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Effects of Gabaculine on Pigment Biosynthesis in Normal and Nutrient Deficient Cells of *Anacystis nidulans*[1]

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ABSTRACT

Pigment biosynthesis in the cyanobacterium, *Anacystis nidulans*, was examined in the presence of gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid). At 20 micromolar, this inhibitor blocked the biosynthesis of both chlorophyll and phycocyanin. Analogs of gabaculine were not effective as inhibitors of chlorophyll or phycocyanin biosynthesis. Iron- and phosphate-deficient cultures were 2- to 4-fold more sensitive to the inhibitor than were normal or nitrate-deficient cultures. Inhibition resulted in the excretion of a mixture of organic acids by the cells. δ-Aminolevulinic acid was a principle component of the mixture, identified by thin layer chromatography. Excretion of δ-aminolevulinic acid occurred following a brief lag after gabaculine addition. It remained linear for nearly 24 hours and was dependent upon illumination. However, high light inhibited excretion. Apparently, gabaculine blocks chlorophyll biosynthesis after the formation of δ-aminolevulinic acid in cyanobacteria.

Recovery of cyanobacteria from iron deficiency is accompanied by a reproducible pattern of photosynthetic membrane repair. When iron is restored to a chlorotic culture, there is a 3 to 5 h lag, followed by the synthesis and insertion of Chl proteins into existing membranes (23). At later times, the assembly of new membrane becomes apparent (27). The factors which regulate the assembly of Chl proteins under these conditions are not well understood. Iron starvation reduces the synthesis of protoporphyrins, presumably by feedback inhibition due to an accumulation of protoheme (5, 8, 28). Since Chl is a component of several thylakoid membrane polypeptides, decreased Chl availability may play a regulatory role in Chl protein assembly.

The differential effects of Chl availability and of intracellular iron during recovery from iron deficiency have previously been examined using two inhibitors of Chl biosynthesis (12). One of these, LA[2], is a competitive inhibitor of ALA dehydratase (17, 18), and effectively blocks protoporphyrin biosynthesis in both normal and iron-deficient cyanobacteria (11). The mechanism by which the second inhibitor, GAB, affects cyanobacteria is virtually unknown (12). This compound interferes with pyridoxal phosphate-linked aminotransferase activity (25, 26), and inhibits Chl biosynthesis in higher plants (9, 10, 15, 29).

In this report, we have examined the effects of GAB on pigment biosynthesis in the cyanobacterium, *Anacystis nidulans*. Our findings consist of three pertinent observations. First, GAB inhibited the accumulation of both Chl and PC. The concentration requirements for inhibition were similar to those of other systems (15, 25). Second, these concentration requirements were influenced by the nutrient status of the cell. Finally, GAB induced the excretion of organic acids, a major excretion product being ALA. This suggested that GAB inhibits Chl biosynthesis in cyanobacteria at a site following ALA formation.

MATERIALS AND METHODS

Cells of *Anacystis nidulans* R2 were grown in shaking culture as previously described (13). Axenic cultures were monitored microscopically and by plating on nutrient agar. Nutrient deficient cells were obtained using modifications of the BG-11 growth medium of Allen (1). To obtain iron-deficient cells, equimolar amounts of ammonium citrate replaced ferric ammonium citrate (14). Nitrate deficiency was induced by adding 10-fold less sodium nitrate (2). Phosphate deficiency was induced by omitting K-phosphate (3). All of the experiments described here were initiated with 3 to 4 d (late log phase) cultures.

Inhibitors were prepared in aqueous solution and were filter-sterilized prior to use. Both ALA and LA were titrated to pH 7.5 with NaOH before sterilization. In some experiments, buffer (0.05 M Tricine, pH 7.5) was added to cultures to ensure that pH changes were not responsible for the results which we observed.

Assays. The Chl and PC content of cultures were estimated from spectra obtained using an SLM/Aminco DW2-C spectrophotometer. Estimates were made using the simultaneous equations of Jones and Myers (16). Cell viability was monitored as plating efficiency on BG-11 solidified by 1% (w:v) agar (13).

To measure excretion of organic acids, cells were harvested and resuspended in normal growth media (BG-11) to a cell concentration of 10^9 cells/ml. Inhibitor was added to these concentrated cultures and incubation occurred (200 ft-c white light at 33°C) for 22 h with vigorous shaking. In some experiments, light intensity was varied using neutral density screens. Cells were harvested by centrifugation and the spent medium was clarified by passage through a 0.2 μm filter.

The filtrate was analyzed in several ways. First, the concentration of the excreted product was estimated using a protocol for the determination of ALA (17). To filtrate (1.0 ml), 0.4 ml of 2 M sodium acetate (pH 4.7) and 0.028 ml acetyl acetone were added. The mixture was placed in a boiling water bath (10 min). The pyrrole product formed by condensation was determined using modified Ehrlich reagent and was quantified by absorption at 552 nm (20). Alternatively, time course experiments required greater sensitivity. ALA was first concentrated from the filtrate by ion exchange chromatography (29), then condensed and assayed as described above.

Second, the major excretion product in the filtrate was identified as ALA by TLC. Three solvent systems were employed

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[2] Abbreviations: ALA, 5-aminolevulinic acid; GAB, gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid); LA, levulinic acid; PC, C-phycocyanin

(19): solvent A (isopropanol/distilled H_2O 7:3 v:v), solvent B (1-butanol/acetic acid/water 4:1:1: v:v:v), and solvent C (ethanol: 34% ammonium hydroxide 7:3 v:v). Standards were prepared as 2 mM aqueous stocks. Filtrate and standards were either spotted directly on silica gel plates, or were first concentrated by ion exchange chromatography using Dowex 50 in the H^+ form (29). Silica gel plates were sprayed once with fluorescamine (20 mg in 100 ml acetone) and twice with 10% ethanolamine in acetone (v:v) before visualization under UV light. The organic acid content of the spots was quantified using a Kontes chromatography scanner in the fluorescence mode. When filtrates from GAB-treated samples were examined, four spots were observed. With each solvent system, the major spot comigrated with an ALA standard. Solvent system A showed the best resolution of the four spots, with R_F values of nearly 0, 35.4, 56.2, and 78.1.

Chemicals. GAB was obtained from Fluka Chemical Corporation. All other compounds were obtained from Sigma Chemical Company.

RESULTS

Inhibition of Pigment Accumulation. GAB markedly inhibited the accumulation of Chl in *A. nidulans*, as shown in Figure 1. At 10 to 15 μM, GAB abolished Chl biosynthesis in normal cells. Similar concentrations abolished PC biosynthesis (data not shown). Interestingly, the concentration of GAB needed to block Chl accumulation was partially dependent upon the nutritional status of the cells (Fig. 1). Normal and nitrate-deprived cells were inhibited by about the same concentrations of GAB. However, iron- and phosphate-deficient cells required 2- to 4-fold less inhibitor.

The effects of GAB and related compounds on Chl and PC content are shown in Table I. Only GAB effectively blocked pigment biosynthesis. At high concentrations, benzocaine inhibited Chl biosynthesis by about 75%. However, the mechanism of benzocaine inhibition was not the same as with GAB (see below). The effects of GAB on cell viability following a 24 h incubation are shown in Table II. At concentrations which completely inhibited Chl biosynthesis, GAB had little effect on cell viability. Higher concentrations diminished cell viability by about 90%. Neither benzoic acid derivatives nor LA had a significant effect on cell viability.

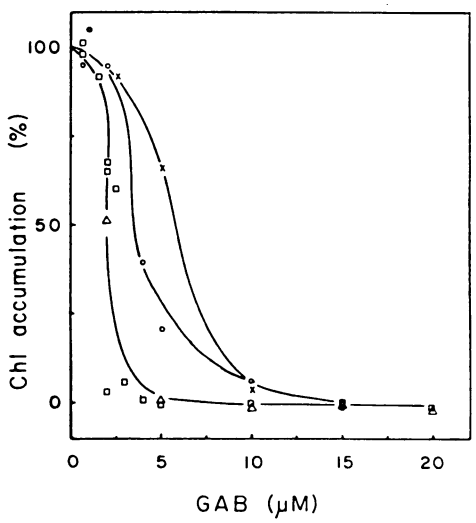

FIG. 1. Effects of GAB on Chl accumulation in *A. nidulans*. One hundred ml of fresh BG-11 media were inoculated with cells in log phase growth. The initial Chl concentration was approximately 0.5 μg Chl/ml. GAB was added and cultures were incubated for 24 h. Chl accumulation during the 24 h incubation is presented as a percentage relative to accumulation in the absence of GAB. Cell types used to initiate this experiment were: (○) grown in complete media, (□) iron deficient, (△) phosphate deficient, and (×) nitrate deficient.

Excretion of ALA Induced by GAB. Experiments with higher plant systems suggest that GAB inhibits the formation of ALA (9, 10, 15, 29). In contrast, LA is a competitive inhibitor of ALA dehydratase (17) and promotes ALA excretion. Thus, we had hoped to use ALA excretion as a method to examine the relationship between LA and GAB. If GAB were to inhibit ALA formation, it would diminish the ALA excretion induced by LA. In contrast to our expectations, however, GAB itself induced the excretion of ALA.

Figure 2 shows the concentration of GAB required to induce ALA excretion by *A. nidulans*. Similar concentrations were needed to fully inhibit pigment biosynthesis (Fig. 1). A reduction in the extent of ALA excretion was observed above 50 to 100 μM GAB, suggesting that GAB had additional effects at higher concentrations. Furthermore, iron- and phosphate-deficient cultures showed a heightened sensitivity to GAB. Nitrate-starved cells, even though they did not accumulate pigment, excreted relatively little ALA. It is likely, therefore, that nitrate starved cells had a lower concentration of nitrogen-rich ALA precursors. Lack of these precursors would reduce ALA biosynthesis, even in the presence of GAB.

Figure 3 shows the time course of ALA excretion and illustrates three points. First, excretion was observed soon after GAB addition to cells. The inhibitor bound relatively quickly to the target enzyme and measurable ALA excretion occurred after 1 to 2 h. Second, following this brief lag, excretion proceeded at a fairly constant rate. Time course experiments were extended past 4.5 h and showed that ALA excretion was linear for nearly 24 h (results not shown). Finally, both iron-deficient and normal cells responded to GAB with similar kinetics of excretion.

Effects of Illumination. The effects of illumination on ALA excretion are shown in Figure 4. The extent of ALA excretion increased with increasing light intensity, suggesting that ALA production required photosynthetic activity. Maximum ALA production was observed at approximately 350 ft-c. Furthermore, higher intensities decreased the extent of ALA production. Since these cultures were not bubbled with CO_2-enriched air during incubation, it is possible that higher illumination caused photoinhibition. It is not clear if GAB, by inhibiting Chl biosynthesis, hastens the onset of photoinhibition.

Inhibition in the Presence of ALA. In higher plants, ALA reportedly can overcome the inhibition imposed by GAB (9, 10). We did not observe this with *A. nidulans*. In the presence of optimal GAB concentrations, ALA was completely ineffective in restoring Chl biosynthesis (Table III).

DISCUSSION

GAB has been described as an irreversible inhibitor of several pyridoxal phosphate-linked transaminase reactions (25). It is one of a class of 'suicide inhibitors,' since latent reactive groups in the inhibitor are activated by the target enzyme. Activation causes an irreversible modification of the enzyme active site (26). GAB is a potent inhibitor of γ-aminobutyric acid-α-ketoglutaric acid transaminase, owing to the β,γ-unsaturation of the inhibitor. Presumably, a *m*-carboxyphenylpyridoxamine phosphate is generated at the active site, and this compound binds so tightly to the enzyme that it can only be liberated upon denaturation (26).

In this report, we have documented the effects which GAB has on pigment biosynthesis in *A. nidulans*. At 10 to 15 μM, GAB suppressed the accumulation of both Chl and PC, presumably by blocking the pathway of protoporphyrin biosynthesis. Approximately the same GAB concentrations are required to inhibit aminotransferase activity (25) and to reduce ALA synthesis by 50% in cell-free systems (29). Considerably higher concentrations

Table I. *Effects of GAB and Related Compounds on Pigment Accumulation in A. nidulans*

The inhibitors were added to iron deficient cells in a volume of 15 ml. Iron was added concurrently to initiate recovery and pigment biosynthesis. The initial culture contained 0.46 μg/ml Chl and 2.81 μg/ml PC, and had a PC/Chl ratio of 6.16. Pigment values in the table represent a gain (or loss) after 24 h. Pigment ratios represent the PC/Chl ratio observed at the end of the experiment.

Inhibitor	Pigment Accumulation				PC/Chl (w/w)
	Chl		Phycocyanin		
μM	*μg/ml*	*% of control*	*μg/ml*	*% of control*	*ratio*
None	1.08	100	13.4	100	10.5
GAB (3-amino-2,3-dihydrobenzoic acid)					
20	−0.06	−5.6	−0.23	−1.7	6.5
100	−0.09	−8.3	−0.21	−1.6	7.1
Benzoic acid					
20	0.85	79	13.1	97	12.1
100	0.80	74	12.5	96	12.5
3-Aminobenzoic acid					
20	0.80	74	13.3	99	12.8
100	0.70	65	11.7	87	12.5
3-Amino-2,5-dichlorobenzoic acid					
20	0.84	78	12.4	93	11.7
100	0.82	76	13.4	100	12.7
5-Amino-2-hydroxybenzoic acid					
20	0.78	72	12.3	92	12.2
100	0.55	51	9.9	74	12.7
Procaine (2-diethylaminoethyl-4-aminobenzoic acid)					
20	0.74	68	12.2	91	12.5
100	0.58	53	10.6	79	12.9
Benzocaine (ethyl-4-aminobenzoic acid)					
20	0.38	35	9.1	68	14.5
100	0.26	28	4.5	34	12.9

Table II. *Cell Viability of A. nidulans following 24 h Incubation with GAB and Selected Unsaturated Analogs*

Cultures containing 10^9 cells/ml were treated with GAB as described in the legend of Figure 2. After 24 h, cells were removed from inhibitor by centrifugation and resuspended in fresh media. Viability was monitored by plating cells in serial dilution and counting the number of colonies formed. Plating efficiency was determined by comparing the number of colonies formed after treatment to those obtained when the cells had not been treated.

Inhibitor Addition	Concentration	Plating Efficiency
	μM	*% of control*
GAB	20	134
	100	7.4
3-Aminobenzoic acid	20	91
	100	86
Benzocaine	20	58
	100	17
LA	50,000	82

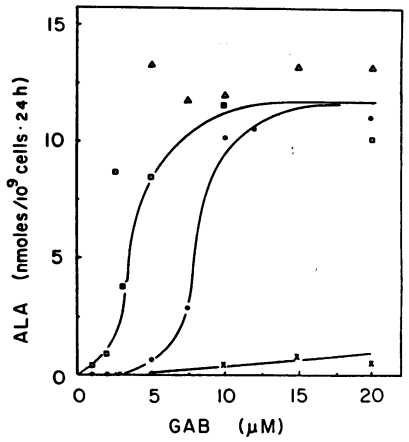

FIG. 2. Effects of GAB on ALA excretion in *A. nidulans*. Cells of various nutritional status were harvested and resuspended in fresh, complete growth media to a concentration of 10^9 cells/ml. These were incubated with GAB for 22 h. Following centrifugation and filtration to remove cells, spent media was assayed for ALA as described in "Materials and Methods." Cell types used in this experiment were: (○) grown in complete media, (□) iron deficient, (△) phosphate deficient, and (×) nitrate deficient.

(about 1 mM) are necessary to block phytochrome biosynthesis in intact, higher plants (10). There were no differential effects on Chl or PC at any inhibitor concentration which we employed. Analogs of GAB do not impose a similar inhibition (Table I).

One important consequence of GAB treatment was the excretion of ALA by inhibited cells (Fig. 2). We were surprised that GAB induced an excretion of ALA. Pigment synthesis in cyanobacteria is thought to be similar to that observed in higher plants. For example, Kipe-Nolt *et al.* (17) demonstrated biosynthesis of ALA in cyanobacteria by monitoring ALA excretion in the presence of LA and suggested that glutamate is the major substrate for ALA synthesis (18). Isotope distribution studies using labeled precursors of ALA have suggested that a 5-carbon pathway of ALA synthesis is operative in cyanobacteria (21).

Furthermore, GAB has been shown to inhibit ALA synthesis in higher plants (9, 10, 15, 29). The pathway from glutamate to ALA in higher plants is under intensive investigation (4), and may proceed via 4,5-dioxovaleric acid (22, 24) or via an activated glutamate (29). Amine transfer is an important component of all models of the glutamate to ALA pathway (4, 22, 24, 29). Because GAB inhibits pyridoxal phosphate-linked aminotransferase ac-

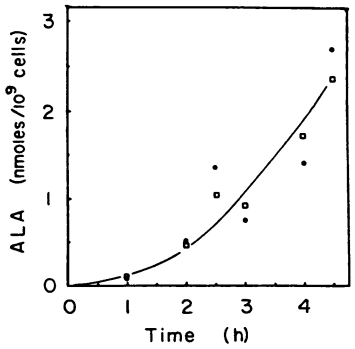

FIG. 3. Time course of ALA excretion induced by gabaculine. Normal (O) and iron deficient (□) cultures were treated with GAB as described in the legend of Figure 2 (7.5 μM GAB to iron stressed cells, 20 μM to normal cells). Aliquots were withdrawn at intervals, and the ALA content of spent media was quantified as described in "Materials and Methods."

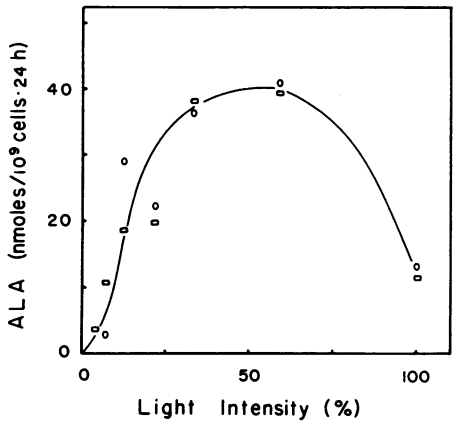

FIG. 4. Effects of illumination on ALA excretion. Normal (O) and iron deficient (□) cells were treated with GAB as described in the legend of Figure 2. Illumination was provided by a bank of cool white fluorescence lights. Light intensity was varied using neutral density screens. Following a 22 h illumination, the ALA content of spent media was quantified as described in "Materials and Methods." Light intensity is expressed as a percentage of maximum light levels (590 ft-c).

Table III. *ALA Does Not Reverse the Inhibitory Effects of GAB in A. nidulans*

GAB and ALA (neutralized to pH 7.0) were added to a cell suspension of (containing 0.69 μg Chl/ml), and were assessed for Chl content after 24 h.

Addition of ALA	No GAB		Plus 20 μM GAB	
	Chl	PC/Chl	Chl	PC/Chl
mM	*μg/ml*	*μg/μg*	*μg/ml*	*μg/μg*
0	2.10	7.05	0.70	7.58
5	2.12	8.77	0.68	7.23
10	1.40	9.72	0.64	6.68

tivity (25, 26), and because ALA synthesis is apparently blocked by GAB in higher plants (9, 10, 15, 29), we anticipated that GAB would inhibit ALA synthesis in cyanobacteria as well. However, our results clearly showed that ALA was excreted during inhibition by GAB. These results argue that the primary site of GAB inhibition, at GAB concentrations which completely prohibit Chl biosynthesis, is on the pathway of ALA utilization. Exogenously supplied ALA was unable to overcome GAB inhibition (Table III).

Our results do not allow us to pinpoint the site of GAB inhibition. However, preliminary experiments suggest that ALA dehydratase activity is not diminished in the presence of the drug (results not shown). Since GAB may irreversibly bind with the target enzyme, we might be able to use the inhibitor to radioactively label the target for observation on polyacrylamide gels. It is possible that there are multiple sites of GAB inhibition in cyanobacterial protoporphyrin biosynthesis. For example, we observed that high GAB concentrations decrease the extent of ALA excretion.

One of the most interesting aspects of our results is the differential effects of nutrient deficiency on GAB inhibition. Cells which were starved for iron and phosphate required less inhibitor (Fig. 1). A similar relationship has been observed between the inhibitor LA and iron deficiency (11). Nitrate starvation lowered the amount of organic acid excretion (Fig. 3).

In cyanobacteria, the nutrient status of the cell profoundly influences pigment content (2, 3, 7, 14, 24). Several control sites have been postulated. Spiller *et al.* (28) demonstrated an iron-requiring enzymatic step in the formation of Mg-protoporphyrin in a higher plant system. In the presence of high concentrations of Co^{2+} and Mn^{2+}, Csatorday and colleagues (6, 7) demonstrated shifts in the relative content of Chl and phycocyanin. High Co^{2+} inhibits the formation of PC, leading to an increase in the Chl/PC ratio. Alternatively, Mn^{2+} depresses the amount of Chl, leading to a decreased Chl/PC ratio. Iron feeding of *Anacystis* leads to an excretion of coproporphyrin III (6).

Iron deficiency represents a special case in cyanobacterial nutrition. In contrast with the results obtained with nitrate (2) or phosphate (3) deficiency, lack of iron causes a shift in Chl absorption peak (from 680 nm to 672 nm) as well as a depression in the relative cellular Chl content. Pakrasi *et al.* (23) correlated the Chl fluorescence characteristics of iron-deficient cells with abnormal Chl-protein patterns on polyacrylamide gels, and suggested that an accumulation of a novel Chl-binding protein was indicative of the iron deficient state. This suggests that iron may play a role in regulating the membrane content of specific Chl-binding proteins.

GAB may be a useful compound in examining the role of iron in Chl-protein biosynthesis. Iron also influences the amount of available intracellular Chl (5, 28), and the relationship between Chl availability and Chl-protein biosynthesis is not clear. GAB will allow the addition of iron to an iron-deficient culture in the absence of Chl biosynthesis. Preliminary experiments have focused on the spectral properties of iron-deficient cells (12). When iron is restored to a deficient culture, Chl synthesis occurs. At the same time, there is a recovery of Chl absorption characteristics toward longer wavelengths. This led to the hypothesis that Chl synthesis and the concomitant synthesis of new Chl-binding proteins were responsible for recovery of normal absorption characteristics. As we have demonstrated here, GAB markedly inhibits Chl biosynthesis. Despite this, GAB does not inhibit the recovery of normal absorption characteristics when iron is restored to deficient cultures (12). Recovery, therefore, must be due to a change in the environment of existing Chl. Experiments monitoring the low temperature fluorescence emission profile of this Chl will be necessary to document this shift.

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