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Dependence of Betaine Stimulation of Vitamin B_{12} Overproduction on Protein Synthesis

YOU-HUA FA,† J. PETER KUSEL,‡ AND ARNOLD L. DEMAIN*

Fermentation Microbiology Laboratory, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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The betaine-stimulated differential synthesis of vitamin B_{12}, i.e., the increase in B_{12} per increase in dry cell weight, by *Pseudomonas denitrificans* was inhibited by rifampin and chloramphenicol but not by benzylpenicillin and carbenicillin at concentrations of antibiotic that inhibit growth. The level of the first enzyme of corrin (and porphyrin) biosynthesis, δ-aminolevulinic acid synthetase, was decreased to a much greater degree by rifampin and chloramphenicol than by the penicillins. These data support the concept that betaine stimulation of B_{12} synthesis is a result of its stimulation of synthesis of δ-aminolevulinic acid synthetase, a labile and presumably rate-limiting enzyme of corrin formation requiring continuous induction. In further support of this hypothesis, it was found that chloramphenicol immediately interfered with both vitamin B_{12} and δ-aminolevulinic acid synthetase formation, no matter when it was added to the system.

Earlier work by Demain and co-workers (4, 5, 7) showed that betaine exerts a major positive effect on vitamin B_{12} and porphyrin overproduction by the industrial B_{12}-producing species *Pseudomonas denitrificans*. In a recent study (J. P. Kusel, Y.-H. Fa, and A. L. Demain, J. Gen. Microbiol., in press), it was found that the presence of betaine increases the specific intracellular content of the first enzyme of corrin and porphyrin biosynthesis, i.e., δ-aminolevulinic acid synthetase (ALAS). In the presence of betaine, the specific activity of the enzyme increased, but upon betaine exhaustion, the specific enzyme activity rapidly decreased. In the absence of betaine, both B_{12} and ALAS decreased from time zero. The simplest interpretation of the data is that betaine stimulates the synthesis of ALAS, the rate-limiting but labile enzyme of both corrin and porphyrin synthesis. If this is correct, the betaine effect should be dependent upon RNA and protein synthesis. This point is established in the present paper.

MATERIALS AND METHODS

Cell cultivation. *P. denitrificans* was grown in a chemically defined medium containing 20 g of sucrose per liter, 4 g each of diammonium phosphate, sodium glutamate, and sodium citrate per liter, plus trace metals (6). Culture volumes of 500 ml were used in 2.8-liter Fernbach flasks at 28°C with continuous agitation (250 rpm, 5-cm orbit) until an optical density equivalent to 5 g of dry cell weight (DCW) per liter was achieved. Cells were centrifuged, washed, and resuspended at 300 to 400 mg of DCW in 100 ml of a replacement medium in 500-ml Erlenmeyer flasks containing 10 g of sucrose per liter, 2 g of diammonium phosphate per liter, and one-fourth the concentration of metals used in the growth medium. Betaine was added at 6 g/liter at this time (t = 0) along with 25 mg of 5,6-dimethylbenzimidazole per liter as a vitamin B_{12} precursor. The flasks were incubated as above; samples were withdrawn for analyses at time zero and at appropriate intervals thereafter.

Vitamin B_{12} assay. Vitamin B_{12} was quantified by an agar-diffusion assay with *Lactobacillus lactis* (Dorner) ATCC 10697 essentially according to Cuthbertson et al. (3), except that samples were applied to penicillin assay disks and placed onto the dry agar surface instead of adding the samples to precut wells. Authentic vitamin B_{12} standards were applied on separate disks in each plating of samples to serve as internal controls. After incubation overnight at 37°C in humidified incubators, the diameters of the growth zones surrounding the disks were determined with a Fisher-Lilly reader. Plots of the logarithm of concentration versus zone diameter were linear, with standards ranging from 0.1 to 3.2 μg/ml and 25 μl of standard applied to each disk.

P. denitrificans cultures were prepared for bioassay of vitamin B_{12} by boiling samples containing 2% $NaNO_2$ and 0.1% KCN at pH 3.5 in a water bath for 3 min, thus converting the unstable B_{12} coenzyme to the more stable

TABLE 1. Effect of chloramphenicol, rifampin, and benzylpenicillin on the differential formation of vitamin B_{12}[a]

Antibiotic added	Concn (mg/liter)	Increase in vitamin B_{12} (mg/liter)	Increase in DCW (g/liter)	Differential increase in vitamin B_{12} (mg/g of DCW)
None		2.32	6.11	0.38
Chloramphenicol	1	2.32	5.04	0.46
	10	0.09	3.34	0.03
Rifampin	1	0.06	2.19	0.03
	10	0.04	2.03	0.02
Benzylpenicillin	1	2.24	5.87	0.38
	10	2.16	5.59	0.39
	100	2.05	5.24	0.39

[a] Antibiotics were added at time zero when the cell concentration was 3.7 g/liter and B_{12} was 0.21 mg/liter. Also added were 6 g of betaine per liter and 25 mg of 5,6-dimethylbenzimidazole per liter. Incubation was for 30 h.

* Corresponding author.
† Present address: Institute of Microbiology, Academia Sinica, Beijing, The People's Republic of China.
‡ Present address: Philip Morris USA, Biomaterials Research Division, Research and Development, Richmond, VA 23261.

TABLE 2. Effect of carbenicillin on the differential formation of vitamin B_{12}[a]

Antibiotic added	Concn (mg/liter)	Increase in vitamin B_{12} (mg/liter)	Increase in DCW (g/liter)	Differential increase in vitamin B_{12} (mg/g of DCW)
None		1.68	2.69	0.62
Chloramphenicol	10	0.06	0.79	0.08
Rifampin	1	0.12	1.47	0.08
Carbenicillin	100	0.39	0.52	0.75
Benzylpenicillin	2,000	0.99	1.60	0.62

[a] Antibiotics were added at time zero when the cell concentration was 3.5 g/liter and B_{12} was 0.36 mg/liter. Also added were 6 g of betaine per liter and 25 mg of 5,6-dimethylbenzimidazole per liter. Incubation was for 14 h, but samples were taken every 2 h, and the results shown are those observed at the time vitamin synthesis stopped and before lysis occurred. These times were 6 h for chloramphenicol, 8 h for rifampin, 10 h for carbenicillin, 12 h for benzylpenicillin, and 14 h for the control.

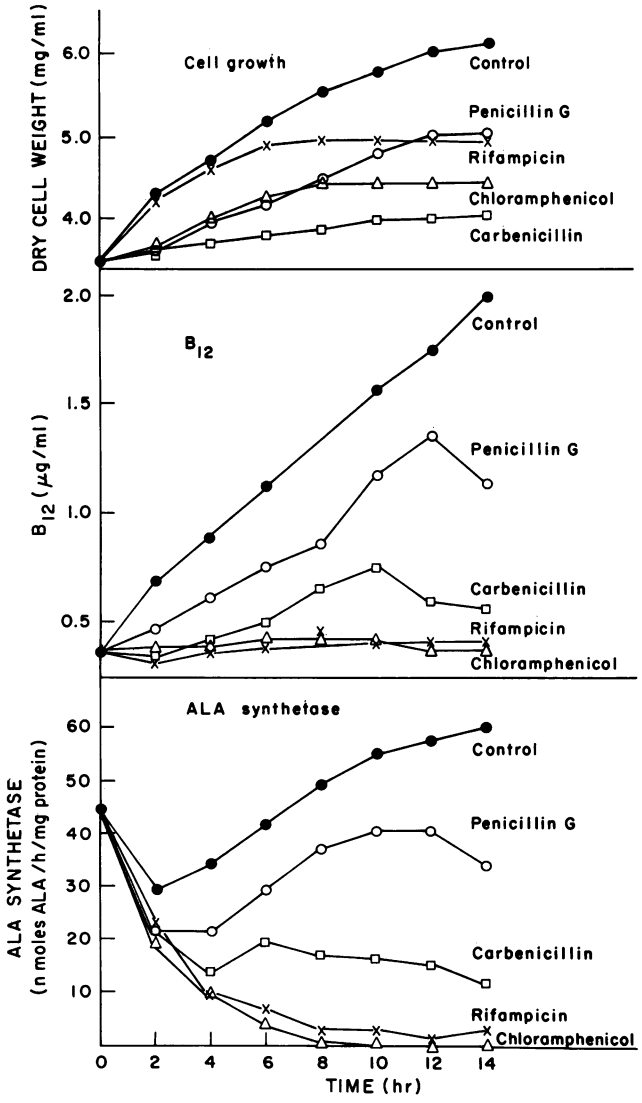

FIG. 1. Effect of antibiotics on growth and formation of vitamin B_{12} and ALAS.

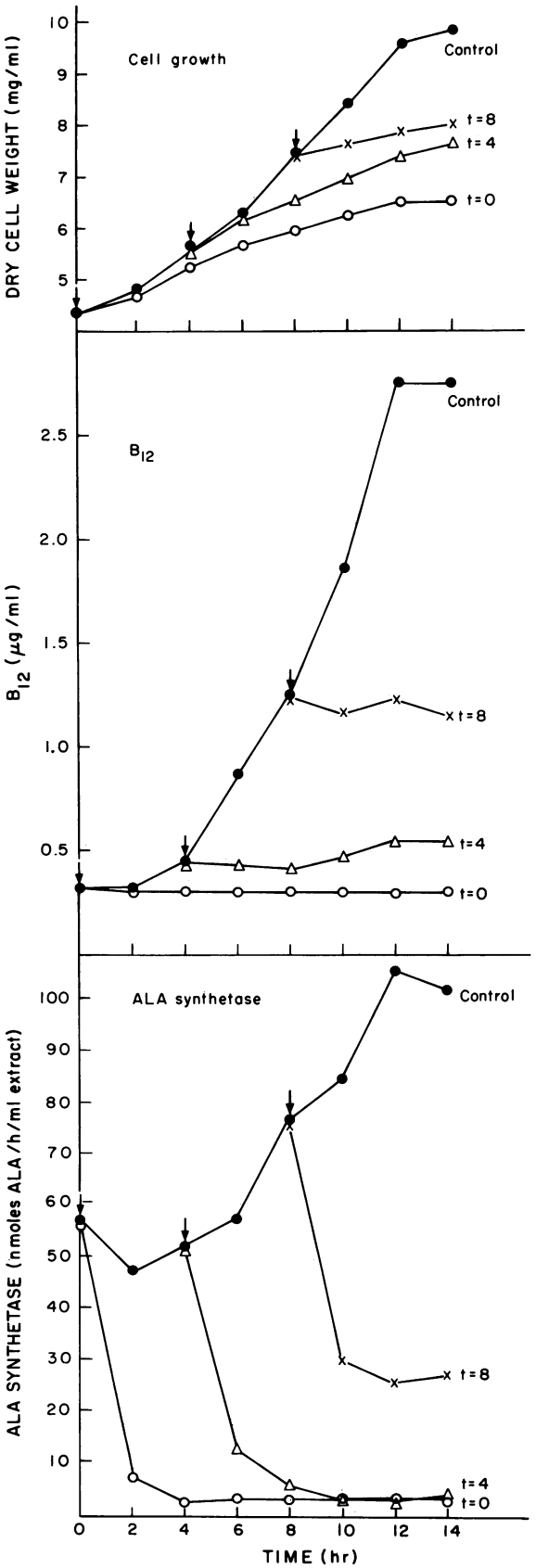

FIG. 2. Effect of time of addition of chloramphenicol on growth and formation of vitamin B_{12} and ALAS.

cyanocobalamin. Samples were frozen until assays could be performed, which was usually within a few days.

Other analyses. ALAS was determined according to Burnham (2). Enzyme assays were done on crude extracts prepared by washing cells twice in a volume of 20 mM Tris-hydrochloride (pH 7.5) buffer equivalent to the broth volume, resuspending them in an equal volume of the same buffer, sonicating with a Branson Sonifier until a 90% reduction in absorbance was attained, and removing debris by centrifugation. Protein was determined by the Bradford (1) procedure with bovine serum albumin as the standard. Reagents for enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Effect of antibiotics on vitamin B_{12} synthesis. To determine whether inhibition of RNA synthesis, protein synthesis, or cell wall synthesis has any effect on betaine-stimulated vitamin B_{12} biosynthesis, rifampin, chloramphenicol, and benzylpenicillin were added individually to cultures of *P. denitrificans* containing the sucrose-inorganic salts replacement culture medium plus 6 g of betaine per liter and 25 mg of the precursor, 5,6-dimethylbenzimidazole, per liter. The heavy cell suspension (3.7 g of DCW per liter) was incubated on the shaker for 30 h, and the changes in vitamin B_{12} and DCW were determined. Since each of the antibiotics inhibited growth and thus the volumetric increase in B_{12} to a greater or lesser extent, we determined the differential production of the vitamin, i.e., micrograms of B_{12} formed per milligram of increase in DCW. Chloramphenicol (inhibitor of protein synthesis) showed a marked inhibition of B_{12} production at 10 mg/liter, whereas rifampin (inhibitor of RNA synthesis) acted similarly at a level as low as 1 mg/liter (Table 1). Benzylpenicillin (inhibitor of cell wall synthesis), on the other hand, showed no effect on differential synthesis of vitamin B_{12} even at 100 mg/liter. Although *P. denitrificans* is very resistant to benzylpenicillin, there was a small degree of growth inhibition (about 15%) at 100 mg/liter, indicating that the penicillin was indeed able to reach its target.

Although the above experiment strongly suggested that mRNA or protein synthesis (or both) was needed for betaine-stimulated vitamin B_{12} production, we felt it necessary to compare chloramphenicol and rifampin with a more potent penicillin, i.e., carbenicillin. We also included a higher level of benzylpenicillin, i.e., 2,000 mg/liter. In this experiment, samples were analyzed every 2 h. The data in Table 2 are given for the point at which B_{12} production ceased and before any cell lysis occurred, out of concern that the data of the first experiment might have been influenced by destruction of B_{12} or by cell lysis or both. Carbenicillin did not inhibit the differential production of B_{12}, even at a concentration that inhibited growth by 80% (Table 2). The higher level of benzylpenicillin inhibited growth by about 40%, but again no differential effect on vitamin synthesis was seen. On the other hand, chloramphenicol at 10 mg/liter (55% growth inhibition) and rifampin at 1 mg/liter (30% growth inhibition) markedly inhibited vitamin B_{12} production.

Effect of antibiotics on ALAS. The specific activity of ALAS was also determined in the above experiment. In the control, the enzyme content dropped for the first 2 h and then increased for the next 12 h (Fig. 1). The early fall in ALAS activity has been reported before (Kusel et al., J. Gen. Microbiol., in press), and appears to be due to the in vivo lability of ALAS coupled with a lag in the betaine stimulatory effect. In the presence of benzylpenicillin, enzyme production started to increase after 4 h and ceased at 10 h. With carbenicillin, there was no net increase, but instead the enzyme maintained itself slightly below the 2-h level. However, when protein or RNA synthesis was inhibited by chloramphenicol or rifampin, respectively, there was a complete disappearance of ALAS beginning at time zero and ending at 8 h. B_{12} production in every case reflected the fate of ALAS. The data in Fig. 1 strongly suggest that this enzyme is very labile in vivo, thus requiring continuous induction, i.e., continuous mRNA and protein synthesis.

Effect of time of addition of chloramphenicol. If we are correct in concluding that ALAS synthesis, and hence B_{12} formation, requires continued induction, chloramphenicol should have a clear negative effect on both processes no matter when it is added. We thus followed growth, B_{12} formation, and ALAS titer in the absence and presence of 10 mg of chloramphenicol per liter added at 0, 4, and 8 h. The results (Fig. 2) are clear. At the moment chloramphenicol is added, ALAS is rapidly lost and B_{12} synthesis stops.

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