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Mechanism of bile-pigment synthesis in algae

^{18}O INCORPORATION INTO PHYCOCYANOBILIN IN THE UNICELLULAR RHODOPHYTE, *CYANIDIUM CALDARIUM*

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The origin of the lactam oxygen atoms of phycocyanobilin from *Cyanidium caldarium* was studied using ^{18}O labelling. By inhibiting photosynthesis, a high ^{18}O enrichment was maintained in the gas phase and the resulting incorporation of label showed that the lactam oxygen atoms were derived from two oxygen molecules. Slow exchange of these oxygen atoms with water was demonstrated directly by using $H_2{}^{18}O$.

Phycocyanobilin (Fig. 1), the light-absorbing chromophore of the phycobiliproteins allophycocyanin and phycocyanin, is a bilitriene-type tetrapyrrole, linked to apoprotein by a thioether bond. Cells of the unicellular rhodophyte, *Cyanidium caldarium*, administered 5-amino[5-^{14}C]laevulinic acid, synthesize radiolabelled phycocyanobilin (Fig. 1) and evolve radiolabelled CO (Troxler, 1972). The phycocyanobilin/CO molar ratio and specific radioactivity ratio suggest that these compounds may originate from the carbon skeleton of haem. The mammalian bile pigments (biliverdin and bilirubin) have very similar structures to phycocyanobilin and are known to be products of haem catabolism, a process that has been extensively investigated in animals, tissue extracts (Schmid & McDonagh, 1975) and in chemical model systems (O'Carra, 1975), but not in algae or in higher plants. From studies on rat liver and spleen, Tenhunen *et al.* (1968) described a specific haem-cleaving enzyme, haem oxygenase (EC 1.14.99.3), which catalyses the conversion of 1 mol of haem into 1 mol of biliverdin and 1 mol of CO. The final stage in bilirubin formation in mammals is the enzymic reduction of biliverdin at the central methine bridge. By using a spleen microsomal haem oxygenase system, alternately with ^{18}O-labelled molecular oxygen and water, Tenhunen *et al.* (1972) found that the two lactam oxygen atoms of bilirubin originate from molecular oxygen. Brown & King (1978) confirmed and extended this observation and defined four possible mechanisms to account for the origin of the two lactam oxygen atoms in bile pigment, as follows: (*a*) the double-hydrolytic mechanism (both lactam oxygen atoms from water); (*b*) the hydrolytic mechanism (one lactam oxygen atom from molecular oxygen and the other from water); (*c*) the one-molecule mechanism (both lactam oxygen atoms from a single oxygen molecule); (*d*) the two-molecule mechanism (both lactam oxygen atoms from molecular oxygen, but from different oxygen molecules). Measurements of isotopically labelled-molecular-oxygen incorporation into bilirubin synthesized, both by living rats (Brown & King, 1978) and by a microsomal haem oxygenase system *in vitro* (King & Brown, 1978), demonstrated haem cleavage by the two-molecule mechanism. Subsequently Chaney & Brown (1978) showed that haem degradation in a non-aqueous chemical model system also occurred by the two-molecule mechanism. It therefore appears that haem degradation to bile pigment in all types of system occurs by a two-molecule mechanism.

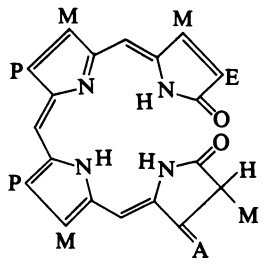

Fig. 1. *Structure of phycocyanobilin*
M, $-CH_3$; A, $=CH-CH_3$; E, $-CH_2CH_3$; P, $-CH_2CH_2CO_2H$.

In assessing the suggestion (Troxler, 1972) that phycocyanobilin in algae is derived from haem, it is therefore important to determine the origin of the

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phycocyanobilin lactam oxygen atoms. In an attempt to carry out such a study, Troxler *et al.* (1979) investigated the incorporation of ^{18}O into phycocyanin synthesized by cells of the alga *C. caldarium*, incubated under an atmosphere containing a mixture of $^{18,18}O_2$ and $^{16,16}O_2$. During this work relatively low incorporations were achieved (approx. 10 atom%), although relatively high initial enrichments were used (approx. 37%). This low incorporation decreased the accuracy of mass-spectral measurements and, in addition, the analysis of the data required two assumptions. First, the decreasing ^{18}O enrichment in the gas phase (typically from approx. 37 atom% to approx. 12 atom%) was attributed to photosynthetic production of $^{16,16}O_2$, resulting in a dilution of the $^{18,18}O_2$, although this was not directly proved. Secondly, since the incorporation of ^{18}O into phycocyanobilin was significantly lower than the final enrichment in the gas phase, an exchange process was assumed between phycocyanin lactam oxygen atoms and water (such a process was demonstrated for phycocyanobilin, but not for phycocyanin itself, in which the chromophore could be protected from exchange by the protein). With these assumptions, a two-molecule mechanism was indicated for phycocyanobilin synthesis. It is clearly important that a definitive determination of the mechanism of bile-pigment synthesis in algae (and, by implication, in plants in general) should be independent of these assumptions. In the present work, therefore, we have tried to prevent photosynthesis during ^{18}O labelling experiments by addition of the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, with the hope of increasing the total incorporation and rendering mass-spectral measurements more accurate. Although it may not be possible to prevent the implied oxygen-exchange reaction, we have attempted to prove its existence in phycocyanin by a direct method using $H_2{}^{18}O$.

Experimental

^{18}O-labelled materials, the culture of *C. caldarium* mutant III-D-2 and the isolation of phycocyanobilin dimethyl ester were as previously described (Troxler *et al.*, 1978, 1979). A sample of 3-(3,4-dichlorophenyl)-1,1-dimethylurea was kindly supplied by Professor H. Woolhouse. For incubations with $^{18,18}O_2$, dark-grown cells, lacking pigments, were suspended in minimal medium in a closed system (Troxler *et al.*, 1979) with and without 3-(3,4-dichlorophenyl)-1,1-dimethylurea (added as a 2mM solution in ethanol to a total concentration of 2μM). After introduction of a measured volume of $^{18,18}O_2$, illumination was commenced and gas samples (10ml) were removed at intervals for analysis by mass spectrometry. In this way, the partial pressures of total oxygen (pO_2) and of $^{18,18}O_2$ ($p^{18,18}O_2$) and the atom% ^{18}O in the gas phase could be determined. Mass spectra of gas samples were obtained on an AEI MS10 spectrometer. No scrambling, i.e. formation of $^{18,16}O_2$, occurs under these conditions.

To investigate possible exchange with water, purified phycocyanin (42mg; Troxler, 1972) was incubated in 10ml of 0.05M-potassium phosphate buffer, pH7.0, containing 10 atom% $H_2{}^{18}O$ and 1% (w/v) NaN_3 at room temperature for 6 days in the dark. Mass spectra of phycocyanobilin dimethyl ester, prepared from phycocyanin (Troxler *et al.*, 1978), were obtained with an AEI MS9 mass spectrometer to give repeat scans (Brown & King, 1978).

Results and discussion

Phycocyanobilin synthesis under $^{18,18}O_2$

The variation in the vlaues of pO_2 and $p^{18,18}O_2$ during synthesis of phycocyanin in the closed system is shown in Fig. 2(*a*). In the control experiments [no 3-(3,4-dichlorophenyl)-1,1-dimethylurea added], the pO_2 decreases initially, presumably due to respiration as the cells utilize stored carbohydrate for synthesis of the photosynthetic apparatus. The value of pO_2 passes through a minimum after about 20h and begins to rise sharply, as photosynthesis becomes dominant. In the culture with 3-(3,4-dichlorophenyl)-1,1-dimethylurea addition, the pO_2 decreases initially due to respiration, but in this case it continues to decline throughout the experiment, showing that oxygen production from photosynthesis is inhibited. Fig. 2(*a*) also shows that the decrease in $p^{18,18}O_2$ in the control experiment is almost identical with that in the experiment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea. This is to be expected because, even when photosynthesis occurs (control), $^{18,18}O_2$ cannot be produced from unlabelled water. Fig. 2(*b*) shows the effect of inhibition of photosynthesis on the atom% ^{18}O in the gas phase. In the control experiment, this value falls rapidly after about 10h as photosynthesis begins, whereas for the experiment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea, the atom% ^{18}O is nearly constant. The slight decrease after about 30h is difficult to explain, but may be due to some degradation of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, either chemically, or as a result of metabolism in algal cells. These experiments therefore demonstrate that the decrease in atom% ^{18}O in the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea is due to photosynthetic oxygen production and that the strategy of using 3-(3,4-dichlorophenyl)-1,1-dimethylurea has been successful, in that a relatively high atom% ^{18}O is maintained throughout the experiment.

An important feature of the ^{18}O labelling approach

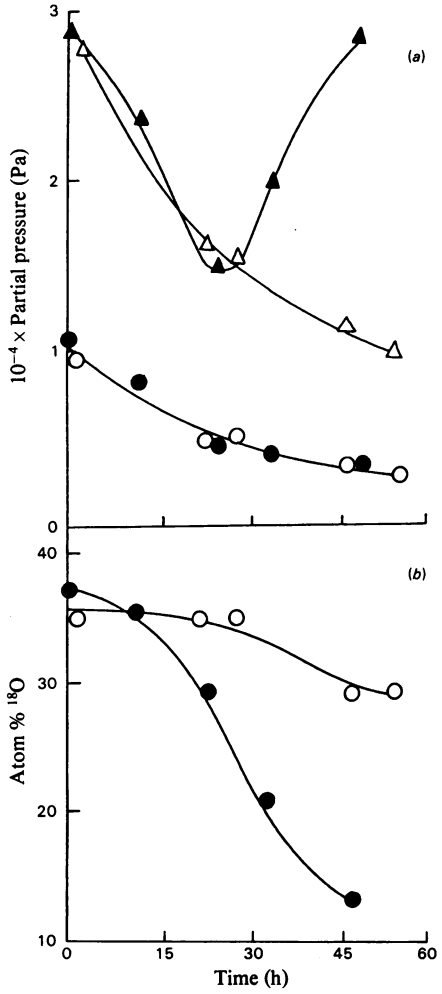

Fig. 2. *Effect of 3-(3,4-dichlorophenyl)-1,1-dimethyurea on photosynthesis in C. caldarium*

(*a*) pO_2 variation in control experiment (▲) and with 3-(3,4-dichlorophenyl)-1,1-dimethylurea added (△); $p^{18,18}O_2$ variation in control experiment (●) and with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (○); (*b*) variation in atom% ^{18}O in control (●) and with 3-(3,4-dichlorophenyl)-1,1-dimethylurea added (○). For experimental details, see the text.

is that the ^{18}O incorporation pattern is different both quantitatively and qualitatively for each of the four mechanisms outlined above (Brown & King, 1978). Specifically, the double-hydrolytic mechanism predicts no incorporation at *m/e* $M+2$ or *m/e* $M+4$ (where M is the mass of the molecular ion), the hydrolytic mechanism predicts incorporation at *m/e* $M+2$, but not at *m/e* $M+4$ and the one-molecule mechanism predicts incorporation at *m/e* $M+4$, but not at *m/e* $M+2$. Only the two-molecule mechanism predicts incorporation at both *m/e* $M+2$ and *m/e* $M+4$. This qualitative distinction is useful where experimental factors preclude a precise quantitative study.

For the ^{18}O labelling experiments with and without 3-(3,4-dichlorophenyl)-1,1-dimethylurea and corresponding incubations under $^{16,16}O_2$, phycocyanobilin dimethyl ester samples were prepared and their mass spectra were determined. The unlabelled material gave a good mass spectrum with a strong molecular ion at *m/e*614, similar to that previously described (Troxler *et al.*, 1978). For the ^{18}O labelling experiments, incorporation at *m/e* M (614), $M+2$ (616) and $M+4$ (618) were determined from repeat scans after correction for the naturally abundant isotopes (Brown & King, 1978) and are shown in Table 1. Because of the falling ^{18}O concentration in the gas phase, meaningful quantitative prediction of the various mechanisms is not possible in the control experiment. On a qualitative basis, significant incorporation occurred at *m/e*616 and apparently also at *m/e*618, indicating a two-molecule mechanism. However, the net incorporation of label (calculated as mole% at *m/e*618 + ½mole% at *m/e*616) is significantly less than the lowest value of the gas-phase enrichment, suggesting a partial exchange reaction. In the experiment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea, Table 1 shows a large incorporation of label at both *m/e*616 and *m/e*618. The extent of incorporation is such that there can be no doubt that, on a qualitative basis, synthesis occurred by a two-molecule mechanism. Table 1 also shows the quantitative predictions for each mechanism, based on the final enrichment in the gas phase. Although there is no doubt that the data clearly reflect a two-molecule mechanism, the incorporations at both *m/e*616 and *m/e*618 are lower than expected, leading to a net incorporation of 22.0%, whereas the predicted value would be at least 29.4%. This loss of label again suggests exchange between the newly synthesized phycocyanin lactam oxygen atoms and water.

$H_2{}^{18}O$ exchange studies

The predicted exchange was studied directly by incubating a solution of unlabelled phycocyanin for 6 days in 10 atom% ^{18}O (see the Experimental section). Data from the mass spectrum of phycocyanobilin dimethyl ester (isolated from the phycocyanin after this period) are shown in Table 1, which also shows the predicted ^{18}O incorporation for complete exchange. Some exchange has clearly occurred because there is a significant incorporation at *m/e*616 and possibly also at *m/e*618. It is also evident from the net incorporation that after 6 days, exchange is only about 50% complete. Thus the environment of phycocyanobilin in native phycocyanin does not completely protect the lactam oxygen atoms from exchange. Although this reaction

Table 1. *^{18}O incorporation into phycocyanobilin dimethyl ester*

Experiment	Mole% found at *m/e*: 614	616	618	Net incorporation (atom% ^{18}O)
Incubation of *C. caldarium* under $^{18,18}O_2$				
Photosynthetically active cells (control; initial atom% $^{18}O = 37.4$, final atom% $^{18}O = 13.0$)	82.5	16.5	1.0	9.3
3-(3,4-Dichlorophenyl)-1,1-dimethylurea-inhibited cells (initial atom% $^{18}O =$ 35.6, final atom% $^{18}O = 29.4$)	61.8	32.0	6.2	22.2
Predictions (based on 29.4% enrichment)				
Double-hydrolytic mechanism	100	0	0	
Hydrolytic mechanism	70.6	29.4	0	
One-molecule mechanism	70.6	0	29.4	
Two-molecule mechanism	49.9	41.5	8.6	
Oxygen exchange between phycocyanin and $H_2{}^{18}O$* (atom% $^{18}O = 10.0$)				
Observed	90.4	9.4	0.2	4.9
Predicted for complete exchange	81.0	18.0	1.0	

* Purified phycocyanin was incubated with $H_2{}^{18}O$ (10.0 atom% ^{18}O) for 6 days in the dark after which phycocyanobilin dimethyl ester was isolated. For experimental details, see the text.

is slow, it would certainly be significant over the time scale of the incubations of *C. caldarium* with labelled oxygen and it seems probable that this accounts for the loss of label during these incubations. Although, in principle, exchange could render distinction between a two-molecule mechanism and a one-molecule mechanism difficult (since a species at *m/e* $M+2$ could be formed from one at *m/e* $M+4$), in the 3-(3,4-dichlorophenyl)-1,1-dimethylurea experiment, the magnitude of the peak at *m/e* 616 is much too high to be explained in this way and the two-molecule mechanism is the only one compatible with all the data.

General discussion

The observation of a two-molecule mechanism has interesting implications for the mechanism of phycocyanobilin synthesis. A metalloporphyrin precursor of algal bile pigment has not been isolated. Bogorad & Troxler (1967) discussed the possible formation of phycocyanobilin from magnesium protoporphyrin-IX versus haem and Hudson & Smith (1975) proposed a scheme for synthesis from a magnesium chlorin precursor, based on several chemical model systems for metallochlorin conversion into bile pigment. However, it is not known whether these model systems occur by the one-molecule mechanism or the two-molecule mechanism and their relevance to phycocyanobilin synthesis is uncertain. Although the possibility of a magnesium-mediated pathway cannot be discounted the weight of present evidence suggests that phycocyanobilin arises from haem in a reaction analogous to mammalian bile-pigment synthesis via haem oxygenase. This is supported by the observations that CO is formed during synthesis of both bilirubin and phytocyanobilin, that both bilirubin and phycocyanobilin are IXα isomers and by the present work, which demonstrates that, like bilirubin, phycocyanobilin is formed by a two-molecule mechanism.

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References

Bogorad, L. & Troxler, R. F. (1967) in *Biogenesis of Natural Compounds* (Bernfeld, P., ed.), pp. 247–313, Pergamon Press, London

Brown, S. B. & King, R. F. G. J. (1978) *Biochem. J.* **170**, 297–311

Chaney, B. D. & Brown, S. B. (1978) *Biochem. Soc. Trans.* **6**, 419–421

Hudson, M. F. & Smith, K. M. (1975) *Chem. Soc. Rev.* **4**, 363–399

King, R. F. G. J. & Brown, S. B. (1978) *Biochem. J.* **174**, 103–109

O'Carra, P. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., ed.), pp. 123–153, Elsevier, Amsterdam

Schmid, R. & McDonagh, A. F. (1975) *Ann. N.Y. Acad. Sci.* **244**, 533–552

Tenhunen, R., Marver, H. S. & Schmid, R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 748–755

Tenhunen, R., Marver, H. S., Pimstone, N. R., Trager, W. F., Cooper, D. Y. & Schmid, R. (1972) *Biochemistry* **11**, 1716–1720

Troxler, R. F. (1972) *Biochemistry* **11**, 4235–4242

Troxler, R. F., Kelly, P. & Brown, S. B. (1978) *Biochem. J.* **172**, 569–576

Troxler, R. F., Brown, A. S. & Brown, S. B. (1979) *J. Biol. Chem.* **254**, 3411–3418