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Porphyrin Overproduction by *Pseudomonas denitrificans*: Essentiality of Betaine and Stimulation by Ethionine

ARNOLD L. DEMAIN[1] AND RAYMOND F. WHITE

Fermentation Research Department, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065

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Ethionine supplementation of a defined medium for growth of *Pseudomonas denitrificans* inhibited vitamin B_{12} overproduction and led to the elaboration of a red pigment. The pigment was shown to be coproporphyrin III. Inhibition by ethionine of cobalamin synthesis is probably due to interference of methylation of the corrin nucleus by methionine. Accumulation of coproporphyrin III is thought to result from interference by ethionine with the activity of methionine in the coproporphyrinogenase reaction; this would inhibit formation of heme, the feedback inhibitor and corepressor of δ-aminolevulinate synthetase, thus allowing unregulated synthesis of coproporphyrinogen III and its degradation product, coproporphyrin III. Betaine, known to be required for vitamin B12 overproduction, was found to be an essential requirement for porphyrin overproduction in the presence of ethionine. Low-level production of porphyrin, which occurs in the absence of ethionine, also required betaine supplementation. Betaine is thus required for overproduction of both corrins and porphyrins in *P. denitrificans*.

We previously reported that betaine or choline is required for overproduction of vitamin B_{12} by *Pseudomonas denitrificans* (2). The requirement is very specific since methionine and other methyl donors, as well as glycine and other catabolic products of betaine, are totally inactive. The activity of betaine is not due to methyl group donation to the corrin ring of vitamin B_{12}; methionine is the precursor of these "extra" methyl groups (R. F. White and A. L. Demain, Biochim. Biophys. Acta, *in press*). It is possible that betaine is acting in a regulatory role as a positive effector of vitamin B_{12} production.

After finding that methionine was the source of the "extra" methyl groups of the corrin ring, we studied the effect of ethionine. As expected, we noted a decrease in synthesis of vitamin B_{12}. Surprisingly, however, the ethionine-supplemented broth had become deep red in color. In the present paper, we describe experiments which show the pigment to be coproporphyrin III. Furthermore, we find betaine to be an absolute requirement for porphyrin overproduction in the presence or in the absence of ethionine. The relationship between corrin and porphyrin biosynthesis will be discussed later (*see* Fig. 5).

[1] Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

MATERIALS AND METHODS

Cultivation. *P. denitrificans* was cultivated on a rotary shaker at 28 C for 5 days in 250-ml Erlenmeyer flasks containing 40 ml of medium previously described (2). L-Ethionine (0.6 mM) was added before sterilization by autoclaving for 15 min at 121 C.

Turbidities of cultures were determined at 1:100 dilution in water with a Beckman DB spectrophotometer at a wavelength of 660 nm.

Vitamin B_{12} assay. Vitamin B_{12} was quantitated by an agar-diffusion assay with *Lactobacillus lactis* Dorner ATCC 10697, with vitamin B_{12} used as standard. Broth samples were prepared for assay by boiling for 3 min with 2.25% $NaNO_2$-0.1% KCN at *p*H 3.0 to 4.0 (H_2SO_4). Although *L. lactis* Dorner also responds to other cobamides and deoxynucleotides, these biological assays agree well with isotope-dilution assays indicating vitamin B_{12} to be the major assayable compound formed in the conditions of these experiments.

Porphyrin quantitation. Total porphyrin in the fermentation broth was estimated by removal of the bacterial cells by centrifugation, dilution of the broth (1:50) with 1 N HCl, and determining the absorbancy at the porphyrin peak at 402 to 406 nm. The extinction coefficient for coproporphyrin (ϵ mM = 489) was used, although other porphyrins or compounds absorbing in this region were undoubtedly present. The 1:50 dilution seems to negate their effect, because broths quantitated

for coproporphyrin after extraction into ethylacetate-glacial acetic acid (3:1, v/v) and back extraction into 1 N HCl were in good agreement with estimates for total porphyrin.

RESULTS

Effect of ethionine. After methionine was found to be the source of the "extra" methyl groups for vitamin B_{12} produced by *P. denitrificans*, we expected to see an inhibition of vitamin synthesis when the methionine analogue, L-ethionine, was added to the fermentation medium. The following was observed: 100 μg of analogue per ml reduced vitamin B_{12} production from 20 down to 13 μg/ml with only a slight depressing effect on growth. Despite this decrease in vitamin B_{12} production, the ethionine-supplemented broths were much redder in color than those of control flasks.

Identification of pigment as coproporphyrin III. Preliminary spectroscopic studies of the ethionine-supplemented fermentation broth revealed a strong absorbance in the Soret region, indicative of porphyrins. In view of this, centrifuged broth was extracted with ethylacetate-glacial acetic acid (3:1). The extract was washed with water and then with 2% sodium acetate and again with water. After extraction into 15% HCl, the *p*H was adjusted to 3.6. The pigment was extracted into ether, washed twice with water, and finally extracted into 0.36% HCl. These extraction characteristics strongly suggested that we were dealing with coproporphyrin III. The absorption spectrum of the isolated porphyrin in ethylacetate-glacial acetic acid is shown in Fig. 1. The four absorption peaks between 500 and 700 nm are typical of coproporphyrin III. After extraction into HCl, the spectrum of Fig. 2 was obtained; the maxima at 548 and 590 nm are also observed with coproporphyrin III.

The isolated material was converted to its methyl ester (3), crystallized from chloroform-methanol, and dissolved in chloroform. The spectrum obtained (Fig. 3) was virtually identical to that of authentic coproporphyrin III tetramethyl ester. Finally, cochromatography of methylated *Pseudomonas* porphyrin and coproporphyrin III tetramethyl ester on paper and on thin-layer plates yielded a single spot in each case with kerosene-chloroform (1:1, v/v) and 2,6-lutidine-water (70:21, v/v) as solvents. The coproporphyrin isomers and uroporphyrin III exhibit different mobilities in this system (3).

Effect of ethionine concentration. The effect of the concentration of L-ethionine is shown in Table 1. Although porphyrin was excreted in the absence of ethionine, its concentration was five times as great in the presence of 100 μg of

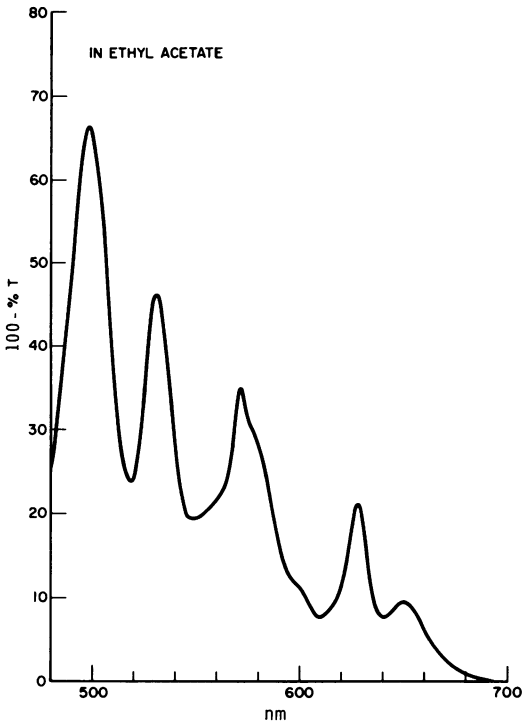

FIG. 1. *Absorption spectrum in ethylacetate-glacial acid (3:1) of porphyrin produced by Pseudomonas denitrificans.*

ethionine per ml (0.6 mM). At this concentration of ethionine, growth rates, cell yields, and broth *p*H values were unaffected (not shown), but vitamin B_{12} production was drastically inhibited. At higher ethionine concentrations, porphyrin and vitamin synthesis were both inhibited. Even at 500 μg of ethionine per ml, the yield of cells was decreased by only about 10%.

Effect of fermentation time. Figure 4 shows the dynamics of visible growth, coproporphyrin synthesis, and vitamin B_{12} production in the presence of ethionine (100 μg/ml). All three processes begin at about 30 hr. Peak cell yield was reached at 70 hr. Production of porphyrin continued until about 85 hr, whereas vitamin biosynthesis continued throughout the experiment.

Effect of betaine on porphyrin overproduction. In all the experiments described up to this point, betaine was included in the medium at a concentration of 1%. Although we knew it to be necessary for vitamin B_{12} production, we did not know whether it affected porphyrin synthesis. Table 2 shows that betaine is obligatory for porphyrin overproduction either in the absence or in the presence of ethionine.

DISCUSSION

Our finding that L-ethionine could stimulate

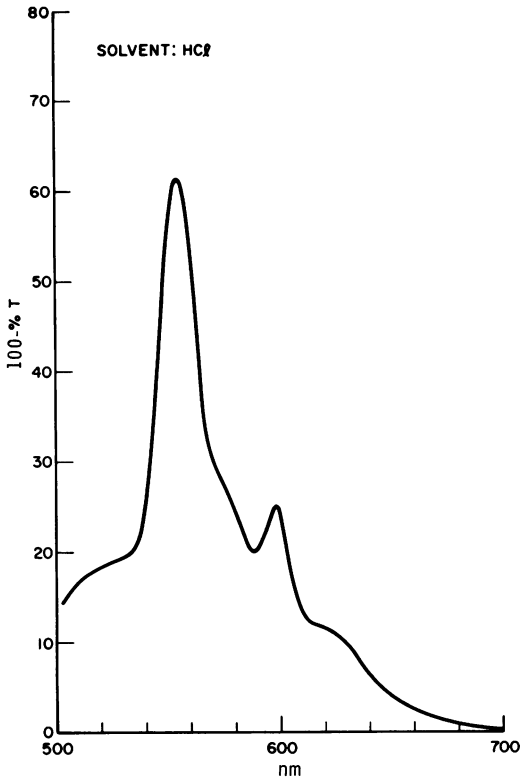

FIG. 2. *Absorption spectrum in 0.36% HCl of porphyrin produced by Pseudomonas denitrificans.*

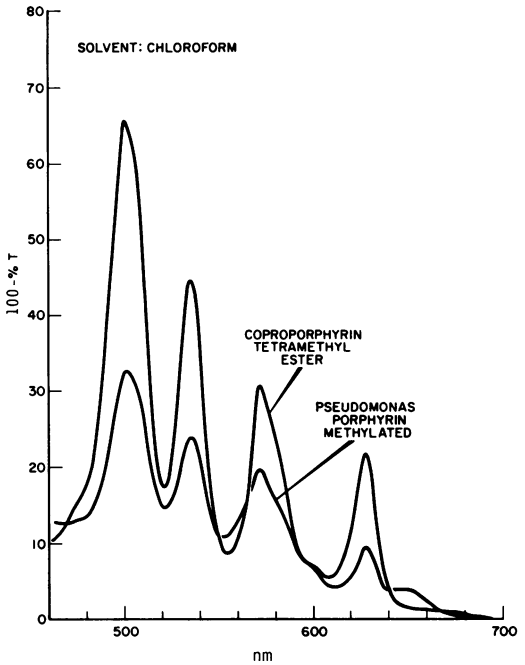

FIG. 3. *Absorption spectra in chloroform of methylated porphyrin produced by Pseudomonas denitrificans and of authentic coproporphyrin III tetramethyl ester.*

porphyrin synthesis was unexpected. Up to the time of our initial report (R. F. White and A. L. Demain, Bacteriol. Proc., p. 4, 1970), such an effect was unknown in nonphotosynthetic organisms. Gibson, Neuberger, and Tait (4) noted in 1962 that methionine antagonism or deficiency, as caused by supplementation of ethionine or threonine, respectively, led to coproporphyrin excretion by illuminated suspension of *Rhodopseudomonas spheroides*. Ethionine also inhibited photosynthetic growth and bacteriochlorophyll production. Since *S*-adenosylethionine was found to inhibit the in vitro methylation of magnesium protoporphyrin, an essential step in bacteriochlorophyll synthesis, it was postulated that blockage of the bacteriochlorophyll branch by methionine deficiency restricts production of some late intermediate which normally exerts negative feedback regulation of porphyrin synthesis; thus, porphyrin synthesis would proceed in an unregulated manner. The odd finding of coproporphyrin accumulation, rather than that of protoporphyrin, was left unexplained until 1969, when Tait (10) reported that methionine was required for the anaerobic conversion of

TABLE 1. *Optimum* L*-ethionine concentration for porphyrin synthesis by P. denitrificans*[a]

Ethionine (μg/ml)	Coproporphyrin[b] (μg/ml)	B_{12} (μg/ml)
0	7.2	14.6
50	34.5	3.6
100	35.9	2.1
150	34.5	1.8
200	18.9	0.9
250	18.9	0.9
500	16.4	0.9

[a] Duration of experiment was 120 hr.
[b] Porphyrin was quantitated by extraction.

coproporphyrinogen III to protoporphyrin by extracts from photosynthetically grown *R. spheroides*. Aerobic conversion did not require methionine. Dark-grown cells failed to carry out the conversion in the presence or absence of methionine. Tait later suggested (11) that methionine (or *S*-adenosylmethionine) may be required to activate coproporphyrinogenase under anaerobic conditions. Jacobs, Jacobs, and Brent (5) recently studied the coproporphyrinogenase reaction in extracts of aerobically grown *Pseudomonas fluorescens*, of anaerobically grown *P. denitrificans*, and of *Escherichia coli* grown in

the presence or absence of air. The conversion of coproporphyrinogen to protoporphyrin proceeded in air in all cases; however, no anaerobic conversion was observed in the presence of methionine.

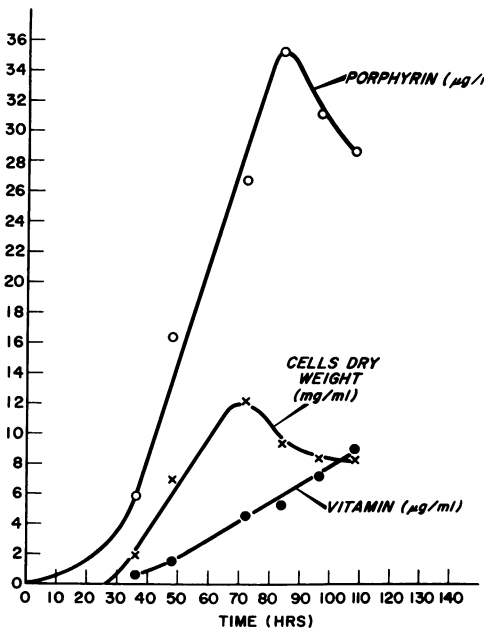

FIG. 4. *Growth and overproduction of coproporphyrin III and vitamin B_{12} in the presence of 100 μg of L-ethionine per ml of medium. The extinction coefficient of coproporphyrin III at 406 nm (*) was used to quantitate porphyrin overproduction.*

Although the above studies (5, 10, 11) would suggest that the methionine requirement for coproporphyrinogenase activity is specific for photosynthetic bacteria synthesizing porphyrins anaerobically, our results with *P. denitrificans* grown aerobically suggest that the methionine effect is of more general significance. Also, Kortstee (6) recently reported that methionine prevents the accumulation of coproporphyrin III by *Arthrobacter globiformis* and enhances heme production. It would appear, then, that failure to observe a methionine or *S*-adenosylmethionine requirement for in vitro coproporphyrinogenase activity is probably due to the use of crude extracts.

The current concept of the branched pathway to corrins and porphyrins is depicted in Fig. 5. Since heme is a feedback inhibitor and core-

TABLE 2. *Absolute requirement of betaine for vitamin B_{12} and porphyrin synthesis by P. denitrificans*[a]

Betaine	L-Ethionine (μg/ml)	Vitamin B_{12} (μg/ml)	Coproporphyrin[b] (μg/ml)
10	0	27.0	5.9
0	0	0	0
10	100	17.5	26.8
0	100	0	0

[a] Duration of experiment was 120 hr.
[b] Porphyrin was quantitated by extraction.

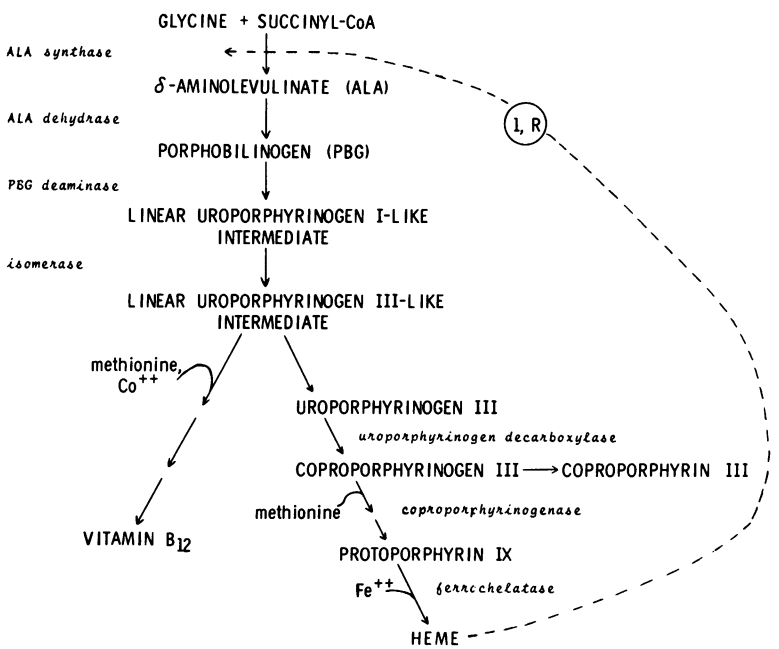

FIG. 5. *Corrin and porphyrin biosynthetic scheme. Dashed line depicts regulation; I, inhibition; R, repression.*

pressor of δ-aminolevulinate synthase (1, 7), it is clear that inhibition of coproporphyrinogenase by ethionine would restrict heme formation and would allow the porphyrin pathway to proceed in an unregulated manner. This would result in overproduction of coproporphyrinogen III. Due to the known instability of coproporphyrinogen III in air, the compound which would accumulate would be its oxidation product, coproporphyrin III. Ethionine also would be expected to inhibit cobalamin production by interfering with methylation of the corrin nucleus.

The most interesting finding of the present study is that betaine is an absolute requirement for porphyrin overproduction, whether or not such accumulation is brought about by ethionine. Thus, betaine is needed by *P. denitrificans* for overproduction of both corrins and true porphyrins. The site of betaine action in the pathway of Fig. 5 is completely unknown. We have not yet tested the activity of choline on porphyrin synthesis but have shown that it can replace betaine for vitamin B_{12} production (2). Although betaine may be a positive effector for some early step of corrin and porphyrin synthesis, we must also consider the possibility that the activities of both methionine and betaine (or choline) in regulating porphyrin synthesis may be indirect and may act via effects on phospholipid synthesis. In a recent note, Kortstee (6) states that phospholipids are required for the conversion of δ-aminolevulinic acid to protoporphyrin IX by extracts of coproporphyrin III-accumulating cells of *A. globiformis*. When this organism is grown with methionine, coproporphyrin is not accumulated, heme synthesis is enhanced, and the cells have a different phospholipid composition. A further involvement of phospholipids with porphyrin biosynthesis is found in *R. spheroides* where the ferrochelatase reaction is markedly stimulated by phospholipids present in the organism (8). Since choline is known to repress phosphatidylcholine biosynthesis in yeast (9), it is possible that, in *P. denitrificans*, growth in choline (and betaine) may so change the phospholipid pattern that heme synthesis is inhibited. This would be expected to derepress the porphyrin-corrin pathway and to lead to increased formation of vitamin B_{12} and porphyrin intermediates. If methionine acts at a different site in the porphyrin pathway, the effect of adding ethionine to the betaine-containing medium might be expected to cause a synergistic accumulation of the porphyrin intermediate but a decreased level of vitamin B_{12}, since ethionine could also inhibit methylation of the corrin nucleus.

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