Biochem. J. (1988) 249, 473–480 (Printed in Great Britain)

Haem synthesis during cytochrome *P*-450 induction in higher plants

5-Aminolaevulinic acid synthesis through a five-carbon pathway in *Helianthus tuberosus* tuber tissues aged in the dark

Danièle WERCK-REICHHART,*‡ Owen T. G. JONES† and Francis DURST*

*Laboratoire d'Enzymologie Cellulaire et Moléculaire (CNRS UA 1182), Institut de Botanique, 28, Rue Geothe, F-67000 Strasbourg, France, and †Department of Biochemistry, The Medical School, University of Bristol, Bristol BS8 1TD, U.K.

Chlorophyll and haem synthesis in illuminated Jerusalem artichoke tuber tissues were very efficiently inhibited by gabaculine (3-amino-2,3-dihydrobenzoic acid). This inhibition seems to be due specifically to a blockade of the pathway for 5-aminolaevulinate biosynthesis which uses glutamate as a substrate (the so-called C_5 pathway) since we could not detect any inhibition of protein synthesis in the treated tissues and there was no effect of gabaculine on the glycine-dependent yeast 5-aminolaevulinate synthase used as a model. In dark-aged artichoke tissues, gabaculine also effectively blocked cytochrome *P*-450 induction, peroxidase activity and 5-aminolaevulinic acid synthesis, thus suggesting the involvement of a C_5 pathway in cytoplasmic and microsomal haemoprotein synthesis in this higher plant. Allylglycine and (2-aminoethyloxyvinyl)glycine, two olefinic glycine analogues which are potential suicide inhibitors of pyridoxal phosphate enzymes, were also demonstrated to be effective blockers of chlorophyll synthesis in artichoke tuber and *Euglena* cells exposed to light.

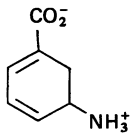
INTRODUCTION

In greening plants, 5-aminolaevulinic acid (5-ALA) for chlorophyll and haem formation is synthesized from the five-carbon skeleton of glutamate by a sequence of at least three choloroplastic enzymes plus one chloroplastic tRNA (the C_5 pathway) (Castelfranco & Beale, 1983; Porra, 1986; Schön *et al.*, 1986). Until now it has, however, been impossible to completely exclude the existence also of a mitochondrial pathway, similar to the animal, bacterial or yeast 5-ALA synthase (i.e. catalysing condensation of succinyl-CoA with glycine), which might be active in the dark and/or involved in the synthesis of cytoplasmic or mitochondrial haemoproteins.

Co-existence of the two pathways has been demonstrated in the photosynthetic phytoflagellate *Euglena gracilis* (Weinstein & Beale, 1983). The C_5 pathway synthetizes porphyrins in the light whereas 5-ALA-synthase is preponderant in dark-grown cells. In illuminated cells, 5-ALA-synthase activity seems to be repressed by a product derived from the five-carbon route (Corriveau & Beale, 1986).

In higher plants, some reports have suggested that 5-ALA synthase may be active in dark-grown leaves (Meller & Gassman, 1982) or chlorophyll-free tissues, like soybean callus (Wider de Xifra *et al.*, 1978) or potato tuber (Ramaswamy & Nair, 1976; Harel, 1978). But more recent data from Chibbar & Van Huystee (1983) show that the haem moiety for cationic peroxidase from cultured peanut cells is derived mainly, if not exclusively, from glutamate. A recent report by Schneegurt & Beale (1986) also indicates that haem *a* in etiolated maize epicotyls is probably formed only via the five-carbon pathway.

Jerusalem artichoke tuber is an achlorophyllian plant tissue in which synthesis *de novo* of microsomal cytochromes *P*-450 and b_5 is induced in the dark by wounding and ageing in water (Benveniste *et al.*, 1977); this induction is amplified in the presence of chemicals like Mn^{2+}, phenobarbital, ethanol, herbicides or clofibrate (Reichhart *et al.*, 1980; Adelé *et al.*, 1981; Salaün *et al.*, 1986). The following experiments were intended to determine which pathway was responsible for the synthesis of the haem moiety of these microsomal cytochromes during induction.



(I)

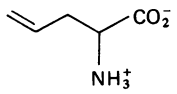
Gabaculine (I), a natural product first isolated from *Streptomyces toyocaenis* (Kobayashi *et al.*, 1976), is a potent irreversible inhibitor of different types of pyridoxal phosphate (PLP)-linked transaminases (Rando, 1977; Burnett *et al.*, 1980; Soper & Manning, 1982). Metabolism of gabaculine by these enzymes leads to specific derivatization of the coenzyme by spontaneous aromatizing to a *m*-anthranilyl-PLP adduct. Recently, gabaculine was also described as a potent inhibitor of chlorophyll synthesis in higher plants and algae (Flint,

Abbreviations used: 5-ALA, 5-aminolaevulinic acid; CA4H, *trans*-cinnamic acid 4-hydroxylase, reduced flavoprotein:oxygen oxidoreductase (4-hydroxylating), EC 1.14.14.1; AVG, (2-aminoethyloxyvinyl)glycine; PLP, pyridoxal phosphate.

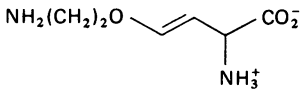
‡ To whom correspondence should be addressed.

1984). This inhibition can be overcome by supplying 5-ALA, and is accompanied by a significant accumulation of glutamate 1-semialdehyde. A more detailed study by Kannangara & Schouboe (1985) shows that the glutamate 1-semialdehyde aminotransferase step of the C_5 pathway is irreversibly inhibited, although the PLP-dependence of this aminotransferase has not yet been demonstrated.

We report here on the effects of gabaculine on chlorophyll and haemoprotein synthesis in Jerusalem artichoke tissues aged in the light and in the dark. The effects of two other mechanism-based inhibitors of PLP enzymes, allylglycine (**II**) and AVG (**III**), on greening plant tissues are also described.



(II)



(III)

MATERIALS AND METHODS

Chemicals

3-Amino-2,3-dihydrobenzoic acid hydrochloride (gabaculine), DL-*C*-allylglycine and L-α-(2-aminoethoxyvinyl)glycine hydrochloride (AVG) were obtained from Fluka, Buchs, Switzerland. Laevulinic acid (free acid), ATP, PLP, NADPH and CoASH were from Sigma, and guaiacol was from Merck. [3-^{14}C]Cinnamic acid was obtained from CEA, Saclay, France and L-[^{35}S]-methionine from Amersham International, Amersham, Bucks., U.K.

Plant material

Jerusalem artichoke (*Helianthus tuberosus* L., var. Blanc Commun) tubers, grown locally, were stored in polyethylene bags at 4 °C in the dark.

For ageing experiments, tubers were sliced (1 mm thick), washed with cold tap water for 30 min and rinsed twice with distilled water before incubation in distilled water containing, when necessary, added inducer or inhibitory compounds. The pH of the ageing solutions, unless otherwise stated, was adjusted to 7 with NaOH or HCl. The incubation medium was kept at room temperature, in the dark, and vigorously bubbled with a stream of hydrated, filtered air.

Euglena and yeast cell cultures

Euglena gracilis Klebs strain Z Pringsheim was grown as described by Beale *et al.* (1981). Cell population densities were determined with a Coulter Counter (Coultronics France). Cells were disrupted by two 20 s sonication periods (Ultrasons Anemasse apparatus used at 20% of its power). *Saccharomyces cerevisiae* cells were grown as described by Volland & Felix (1984).

Preparation of microsomes

Microsomal pellets were prepared by homogenizing aged tuber slices at 4 °C with an Ultra-Turrax homogenizer in a 2 vol. of 0.1 M-sodium phosphate (pH 7.4) containing 15 mM-2-mercaptoethanol, 1 mM-EDTA and 0.2% bovine serum albumin. The homogenate was filtered through four layers of cheesecloth and centrifuged for 15 min at 10000 *g*. Microsomes were sedimented by centrifugation at 100000 *g* for 60 min and the resulting pellets were resuspended in 0.1 M-sodium phosphate buffer (pH 7.4) containing 30% (v/v) glycerol/1.5 mM-2-mercaptoethanol in a Potter–Elvehjem homogenizer and stored at −20 °C.

Chlorophyll and haem determination

Samples (1 g) of aged tissue, washed with distilled water, were ground in 80% (v/v) acetone containing 100 μM-KOH in an Ultra-Turrax homogenizer. The resulting suspension was centrifuged for 5 min at 5000 *g*. Chlorophyll contents of the supernatants were estimated according to Arnon (1949). For haem determinations, pellets, after acetone extraction, were dried under vacuum and dissolved in 2 ml of 0.2 M-KOH containing 3% sodium cholate. Pyridine (1 ml) was added and the samples centrifuged for 5 min at 5000 *g*. Dithionite reduced-minus-oxidized difference spectra of the pyridine haemochrome complex in the supernatant were recorded between 600 and 500 nm. Haem was calculated from the absorbance maximum at 556 nm using an absorption coefficient of 20.7 $mM^{-1} \cdot cm^{-1}$ (Castelfranco & Jones, 1975).

The presence of KOH in acetone used for extraction was essential for accurate haem determination in uninduced tissues. In tissues aged on $MnCl_2$ solutions, haem determination was impossible (by fluorescence or the pyridine-haemochrome assay), due to the presence of an excess of interfering phenolic compounds.

5-ALA accumulation

Rates of 5-ALA synthesis were determined *in vivo* by the method of Beale (1970). Laevulinic acid, a competitive inhibitor of 5-ALA dehydratase (Nandi & Shemin, 1968), blocks utilization of 5-ALA by the plant tissues. 5-ALA, accumulated in the presence of the inhibitor, can be extracted and quantitated. Preliminary experiments showed that 8 h incubation in the presence of 10 mM-laevulinic acid provided optimal conditions for the estimation of 5-ALA accumulation rates in ageing artichoke tissues. Laevulinic acid, adjusted to a pH of 6.5 with NaOH, was added directly to the ageing medium. After 8 h, 6 g samples of washed tissue were ground at 4 °C in 10 ml of Tris/HCl buffer (0.05 M, pH 8.5) containing 0.01 M-$MgCl_2$ and 15 mM-2-mercaptoethanol using an Ultra-Turrax homogenizer. The extract was filtered thorugh four layers of cheesecloth. The filtered extract (1 ml) was added to 250 μl of cold 20% trichloroacetic acid, mixed and centrifuged for 5 min at 8000 *g*. Supernatant (1 ml) was then adjusted to pH 4.6 with about 500 μl of 1 M-sodium acetate, mixed with 40 μl of acetylacetone and heated for 20 min at 95 °C to form a pyrrole derivative (Mauzerall & Granick, 1956). After cooling, 1.5 ml of Ehrlich reagent was added and the mixture was centrifuged for 5 min at 5000 *g*. The absorbance of the clear supernatant was read at 553 nm 10 min after addition of the reagent. Results were calculated from a calibration curve obtained by adding known amounts of 5-ALA to a similar plant extract.

Yeast 5-ALA synthase measurements

A crude yeast extract was used for 5-ALA synthase assays, according to Volland & Felix (1984), using chemically synthesized succinyl-CoA (Simon & Shemin, 1953) as substrate. In some assays PLP was omitted or its concentration reduced to avoid direct interaction between exogenous PLP and inhibitors.

Microsomal enzyme determinations

trans-Cinnamic acid 4-hydroxylase (CA4H) was assayed as previously described (Reichhart *et al.*, 1980). However, incubation times longer than 10 min, and preincubation of the microsomes in the presence of NADPH, were avoided in order to limit auto-catalysed inactivation of the enzyme.

NADPH–cytochrome *c* reductase was measured as described by Benveniste *et al.* (1986), with addition of 5 mM-KCN to the reaction mixture to minimize cytochrome *c* reoxidation by contaminating mitochondria.

Cytochrome b_5 concentrations were determined from the $Na_2S_2O_4$-reduced-minus-oxidized difference spectra, using an absorption coefficient of 171 $mM^{-1} \cdot cm^{-1}$ for $A_{424-409}$ (Omura & Takesue, 1970).

Contents of cytochrome *P*-450 were calculated from $A_{450-490}$ in a dithionite-reduced-CO-minus-reduced difference spectrum with an absorption coefficient, determined by Omura & Sato (1964), of 91 $mM^{-1} \cdot cm^{-1}$.

Total haem content of microsomes was determined at 557 nm from the peak intensity in reduced-minus-oxidized spectra. An absorption coefficient of 20 $mM^{-1} \cdot cm^{-1}$ was used (Bendall *et al.*, 1971).

Other assays

Peroxidase activity was determined (using guaiacol as a substrate) by the method of Pütter (1974), in a crude plant extract prepared as follows: 5 g samples of artichoke tissue were extracted at 4 °C in 10 ml of sodium phosphate buffer (0.1 M, pH 7.4) using an Ultra-Turrax homogenizer. The homogenate was filtered and assayed immediately. The change in absorbance at 436 nm was followed in the presence of 100 μM-H_2O_2 and 300 μM-guaiacol. An absorption coefficient of 25.5 $mM^{-1} \cdot cm^{-1}$ was used for calculations.

Proteins were estimated by the method of Schacterle & Pollack (1973) using bovine serum albumin as a standard.

Respiration measurements were made on calibrated (1 mm thick, 12 mm diameter) disks of aged tuber tissue by polarography using a Clark oxygen electrode.

[^{35}S]Methionine incorporation into proteins was determined as follows: 5 g samples of aged slices were transferred to Erlenmeyer flasks with 25 ml of ageing medium containing 370 KBq of [^{35}S]methionine (sp. activity 19.5–41 TBq/mmol). After 2 h incubation in a rotatory agitator, tissues were washed, dried and homogenized at 4 °C in 10 ml of phosphate buffer (0.1 M, pH 7.4) containing 15 mM-2-mercaptoethanol. Cold 100% trichloroacetic acid was added to the filtered extract to obtain a final 5% (v/v) concentration. After 10 min on ice, precipitated proteins were pelleted by centrifugation (5 min at 3000 *g*) and washed twice with 5% trichloroacetic acid. The final pellet was then dissolved in 5 ml of 0.1 M-NaOH. Protein content and radioactivity were measured in this solubilized pellet. To measure [^{35}S]methionine incorporation into *Euglena* proteins, approx. 10^6 cells were given a 2 h pulse (approx. 1.85 MBq in 100 ml of medium).

RESULTS

Effect of gabaculine on artichoke tissues exposed to light

Fig. 1 shows that 50 μM-gabaculine was sufficient to completely suppress chlorophyll synthesis in artichoke tissues aged 4 days in the light. This inhibition was accompanied by a decrease in carotenoid formation. Gabaculine proved much less effective if given at pH 4 (dissolved in distilled water) than at pH 7. Measured P_i of gabaculine is about 6.8. This probably means that the neutral or negatively-charged form of the molecule penetrates more efficiently into the cells. Gabaculine inhibition *in vivo* might be further influenced by intracellular and intrachloroplastic pH. It should be noted that the highest inactivation rate for γ-butyric acid transaminase *in vitro* by gabaculine was obtained at pH 8.1 by Rando (1977).

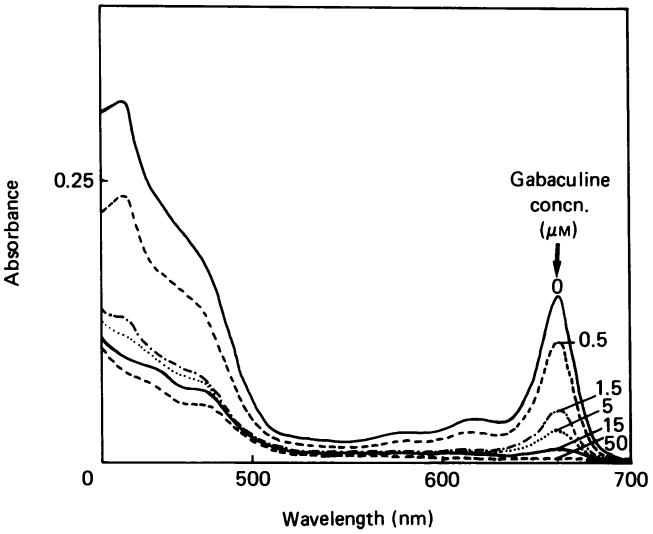


Fig. 1. Effect of gabaculine on chlorophyll synthesis in artichoke slices aged in the light

Jerusalem artichoke tuber slices were aged 4 days in the light in water containing 0–50 μM-gabaculine. Chlorophyll extractions and measurements were as described in the Materials and methods section.

Haem content was less reduced than chlorophyll content: 50 μM-gabaculine decreased the haem content by only 84% (Table 1). This reflects both the light-induced synthesis of chloroplastic haem and the turnover of extrachloroplastic haemoproteins (intact tuber tissue already contains significant amounts of haem).

Concentrations of gabaculine as high as 150 μM did not affect respiration of artichoke tissues. This could be explained either by the existence of a mitochondrial gabaculine-insensitive 5-ALA synthase, or by a very slow turnover of respiratory cytochromes. Indeed, turnover rates as low as 1300 h were reported for the respiratory cytochromes in rat liver (Granick & Beale, 1978).

Inhibition of chlorophyll synthesis by other mechanism-based inactivators for PLP enzymes

Many amino acids bearing olefinic functional groups inhibit PLP enzymes more or less specifically (Walsh, 1982). Among them, allylglycine was assumed to be a suicide inhibitor for glutamate decarboxylase, whereas AVG was an inhibitor of a PLP enzyme involved in production of the fruit-ripening hormone, ethylene. We tested these commercially available compounds for their ability to inhibit chlorophyll synthesis in illuminated tuber tissues, since we were looking for possible specific

Table 1. Effect of gabaculine on haem content and respiration of illuminated artichoke tissues

Measurements were made on the same tissues as those described in Fig. 1. Haem was estimated from a pyridine haemochrome assay; respiration of calibrated disks of tuber tissues was measured with a Clark electrode. Values are means of duplicate experiments. Haem content after 4 days ageing in the dark: 522±43 pmol/g fresh wt.; in freshly sliced tissues: 806±106 pmol/g fresh wt.

Gabaculine concentration (μM) ...	0	0.5	1.5	5	15	50	150
Haem content (pmol/g fresh wt.)	1956±215	1757±254	1050±37	739±159	652±0	304±14	195±21
Decrease in haem content (%)	0	9	46	62	67	84	90
Respiration (μl of O_2/min per disk)	1.275±0.005	1.250±0.030	1.350±0.025	1.220±0.020	1.150±0.050	1.440±0.025	1.290±0.020

Table 2. Effect of allylglycine and AVG on chlorophyll synthesis in illuminated artichoke tissues

Tuber slices were aged in water containing different inhibitor concentrations. pH was maintained at 7. Chlorophyll was measured after 4 days exposure to 2500 lux white light. Values are means of duplicate experiments.

Allylglycine concentration (μM) ...	0	1	10	100	1000	10000
Chlorophyll content (mg/g fresh wt)	18±14	19.5±3.2	21.7±0.9	15.05±1.45	12.2±2.7	4.4±0.6
Change in chlorophyll synthesis (%)	0	+8	+20	−16	−32	−76

AVG concentration (μM) ...	0.01	0.1	1	10	100
Chlorophyll content (mg/g fresh wt.)	14.25±1.45	17.35±0.05	18.85±3.55	22.7±2.3	9.7±0.4	0.8±0.4
Change in chlorophyll synthesis (%)	0	+22	+32	+59	−32	−94

glycine-like inhibitors for the mitochondrial 5-ALA synthase pathway. Both compounds inhibited the synthesis of chlorophyll (Table 2). A 94% inhibition was achieved by 100 μM-AVG, whereas only a 76% chlorophyll decrease was observed with 10 mM-allylglycine. This chlorophyll synthesis inhibition was accompanied by a decrease in haem content in proportions similar to those obtained with gabaculine. No respiration inhibition was observed.

At very low concentrations both compounds produced a stimulation of chlorophyll synthesis. This stimulation was reproducible and observed also with very low gabaculine concentrations ($\leqslant 0.1$ μM).

The effect of both inhibitors was also tested on the greening of a culture of *Euglena*. Allylglycine was inhibitory at mM concentrations, but AVG was ineffective (or caused a slight stimulation) at the highest concentration tested (200 μM). The permeability of *Euglena* cells to AVG was not investigated.

Effect of gabaculine on protein synthesis

Many PLP-dependent transminases are involved in amino acid biosynthesis. For this reason it was important to check if gabaculine was acting through protein synthesis inhibition. [^{35}S]Methionine incorporation into trichloroacetic acid-precipitable proteins was taken as an index of protein synthesis efficiency. After 24 or 48 h ageing in 1, 10 or 100 μM-gabaculine, artichoke slices were given a 2 h [^{35}S]methionine pulse: absorption of the radioactivity by the tissues, whole protein content and ^{35}S incorporation into these proteins were measured. A slight decrease in [^{35}S]methionine absorption by the tissues especially at low gabaculine concentrations (1 μM) was observed. However, whole protein content or ^{35}S incorporation into trichloroacetic acid-precipitable proteins were not significantly modified. Similarly, no inhibition of protein synthesis by gabaculine in greening *Euglena gracilis* was detected. (In *Euglena* grown for 3 days in the dark in the presence of 100 μM-gabaculine, 50% enhancement of ^{35}S incorporation into proteins was recorded).

Effect of mechanism-based inactivators of PLP enzymes on yeast 5-ALA-synthase activity *in vitro*

No 5-ALA synthase activity could be detected using colorimetric (Volland & Felix, 1984) or radiochemical (Strand *et al.*, 1972) techniques, either in crude extracts or in purified mitochondria prepared from artichoke

tissues induced by ageing, phenobarbital or Mn^{2+} pretreatment. In our hands, the detection limits of these methods was approx. 0.2 nmol of 5-ALA formed per assay. A crude enzyme preparation from yeast was therefore used to test the effect of gabaculine and other inhibitors on the PLP-dependent mitochondrial 5-ALA synthase [succinyl-CoA:glycine *C*-succinyl transferase (decarboxylating)]. Gabaculine, allylglycine and AVG concentrations as high as 5 mM produced no inhibition of yeast 5-ALA synthase activity when added in the enzymatic test. Moreover, preincubation of the enzyme preparation with 10 mM solutions of inhibitors for 20–60 min at 30 °C or for 60 min at 4 °C before activity measurements, did not cause any inhibition.

Inhibition by gabaculine of cytochrome *P*-450 and ALA synthesis in the dark

Fig. 2 shows the effect of increasing gabaculine concentrations (0–250 μM) on cytochrome *P*-450 content and CA4H activity in Jerusalem artichoke tissues induced by 48 h ageing in Mn solution in the dark. CO-binding spectra and CA4H activity show parallel inhibition.

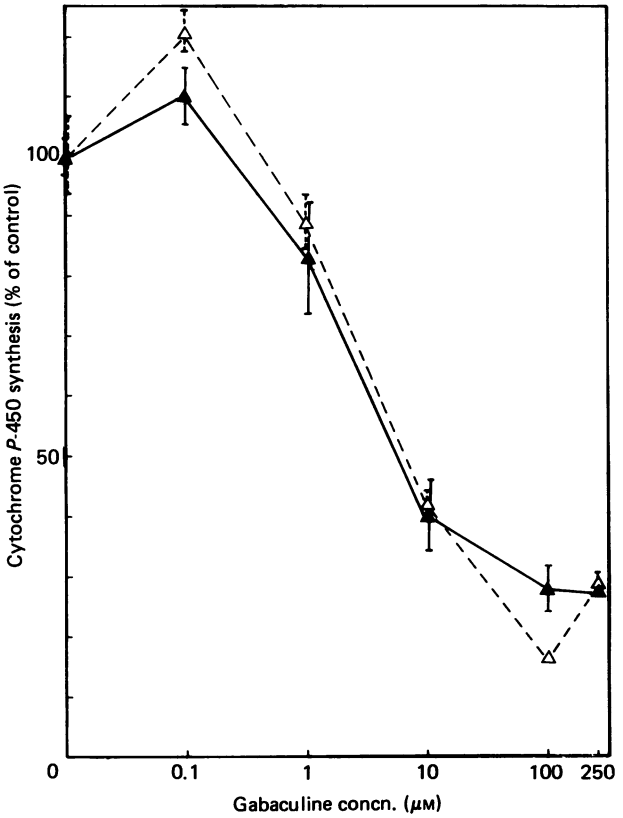


Fig. 2. Effect of gabaculine on cytochrome *P*-450 induction in artichoke tissues aged in the dark

Artichoke tissues were aged 48 h in the dark in water containing 25 mM-$MnCl_2$. Microsome preparation, cytochrome and CA4H measurements were as described in the Materials and methods section. ▲, CO-binding spectrum ($n = 2$, 100% = 102±3 pmol/mg of protein); △, CA4H activity ($n = 3$, 100% = 72±5 pkat/mg of protein).

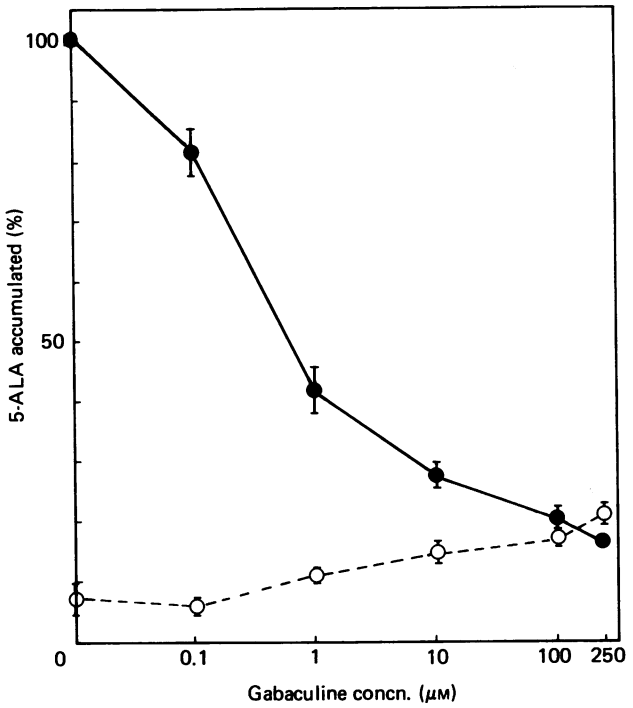


Fig. 3. ALA accumulation in artichoke tissues aged in the dark in the presence of different gabaculine concentrations

Tissues were aged 20 h in water containing different gabaculine concentrations. ALA content was determined before the addition of 10 mM-laevulinate, pH 6.5 (○) and then measured again after 8 h further incubation (●). ALA extractions and measurements were as described in the Materials and methods section (100% = 1.807± 0.016 nmol of ALA/mg of protein.)

Maximum (i.e. approx. 75–80%) inhibition was attained with 100 μM-gabaculine. With very low gabaculine concentrations (0.1 μM) a slight stimulation (20%) of cytochrome *P*-450 synthesis was, however, observed.

This cytochrome *P*-450 inhibition appears to be due to inhibition of haem synthesis since a concomitant inhibition of ALA synthesis was observed in tissues aged in the presence of gabaculine (Fig. 3). ALA accumulation rates were measured between 20 and 28 h ageing, since we showed in a preliminary experiment that this was the period of maximum ALA synthesis rates in tissues aged in water or in $MnCl_2$ solutions. No stimulation of ALA synthesis was observed in tissues incubated in 0.1 μM-gabaculine.

Measurements of ALA content of the gabaculine-treated tissues after 20 h ageing in the absence of laevulinic acid (Fig. 3) show an apparent increase of the ALA pool. A similar observation was made previously by Kannangara & Schouboe (1985) in illuminated barley leaves exposed to gabaculine. These authors stated that glutamate 1-semialdehyde could interfere with ALA in the Ehrlich colorimetric test. Thus, as we made no attempt to separate these compounds, the apparent increase of the ALA pool in our experiments could be due to the interference of the accumulating glutamate 1-semialdehyde when glutamate 1-semialdehyde aminotransferase is blocked by gabaculine.

Table 3. Components of the microsomal electron-transport chains in induced artichoke tissues treated with gabaculine

Activities and cytochrome content were measured in microsomes prepared from freshly sliced artichoke tissues (control) and from tissues aged in water containing 25 mM-$MnCl_2$ for 72 h in the dark and in the presence of indicated gabaculine concentrations.

Gabaculine concentration (μM) ...	Control	0	0.1	1	10	100	250
Cytochrome *P*-450* (pmol/mg of protein)	11±0.9	94±13	160±8	72±7	42±2.6	24±2.5	19±2.7
CA4H* (pkat/mg of protein)	0.43±5	53±2.4	82±4.6	54±3	33±1.8	10.8±1.2	11.7±1.2
Cytochrome b_5† (pmol/mg of protein)	149±6.6	649±20	544±30	309±1	295±20	244±14	244±4.5
NADPH–cytochrome *c* reductase† (pkat/mg of protein)	72±2.5	3123±172	2919±101	2596±51	2755±205	2277±42	2210±14

* $n = 3$; † $n = 2$.

Table 4. Haem and haemoprotein content of gabaculine-treated artichoke tissues aged in the dark

Artichoke tissues were aged 4 days in distilled water (pH 7) containing gabaculine at indicated concentrations. Haem content was measured in 1 g of acetone-extracted tissues, guaiacol peroxidase activity in a crude extract prepared in the absence of SH compounds and cytochromes *P*-450 and b_5 in microsomes. Guaiacol peroxidase activity in uninduced freshly sliced tubers was 197±2.5 pkat/mg of protein. Haem content was 806±106 pmol/g fresh wt., and other control values are the same as those in Table 3. Values are means of duplicate experiments. N.d., not detectable.

Gabaculine concentration (μM) ...	0	0.3	1	3	10	30	100
Total haem content (pmol/g fresh wt.)	522	291	378	144	144	72	57
Guaiacol peroxidase (pkat/mg of protein)	2416±102	1882±37	970±16	283±4	256±3	210±6.5	181±1.5
Cytochrome *P*-450 (pmol/mg of protein)	41.7±0	42.4±1.5	42.8±1.4	19.8±1.1	10.9±0.6	5.7±0	N.d.
Cytochrome b_5 (pmol/mg of protein)	178±3	231±8	185±2	174±15	130±13	129±19	92±16

Comparative effects of gabaculine on microsomal electron transport components, peroxidase and whole haem content in the dark

Maximal induction of the microsomal mixed-function-oxidase system was obtained after 72 h ageing of artichoke slices in the dark in a 26 mM-$MnCl_2$ solution. Table 3 compares the effects of gabaculine on cytochrome *P*-450 and its capacity to hydroxylate cinnamic acid, cytochrome b_5 content and NADPH–cytochrome *c* reductase activity in Mn-induced tissues. Gabaculine (100 μM) produced approx. 85% inhibition of cytochrome *P*-450 and of cytochrome b_5 biosynthesis, whereas NADPH–cytochrome *c* reductase was only slight affected (28% inhibition). Thus, blockade of the five-carbon pathway for haem production results in a strong inhibition of microsomal haemoprotein synthesis in plant tuber tissues kept in the dark. It must be stressed that, in this experiment, only cytochrome *P*-450 and its hydroxylating activity were increased at the lowest concentration of gabaculine (0.1 μM) (+180% and +150% respectively), while cytochrome b_5 was inhibited by 20%.

Measurements of haem content and peroxidase activity of artichoke tissues aged on Mn are not possible because the very high concentrations of phenolics that accumulate in Mn-treated tissues prevent accurate spectroscopic haem measurements and also because of the strong interference by Mn of tetraguaiacoquinone formation from guaiacol by peroxidase. This led us to measure haem content and peroxidase activity, as well as cytochromes *P*-450 and b_5 contents in tissues aged 4 days in distilled water in the dark (Table 4). In such long-term experiments, results not only express enzyme biosynthesis but also reflect turnover rates, since induction maxima for water-aged tissues are obtained between 24 and 48 h.

Haem content was much lower in dark-aged tissues than in illuminated slices (cf. Table 1); however, similar inhibitions were observed in both cases, using the same gabaculine concentrations. Peroxidase appeared highly inducible (> 10-fold) by ageing and its activity was very efficiently decreased by gabaculine. An inhibition of

peroxidase activity of 91% was observed in tissues aged on 30 μM-gabaculine. At the same time, total haem or cytochrome *P*-450 content decreased by 86% and cytochrome b_5 by only 28%.

DISCUSSION

Our results show that gabaculine appears to be a useful, efficient (at micromolar concns.) and selective inhibitor of tetrapyrrole biosynthesis in plant tissues which are normally non-chlorophyllous. Chlorophyll as well as haem synthesis was inhibited in illuminated root tissues, but haem synthesis inhibition was also observed in the dark. This inhibition is not attributable to an effect on protein synthesis since (1) [^{35}S]methionine incorporation into treated tissues was not appreciably modified and (2) formation of non-haemoprotein enzymes (NADPH–cytochrome *c* reductase for example) was only slightly affected.

Kannangara & Schouboe (1985) showed that gabaculine blocks 5-ALA formation from glutamate by inactivating glutamate 1-semialdehyde aminotransferase in etiolated barley exposed to light. It is confirmed here that, even in tuber tissues kept in the dark, gabaculine acts as an inhibitor of 5-ALA synthesis. It is unlikely that blockade of 5-ALA formation is due to the inhibition of a mitochondrial 5-ALA synthase as the yeast enzyme appears completely insensitive to gabaculine inhibition.

Jerusalem artichoke tuber is a white plant tissue containing plastids in their most rudimentary form (proplastids): 3 days of intense illumination is necessary to allow plastid differentiation and visible chlorophyll synthesis. These tissues appear to synthesize haem mainly through a five-carbon glutamate-dependent pathway, even when kept in the dark in conditions allowing no chloroplast differentiation. This implies that the five-carbon pathway for 5-ALA synthesis must be one of the enzymatic systems kept active and inducible independently of light, in rudimentary, chlorophyll-free forms of plastids. However, the existence of a glutamate-dependent pathway for 5-ALA formation in another cellular compartment cannot, at present, be excluded. It is noteworthy that Chibbar & Van Huystee (1986) showed that, even if the haem moiety of cationic peroxidase of peanut cells is derived from glutamate, most of this haem originates from mitochondria. This might, however, only mean that the final steps for haem synthesis occur in the mitochondria.

We tested allylglycine and AVG as potential selective inhibitors of the mitochondrial 5-ALA synthase, as they were both olefinic glycine derivatives. Neither of these compounds had any inhibitory effect on yeast 5-ALA synthase activity. However, both efficiently inhibited greening of illuminated artichoke tuber tissues: AVG was more effective (at μM concentrations) than allylglycine (active at mM concentrations). This would confirm the PLP-dependence of the glutamate 1-semialdehyde aminotransferase, but it also suggests that PLP is fairly accessible in the active site. In contrast, 5-ALA synthase appears to have a very restricted pocket for substrate fixation.

An increase in chlorophyll and haem contents was repeatedly found in tissues treated with very low concentrations of all inhibitors tested. It is unclear if this results from increased biosynthesis of tetrapyrrole due to a feedback mechanism, or from a slower breakdown.

Gabaculine, a specific inhibitor of the five-carbon pathway of 5-ALA synthesis in artichoke tissues, blocks both cytochrome *P*-450 and cytochrome b_5 induction, as well as peroxidase induction when tuber slices are aged in the dark. This suggests that the haem necessary for the synthesis of the most abundant cytoplasmic and microsomal haemoproteins is made mainly by a glutamate-dependent pathway in tuber tissues. These data corroborate the results of Chibbar & Van Huystee (1983) concerning the peroxidase of peanut cells. We cannot yet exclude the existence of a minor 5-ALA-synthase-type pathway, since 10–20% of haem synthesis appears resistant to gabaculine in our experiments (see Fig. 2, Tables 3 and 4). But this apparent resistance to gabaculine inhibition could be due to haem linked to (or released from) haemoproteins slowly turning over (e.g. cytochrome b_5, mitochondrial cytochromes).

Sensitivity of the different haemoproteins to gabaculine inhibition gives some information about their relative stability. Peroxidase, which was affected to a greater extent and by lower gabaculine concentrations, appears to have the most rapid turnover. As in animals, cytochrome *P*-450 seems to have a much shorter half-life in higher plants than cytochrome b_5: half-lives of 7–34 h were described for the haem moiety of animal cytochrome *P*-450 (Gasser *et al.*, 1982; Sadano & Omura, 1983*a*; Parkinson *et al.*, 1983), whereas the estimated half-life for cytochrome b_5 was about 40 h (Sadano & Omura, 1983*b*).

At very low concentrations (< 1 μM), gabaculine produces an increased cytochrome *P*-450 induction in ageing tuber tissues. This was true even for concentrations high enough to partially inhibit the synthesis of haem and inhibit 5-ALA accumulation.

Mechanisms of this cytochrome *P*-450 increase remain unclear, but it appears that cytochrome *P*-450 may be increased in conditions of limited haem availability. Thus, cytochrome *P*-450 apoprotein seems to be able to trap haem efficiently (through high affinity or cellular compartmentation) even when its synthesis is restricted.

Artichoke cytochrome *P*-450 has now been purified (Gabriac *et al.*, 1985). Use of specific antibodies will help in understanding better the mechanisms of induction at the apoprotein level. It is important to emphasize that higher plants, unlike animals, yeasts or bacteria, appear to synthesize haem for cytochrome *P*-450 induction through a glutamate-dependent pathway, and not via a mitochondrial 5-ALA synthase. This is likely to have significant consequences for the regulation mechanisms concerned.

We thank Dr. C. Volland of the Laboratoire de Biochimie des Porphyrines, Université Paris VII for providing yeast ALA synthase and for her help with enzyme measurements. Dr. W. T. Griffiths, Dr. P. M. Wood and Dr. P. A. Castelfranco are gratefully acknowledged for helpful discussion and friendly encouragement as this work was initiated. D.W.-R. is grateful to the British Council for a postdoctoral bursary allowing her stay at Bristol University.

REFERENCES

Adelé, P., Reichhart, D., Salaün, J. P., Benveniste, I. & Durst, F. (1981) Plant Sci. Lett. **22**, 39–46

Arnon, D. I. (1949) Plant Physiol. **24**, 1–15

Beale, S. I. (1970) Plant Physiol. **45**, 504–506

Beale, S. I., Foley, T. & Dzelzkalns, V. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 1666–1669

Bendall, D. J., Davenport, H. E. & Hill, R. (1971) Methods Enzymol. **23**, 327–344
Benveniste, I., Salaün, J. P. & Durst, F. (1977) Phytochemistry **16**, 69–73
Benveniste, I., Gabriac, B. & Durst, F. (1986) Biochem. J. **235**, 365–373
Burnett, G., Yonaha, K., Toyama, S., Soda, K. & Walsh, C. (1980) J. Biol. Chem. **255**, 428–432
Castelfranco, P. A. & Jones, O. T. G. (1975) Plant Physiol. **55**, 485–490
Castelfranco, P. A. & Beale, S. I. (1983) Annu. Rev. Plant Physiol **34**, 241–278
Chibbar, R. M. & Van Huystee, R. B. (1983) Phytochemistry **22**, 1721–1723
Chibbar, R. M. & Van Huystee, R. B. (1986) Phytochemistry **25**, 585–587
Corriveau, J. L. & Beale, S. I. (1986) Plant Physiol. Suppl. **80**, 52
Flint, D. H. (1984) Plant Physiol. Suppl. **75**, 170
Gabriac, B., Benveniste, I. & Durst, F. (1985) C.R. Hebd. Seances Acad. Sci. **301**, 753–758
Gasser, R., Hauri, H. P. & Meyer, U. A. (1982) FEBS Lett. **147**, 239–242
Granick, S. & Beale, S. I. (1978) in Advances in Enzymology (Meister, A., ed.), vol. 46, p. 33–203, Wiley, New York
Harel, E. (1978) in Chloroplast Development (Akoyunoglou, G. & Argyroudi-Akoyunoglou, J. H., eds.,), pp. 33–44, Elsevier Biomedical Press, Amsterdam
Kannangara, C. G. & Schouboe, A. (1985) Carlsberg Res. Commun. **50**, 179–191
Kobayashi, K., Mishima, H., Kurihara, H., Miyazawa, S. & Tehara, A. (1976) Tetrahedron Lett. 537–540
Mauzerall, D. & Granick, S. (1956) J. Biol. Chem. **219**, 435–446
Meller, E. & Gassman, M. L. (1982) Plant Sci. Lett. **26**, 23–29
Nandi, D. L. & Shemin, D. (1968) J. Biol. Chem. **193**, 1236–1242
Omura, T. & Sato, R. (1964) J. Biol. Chem. **239**, 2370–2378
Omura, T. & Takesue, S. (1970) J. Biochem. (Tokyo) **67**, 249–257
Parkinson, A., Thomas, P. E., Ryan, D. E. & Levin, W. (1983) Arch. Biochem. Biophys. **225**, 216–236
Porra, R. J. (1986) Eur. J. Biochem. **156**, 111–121
Pütter, J. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), p. 685, Verlag Chemie, Weinheim, and Academic Press, New York and London
Ramaswamy, M. K. & Nair, P. M. (1976) Indian J. Biochem. Biophys. **13**, 394–397
Rando, R. R. (1977) Biochemistry **16**, 4604–4610
Reichhart, D., Salaün, J. P., Benveniste, I. & Durst, F. (1980) Plant Physiol. **66**, 600–604
Sadano, H. & Omura, T. (1983*a*) Biochem. Biophys. Res. Commun. **116**, 1013–1019
Sadano, H. & Omura, T. (1983*b*) J. Biochem. (Tokyo) **93**, 1375–1383
Salaün, J. P., Simon, A. & Durst, F. (1986) Lipids **21**, 776–779
Schacterle, G. R. & Pollack, R. L. (1973) Anal. Biochem. **51**, 654–655
Schneegurt, M. A. & Beale, S. I. (1986) Plant Physiol. Suppl. **80**, 52
Schön, A., Krupp, G., Gough, S., Berry-Lower, S., Kannangara, C. G. & Soll, D. (1986) Nature (London) **322**, 281–284
Simon, E. J. & Shemin, D. (1953) J. Am. Chem. Soc. **75**, 2520
Soper, T. S. & Manning, J. M. (1982) J. Biol. Chem. **257**, 13930–13936
Strand, C. J., Swanson, A. L., Manning, J., Branch, S. & Marver, H. S. (1972) Anal. Biochem. **47**, 457–470
Volland, C. & Felix, F. (1984) Eur. J. Biochem. **142**, 551–557
Walsh, C. (1982) Tetrahedron **38**, 871–909
Weinstein, J. D. & Beale, S. I. (1983) J. Biol. Chem. **258**, 6799–6807
Wider de Xifra, E. A., Stella, A. M., Battle, A. M. Del C. (1978) Plant Sci. Lett. **11**, 93–98

Received 18 March 1987/22 June 1987; accepted 23 September 1987