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The effect of *N*-methylprotoporphyrin IX on the synthesis of photosynthetic pigments in *Cyanidium caldarium*

Further evidence for the role of haem in the biosynthesis of plant bilins

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N-Methylprotoporphyrin IX strongly inhibits synthesis of phycocyanobilin, but not chlorophyll *a*, in the dark. In the light, both phycocyanin and chlorophyll *a* synthesis are inhibited in parallel. These results are consistent with the intermediacy of haem in algal bilin synthesis and suggest a control mechanism for chlorophyll *a* synthesis, previously unknown.

The degradation of haemoproteins to produce bile pigments is a well-known metabolic pathway in animals (Jackson, 1974; Schmid & McDonagh, 1975; Schmid & McDonagh, 1979; Brown & Troxler, 1982). These bilins (biliverdin and bilirubin) are transient metabolites with no apparent function, other than the provision of a pathway for elimination of unwanted haem. In plants and blue–green algae, however, bilins very similar in structure to the animal bile pigments play important functional roles in photosynthesis and photomorphogenesis (Troxler, 1975). The plant bilins occur in association with proteins (phycobiliproteins), as exemplified by phycocyanin and phycoerythrin, which function as photosynthetic antennae pigments in plants and certain algae (O'Carra & O'hEocha, 1977).

It is well established that the plant bilins are formed via the porphyrin biosynthetic pathway (Troxler, 1972), but it is only recently that direct evidence has been obtained for a precursor–product relationship between haem and plant bilins (Troxler *et al.*, 1979; Brown *et al.*, 1980, 1981). Prevously it was thought that the pigments might be formed via the magnesium branch of the porphyrin synthesis pathway (Hudson & Smith, 1975). The finding that plant bilins are formed from haem implies a necesary role in phycobiliprotein chromophore synthesis for both the enzyme that inserts iron into protoporphyrin IX, protohaem ferro-lyase (ferrochelatase, EC 4.99.1.1) and for the enzyme that catalyses the haem macrocyclic-ring-cleavage step, haem oxygenase (EC 1.14.99.3). Although significant haem oxygenase activity has not yet been detected in plant or algal systems, the presence of ferrochelatase has recently been demonstrated in cells of the unicellular rhodophyte *Cyanidium caldarium* (S. B. Brown, J. A. Holroyd & O. T. G. Jones, unpublished work). This organism produces phycocyanin and chlorophyll *a* when grown photosynthetically, but may also be grown aerobically in the dark when there is no formation of photosynthetic pigments. This is a particularly convenient system for study of algal bilin synthesis, since such dark-grown cells may be induced to produce pigment in two ways. First, suspension in minimal medium and exposure to light results in rapid synthesis of phycocyanin and chlorophyll *a*, without significant further cell division. Such cells are photosynthetically competent. Secondly, dark-grown cells may be resuspended in medium containing glucose and 5-aminolaevulinate in the dark, when the cells excrete into the medium large quantities of porphyrins and phycocyanobilin, the bilin chromophore of phycocyanin (Troxler & Bogorad, 1966). It has recently been shown that such cells also synthesize chlorophyll *a* in the dark under these conditions, although the chlorophyll does not appear to be photosynthetically functional when these cells are exposed to light (Troxler & Brown, 1980).

Recently NMP has been shown to be a potent inhibitor of ferrochelatase (De Matteis *et al.*, 1980; Tephly *et al.*, 1981). Initial work with NMP has been concerned with mammalian systems, where there is a relatively large haem turnover, owing to meta-

Abbreviation used: NMP, *N*-methylprotoporphyrin IX.

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bolism of haemoglobin and liver haem. As yet, however, no detailed study has been carried out on plant or algal systems synthesizing phycobiliproteins, although these also might be expected to involve substantial haem turnover and relatively high activities of ferrochelatase. In plant systems, it is also possible that NMP might inhibit the formation of chlorophyll via the insertion of magnesium into protoporphyrin IX. However, in a very recent study, Houghton *et al.* (1982) have shown that NMP inhibits haem synthesis (destined for cytochromes) but not chlorophyll synthesis in *Rhodopseudomonas spheroides.*

In the present study, we describe the effects of NMP on phycocyanobilin and chlorophyll *a* synthesis in cells of *C. caldarium.*

Experimental

Materials

All materials obtained commercially were of analytical reagent grade where possible. Solvents were redistilled before use. NMP dimethyl ester was prepared by methylation of protoporphyrin IX dimethyl ester with methyl iodide at 95°C for 24h, broadly by the method of De Matteis *et al.* (1980). This material appeared identical with a sample kindly provided by Dr. F. De Matteis and with another prepared independently by one of us (K.M.S.), as judged by its electronic spectrum and behaviour on t.l.c. The methylated product is a mixture of all four possible isomers (Kunze & Ortiz-de Montellano, 1981). Both NMP free acid and its dimethyl ester were used in the present study (see below). To prepare stock solutions of NMP, an appropriate quantity (weighed approximately) of the dimethyl ester was dissolved in a known volume of chloroform and a small sample (usually 0.1ml) diluted with chloroform. The electronic spectrum of this diluted solution showed a strong Soret peak at 418nm ($\varepsilon = 126 \times 10^3 M^{-1} \cdot cm^{-1}$; De Matteis *et al.*, 1982). The concentration of the diluted solution (and hence the total quantity of NMP in the original solution) could thereby be determined. This solution was reduced to dryness under N_2. For experiments requiring NMP dimethyl ester, this residue could be directly dissolved in appropriate medium to yield a stock solution of known concentration. For experiments requiring NMP free acid, the residue was dissolved in approx. 0.5ml of 6M-HCl and allowed to stand at room temperature for 4h. The HCl was removed in a stream of N_2. The resultant NMP free acid was dissolved directly in appropriate medium to yield a stock solution of known concentration.

Cells of *C. caldarium* (Allen, 1959) were grown in acid medium as previously described (Brown *et al.*, 1981). *C. caldarium* mutant III-D-2 was used in the present work, since this strain produces more pigment per cell than the wild-type. When required, cells were disrupted by use of a French pressure cell (American Instrument Co. Inc., Silver Springs, MD, U.S.A.) at 20000lbf/in^2 (1.38×10^5kPa) internal pressure.

Incubation of C. caldarium with NMP in the dark

Dark-grown cells of *C. caldarium* were collected by centrifugation (5000*g* for 15min) and washed with water. Several cultures were prepared, varying only in the concentration of NMP free acid or NMP dimethyl ester. Each contained 3ml of packed *C. caldarium* cells, at 0.54g of glucose, 0.048g of 5-aminolaevulinic acid and appropriate volume of stock NMP solution, suspended in 30ml of medium, pH2 (Troxler, 1972) in a 100ml conical flask. Cultures were maintained in a shaking incubator at 37–40°C in the dark, except when samples were being taken. A 2ml sample was withdrawn from each flask periodically and cells were collected by centrifugation for 10min (bench centrifuge). The spectrum of the cell-free supernatant in the range 350–700nm was determined on a Unicam SP8-100 spectrophotometer. Phycocyanobilin was then extracted from the supernatant (Brown *et al.*, 1981) by addition of 2ml of chloroform and mechanical agitation followed by centrifugation for 5min (bench centrifuge) to aid separation of the phases. The spectrum of the extracted bilin was measured (in chloroform) in the wavelength range 350–700 nm.

The pelleted cells obtained after initial centrifugation of each sample were washed with water and resuspended in 2ml of water. After thorough mixing, 0.1ml of this suspension was added to 1.5ml of water. The spectrum of this whole-cell suspension was measured in the wavelength range 350–750nm against a reference containing cells that had been similarly obtained at time zero (5-aminolaevulinic acid, glucose and NMP added) but that had been stored at 2°C to prevent pigmentation. This technique considerably reduces scattering and has been shown to reveal peaks at 620nm and 675nm due to phycocyanin and chlorophyll *a* respectively (D. I. Vernon, unpublished work). Increase in absorption at 620nm and 675nm may therefore be used to monitor phycocyanin and chlorophyll *a* synthesis.

A culture containing the maximum concentration of NMP used (20μM) and a control culture (without NMP) were analysed for porphyrin content after several days of incubation. Cells were collected by centrifugation (see above) and the supernatant extracted with chloroform until no further phycocyanobilin could be removed. Talc (0.2g; BDH, Poole, Dorset, U.K.) was added to the aqueous supernatant and the mixture agitated vigorously. After centrifugation (bench centrifuge), 0.2g of talc was again added to the aqueous supernatant and the

procedure was repeated until no residual pigment in solution was evident. The combined talc fractions were dried in air and suspended in acetone/conc. HCl (100:1, v/v). After centrifugation the acetone extract was reduced to dryness under N_2 and the residual porphyrins separated by paper chromatography as described by Troxler & Bogorad (1966), using appropriate pure compounds as markers to identify uroporphyrin III, coproporphyrin III and protoporphyrin IX. The intracellular porphyrin content was studied in a similar way by disrupting the cells with a French press (see above) and adsorbing the porphyrins on talc as described above.

Incubation of C. caldarium with NMP in the light

Dark-grown cells of *C. caldarium* were collected as described above and a number of cultures prepared, again varying only in the concentration of NMP free acid or its dimethyl ester. Each contained 3ml of packed cells suspended in 60ml of medium, pH2 (Troxler, 1972) in a 250ml conical flask. Cultures were maintained on a shaking water bath at 37°C, illuminated by two 40W fluorescent tubes (approx. 5400lx). Pigmentation was monitored by withdrawing 2ml samples periodically and determining phycocyanin and chlorophyll *a* content by direct spectrophotometric measurement of cells as described above.

Results

Dark incubations of C. caldarium with 5-aminolaevulinic acid

Spectra of the supernatant solutions after centrifugation of culture samples at various times after incubation with 5-aminolaevulinic acid and NMP in the dark showed a progressive increase in absorption around 400nm, with development of a sharp peak at 401nm. These cell suspensions also showed a corresponding increase in fluorescence, confirming the expected production of porphyrins (Troxler & Bogorad, 1966). The increases in absorbance at 401nm with time for a control incubation (no NMP) and incubations containing various concentrations of NMP free acid (up to 20μM) were almost identical. This indicates that there is very little influence of NMP free acid on the excretion of porphyrins into the suspension medium. Similar results were obtained when NMP dimethyl ester was used in corresponding experiments.

Fig. 1 shows results from the same experiments, when the cell-free supernatant solutions were extracted with chloroform and the absorbance of the resulting extracts monitored at 610nm, corresponding to the absorption maximum for phycocyanobilin. It may be seen from Fig. 1(*a*) that NMP free acid strongly inhibits the synthesis of phycocyanobilin. This effect is apparent at 1.0μM-NMP

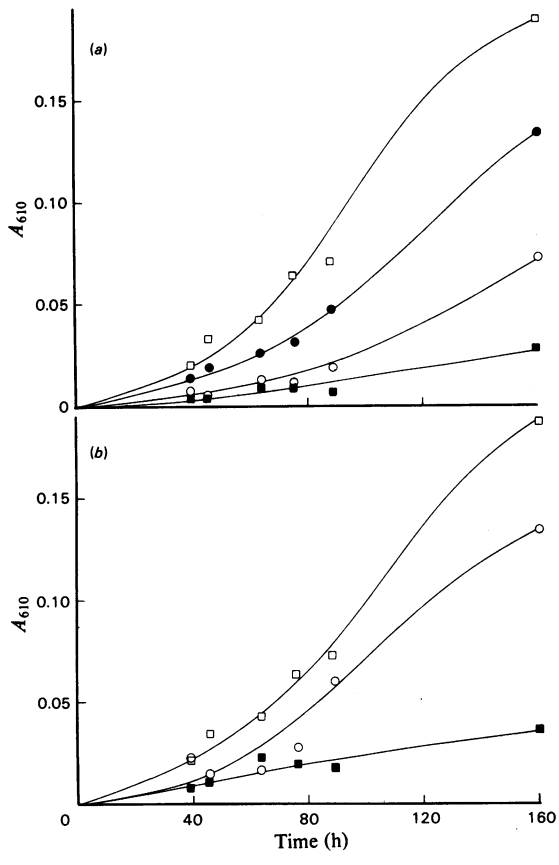

Fig. 1. *Effect of NMP on phycocyanobilin excretion by C. caldarium in the dark*

Excretion of phycocyanobilin into the medium was monitored by measurement of absorption at 610nm in chloroform extracts as described in the Experimental section. (*a*) Concentrations of NMP free acid were: □, zero (control); ●, 1μM; ○, 5μM; ■, 20μM. (*b*) Concentrations of NMP dimethyl ester were: □, zero (control); ○, 5μM; ■, 20μM.

free acid. When NMP dimethyl ester was used in otherwise identical incubations, the results shown in Fig. 1(*b*) were obtained. Again, a strong inhibitory effect is apparent, though possibly somewhat less than with the free acid.

The synthesis of phycocyanin and chlorophyll in these experiments was measured as described in the Experimental section. There was no significant increase in absorption at 620nm, indicating no detectable phycocyanin synthesis in the cells, in agreement with previous studies (Troxler & Brown, 1980). However, as expected, there was significant chlorophyll *a* synthesis (Troxler & Brown, 1980), as indicated by the progressive increase in absorbance

at 675nm, shown in Fig. 2(*a*) for incubations containing various concentrations of NMP free acid. There is apparently a small effect of NMP on chlorophyll synthesis, but this is much less acute than the effect on phycocyanobilin synthesis. Indeed, for 10μM-NMP free acid, after about 90h inhibition is hardly apparent. Similar experiments with NMP dimethyl ester revealed even less inhibitory effect (Fig. 2*b*).

The nature and quantity of porphyrins excreted into the medium and retained within the cells were investigated at the completion of incubations as described in the Experimental section. For the control incubation, the porphyrins excreted into the medium consisted chiefly of uroporphyrin III, coproporphyrin III and porphyrins with intermediate numbers of carboxy groups (Troxler & Bogorad, 1966). A similar pattern was found for the porphyrins excreted into the medium from cells incubated with 20μM-NMP free acid. The total concentration of porphyrins was approximately equal in each case. However, when the cells were disrupted and the intracellular porphyrin content examined, significant differences were found between control and NMP-treated cells. Control cells contains little porphyrin and no detectable protoporphyrin IX, but cells incubated in 20μM-NMP free acid contained substantial quantities of protoporphyrin IX.

Synthesis of photosynthetic pigments by C. caldarium in the light

C. caldarium cells were resuspended in minimal medium and incubated in the light (see the Experimental section) with various concentrations of NMP dimethyl ester. The spectrum of a control incubation (no NMP dimethyl ester) showed a progressive development of peaks at 620nm and 675nm corresponding to phycocyanin and chlorophyll respectively. The increase of absorbance at 620nm during incubation in the light is shown in Fig. 3 as a function of NMP dimethyl ester concentration. As with the formation of phycocyanobilin in the dark, it may be seen that NMP dimethyl ester effects a strong inhibition of phycocyanin synthesis. A similar, though somewhat less marked, inhibition was observed with NMP free acid. Bearing in mind the observation that chlorophyll synthesis in the

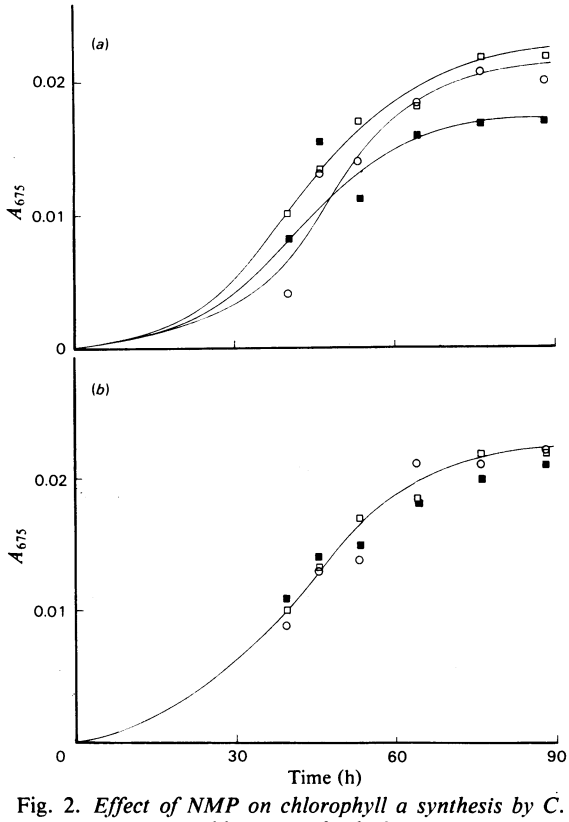

Fig. 2. *Effect of NMP on chlorophyll a synthesis by C. caldarium in the dark*

Chlorophyll *a* synthesis was measured as the increase in absorption at 675nm as described in the Experimental section. (*a*) Concentrations of NMP free acid were: □, zero (control); ○, 10μM; ■, 20μM. (*b*) Concentrations of NMP dimethyl ester were: □, zero (control); ○, 5μM; ■, 20μM. Because of the closeness of the data, only the line referring to the control points has been drawn.

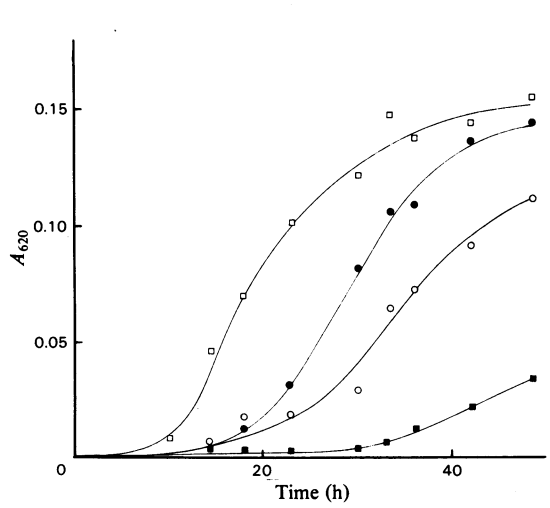

Fig. 3. *Effect of NMP dimethyl ester on phycocyanin synthesis by C. caldarium*

Synthesis of phycocyanin was monitored by measurement of absorption at 620nm as described in the Experimental section. Concentrations of NMP dimethyl ester were: □, zero (control); ●, 0.5μM; ○, 5μM; ■, 20μM.

dark was influenced very little by NMP, it might be expected that during light-induced pigmentation, chlorophyll synthesis would have greatly exceeded that of phycocyanin. However, a strong inhibition of chlorophyll synthesis by NMP dimethyl ester was observed when cells were illuminated. Indeed, the ratio of absorbance at 675 nm to that at 620 nm was almost constant in every incubation, i.e. independent of time and independent of NMP concentration. These results clearly show that inhibition of chlorophyll synthesis almost exactly parallels that of phycocyanin synthesis.

Discussion

Our experiments indicate that both NMP free acid and NMP dimethyl ester strongly inhibit bilin synthesis both in the light and in the rather more artificial system where addition of 5-aminolaevulinic acid promotes synthesis of phycocyanobilin in the dark. This result implies a requirement for ferrochelatase activity and therefore strongly supports previous conclusions (Brown *et al.*, 1981) that haem is a necessary intermediate in algal bilin synthesis. Recent experiments by Beale (1982) are consistent with these findings. In the dark system, it has been well established (Troxler & Bogorad, 1966) that porphyrins accumulate during the incubation. It appears from the present work that porphyrins with more than two carboxy groups (chiefly uroporphyrin III and coproporphyrin III) are excreted into the suspending medium, whereas protoporphyrin (which is dicarboxylic) is retained preferentially within the cells. Our results show a marked increase in intracellular protoporphyrin IX in the presence of NMP. This accumulation might be expected, since the enzyme utilizing protoporphyrin IX is inhibited. Correspondingly, the formation ot porphyrins before protoporphyrin IX in the biosynthetic pathway is almost unaffected by NMP.

The synthesis of chlorophyll *a* in the dark is hardly influenced by NMP. This result suggests that NMP does not inhibit individual steps of chlorophyll biosynthesis, including the step at which magnesium is inserted into protoporphyrin IX. This conclusion is also supported by the work of Houghton *et al.* (1982) who found that bacteriochlorophyll synthesis in *R. spheroides* did not appear to be inhibited by NMP. In the light however, chlorophyll *a* synthesis was not only inhibited by NMP, but the inhibition was precisely parallel to that of phycocyanin synthesis. A possible explanation is that the pathways of biosynthesis of chlorophyll in the dark and in the light differ at some stage and that NMP happens to act at that stage in the light. Alternatively, it is also possible that some photodegradation product of NMP might be formed in the light and might inhibit chlorophyll synthesis. However, both of these explanations seem unlikely in view of the precisely parallel inhibition of phycocyanin and chlorophyll synthesis. A more plausible explanation is that the primary inhibition lies in phycocyanin synthesis and that chlorophyll synthesis is strictly co-ordinated with that of phycocyanin. This would involve a control mechanism by which chlorophyll synthesis is not able to proceed in advance of phycocyanin synthesis. The nature of such a control mechanism is unknown.

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