl-2-methylpyrimidine has been recently reviewed (3). Pantothenate is proposed to donate three carbon (C-2, -3, and -5) and one nitrogen (N-4) atom, with two of those carbons (C-2 and -3) and the nitrogen ultimately coming from aspartate. Common nitrogen donors are proposed to account for the remaining two nitrogen atoms.

supported by the slight aspartate auxotrophy observed with *panR* mutants (unpublished data). A mutation leading to aspartate auxotrophy (*asp-544*::Tn*10*) was introduced into a *panR* strain by P22 transduction to construct DM132 (*purF panR asp*) and DM142 (*purF purI panR asp*). The *asp-544*::Tn*10* mutation was introduced to minimize the dilution of label by endogenously synthesized aspartate. The following three lines of evidence support the hypothesis that the pathway is as presented in Fig. 4.

(i) Thiamine was partially purified from both strains DM132 and DM142 as described in Materials and Methods. The purified thiamine-containing fractions were applied to silica TLC plates and developed with three different solvent systems (see Materials and Methods). A single radioactive spot was measured in each solvent system. For samples from both DM132 and DM142, the relevant mobilities were R_{fa} = 0, R_{fb} = 0.57, and R_{fc} = 0.68. In all cases, the biological activity of thiamine (as described in Materials and Methods) was associated with the radioactive spot. The biologically active spot did not have the same R_f as did commercial thiamine HCl. This may indicate that the biologically active compound purified from the cells was α-hydroxyethyl thiamine, a derivative which is often isolated and which is able to satisfy the requirement of a *thi* mutant (4).

(ii) An additional experiment was done to quantitate the radioactivity and correlate it with biological activity. Parallel TLC plates were run with a sample from strain DM132 (*purF panR asp*) and developed in a 10:2:88 mixture of pyridine-acetic acid-water, and the developed plates were cut in horizontal strips 1 cm in width. The strips from one plate were used for scintillation counting, and those from the other plate were extracted and used for bioassays (see Materials and Methods). In such an experiment, ~70% of the radioactive counts were associated with biological activity. An additional 20% of the counts were in the two fractions flanking the biologically active peak, and the remaining 10% of the counts were in the trailing shoulder of the peak. I believe that this is also radiolabeled thiamine, but in amounts too low to satisfy the auxotrophic requirement of the tester strain. The broad peak of radioactivity is a characteristic of the solvent systems that were required to move thiamine from the origin.

(iii) Quantitation of thiamine by the thiochrome assay (see Materials and Methods) indicated a recovery of 2.2 and 2.7 μg of thiamine per g of cells from DM132 and DM142, respectively. The specific activities of the recovered thiamine were 5.5 and 5.7 μCi/μmol, on the basis of the assumption that the purified fraction contained only one radioactive compound and that it was thiamine. The specific activity of the added aspartate was 17 μCi/μmol. These results are therefore consistent with at least one carbon from aspartate being donated to thiamine through the APB pathway. The model shown in Fig. 4 predicts that two of the four carbons from aspartate should be incorporated into thiamine by this pathway; this would predict a specific activity of ~8 μCi/μmol for the isolated thiamine. Determining whether aspartate is a direct precursor of thiamine via pantothenate will require further labeling studies.

[^{14}C]aspartate labels the pyrimidine moiety of thiamine. To clearly demonstrate that pantothenate-dependent synthesis of thiamine was replacing the purine pathway, I determined which moiety (pyrimidine or thiazole) of thiamine was being labeled by [^{14}C]aspartate. This was done by combining radiolabeled thiamine (1.5 μg) recovered from DM142 with carrier thiamine (1 mg). This solution was cleaved with sodium bisulfite, yielding the two components of thiamine. The hydrolysate was separated on a silica TLC plate and developed with acetonitrile-water (40:10, pH 7.85). Control experiments showed that with this solvent system thiamine has an R_f of 0.05, 4-amino-5-hydroxymethyl-2-methyl pyrimidine has an R_f of 0.14, and thiazole has an R_f of 0.6. The identities of these compounds were also confirmed by the ability of the appropriate spot to satisfy the requirement of a thiazole- and/or pyrimidine-specific mutant (21) in a bioassay (see Materials and Methods).

For the radiolabeled sample, no radioactivity above background were obtained at the R_f corresponding to thiazole (detected by iodine staining). This fraction was biologically active and corrected the requirement of a thiazole-specific mutant but not that of a pyrimidine-specific mutant. The majority (56%) of the radioactivity remained at the origin. This fraction had very weak biological activity as thiamine, that is, it satisfied both pyrimidine- and thiazole-specific mutants. The remainder of the radioactivity was in a fraction from an R_f of 0.02 to 0.13. This fraction had significantly more biological activity of thiamine than did the origin. This result suggests that the majority of the radioactivity at the origin is in pyrimidine sulfonic acid (the pyrimidine derivative that results from the bisulfite cleavage), which is not biologically active (18). Pyrimidine sulfonic acid would be expected to have a different relative mobility than the 4-amino-5-hydroxymethyl-2-methyl pyrimidine of the control experiments because of the additional sulfonic acid side chain. None of the solvent systems tested separated the pyrimidine moiety completely from the thiamine moiety. However, in all cases the thiazole, which was clearly separable, had no radioactive counts associated with it. These results support the hypothesis that aspartate is a specific precursor of the pyrimidine moiety of thiamine through the APB pathway. This is consistent with the genetic data indicating that the role of the de novo purine genes (which synthesize the pyrimidine moiety) is bypassed by the APB pathway. Since it is known that aspartate is a precursor to pantothenate, it is plausible to suggest that pantothenate is a precursor of the pyrimidine synthesized via the APB pathway.

DISCUSSION

The physiological studies presented here uncover hitherto unknown metabolic capabilities of *S. typhimurium* with respect to the biosynthesis of the pyrimidine moiety of thiamine. Herein I described the identification and initial characterization of a new biosynthetic pathway for the synthesis of the pyrimidine moiety of thiamine, designated APB. The existence of this pathway is clearly indicated by the thiamine-independent growth of *purF*, *-G*, *-D*, and *-I* mutants, i.e., the APB pathway does not require any genes in the de novo purine biosynthetic pathway needed for the synthesis of AIR.

A distinct characteristic of the APB pathway is that it is only functional under anaerobic conditions (Fig. 2). At present, it is unclear how oxygen exerts its negative effect. The effect may be brought about by transcriptional regulation of the APB genes, or it may reflect oxygen lability of either enzymes or intermediates in the pathway.

The distinction between the alternative *purF* function and the APB pathway was clearly demonstrated by the thiamine-independent growth of *purF* mutants under aerobic growth conditions. These are conditions under which the APB pathway is not functional (Table 3). Another distinguishing feature is that the alternative *purF* function requires func-

tional *purG*, *-D*, and *-I* gene products for the synthesis of thiamine. An interesting feature of this alternative *purF* function is that it appears to require exogenous pantothenate and/or low growth temperature conditions. The effects of pantothenate and temperature are not additive, suggesting that they may affect the same regulatory system. The effect of pantothenate on the *purF* alternative is a subtle one, as it affects only growth yield and not growth rate. The requirement of the alternative *purF* function for pantothenate is also in contrast with the APB pathway, the activity of which does not require either pantothenate or low growth temperature.

Although the current data suggest that the alternative *purF* function and the APB pathway are phenotypically separable, it is still formally possible that the two are somehow connected. This possibility needs to be explored by further genetic and biochemical studies.

One interpretation of the genetic data is that the APB pathway synthesizes AIR, which can then be converted by the standard route of thiamine biosynthesis to 4-amino-5-hydroxylmethyl-2-methylpyrimidine. If this is the case, the production of AIR through the APB pathway does not satisfy the purine requirements of the cell, since all purine mutants are auxotrophic under anaerobic growth conditions. There is currently no data to rule out the possibility that the APB pathway produces an intermediate subsequent to AIR in the formation of 5-hydroxylmethyl-2-methylpyrimidine.

The results of tracing studies allow me to postulate that pantothenate is a precursor to the pyrimidine moiety of thiamine via the APB pathway (Fig. 4). Further tracing studies utilizing specifically labeled pantothenate are required to further test this model.

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