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The effect of gabaculine on tetrapyrrole biosynthesis and heterotrophic growth in *Cyanidium caldarium*

Jennifer D. HOUGHTON, Larry TURNER and Stanley B. BROWN*
Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

Pigment synthesis in four strains of the unicellular red alga *Cyanidium caldarium* with different pigment-synthesizing patterns was inhibited in the presence of gabaculine (3-amino-2,3-dihydrobenzoic acid). Parallel inhibition of light-induced chlorophyll and phycocyanin synthesis was observed in strain III-D-2, which only synthesizes pigments in the light. Similar parallel inhibition was observed in the dark in mutant CPD, which is able to synthesize chlorophyll and phycocyanin in the absence of light. Inhibition of pigment synthesis in all strains was overcome by addition of 5-aminolaevulinic acid. Inhibition of phycocyanin synthesis in mutant GGB (unable to synthesize chlorophyll) and inhibition of chlorophyll synthesis in mutant III-C (unable to synthesize phycocyanin) were also observed. Gabaculine also inhibited the heterotrophic growth of *C. caldarium* in the dark. However, inhibition was overcome after an extended lag period, following which cell growth proceeded at a similar rate to that of control cells not exposed to gabaculine. Heterotrophic growth in cells pre-exposed to gabaculine was not inhibited by subsequent exposure. Possible mechanisms for this adaptation are discussed.

INTRODUCTION

In recent years it has become clear that the biosynthesis of the tetrapyrrole precursor 5-aminolaevulinate (ALA) in many plant systems is different from that in animals. In animals ALA destined for haem is formed by condensation of glycine and succinate, catalysed by the enzyme ALA synthase (EC 2.3.1.37). In plants, it is well established that ALA synthesis may occur via the so-called '5-carbon pathway' from glutamate (Castelfranco & Beale, 1983; Mayer *et al.*, 1987; Kannangara *et al.*, 1988). In this pathway, the intact 5-carbon skeleton of glutamate is transformed by a multi-enzyme pathway involving three enzyme components and a tRNA. Glutamyl tRNA and glutamate 1-semialdehyde are intermediates in the reaction sequence (Kannangara *et al.*, 1988). The 5-carbon pathway of ALA synthesis has been shown to operate in a wide variety of plants, algae and bacteria. However, there has been much debate as to whether all plant tetrapyrrole synthesis occurs by the 5-carbon pathway or whether, under some circumstances, plants may also use the classical ALA synthase system. In particular, it has been suggested that chlorophyll synthesis may occur by the 5-carbon pathway and haem synthesis may occur by the ALA synthase pathway (Meller & Gassman, 1982).

For some time gabaculine (3-amino-2,3-dihydrobenzoic acid) has been recognized as an inhibitor of pyridoxal phosphate-requiring enzymes (Walsh, 1984). Recently it has been shown to be a remarkably specific inhibitor of the 5-carbon pathway of ALA synthesis in a number of photosynthetic organisms (Flint, 1984; Corriveau & Beale, 1986; Werck-Reichhart *et al.*, 1988), and has proved useful in distinguishing between ALA synthesis via the 5-carbon pathway and the ALA synthase pathway. Gabaculine is now known to inhibit the final step in the proposed mechanism of 5-carbon ALA synthesis from glutamate catalysed by the enzyme glutamate-1-semialdehyde aminotransferase (Hoober *et al*, 1988). For higher plants there is a growing body of literature to suggest that all ALA is synthesized via the 5-carbon pathway, whether required for bulk chlorophyll synthesis in the light, or for the relatively smaller amounts of haemoproteins synthesized even in the absence of light (Schneegurt & Beale, 1986; Werck-Reichhart *et al.*, 1988). However, in *Euglena gracilis*, for example, it is known that the two pathways of ALA synthesis co-exist, with light favouring production by the carbon pathway, whilst the ALA synthase route is the major one in cells growth in the dark, providing mitochondrial haems for heterotrophic growth (Weinstein & Beale, 1983).

Cyanidium caldarium is a unicellular alga, capable of growing both heterotrophically in the dark (usually without synthesis of pigments) and photosynthetically in the light, when it synthesizes both chlorophyll *a* and phycobiliproteins (phycocyanin and allophycocyanin). These pigments are also formed without significant cell growth when unpigmented dark-grown cells are exposed to light (Brown *et al.*, 1981), and this represents a very convenient system for study of pigment synthesis. The phycobiliproteins, which act as accessory pigments, have been shown to be synthesized via haem (Brown *et al.*, 1981; Brown & Troxler, 1982). This type of organism is therefore of special interest from the point of view of ALA formation, since it synthesizes large quantities of both chlorophyll and haem.

Mutants of *C. caldarium* exist in which either phycocyanin or chlorophyll synthesis is blocked, while synthesis of the other pigment is still inducible by light. In addition, a mutant strain of the organism exists which is capable of synthesizing both chlorophyll and phycocyanin even in the absence of light. We report here on the effects of gabaculine on chlorophyll and phycocyanin

Abbreviation used: ALA, 5-aminolaevulinate.
* To whom correspondence should be addressed.

Table 1. Growth and pigmentation characteristics of strains of *C. caldarium*

For details of growth and pigmentation conditions see the text and Brown *et al.*, 1981. Abbreviations used: Chl, chlorophyll; PC, phycocyanin.

	Heterotrophic growth		Exposure of heterotrophically grown cells to light		Photosynthetic growth	
Strain	Chl	PC	Chl	PC	Chl	PC
II-D-2	−	−	+	+	+	+
CPD	+	+	+	+	+	+
III-C	−	−	+	−	+	−
GGB	−	−	−	+	not possible	

synthesis in *C. caldarium* under the different biosynthetic demands of the mutants, and on its inhibition of heterotrophic growth in this organism.

EXPERIMENTAL

Materials

C. caldarium strains III-D-2 and CPD were gifts from Prof. R. F. Troxler (Boston University School of Medicine, Boston, MA, U.S.A.) and strains III-C and GGB from Dr. S. I. Beale (Division of Biology and Medicine, Brown University Providence, Rhode Island, U.S.A.). Their growth and pigmentation characteristics are shown in Table 1. Gabaculine was obtained from Fluka Chemicals Ltd., Glossop, U.K. and 5-aminolaevulinic acid hydrochloride was obtained from Sigma, Poole, U.K. All other materials used were Analytical Reagent Grade, where these were available.

Cell culture

Bulk quantities of cells were grown heterotrophically by incubation in the dark in minimal medium supplemented with 1 % glucose at 40 °C as previously described (Brown *et al.*, 1981). Pigment synthesis was induced in dark-grown cells by resuspension of packed cells (1 vol.) in glucose-free medium (20 vols.), followed by incubation under 6 × 20 W fluorescent tubes at 30 °C for 48 h following the detailed procedures described earlier (Brown *et al.*, 1981). The effect of gabaculine on heterotrophic growth was studied by addition of a concentrated solution of the inhibition to the growth medium. Studies on the effect of gabaculine were performed by addition of a stock solution of gabaculine to duplicate 8 ml volumes of cells in 20 ml foam-stoppered universal bottles, incubated in the light as described above. In some experiments, exogenous ALA was added at the same time as gabaculine. Absorbance at 580 nm was used to measure cell density based on the light scattering due to cells present in the culture, as interference by pigment absorption at this wavelength is minimal.

Determinations of pigment concentrations

Whole-cell absorption spectra were carried out as described previously (Houghton *et al.*, 1986). Measurements of the overlapping absorbance peaks of phycocyanin and chlorophyll were corrected using the simultaneous equations of Beale & Chen (1983), and concentrations were calculated using the absorption coefficient of Troxler (1972).

RESULTS AND DISCUSSION

Fig. 1 shows typical intracellular accumulation of both chlorophyll and phycocyanin which occurs over a 125 h period, following exposure of heterotrophically grown cells to light. Because the precise progress of pigmentation varied from one batch of cells to another, all comparative studies were performed on the same batch of heterotrophically grown cells. Fig. 1 shows that chlorophyll and phycocyanin synthesis occur approximately in parallel and that synthesis was still occurring after 3 days. To study the effect of gabaculine, an incubation period of 48 h was chosen. This time corresponds to a stage at which a good deal of pigmentation has occurred, but substantial synthesis is still continuing.

Fig. 2 shows the effect of various gabaculine concentrations on pigment accumulation in III-D-2 cells after 48 h. It is clear that marked inhibition of both chlorophyll and phycocyanin synthesis occurred and, moreover, the inhibition occurred approximately in parallel for both

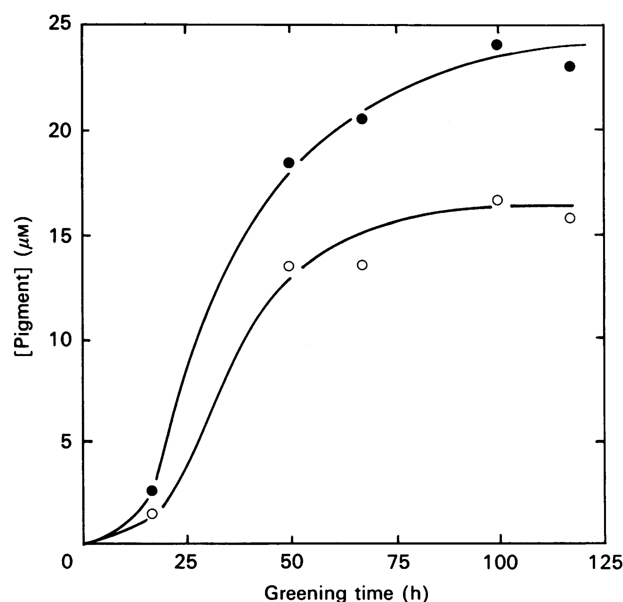

Fig. 1. Pigmentation in *C. caldarium* following exposure of heterotrophically grown cells to light

III-D-2 cells were grown heterotrophically and exposed to light as described in the text and by Brown *et al.* (1981). Chlorophyll (●) and phycocyanin (○) concentrations were determined by whole-cell spectroscopy.

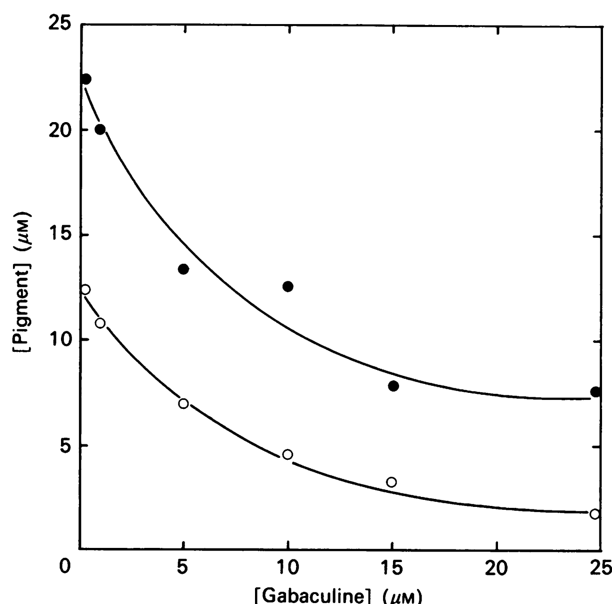

Fig. 2. Effect of gabaculine on chlorophyll and phycocyanin synthesis in *C. caldarium*

Cells of mutant III-D-2 were grown heterotrophically as described in the Experimental section and illuminated in the presence of gabaculine at the concentrations indicated for 48 h. Chlorophyll (●) and phycocyanin (○) concentrations were determined by whole-cell spectroscopy. Each point represents the mean of two duplicate incubations.

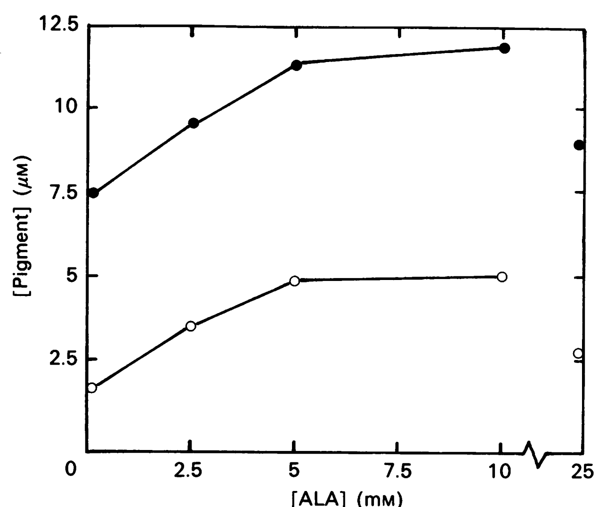

Fig. 3. Effect of ALA on chlorophyll and phycocyanin synthesis in gabaculine-treated *C. caldarium*

III-D-2 cells were grown heterotrophically as described in the Experimental section and incubated in the presence of a fixed concentration of gabaculine (25 μM) with varying concentrations of ALA for 48 h in the light. Chlorophyll (●) and phycocyanin (○) concentrations were determined by whole-cell spectroscopy. Each point represents the mean of two duplicate incubations.

pigments, with 50% inhibition being achieved at about 10 μM-gabaculine. Similar results were obtained for inhibition in mutant CPD in the light. In mutant III-C (which synthesizes only chlorophyll), 50% inhibition of pigment synthesis was produced by 25 μM-gabaculine, whilst in mutant GGB (which synthesizes only phycocyanin) 50% inhibition was observed at only 5 μM-gabaculine. In strain CPD, capable of synthesizing phycocyanin and chlorophyll in the absence of light, gabaculine also inhibited pigment synthesis in the dark, under otherwise similar experimental conditions.

Addition of ALA to gabaculine-treated cells

If the inhibition of pigment synthesis observed in Fig. 2 is due to the effect of gabaculine preventing ALA synthesis, then addition of exogenous ALA might be expected to counteract the influence of gabaculine. Fig. 3 shows the effect of varying concentrations of ALA on pigments accumulation after 48 h in III-D-2 cells, incubated with 25 μM-gabaculine. In this experiment, control cells, i.e. cells which had not received gabaculine or ALA, produced pigment at concentrations of 22 and 12.5 μM for chlorophyll and phycocyanin respectively. It is clear that, whilst at very low ALA concentrations gabaculine inhibition is marked, this inhibition is progressively overcome by addition of up to 10 mM-ALA. Further addition of ALA, however, leads to a decrease in pigmentation, due to toxic effects of ALA itself at high concentrations. Such a decrease is also seen in cells incubated with ALA but not gabaculine (results not shown). For the other three strains of *C. caldarium*, ALA was also found to counteract the effect of gabaculine. These data confirm that the inhibitory effect on pigment formation is a consequence of gabaculine inhibition of ALA synthesis.

Inhibition of heterotrophic growth by gabaculine

Heterotrophic growth of *C. caldarium* does not require chlorophyll or phycocyanin, but it does require small quantities of cytochromes, the prosthetic groups of which are synthesized via ALA. It was therefore of interest to determine if gabaculine inhibits heterotrophic growth of *C. caldarium*. Fig. 4 shows growth curves for strain III-D-2 both without gabaculine and with gabaculine at a concentration (10 μM) which was sufficient to cause 50% inhibition of pigment synthesis in the light. The control cells without gabaculine showed a typical lag period of 8–12 h followed by a steady increase in cell density. In the gabaculine-treated cells, however, growth did not occur for 12 days. After this lag period, growth resumed at a rate comparable with that observed in control cells not exposed to gabaculine. This was true not only of strain III-D-2, which requires cytochromes for respiratory growth, but also strain CPD, which synthesizes chlorophyll and phycocyanin even in the dark. Similar effects were shown by the other mutant strains, with the gabaculine-induced lag phase varying between 9 and 14 days. Moreover, when these gabaculine-treated cells were allowed to grow exponentially for 4 days and then harvested, resuspended in gabaculine-free medium and exposed to light, pigment synthesis (characteristic of the particular strain) occurred at a similar rate to that in untreated cells.

Under conditions otherwise identical with those described above where gabaculine inhibited heterotrophic growth of mutant III-D-2, addition of 10 μM-ALA restored normal growth characteristics.

When an inoculum of cells, which had grown following gabaculine adaptation, was incubated in fresh gabaculine-containing medium, their growth curve was typical of control cells in gabaculine-free medium. Thus the ability of cells to grow normally after 9–14 days exposure to gabaculine appears to be related to changes

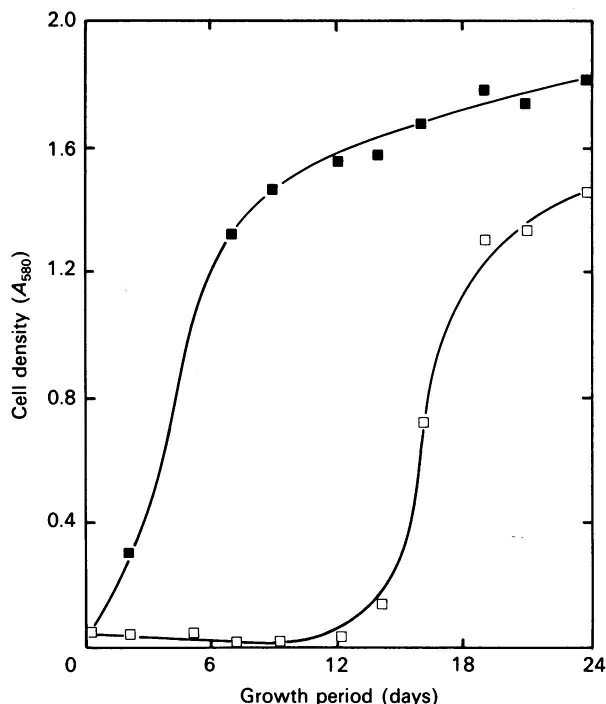

Fig. 4. Inhibition of heterotrophic growth of *C. caldarium* by gabaculine

III-D-2 cells were prepared for heterotrophic growth as described in the Experimental section and incubated in the presence (□) and absence (■) of gabaculine (10 μM) for up to 14 days. Cell density was estimated by absorbance due to light scattering at 580 nm.

in the cells themselves, rather than de-activation of the gabaculine. This was further supported by the fact that medium, collected from cultures in which cells had grown after a prolonged gabaculine-induced lag period, was still capable of inhibiting heterotrophic growth in a culture freshly inoculated with untreated cells.

General discussion

Our results show that gabaculine is capable of inhibiting not only chlorophyll synthesis, but also phycocyanin and hence, haem synthesis in *C. caldarium* both in the light and, in the case of mutant CPD, in the dark. The observation that this inhibition can be overcome by addition of exogenous ALA is strong evidence that gabaculine is acting primarily on the synthesis of ALA. It has been clearly demonstrated that, for organisms known to contain ALA synthase, gabaculine does not inhibit this enzyme up to concentrations of 5 mM, considerably in excess of those used in this work (Corriveau & Beale, 1986; Werck-Reichhart *et al.*, 1988). Our results, therefore, strongly imply that the 5-carbon pathway is the normal route of ALA formation for both chlorophyll and phycocyanin via haem. Heterotrophic growth was also inhibited by gabaculine and, since normal growth is restored by addition of exogenous ALA (see the Results section), it seems probable that gabaculine is acting to inhibit cytochrome formation, suggesting that the ALA destined for cytochrome haem is also normally produced by the 5-carbon pathway. These conclusions are consistent with previously published reports of the involvement of the 5-carbon pathway in haem synthesis in *C. caldarium* using radiolabelling techniques (Jurgenson *et al.*, 1976; Troxler & Offner, 1979; Weinstein & Beale, 1984).

It is intriguing that inhibition of heterotrophic growth by gabaculine was overcome in cells after prolonged exposure and that the phenomenon appears to be due to cell adaptation rather than medium detoxification. Adaptation to heterotrophic growth in the presence of gabaculine has not been previously observed, though small levels of haem-synthesizing activity, apparently resistant to gabaculine, have been reported (Gardner & Gorton, 1985; Werck-Reichhart *et al.*, 1988), and u.v.-induced gabaculine-resistant mutants of *Chlamydomonas reinhardtii* are now known (Kahn & Kannangara, 1987). In this case, the mutation is believed to involve the enzyme glutamate-1-semialdehyde aminotransferase, the activity of which was up to 4-fold higher in gabaculine-resistant cells than in the wild-type. The present data do not permit evaluation of the mechanisms of adaptation in *C. caldarium* and further work is being undertaken to determine whether this is also due to changes in the activity of this enzyme.

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