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Identification of the vitamers of vitamin B_6 excreted by a yeast mutant growing in a glucose minimal culture medium

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Abstract

It has been reported that the only vitamers of vitamin B_6 excreted by a yeast mutant growing in a fairly complete culture medium were pyridoxine, pyridoxal and pyridoxamine. In this work, evidence is presented that when the same mutant grows in a glucose minimal culture medium it excretes in addition pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate. Differences in the activities of acid phosphatase(s) were found in crude extracts from yeast mutant cells growing in the two culture media. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Vitamins; Pyridoxine; Pyridoxal 5′-phosphase

1. Introduction

Vitamin B_6 was discovered more than 60 years ago, yet its biosynthetic pathway has not been fully clarified [1]. Evidence in the last few years shed light on the studies of the biosynthetic pathway of vitamin B_6 in a bacterium, *E. coli*, when the two immediate precursors of vitamin B_6 were identified [2,3]. However, not much is known about its biosynthetic pathway in yeast [4,5,6,7], which has been reported to be different than that found in *E. coli* [4]. Differences in the biosynthesis of another B vitamin, thiamin, between procaryotes and eucaryotes have also been found [8–10].

Studies on the biosynthesis of vitamin B_6 have been hampered by the lack of a system that produces workable quantities. The isolation of mutants of the yeast *S. marxianus* which are resistant to isonicotinic acid hydrazide (INH) and which when grown in a complete culture medium excrete fairly large quantities of vitamin B_6 has been reported [11,12]. The vitamin B_6 vitamers excreted in the culture medium were identified as pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM) [12]. None of the corresponding 5′-phosphate esters, pyridoxine 5′-phosphate (PNP), pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) were present in the culture media [12–14].

We wanted to study if certain labeled amino acids are incorporated into vitamin B_6. However, the culture media we have been using contain amino acids [12–15]. Therefore, the mutant yeast was grown in a minimal culture medium with glucose as the sole carbon source, and ammonium chloride as the sole nitrogen source. To our surprise, under these growth conditions the excreted vitamers of vitamin

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B_6 in the culture medium were different than those excreted in the complete culture medium.

The purpose of this report is to present evidence concerning the identity of the vitamers which are excreted by the yeast mutant when it grows in a glucose minimal culture medium.

2. Experimental

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin, phosphatase acid from potato type IV-S, nicotinic acid, d-biotin, PN, PL, PM, PLP and PMP were obtained from Sigma (St. Louis, MO, USA). The synthesis of PNP has been reported previously [16]. All other chemicals used were of reagent grade.

2.2. Culture media

Yeast mutant P-131 [12] was grown either in the complete culture medium [15] omitting the addition of thiamine and PN, but adding nicotinic acid (0.2 mg/l) or in a minimal culture medium consisting of (g/l): glucose, 30; KH_2PO_4, 3; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.125; $MnSO_4 \cdot H_2O$, 0.0025; $FeCl_3 \cdot 6H_2O$, 0.0025; $CaCl_2 \cdot 2H_2O$, 0.065; nicotinic acid, 0.001; and d-biotin, 20 μg/l. The volume was made to 1 liter with glass-distilled water and the pH of the solution was 4.2. The yeast mutant was grown on agar slants of medium A [11] supplemented with nicotinic acid (1 μg/ml). The yeast mutant requires nicotinic acid for growth while the parent does not [17]. Cells from a 24-h agar slant culture at 29°C, were suspended in sterile 0.9% sodium chloride solution, centrifuged and resuspended in a similar sterile solution. Conical flasks (300 ml) containing 50 ml of culture medium, covered with aluminum foil, were steamed for 20 min, cooled and inoculated aseptically, all with the same volume of inoculum. The inoculum for the parent culture was prepared the same way except that it was grown on agar slants of medium A (nicotinic acid was not added). The flasks were incubated at 29°C in a model G25 gyrotory shaker New Brunswick Scientific (New Brunswick, NJ, USA) at 70 rpm. Aliquots (1 ml) each were removed aseptically, every day, to measure growth and to determine high-performance liquid chromatography (HPLC) profile. Samples were injected into HPLC after removal of the cells by centrifugation in a Beckman Microfuge E (Fullerton, CA, USA). For production of larger quantities of cells, 500-ml cultures were grown in Fernbach flasks under the same conditions. Yeast cells were collected by centrifugation in a Marathon 10 K centrifuge Fisher Scientific (Pittsburgh, PA, USA) for 10 min at 2000 *g*.

2.3. High-performance liquid chromatography

The HPLC system used and conditions have been reported [14]. The HPLC column used for this work, was the same one reported before which, however, has been regenerated three times since first used according to suggested procedures [14]. Therefore, the peaks are wider than those in the original report and the retention times a little different (PL, PN) depending on how long the column was used without regeneration and on the temperature in the laboratory. Solution of standards were injected to confirm the retention times.

2.4. Crude cell extracts

Parent or mutant yeast cells, 0.5–0.8 g wet weight, were washed twice with 0.9% NaCl and placed in mini-beat beater vials to which were added 1 ml of 0.1 *M* potassium phosphate buffer pH 7.0, 0.05 m*M* in PMSF. The vials were filled with 0.5 mm diameter zirconium beads. The cells were disrupted by three, 1-min agitations in a mini-bead beater Biospec Products (Bartlesville, OK, USA) with cooling between agitations. The vials were centrifuged in a frozen head of Microfuge E for 2 min and the supernatant kept in a freezer at −30°C and used, within two weeks, as crude cell extract.

2.5. Hydrolysis of phosphate esters

To 2 ml of cell free culture media of the yeast mutant that had grown for six days in glucose minimal culture medium was added sodium acetate till the pH was changed from 2.6 to 4.7 and placed in

a 25-ml conical flask after removing 0.1 ml for the HPLC profile (0 time). Then a few particles of acid phosphatase were added, the flask stoppered and incubated at 37°C and 70 rpm. Aliquots of 0.1 ml were removed at various time intervals and immediately injected into HPLC.

2.6. Phosphatase activity in crude cell extracts

To 2.3 ml of 0.1 *M* sodium acetate buffer pH 4.5 in a 25-ml conical flask was added 50 nmoles of PLP and 0.2 ml of crude cell extract made from parent or mutant cells that had grown in the complete culture medium or mutant cells that had grown in the glucose minimal culture medium. All yeast cells had been collected at their corresponding logarithmic phase of growth. Immediately after mixing the contents of the small conical flask, 0.1 ml was removed and added to 0.3 ml of 1 *M* perchloric acid, vortex-mixed, centrifuged for 5 min in Microfuge E to remove the precipitated protein, the supernatant decanted into another tube and a sample injected into HPLC. The conical flask was stoppered and incubated at 37°C and 70 rpm. Aliquots of 0.1 ml were removed at various time intervals and treated the same way.

2.7. Growth of yeast mutant in presence of PL, PN or PM

To cultures of the yeast mutant at the logarithmic phase of growth (3rd day) was added aseptically PL, PN or PM solution to make their concentrations in the minimal culture medium 10 μ*M*. Immediately, an aliquot was removed and another one the next day to determine the HPLC profiles and compare them to that of the cell-free culture medium to which nothing was added.

The protein concentration in the cell extracts was determined by the Lowry method [18], using bovine serum albumin as standard.

All experiments were carried out under conditions of subdued light and were repeated at least twice, except for the growth experiments in the glucose minimal culture medium which were repeated more than ten times.

3. Results and discussion

When the yeast mutant was growing in a fairly complete culture medium [13], the pH of the medium after 3–5 days was around four due to the presence of the potassium citrate–citric acid buffer. The HPLC profile of a cell-free culture medium of the yeast mutant after it was grown for 5 days has been reported [13,14]. The only vitamers of vitamin B_6 present were PM, PL and PN. However, when the yeast mutant grew in the glucose minimal culture medium the pH of the medium was around three after three days and around 2.5 after six days. The mutant in this medium was growing, approximately, half as fast as in the complete medium.

In Fig. 1(A) is presented the chromatogram obtained from cell-free culture medium of the yeast mutant after it had been grown for six days in the glucose minimal culture medium. The retention time of the peaks suggested that the vitamers were PM, PL and PN. Two other fairly large peaks could be seen that had retention times close to PMP and PLP/PNP [14] which, initially, were not considered to be those vitamers since the phosphorylated forms of the vitamin were not excreted when the mutant was growing in fairly complete culture media [12–14]. However, when an aliquot similar to the one that is presented in Fig. 1(A) was injected into HPLC without post-column derivatization, the peak with retention time close to that of PLP/PNP was very much reduced indicating that it was probably PLP and not PNP (B). PNP was the vitamer excreted by a mutant of *E. coli* that lacked PNP/PMP oxidase activity [19]. The next step was to show that the two peaks suspected to be PMP and PLP were phosphate esters by hydrolyzing them with the aid of acid phosphatase. When the phosphatase test was applied to cell-free minimal culture medium, as reported in the experimental section, the hydrolysis of PLP was almost complete after 3 h, however only 30% of PMP was hydrolyzed during that period. In that culture medium the concentration of phosphate, which inhibits hydrolysis, was about 22 m*M*. Therefore, the yeast mutant was grown in minimal culture medium whose KH_2PO_4 concentration was reduced from 3 to 1g/l, i.e. the phosphate concentration was about 7.3 m*M*. When the phosphatase test was repeated under the same conditions, PLP was hydro-

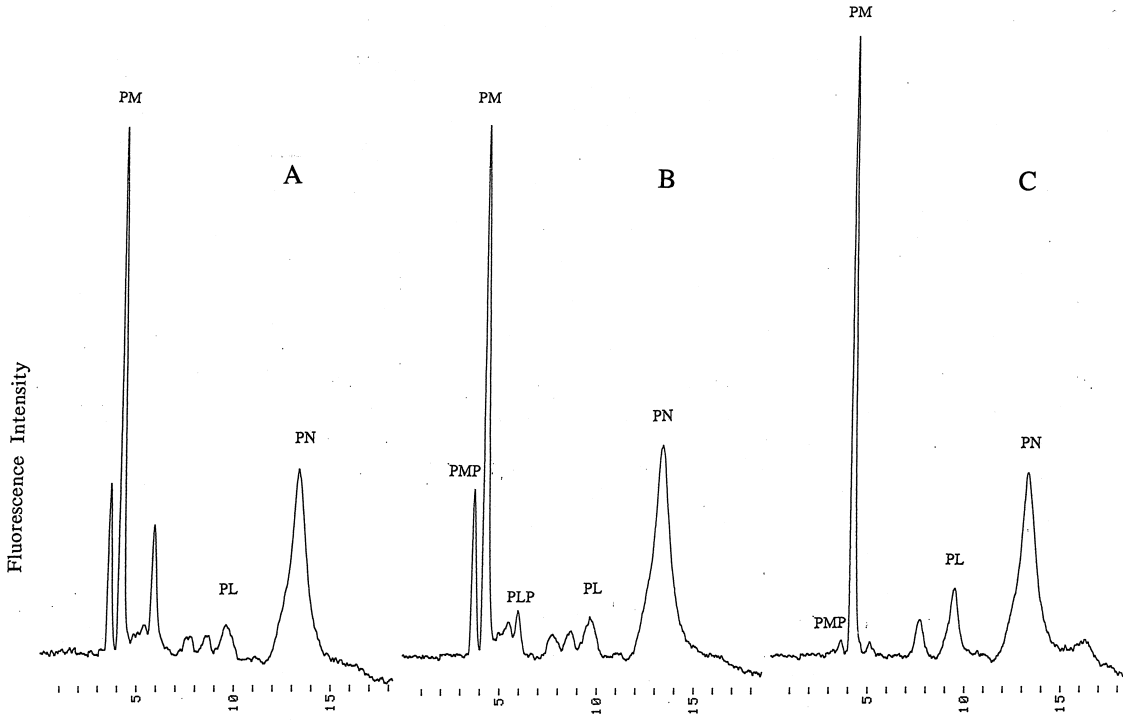

Fig. 1. HPLC tracing of cell-free glucose minimal culture medium of yeast mutant after it had grown for 6 days; with (A and C), without (B) post-column derivatization and after incubation with potato acid phosphatase (C). The time scale is given in min.

lyzed and PMP was almost completely hydrolyzed within 5 h (C) and the quantities of PM and PL were increased accordingly. Apparently, there were other phosphate esters present in the cell-free culture medium which were hydrolyzed under these conditions, since peaks were eliminated and new peaks appeared. Mutants of *E. coli* resistant to INH also excrete vitamers of vitamin B_6 in the culture medium i.e. PMP, PLP and PM [20], while this yeast mutant excretes, in addition to these three, also PL and PN.

A few postulations can be put forward to explain the reason(s) for the yeast mutant to excrete different vitamers of vitamin B_6 depending on the culture medium it was growing in. One reason, it was thought, could be the acid phosphatase activity of the yeast mutant cells depending on what culture media they were growing in. To test this hypothesis, PLP was added to crude cell extracts made from mutant yeast cells which had been grown in the two different media. In Fig. 2 are presented the chromatograms obtained, without post-column derivatization, when PLP was added to extracts from cells grown in complete culture medium at 0 time (A) and after 2.5-h incubation (B). It can be seen that in addition to hydrolysis of PLP, there was also transamination of PLP to PMP. In Fig. 3 are depicted the corresponding chromatograms obtained from mutant yeast cells that had grown in minimal culture medium at 0 time (A) and after 2.5 h incubation (B). It is obvious that much less PLP was hydrolyzed and there was also much less transamination relative to the chromatograms in Fig. 2. To have some kind of comparison, this assay was carried out with extracts from parent yeast cells that had grown in the fairly complete culture medium. The corresponding chromatograms are presented in Fig. 4 at 0 time (A) and after 2.5 h incubation (B). It can be seen that the reduction of the PLP peak is larger than that of Fig. 3 (B). A rough estimation of the specific activity of acid phosphatases in the crude cell extracts, calculated as loss of PLP, was (nmoles/mg protein/h): 1.7, 2.7 and 6.1 respectively for mutant yeast grown in glucose minimal medium, parent, and mutant yeast grown in the fairly complete medium. The

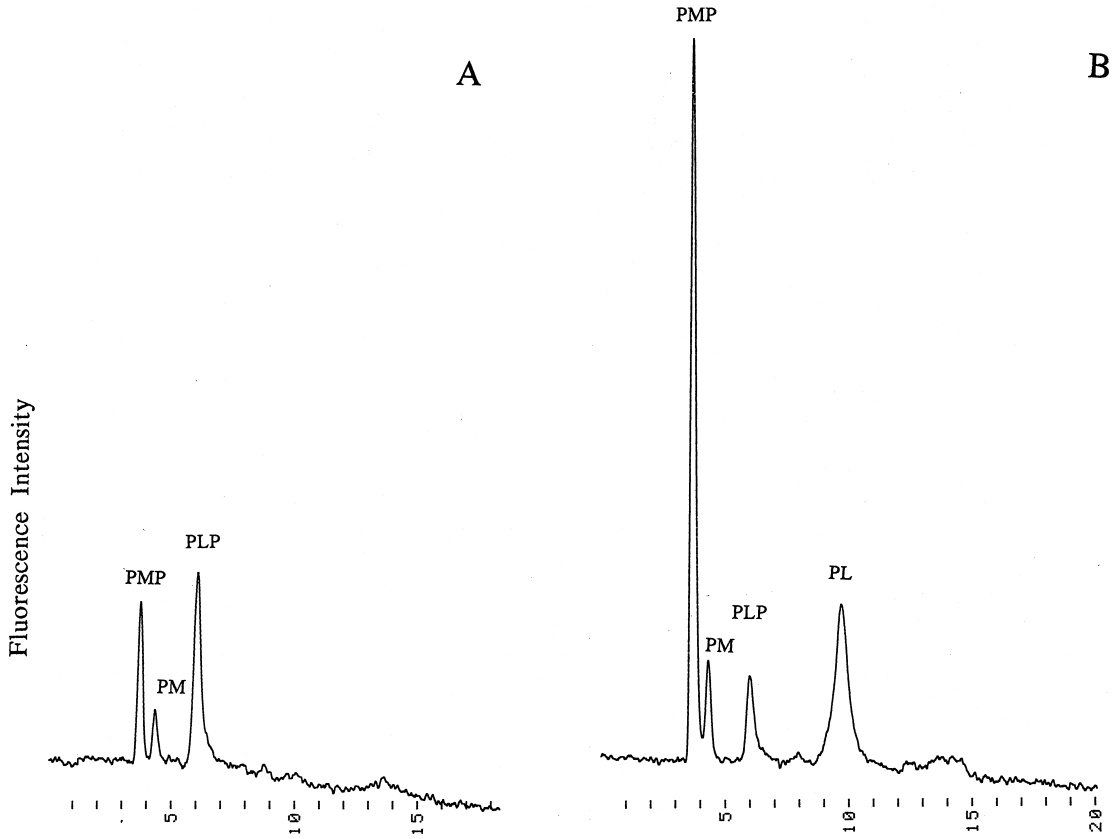

Fig. 2. HPLC tracing, without post-column derivatization, of PLP added to crude cell extracts obtained from yeast mutant which had grown in the fairly complete culture medium at 0 time (A) and after 2.5 hs incubation (B).

specific activities were calculated as if PLP were only hydrolyzed to PL. However, as can be seen from the chromatograms, some PLP was transaminated to PMP. The fact that the activity of acid phosphatases in crude extracts from mutant yeast cells grown in glucose minimal culture medium was more than three times smaller than that of cells grown in the fairly complete culture medium could explain the excretion of PLP and PMP in that culture medium. Of course, other explanations cannot be excluded e.g. defective cell membrane due to lack of a constituent which might be present (or biosynthesized) in the complete medium, the very low pH of the glucose minimal culture medium etc.

Since the three phosphate esters of vitamin B_6 are not effective growth factors for yeast [21–23] or several bacteria [23] it has been assumed that in general they do not cross cell membranes. However, excretion of phosphate esters of vitamin B_6 by resting yeast cells [24] and growing bacteria [20,25,26] has been reported. Therefore, the excretion of phosphate esters of vitamin B_6 by growing mutant yeast cells should not be unexpected.

The following criteria were taken as indicators that the presence of PLP and PMP in the cell-free culture media was due to excretion from the cells and not to lysing of the cells and releasing them into the medium. First, the quantity of PMP present in the cell-free culture media is approximately one third of that of PLP (see [14] for detector fluorescence response), while in the cells the quantity of PMP is approximately four times as that of PLP (Fig. 5). In this figure is presented the HPLC profile of the perchloric acid extracts of yeast mutant cells without post-column derivatization (A) and with post-column derivatization (B). A rough estimate of the vitamin

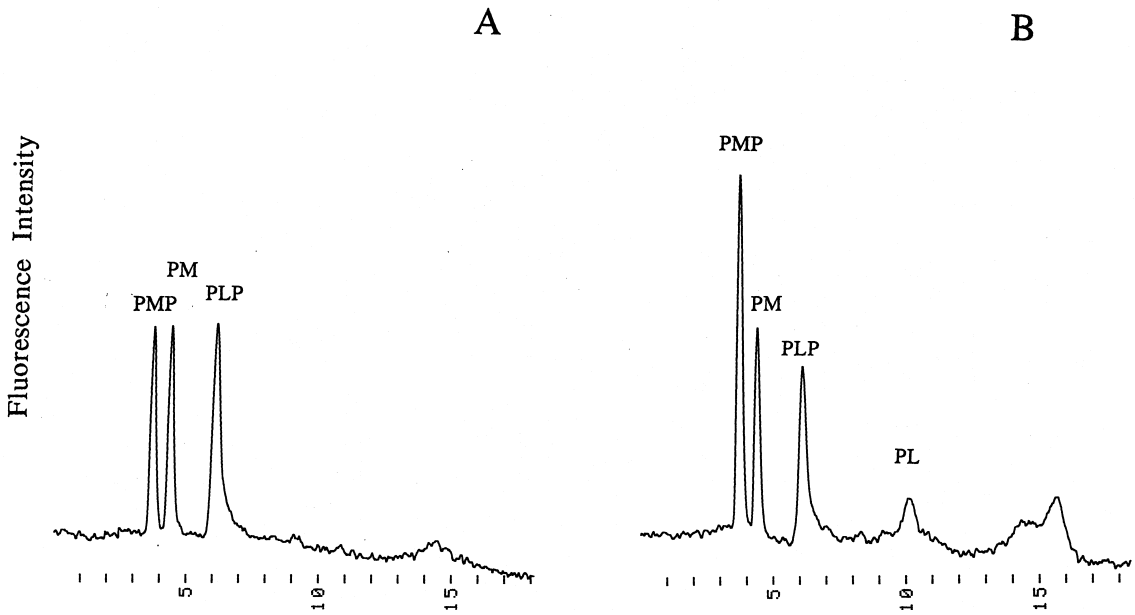

Fig. 3. HPLC tracing, without post-column derivatization, of PLP added to crude cell extracts obtained from yeast mutant which had grown in minimal culture medium, at 0 time (A) and after 2.5 hs incubation (B).

B_6 content was 446 nmoles/g dried cells (PMP 30%, PM 22%, PLP 7%, PN 41%) while for the parent yeast was 23 nmoles/g dried cells (PMP 91%, PM 9%). We could not measure any PLP in the cells of commercial baker's yeast [14] or in the cells of the parent yeast. Second, the PLP and PMP contents of

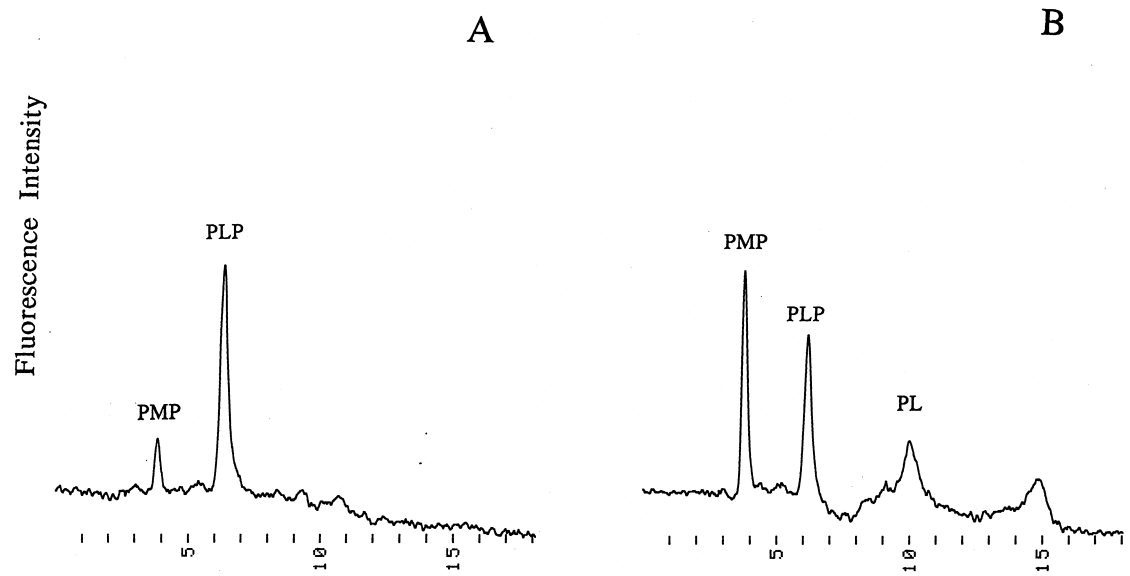

Fig. 4. HPLC tracing, without post-column derivatization, of PLP added to crude cell extracts obtained from parent yeast which had grown in the fairly complete culture medium, at 0 time (A) and after 2.5 hs incubation (B).

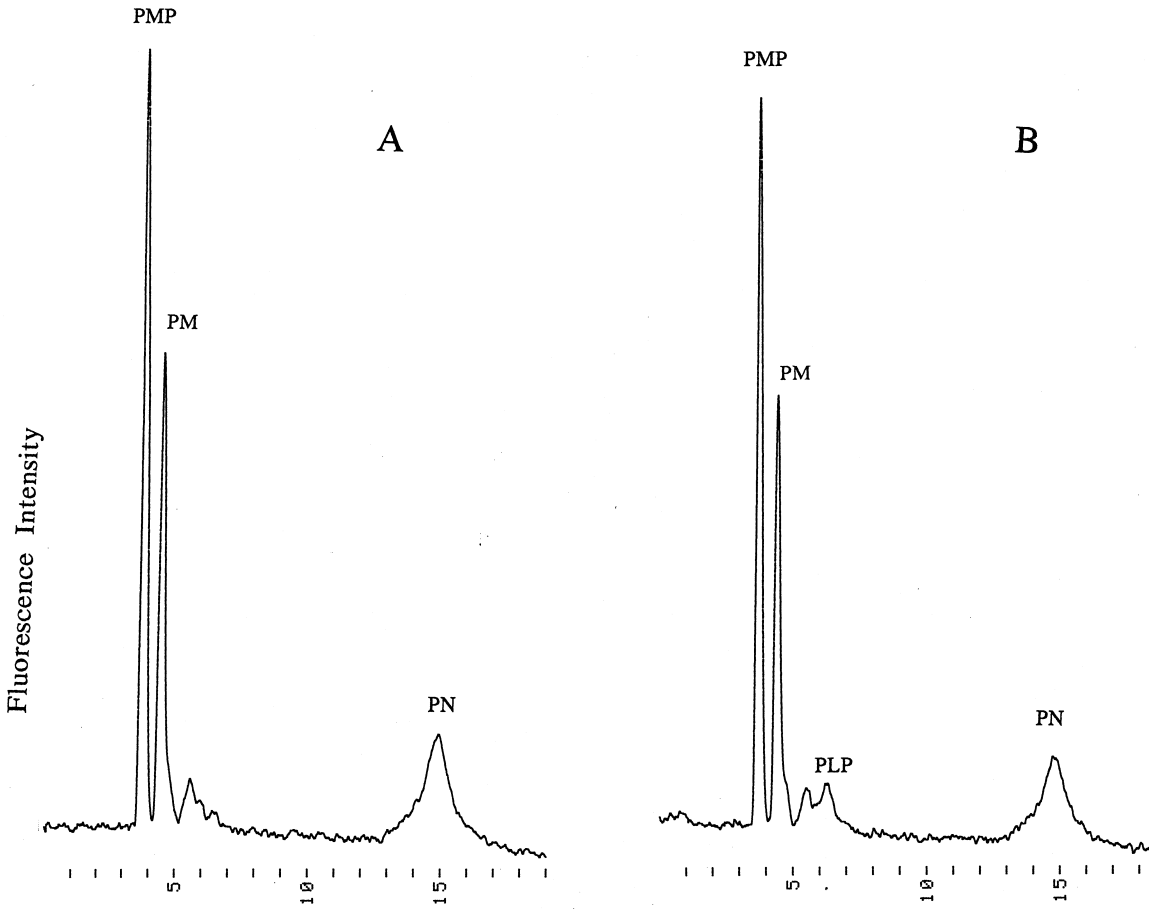

Fig. 5. HPLC tracing of perchloric acid extracts obtained from yeast mutant cells that had grown in the fairly complete culture medium, without (A) and with (B) post-column derivatization.

cell-free culture media were both increasing during the logarithmic phase of growth and the PLP content started decreasing at the end of the stationary phase due to its oxidation to 4-pyridoxic acid 5′-phosphate [14] while that of PMP remained constant. Third, no turbidity appeared when crystals of trichloroacetic acid or drops of 70% perchloric acid were added to cell-free culture media (from the stationary phase) that had been concentrated to one tenth its volume, by lyophilization, which indicated absence of protein.

In order to see the effect of exogenous vitamin B_6 on its de novo synthesis by mutant yeast cells growing in glucose minimal medium during the logarithmic phase, PM, PL or PN was added to the medium to make their concentration 10 μM. The HPLC profile of their cell-free cultures was taken immediately after addition of the vitamers and also the next day to see if the quantities of the excreted PLP or PMP were different than those of the culture which had nothing added (control). No changes in the excreted quantities of PLP or PMP were observed in the HPLC profiles. The only change that could be seen was with the culture that had PL added. During the 24-h period, PL was taken up by the cells, reduced it to PN and excreted it into the culture medium almost quantitatively Fig. 6, A–C. It has been reported [27, 28] that a NADP-specific PN dehydrogenase exists in baker's and brewer's yeasts. However, the equilibrium position of the reaction PL↔PN greatly favors PN formation. The quantities of added PM or PN in the corresponding cultures

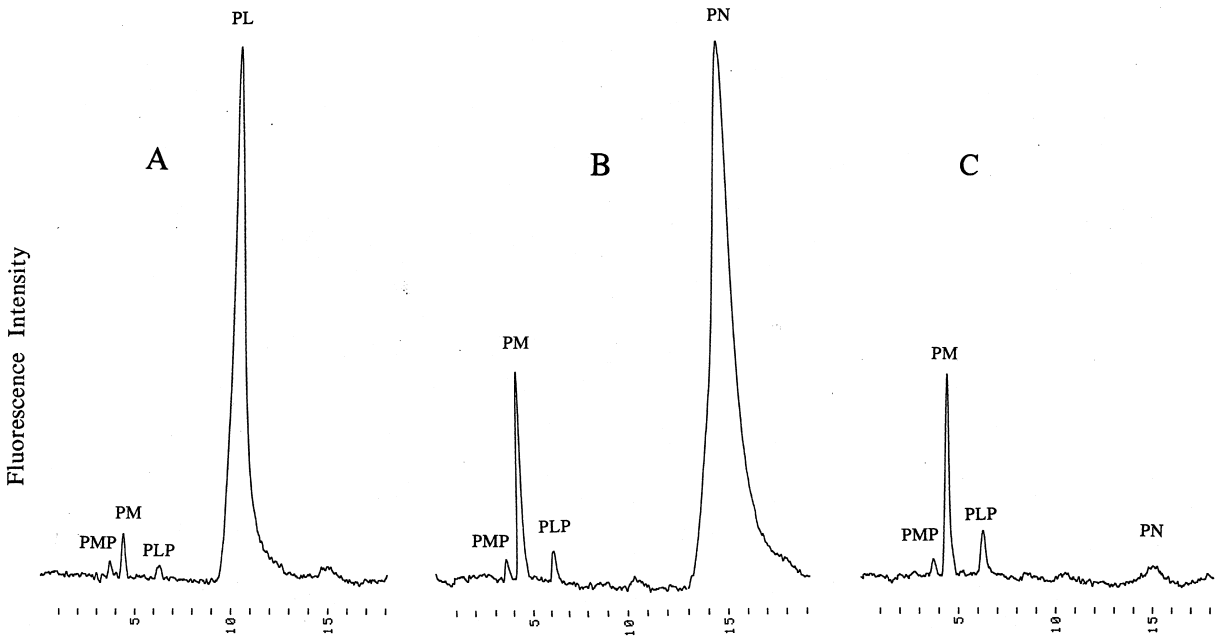

Fig. 6. HPLC tracing, with post-column derivatization, of cell-free minimal culture media of the yeast mutant growing during the logarithmic phase, immediately after PL was added (A), next day (B) and control (C).

were the same on the 4th day as on the 3rd day, i.e. PM or PN were not further metabolized by the mutant yeast cells. It is known, that PN and PL are taken up by resting yeast cells in the pH range of 2.5–5 while PM is more effectively taken up at a higher pH 5.5-6.5 [24]. Since the quantity of PLP excreted by the mutant yeast is not influenced by exogenous vitamin B_6, it seems that, for this mutant, the salvage pathway (PN→PNP→PLP or PL→PLP) does not affect the de novo synthesis of PLP.

Since this yeast mutant overproduces vitamin B_6, it could mean that it has lost its ability to regulate the level of PLP in the cell and therefore excretes the excess amount in the culture medium (glucose minimal medium, not very active phosphatases) or hydrolyzes it and then excretes it (fairly complete culture medium, very active phosphatases). Yeast, in general, does not contain PN [14,29], however, this yeast mutant excretes in the culture media and its cells contain PN in perchloric acid extracts obtained as has been reported [14], from the same yeast mutant cells that had been grown in the fairly complete culture medium and used to measure the activity of acid phosphatases. Reasonable explanations for this discrepancy are the following. If PNP is the first vitamer of vitamin B_6 biosynthesized, as is the case for *E. coli* [2,3], then the presence of PN in the cells as well as in the culture media of the yeast mutant might be due to the increased activity of phosphatases. Another explanation could be that the PLP which is formed by oxidation of PNP or PMP or by being the first vitamer biosynthesized in the yeast mutant, is hydrolyzed to PL which in turn is reduced to PN as shown previously.

Future work will, hopefully, give an answer to which vitamer of vitamin B_6 is biosynthesized first in yeast.

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