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Vitamin B_6 Requirements of Nutritionally Variant *Streptococcus mitior*

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The growth rate of three vitamin B_6-dependent *Streptococcus mitior* (B6DS) and two non-B6DS strains in Todd-Hewitt broth, with and without vitamin B_6 supplementation, was examined. Even in optimally supplemented culture media, the growth rate of the three B6DS strains was much slower than that of comparable non-B6DS strains. Uptake studies with [^{3}H]pyridoxine suggest that these B6DS strains cannot assimilate pyridoxine. Although not transported intracellularly, pyridoxine inhibited the growth of B6DS strains in media supplemented with other vitamin B_6 analogs, probably by binding to the vitamin B_6 transport system and inhibiting the uptake of the other vitamin B_6 analogs.

In recent years, there has been an increasing awareness of the involvement of nutritionally deficient viridans streptococci as a significant cause of systemic disease in humans. In one study, pyridoxal (P-al)-dependent *Streptococcus mitior* (*mitis*) accounted for 5 to 6% of streptococcal endocarditis at The New York Hospital (9). These organisms are probably similar to the satelliting streptococci (4, 7) and thiol-requiring mutants (2) described previously. Previous studies from this laboratory (1, 9) have shown that the active forms of vitamin B_6, either P-al or pyridoxamine (P-amine) but not pyridoxine (P-ine), replace the requirement for certain sulfhydryl (thiol) compounds. In a separate study, Cooksey et al. (3) demonstrated that all 25 nutritionally variant streptococci that they examined grew in Todd-Hewitt broth (THB) supplemented with 0.001% P-al. Because of the need for vitamin B_6 supplementation of culture media for the isolation and cultivation of the vitamin B_6-dependent streptococci (5), the underlying mechanism responsible for the supplemental activity of the vitamin B_6 analogs was investigated.

Three nutritionally variant (Br, Fe, and Gu) and two non-nutritionally variant (no. 27 and 591) streptococcal strains were selected for these studies. These strains were isolated from cases of bacterial endocarditis seen at The New York Hospital and were subsequently classified as *S. mitior* because of their biochemical reaction profile and the low content of rhamnose in their cell walls (9). The growth rate of these strains in various media was examined. For these experiments, bacterial strains were grown overnight in THB containing 10.0 μg of P-al per ml at 37°C with agitation, pelleted by centrifugation at 10,000 × *g* for 10 min, washed twice with THB, and resuspended and diluted to a final concentration of approximately 2×10^4 to 5×10^4 bacteria per ml in THB with or without the indicated vitamin B_6 analog at a final concentration of 0.001% (or 10 μg/ml). These cultures were then incubated at 37°C with agitation and were periodically checked for growth by recording the optical density at 550 nm or determining the number of colony-forming units per milliliter.

The growth rate of strain 27 (a representative non-vitamin B_6-dependent *S. mitior* [B6DS] strain) in various media is shown in Fig. 1. Although the strain was capable of growing in unsupplemented THB, maximal growth was enhanced by the addition of 0.001% P-al, P-amine, or P-al phosphate. In contrast, the addition of P-ine or P-amine phosphate did not appear to enhance the growth observed when THB was used alone. Maximal growth was observed at 24 h in THB with P-al. Similar results were observed with strain 591 (data not shown).

Similar studies were performed with the three B6DS strains, and the results with strain Fe are shown in Fig. 2. No growth was observed in unsupplemented THB or in THB with 0.001% P-ine or P-amine phosphate. Optimal growth was observed in THB supplemented with 0.001% P-al phosphate, followed in order by 0.001% P-al and P-amine. Although the maximal growth obtained with the B6DS strains compares with that of the non-B6DS strains, it required almost twice as long an incubation time for the B6DS strains to reach this maximum (40 h compared with 24 h), even though the starting inocula were

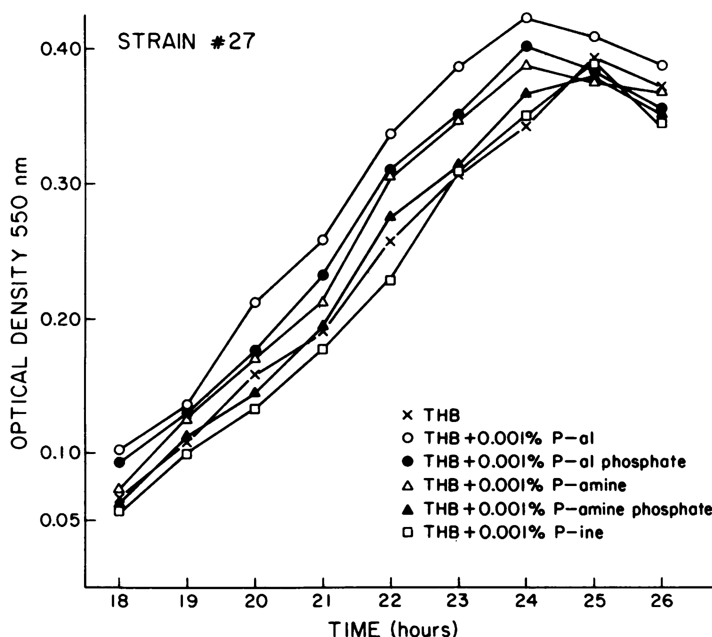

FIG. 1. Growth of strain 27 in THB with and without supplements at 37°C with agitation. Values represent the mean of three determinations.

equivalent. Although not shown, similar growth curves were obtained for strains Br and Gu.

The inability of P-ine to support the growth of vitamin B_6-dependent streptococci in these studies agrees with previous studies reported by this laboratory (1, 9). There are at least two possible explanations for the inability of P-ine to support the growth of these B6DS isolates: (1) either P-ine is not transported intracellularly by these isolates or (2) once internalized, it cannot be converted to the active coenzyme form, P-al phosphate. To determine whether these strains could transport P-ine intracellularly, a protocol similar to that described by Shane and Snell (11) was followed. The five streptococcal strains described above and one *Streptococcus faecalis* strain were grown in THB containing 10.0 μg of P-al per ml for 18 h at 37°C with agitation, pelleted by centrifugation at 10,000 × *g* for 10 min, washed twice with 0.85% NaCl, and resuspended to a concentration of approximately 10^9 organisms per ml in the following salt solution (grams per liter of distilled water): glucose, 10; KH_2PO_4, 0.56; $(NH_4)_2SO_4$, 1.88; KCl, 0.42; $CaCl_2 \cdot 2H_2O$, 0.125; $MgSO_4 \cdot 7H_2O$, 0.125; $FeCl_3 \cdot 6H_2O$, 0.0026; and $MnSO_4$, 0.0026. This suspension was then placed in a shaking water bath at 30°C. After 30 min, 10 μCi of $[^3H]$P-ine (Amersham Corp., Arlington Heights, Ill.) was added to the bacterial suspension, and the mixture was shaken at 30°C for up to 1 h. At various time points, 1-ml aliquots were removed and filtered through 0.2-μm filters, and the filters were washed six times with 5 ml of ice-cold distilled water. Afterwards, the filters were removed, placed in scintillation vials, allowed to air dry, and then counted in 10 ml of Aquasol scintillation fluid (New England Nuclear Corp., Boston, Mass.) with a Beckman model 8000 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). *Saccharomyces uvarum* ATCC 9080 was included in these experiments as a positive control for P-ine uptake, as previously reported by Shane and Snell (11); *S. uvarum* was grown in a medium containing 20 g of peptone, 20 g of glucose, and 10 g of yeast extract per liter of distilled water. *S. faecalis* was employed as a negative control for P-ine uptake since it reportedly cannot assimilate this compound (8).

The uptake of radioactively labeled P-ine by the five test strains and two control strains is shown in Fig. 3. There was no uptake of $[^3H]$P-ine by *S. faecalis* and relatively little uptake by any of the three B6DS isolates examined (Br, Fe, and Gu). The two non-B6DS strains (no. 27 and 591) assimilated approximately 0.30 pmol of $[^3H]$P-ine per 10^9 bacteria (three to four times that of the B6DS isolates). This uptake was, however, 13 times less than that observed with *S. uvarum*, the positive control for P-ine uptake.

Although P-ine was not taken up by these B6DS isolates, preliminary studies had indicated that the addition of P-ine might inhibit the

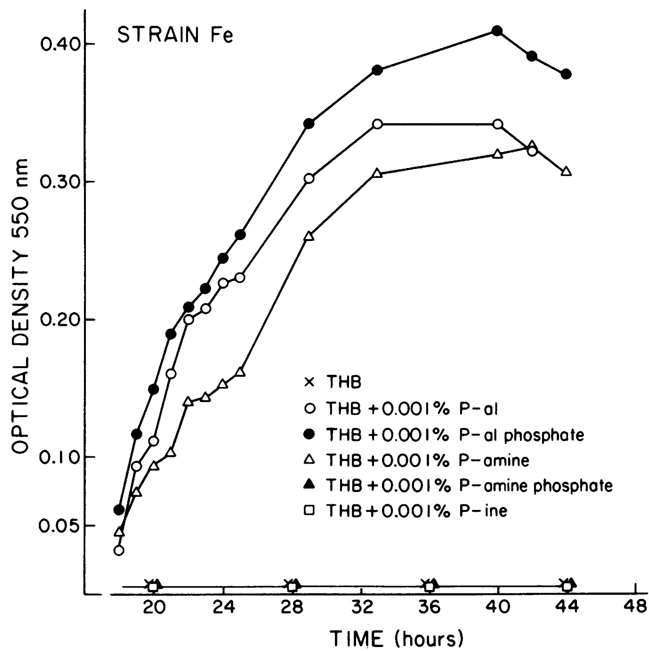

FIG. 2. Growth of strain Fe in THB with various vitamin B_6 analogs at 37°C with agitation. Values represent the mean of three determinations.

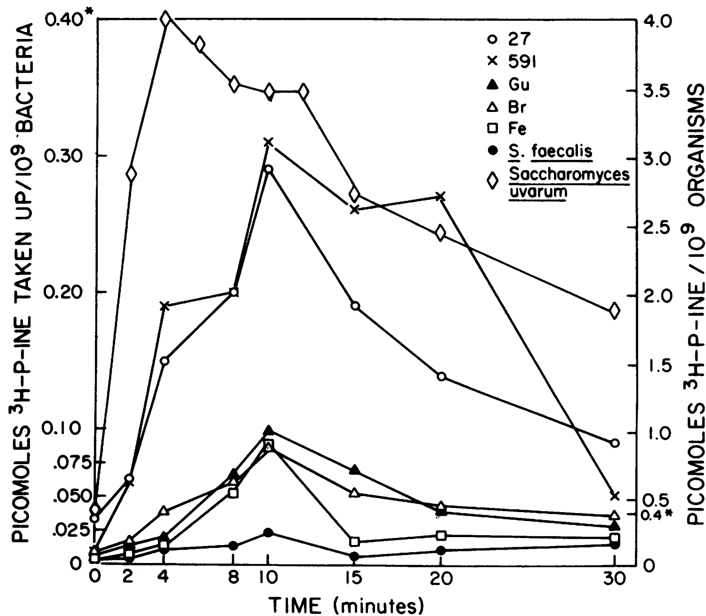

FIG. 3. The uptake of [^{3}H]P-ine hydrochloride by *S. uvarum*, *S. faecalis*, two non-B6DS strains (no. 27 and 591), and three B6DS isolates (Br, Fe, and Gu). After overnight growth, the strains were pelleted, washed, resuspended in a salt solution, and incubated for 30 min. Afterwards, labeled P-ine was added, and samples were removed at indicated times and filtered; the filters were washed extensively and air dried, and the radioactivity on the filters was determined. The results for all of the streptococcal strains are expressed as picomoles of P-ine taken up by 10^9 bacteria, and values for this are shown on the left-hand ordinate. Values for *S. uvarum* are expressed on the right-hand ordinate.

growth of these strains in THB with P-al. With a starting inoculum of approximately 2×10^4 to 5×10^4 bacteria per ml, strains Br, Fe, and Gu were incubated in THB or THB supplemented with various concentrations of P-al, P-amine, or P-al phosphate (ranging from 0.1 to 20 μg/ml) in the presence or absence of increasing concentrations of P-ine (ranging from 0.1 to 1,000 μg/ml). These mixtures were then incubated for 48 h at 37°C with agitation. Bacterial growth was measured by turbidity as well as by determining the number of colony-forming units per milliliter. The results indicated that a ratio of P-ine to P-al of 50:1 inhibited the growth of all three strains in media supplemented with 0.1 and 1.0 μg of P-al per ml. At concentrations of 5.0 μg or greater of P-al per ml, however, a ratio of P-ine to P-al of 200:1 did not inhibit growth, probably because enough P-al was able to enter the cell and support growth (0.1 μg of P-al per ml is sufficient to support the growth of these B6DS strains). Similar results were observed with P-amine and P-al phosphate. This inhibition might be due to competition for uptake of the various vitamin B_6 forms at the cell surface. The binding of P-ine to the transport mechanism, although P-ine is not itself transported, could inhibit the uptake of other vitamin B_6 forms. Mulligan and Snell have previously reported that although not transported itself, P-ine is a competitive inhibitor of P-al transport by *S. faecalis* (8).

P-ine did not, however, inhibit the growth of two non-B6DS strains in P-al-supplemented media. The uptake studies with these strains demonstrated that P-ine was taken up slightly by these strains, suggesting that one difference between these strains and the B6DS strains involves the interaction of P-ine with the transport system. Although taken up, P-ine did not enhance the growth rate compared with that observed in unsupplemented THB. Once inside a cell, P-ine can either be converted to P-al via P-ine oxidase or phosphorylated to P-ine phosphate. All three vitamin forms can be phosphorylated by the same enzyme, P-al kinase, although the affinity of this enzyme for the three substrates can vary significantly (10). P-ine phosphate would then be converted to P-al 5-phosphate by P-ine phosphate oxidase (10). Repeated attempts to detect either P-ine oxidase or P-ine phosphate oxidase in any of the streptococcal strains described in this paper have been unsuccessful (N. L. Schiller, unpublished data). The absence of these enzymes would suggest that even if transported intracellularly, P-ine would not be a suitable vitamin B_6 analog for these strains. This would be consistent with the observations of Koser (6), who reported that most streptococcal species seem to prefer P-al or P-amine to P-ine for growth, and it also would explain why P-ine did not enhance the growth rate of strains 27 and 591 over that observed in unsupplemented THB. Further examination of these enzymes in these streptococcal strains is in progress.

In summary, these studies support the previous reports (1, 5, 9) which stress that culture media must be supplemented with either P-al or P-amine but not P-ine for the isolation and in vitro cultivation of vitamin B_6-dependent streptococci. It should be noted that even in optimally supplemented media, the growth rate of these B6DS strains is much slower than that of comparable non-B6DS strains, and hence longer incubation times are needed to isolate these organisms. Although not assimilated by these B6DS isolates, P-ine does inhibit the growth of these strains in media supplemented with P-al or P-amine. This inhibition probably occurs by the binding of P-ine to the vitamin transport system, and although not itself transported, P-ine inhibits the entry of the usable vitamin B_6 forms.

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