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N-Methyl Mesoporphyrin IX Inhibits Phycocyanin, but Not Chlorophyll Synthesis in *Cyanidium caldarium*[1]

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ABSTRACT

The ability of *N*-methyl mesoporphyrin IX (NMMP) to block heme synthesis by specifically inhibiting enzymic iron insertion into protoporphyrin IX was exploited to test whether heme is a precursor of the bilin chromophore of phycocyanin (PC). A strain of the unicellular rhodophyte *Cyanidium caldarium* which forms normal amounts of both chlorophyll (Chl) and PC in the dark was employed to avoid phototoxic effects of exogenous porphyrins. Relative Chl and PC content were assayed spectrophotometrically on whole cell suspensions.

When cells were grown in the dark on a glucose-based heterotrophic medium at 42°C, neither division rate nor Chl synthesis was affected by NMMP up to 3.0 micromolar and for as long as 72 hours. NMMP had a dose-dependent inhibitory effect on PC synthesis. PC to Chl absorbance ratios, relative to control cell values, were 100%, 89%, 86%, and 50% in cells grown for 48 hours with 0.3, 1.0, 3.0, and 10.0 micromolar NMMP, respectively. NMMP also caused the accumulation of intracellular protoporphyrin.

The ability of NMMP to cause intracellular accumulation of protoporphyrin and to block PC synthesis specifically while allowing normal Chl formation is consistent with its action as a specific inhibitor of enzymic iron chelation, and supports the role of heme as a precursor to the phycobilins.

Biliproteins are the major light-harvesting pigments in blue-green, red, and cryptomonad algae. The biliproteins are composed of open-chain phycobilin chromophore molecules which are covalently bound to proteins. Although the pigments are present in high abundance in the organisms in which they occur, their biosynthesis is poorly understood. Structural considerations suggest that phycobilins arise from macrocycle ring-opening of a porphyrin or metalloporphyrin precursor, but the relevant enzymic steps have not been observed *in vitro* (Fig. 1).

Recently, Brown *et al.* (5) reported that exogenous ^{14}C-heme was incorporated into phycocyanobilin, the bilin chromophore of PC[2], but not into Chl, in greening cultures of the unicellular rhodophyte *Cyanidium caldarium*. This result suggests that heme is a metabolic precursor to the phycobilins. Others have proposed that the phycobilins might arise from the Mg-porphyrin branch of the tetrapyrrole pathway leading to Chl (1, 9). Possible biosynthetic paths to phycobilins are illustrated in Figure 2.

N-Methylated meso- and proto-porphyrins IX (Fig. 1) were recently reported to be very potent competitive inhibitors of the last step in the heme biosynthetic pathway—the insertion of iron into the porphyrin nucleus catalyzed by the enzyme ferrochelatase (Fig. 2) (7, 10). NMMP was recently found to inhibit heme synthesis and induce ALA synthase activity, while having no effect on Chl synthesis, when administered to *Euglena gracilis* (3). This indicates that, *in vivo*, the inhibitory effect of NMMP is restricted to iron insertion into protoporphyrin IX, and that Mg insertion, which leads to Chl, is not affected. We have now exploited the inhibitory specificity of NMMP to demonstrate the dependence of phycocyanobilin synthesis on heme formation in *Cyanidium caldarium.*

Portions of this work were previously reported in abstract form (2).

MATERIALS AND METHODS

Cells. Wild-type cells of *Cyanidium caldarium* and a series of mutants (11) capable of forming only Chl (III-C), phycobilins (GGB), or neither pigment type (GGB-Y) were a generous gift from Dr. R. F. Troxler (Boston University School of Medicine). Wild-type cells form Chl and PC only in the light. From wild-type cells, we obtained a spontaneous mutant line (CPD) which forms high amounts of Chl and PC in light or complete darkness.

Growth Conditions. Cells were grown axenically at 42°C in a pH 2 glucose-based heterotrophic liquid medium (Table I) and were kept suspended by orbital shaking. Cells were counted with a Coulter model ZBI instrument (Coulter Electronics). Exponential cell population doubling time was approximately 12 h.

Porphyrins. NMMP was synthesized from the corresponding porphyrin dimethyl ester by incubation in complete darkness for 3 d at room temperature in dry dichloromethane and methyl fluorosulfonate (13) followed by silica gel column chromatographic purification and ester hydrolysis overnight at room temperature in 6 N HCl. Overall yield was approximately 60%. Concentration was calculated from the absorption at the peak wavelength of 406 nm in 0.1 N HCl, using the molar absorption coefficient of 4.06×10^5 at 404 nm reported for mesoporphyrin dication (15).

Incubations. Strain CPD cells were incubated with NMMP in the dark to avoid possible phototoxic effects of the porphyrins which can occur in the light. The cultures were inoculated at a cell density such that harvesting would occur in the exponential phase of growth in all cases. NMMP was added directly to the cell cultures from a concentrated solution in 0.1 N HCl.

***In Vivo* Pigment Analysis.** Because PC cannot be quantitatively extracted from cells, an *in vivo* quantitative spectrophotometric pigment assay was employed. Cells were washed with water, then were resuspended in stirred cuvettes containing water with 0.033% TiO_2 and 0.1% Tween 80 to assure uniform light scattering. The reference cuvette contained all components except cells. Apparent light absorption was measured at a nonabsorbing wavelength (750

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[2] Abbreviations: PC, phycocyanin; NMMP, *N*-methyl mesoporphyrin IX.

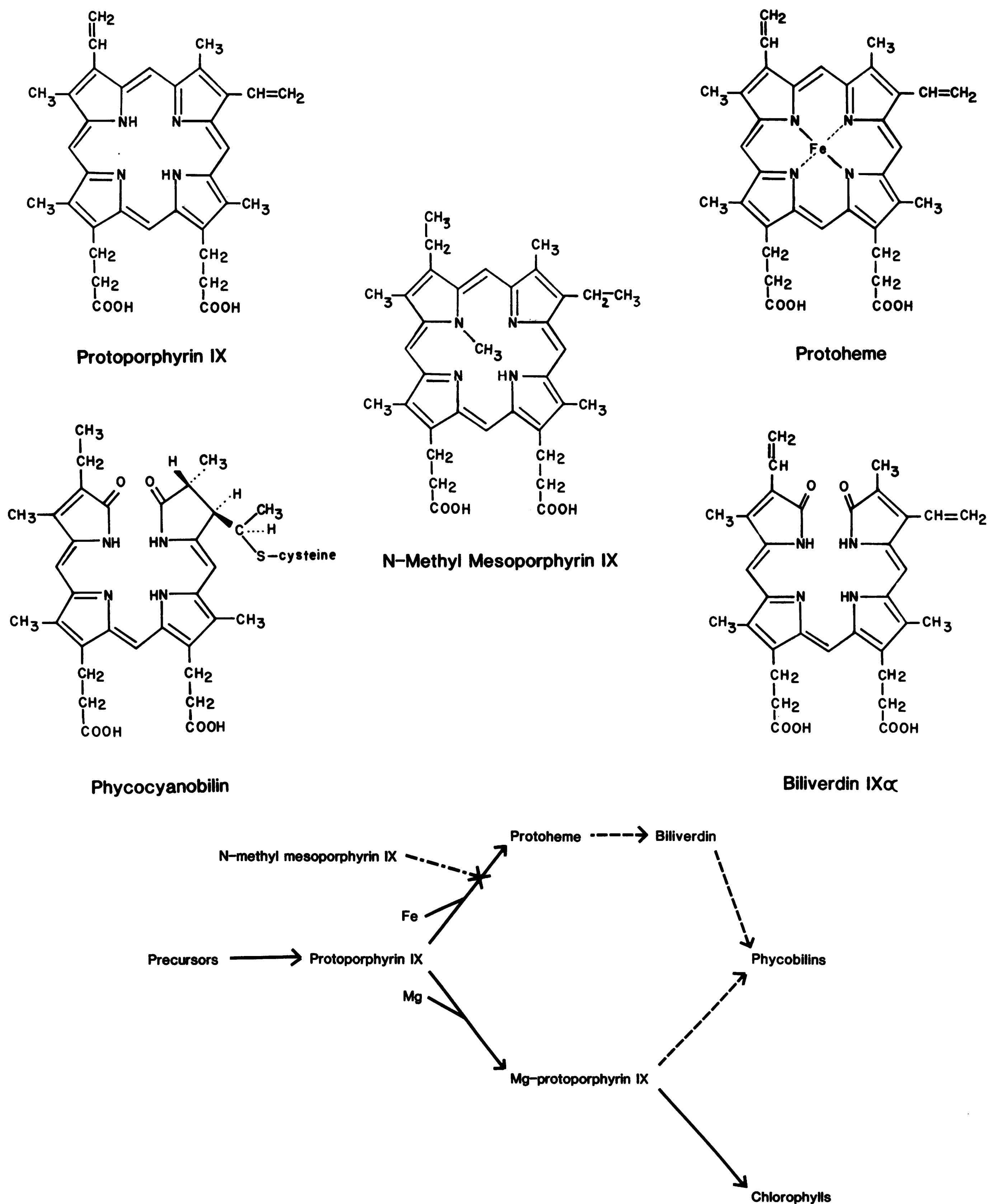

FIG. 1. Structural formulas of the porphyrins and bilins discussed in the text. Phycocyanobilin may have an additional ester linkage to the apoprotein via one of the propionate groups. Only one of four possible structural isomers of NMMP is shown. The synthetic product is a mixture of all four isomers.

FIG. 2. Possible routes to phycocyanobilin, and site of inhibition of heme formation by NMMP.

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Table I. *Composition of the Medium for Heterotrophic Growth of C. caldarium*

Dextrose is autoclaved separately as a 3 M solution and added to cool medium at a final concentration of 5.0×10^{-2} M. Final pH is approximately 2.0.

Ingredient	Final Concn.
	M
$(NH_4)_2SO_4$	1.5×10^{-2}
KH_2PO_4	5.0×10^{-3}
$MgSO_4$	2.5×10^{-3}
$CaCl_2$	1.0×10^{-4}
The following ingredients are prepared as a 1,000-fold concentrate:	
H_2SO_4	2.5×10^{-3}
$FeCl_3$	5.0×10^{-5}
$ZnSO_4$	1.0×10^{-5}
$MnCl_2$	1.0×10^{-5}
H_3BO_3	1.0×10^{-6}
$CuSO_4$	1.0×10^{-6}
Na_3VO_4	1.0×10^{-6}
$(NH_4)_6Mo_7O_{24}$	1.0×10^{-6} (Mo)
$CoCl_2$	1.0×10^{-7}

nm) and at the *in vivo* absorbance peaks of Chl (677 nm) and PC (618 nm). Analysis was performed using a Varian-Cary model 219 spectrophotometer. The *in vivo* absorbance ratio of each pigment type was determined by the use of mutant cells containing only one pigment, and equations were derived from these data to allow calculation of relative concentration of Chl and PC (see "Results").

Porphyrin Extraction. Porphyrins were extracted by an adaptation of the method outlined by Fuhrhop and Smith (8). Cells were washed twice with water and resuspended in 1 ml glacial acetic acid. Then 3 ml ethyl acetate were added with vigorous mixing. The solvent was collected after centrifugation, and the extraction was repeated twice. The combined solvent fraction was extracted twice with 5-ml portions of saturated sodium acetate. The combined saturated sodium acetate layers were back-extracted with 2 ml ethyl acetate which was then combined with the original ethyl acetate fractions, and then extracted with 1 ml 3% sodium acetate. Porphyrins were then extracted from the ethyl acetate fraction with two 3-ml portions of 5 N HCl. The combined HCl extracts were neutralized to pH 4 with 5 N NaOH and saturated sodium acetate. Finally, the porphyrins were extracted with three 2-ml portions peroxide-free diethyl ether. The ether solution was subjected to HPLC as described below.

HPLC. Porphyrin analyses were performed on a Varian model 5000 instrument (Varian Associates, Inc.) equipped with a Rheodyne model 7125 sample injector. Separation was achieved by reverse-phase chromatography with a 4.6- × 150-mm column of octadecylsilane on 5-μm silica particles. Column temperature was set at 30°C and the solvent was 90% methanol, 5% water, and 5% glacial acetic acid (by volume). Flow rate was 2 ml/min. Porphyrins were detected by fluorescence (Varian Fluorichrom) with excitation occurring at 400 nm and emission monitoring at 580

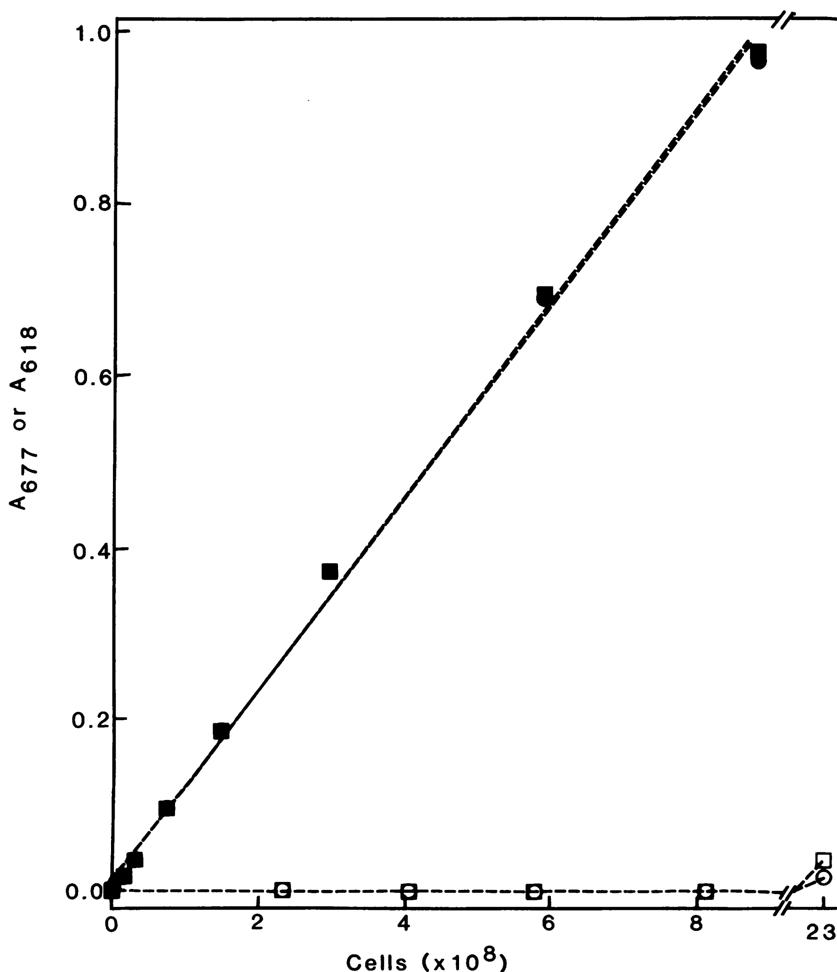

FIG. 3. *In vivo* net light absorption *versus* cell number, of wild-type (●,■) and nonpigmented mutant (○,□) cells at 677 nm (●,○) and 618 nm (■,□), the respective *in vivo* absorption maxima of Chl and PC.

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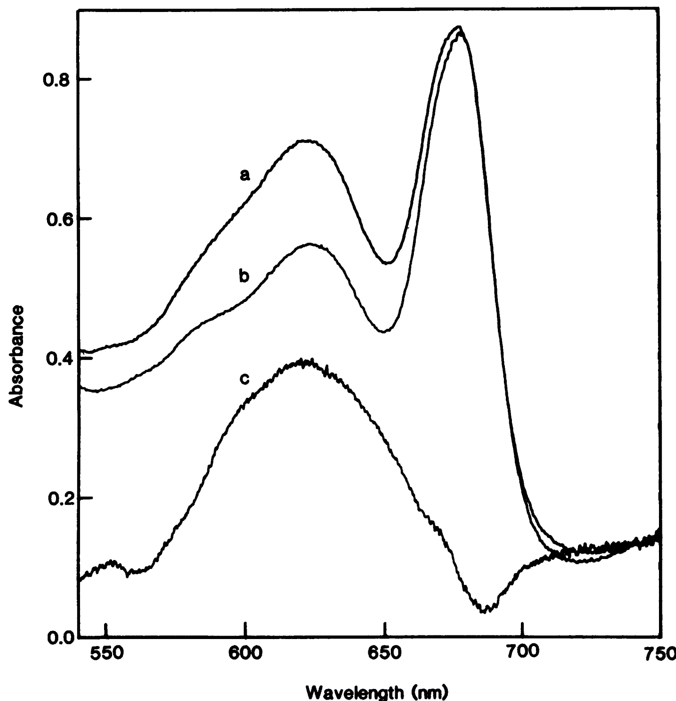

FIG. 4. *In vivo* light absorption spectra of CPD strain control cells (a), cells grown for 48 h with 3.0 μM NMMP (b), and the difference spectrum of control cells *versus* 3.0 μM NMMP-treated cells (c). The difference spectrum is shown expanded 2-fold.

nm and longer wavelengths.

Chemicals. An initial sample of NMMP (as the dimethyl ester) was a generous gift from F. DeMatteis (Medical Research Council, Surrey, England). Methyl fluorosulfonate was from Aldrich. Other chemicals were obtained from Sigma and Fisher.

RESULTS

Validation of *In Vivo* Pigment Analysis. Because it is impossible to quantitatively extract PC from *C. caldarium* cells, it was necessary to develop a quantitative *in vivo* analysis method. The previously employed opal glass method (11, 14) was deficient because it is not possible to precisely match the wavelength-dependent light scattering qualities of cell suspensions with the opal glass reference light scatterer. We were able to overcome this difficulty by adding a potent light scatterer to cell suspensions, at a concentration sufficient to cause so much light scattering that any additional scattering contribution due to the cells was insignificant. TiO_2 is inert, water-insoluble, highly scattering, and it does not absorb light within the visible wavelength range. A slight tendency to clump and adhere to some surfaces was overcome by the addition of 0.1% Tween 80 to TiO_2 suspensions. A concentration of 0.033% TiO_2 (w/v) was sufficient to cause uniform light scattering. Before each series of measurements, the stock TiO_2-Tween mixture was dispersed by sonication and kept suspended by stirring.

Light absorbance readings at 618, 677, and 750 nm were recorded in stirred cuvettes containing cells of strain CPD in aqueous TiO_2-Tween mixture at various cell densities. The reference cuvette contained stirred TiO_2-Tween mixture without cells. Net *A* at 618 and 677 nm (after subtraction of the reading at 750 nm) were plotted against cell concentration. Both sets of points yielded straight lines within the range 0 to 9×10^8 cells/ml, and both curves extrapolated to zero net absorbance at zero cell concentration (Fig. 3). An identical experiment performed with unpigmented strain GGB-Y cells revealed no net absorption at either 618 or 677 nm within the same range of cell concentration, and only very slight net absorption at extremely high cell concentrations (Fig. 3). It can therefore be concluded that the net absorption at 618 and 677 nm in suspensions of strain CPD cells is due to the pigments PC and Chl.

In vivo spectra of cell suspensions of strains III-C and GGB, which contain only Chl and PC, respectively, indicated absorption maxima of 618 nm for PC and 677 nm for Chl. At 618 nm, the net absorption of strain III-C cells (due to Chl) was 35.8% of the value at 677 nm, and at 677 nm the net absorption of strain GGB cells (due to PC) was 7.9% of the value at 618 nm. From these quantities, the following equations can be written:

$$A_{618} = A_{618} \text{ (due to PC)} + 0.358\, A_{677} \text{ (due to Chl)}$$

$$A_{677} = A_{677} \text{ (due to Chl)} + 0.079\, A_{618} \text{ (due to PC)}$$

Simultaneous solution of the equations yields the results:

$$A_{618} \text{ (due to PC)} = 1.03\, A_{618} - 0.37\, A_{677}$$

$$A_{677} \text{ (due to Chl)} = 1.03\, A_{677} - 0.082\, A_{618}$$

Effects of NMMP on Pigment Composition. Strain CPD cells were grown in the dark for 48 h in the presence or absence of 3 μM NMMP. Cells were harvested, resuspended in TiO_2-Tween, and the net absorption spectra were recorded. Compared to the absorption at 677 nm (due mainly to Chl), the absorption at 618 nm was markedly lower in the NMMP-treated cells (Fig. 4). The difference spectrum of approximately equal concentrations of untreated *versus* treated cells reveals a peak difference at 618 nm, which is the *in vivo* absorbance peak of PC. The fractional difference in PC content is somewhat greater than the fractional difference in light absorption at 618 nm, because a significant fraction of the absorption at this wavelength is due to Chl. NMMP at 100 μM had no measurable effect on the net absorption at 618 and 677 nm of broken cell extracts in pH 6.8, 100 mM K-phosphate (data not shown). Because NMMP has a greater absorption at 618 nm than at 677 nm, the effect of any residual NMMP in washed cell suspensions would cause an increase in the A_{618}/A_{677} ratio, rather than the observed decrease.

Cells were grown for 24, 48, and 72 h with 0, 0.25, 1.0, and 2.5 μM NMMP. Cell number was determined for each sample, and relative contents of Chl and PC per ml of culture were quantitated by *in vivo* spectrophotometry. NMMP had no effect on cell growth or Chl synthesis within the range of concentration and growth period examined (Fig. 5). The relative ratio of PC to Chl was decreased in NMMP-treated cells, and the extent of the decrease was both concentration- and time-dependent (Table II). The decrease was greater at high NMMP concentrations at all time periods, and the cells appeared to recover from lower concentrations of NMMP more rapidly.

At higher concentrations of NMMP, a slight degree of inhibition of growth and Chl synthesis was observed at 48 h, but the formation of PC was inhibited much more strongly than growth or Chl formation (Table III).

Effect of NMMP on Porphyrin Accumulation. Strain CPD cells were grown in complete darkness for 24 h in the presence or absence of 3 μM NMMP. Cells were harvested and porphyrins were extracted as described in "Materials and Methods." Reverse-phase HPLC revealed an increased level of a fluorescent component in the treated cells. The peak elution time of the accumulated fluorescent product was identical to that of protoporphyrin IX, and differed from those of NMMP and mesoporphyrin IX (Fig. 6). Under these chromatographic conditions, uroporphyrin III and coproporphyrin III had peak elution times of 1.25 and 4.85 min, respectively. Chl degradation products would not be expected to appear in the porphyrin-containing cell extracts. However, for purposes of comparison, the HPLC retention times for pheophorbide *a*, pyropheophorbide *a*, and pheophytin *a* are 3.8, 5.5, and

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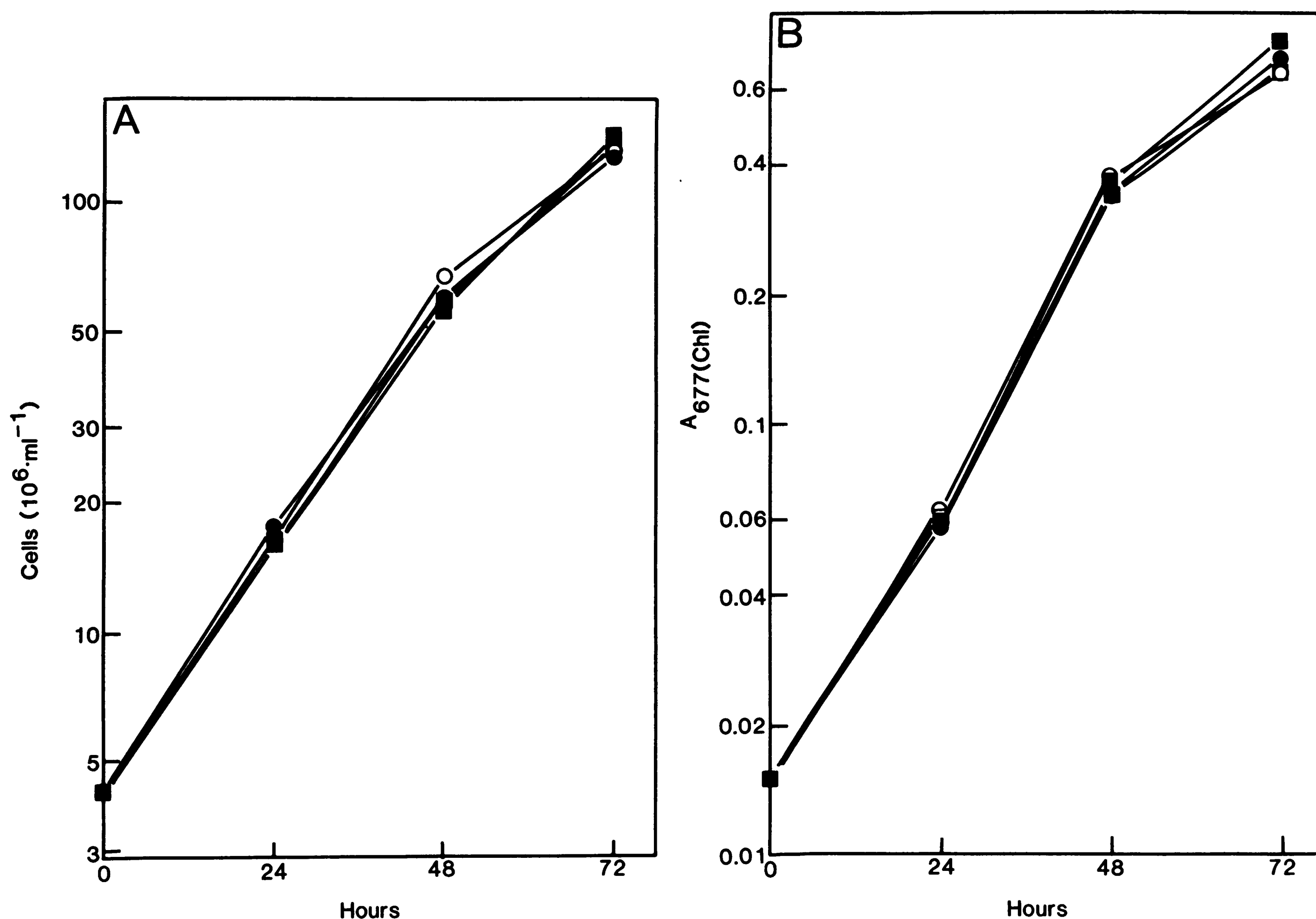

FIG. 5. A, Number of cells *versus* hours of growth at various concentrations of NMMP: (●), control; (○), 0.25 μM: (■), 1.0 μM; (□), 2.5 μM. B, Chl *versus* hours of growth at various concentrations of NMMP: (●), control; (○), 0.25 μM; (■), 1.0 μM; (□), 2.5 μM.

Table II. *Effects of NMMP on Relative Ratio of PC to Chl Synthesis in C. caldarium Strain CPD Cells during 72 Hours of Treatment*

NMMP Concn.	PC Synthesis:Chl Synthesis		
	24 h	48 h	72 h
μM		*relative ratio*	
0.00	1.00	1.00	1.00
0.25	0.79	0.89	0.91
1.00	0.63	0.85	0.88
2.50	0.68	0.81	0.78

Table III. *Effects of NMMP on Rates of Growth, Chl Synthesis, and PC Synthesis in C. caldarium Strain CPD Cells during 48 Hours of Treatment*

NMMP Concn.	Relative Growth Rate	Relative Chl Synthesis	Relative PC Synthesis	PC Synthesis: Chl Synthesis
μM				*ratio*
0.0	1.00	1.00	1.00	1.00
0.3	1.04	1.00	1.00	1.00
1.0	1.14	1.10	0.98	0.89
3.0	1.16	1.00	0.86	0.86
10.0	0.97	0.92	0.46	0.50

67.1 min, respectively. Quantitation by means of net peak height comparisons with chromatograms of standard solutions yielded protoporphyrin IX values of 77 and 650 pmols·(10^8 cells)$^{-1}$ for control and NMMP-treated cells, respectively.

DISCUSSION

Although there is little direct *in vitro* evidence to support the role of heme as an intermediate in the biosynthesis of biliprotein chromophores, its participation is strongly suggested by the obvious similarity of the structures of the phycobilins to that of biliverdin IX α, the first product of heme catabolism in animals. Moreover, it has been shown that, as is the case in animal heme catabolism, bilin and CO are produced *in vivo* in equimolar amounts (16), and that each lactam O atom in the bilin is derived from a separate molecule of molecular O_2 (4). Nevertheless, neither of the two key reactions of the proposed biosynthetic scheme—the enzymic insertion of iron into protoporphyrin IX and the oxidative macrocycle ring cleavage at the α-meso position—has been reported in extracts of any blue-green, red, or cryptomonad alga, in spite of the fact that these organisms are capable of synthesizing phycobilins at very high rates *in vivo*.

One report on successful incorporation of small amounts of ^{14}C from exogenous labeled heme into the phycocyanobilin of the blue-green alga *Anacystis* (12) could not be confirmed by others (6). Later, *C. caldarium* was reported to incorporate significant quantities of ^{14}C from exogenous heme into phycocyanobilin (5). The lack of simultaneous ^{14}C incorporation into Chl indicated that the route of incorporation was direct. Exogenous nonlabeled heme caused a decrease in the incorporation of [^{14}C]δ-aminolevulinic acid into phycocyanobilin (5). While these results strongly suggest that heme is a precursor to the phycobilins, the possibility

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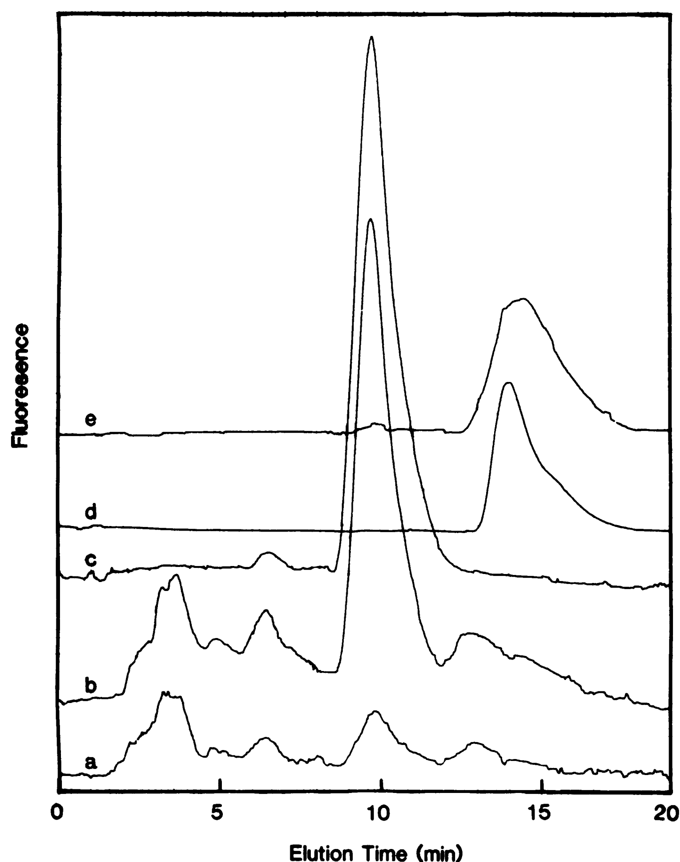

FIG. 6. Reverse-phase HPLC fluorescence elution profiles of extracts from control cells (a), cells grown for 24 h with 3.0 μM NMMP (b), and standard samples of protoporphyrin IX (c), mesoporphyrin IX (d), and NMMP (e).

of an indirect route was not completely ruled out. For example, the exogenous ^{14}C-heme might be able to permeate an intracellular region where phycobilin synthesis occurs, and be unable to permeate another region where Chl synthesis takes place. Thus, it is important to obtain additional types of evidence for the precursor role of heme in phycobilin synthesis.

NMMP and related *N*-alkylated porphyrins were recently discovered to be powerful competitive inhibitors of enzymic heme synthesis. They act as competitive inhibitors of the last step of the heme biosynthetic pathway, insertion of ferrous iron into protoporphyrin IX by the enzyme ferrochelatase (7, 13). In the phytoflagellate *Euglena gracilis*, growth in the presence of μM concentrations of NMMP caused induction of δ-aminolevulinic acid synthase activity, inhibition of ^{59}Fe incorporation into heme, and lowering of the level of extractable heme (3). Importantly, Chl synthesis was not affected by NMMP, indicating that the inhibition was restricted to iron chelation, and that magnesium chelation, which is the first step unique to Chl synthesis, was unaffected under the conditions employed.

NMMP has now been found to inhibit PC formation in *C. caldarium* under conditions where neither growth nor Chl synthesis is affected. As in the case of *Euglena*, a specific inhibitory action on enzymic iron chelation is indicated by the absence of inhibition of the formation of magnesium-containing tetrapyrroles. The induction of protoporphyrin accumulation in treated cells provides additional support for the proposed role of the NMMP. The accumulation of intracellular protoporphyrin also provides an explanation for the apparent ability of the cells to partially overcome the inhibitory effects of NMMP on PC synthesis at longer incubation times. Protoporphyrin at high concentrations would be expected to compete more effectively with NMMP for sites on the enzyme ferrochelatase. The true intracellular concentrations of NMMP are probably much lower than the administered concentrations, because of limited solubility and limited permeability of the cells to the compound.

NMMP did not cause a detectable decrease in the extractable heme levels in *C. caldarium* (Beale and Chen, unpublished), even though the drug did induce a drop in heme level in *E. gracilis* (3). This difference might be due to the fact that, whereas heme is the major end product of the heme pathway in *Euglena*, a relatively minor proportion of the heme becomes an end product in *C. caldarium*, and most of the heme synthesized is further metabolized to phycobilins. Under our experimental conditions where phycobilin synthesis is inhibited by 50% at most, there is still sufficient heme synthesis occurring to satisfy the cellular requirements for heme as an end product.

In conclusion, the ability of NMMP, a specific inhibitor of iron incorporation into heme, to inhibit PC synthesis under conditions where Chl formation is unaffected, provides evidence indicating a precursor role for heme in the synthesis of phycobilins, and that the phycobilins arise from the iron branch, rather than the magnesium or free porphyrin branches, of the tetrapyrrole biosynthetic pathway. NMMP may be useful in future studies of the intermediates between heme and phycobilins, and on the coordinated regulatory mechanisms of bilin and apobiliprotein synthesis.

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