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MECHANISM OF PHOTO-OXIDATION OF BACTERIOCHLOROPHYLL-C DERIVATIVES

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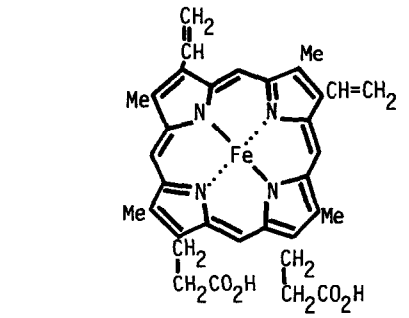
Summary: Photo-oxidation of pheophorbides related to the bacteriochlorophylls-c to give open-chain acetylbilitrienes is shown to proceed by a mechanism involving only one oxygen molecule.

Degradation of protoheme (1) to give protobiliverdin (2) is an important natural process occurring in animal tissues, and it represents the main pathway for catabolism of heme-protein heme.[1] Though heme degradation has been extensively studied because of its health-related aspects, the most abundant tetrapyrrole in the biosphere is chlorophyll, and we have no idea how the millions of tons of chlorophyll pigments are degraded annually in senescent leaves. Several studies have shown that chlorophylls from green plants are readily photo-oxidized[2,3], but the process appears to be complex and no bile pigment analog has yet been isolated from such systems. On the other hand, recent studies have been carried out on the methyl pheophorbides derived from bacteriochlorophylls-c obtained from *Chloropseudomonas ethylicum*[4] and *Chlorobium limicola*[5,6]. These bacteria are obligate anaerobes which produce chlorophylls (*Chlorobium* chlorophylls '660') in the form of a homologous mixture; the structures of the methyl pheophorbides obtained from the natural chlorophylls by treatment with sulfuric acid in methanol are shown in (3). Photo-oxidation of the pheophorbides (3) gives a mixture of stable acetylbilitrienes (4) which resemble biliverdin. A synthetic analog (5)[7] also undergoes photo-oxidative ring cleavage, and it is possible that this process may represent a pathway for natural chlorophyll degradation in these organisms.

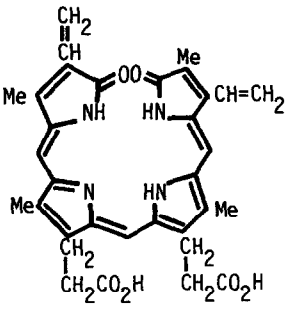
The mechanism for degradation of heme to bile pigment has been studied using ^{18}O labelling in the molecular oxygen to which the system is exposed. In living rats[8], in algal cells[9], in the heme oxygenase system[10], and in chemical model systems[11,12], these studies showed that the lactam oxygen atoms inserted into the product bile pigment were derived from two

different oxygen molecules *(Two-Molecule Mechanism)* rather than from a single oxygen molecule *(One-Molecule Mechanism)* as had been universally assumed. The method depends upon the use of ^{18}O enriched oxygen containing $^{18,18}O_2$ and $^{16,16}O_2$, but none of the mixed species $^{18,16}O_2$. The labelling method should be equally applicable to photo-oxidation of the bacteriochlorophylls-c, and their derivatives (3) and (5). In order to confirm proposals[3] about the origin of the lactam oxygen atoms and the unique regioselectivity of the photocleavage such that only material with the acetyl group on ring D is obtained, Risch and Belter[6] carried out some ^{18}O labelling experiments. However, from their data it was not clear whether the reaction proceeded via a *One-Molecule Mechanism* or a *Two-Molecule Mechanism*. In view of the finding of a *Two-Molecule Mechanism* for heme degradation, we now report ^{18}O labelling studies on the production of photobilins (4) and (6).

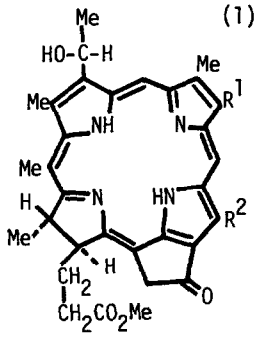
Reactions were carried out in a closed system into which an appropriate ^{18}O atmosphere (32 atom % enrichment) could be introduced. Photo-oxidation was achieved by exposure to two 60 W tungsten lamps (approx. 20 cm distant) supplemented by sunlight. The progress of the reaction was monitored by carrying out a blank reaction under similar illumination conditions, but using $^{16,16}O_2$, and testing samples periodically by TLC. When photo-oxidation was complete (18 h) the reaction mixture was applied to silicagel G TLC plates which were developed with 2% MeOH in CH_2Cl_2. The mass spectrum for the unlabelled photobilin showed a major molecular ion at m/e 626 (R^1= Et, R^2= Et) with homolog molecular ions at m/e 612, 640, and 654. The principal fragment peaks in the spectrum corresponded to loss of $COCH_3$, giving an ion at m/e 583 (homologs at m/e 569, 597, and 611). The spectrum of material isolated from an experiment carried out under a mixture of $^{18,18}O_2$ and $^{16,16}O_2$ shows incorporation of label at m/e M+4 (where M = mass of molecular ion), but no incorporation at M+2. This applies for each molecule in the homologous series. These observations immediately suggest photo-oxidation by a *One-Molecule Mechanism*, which requires incorporation to give m/e M+4, but not M+2. The data for the main species at m/e 626 were analyzed quantitatively by carrying out repeat scans and making due allowance for naturally abundant isotopes as described in detail elsewhere.[8] The results are shown in Table 1, along with the expected incorporations for both *One-Molecule* and *Two-Molecule Mechanisms*. It is clear that there is good quantitative correlation of the experimental data with a *One-Molecule Mechanism*, in contrast to that found in heme catabolism. For the fragments corresponding to loss of $COCH_3$ (mass "F"), there is incorporation at m/e F+2 but little or no incorporation at m/e F+4.



(1)

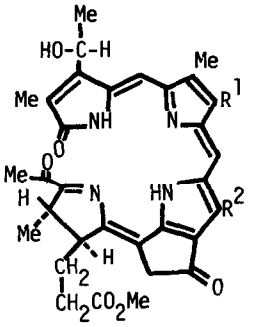


(2)



(3)

$h\nu/O_2$



(4)

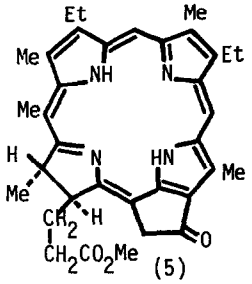
Major Photobilin Homologs:

R^1= Et, R^2= Me; M^+= m/e 612

R^1= Et, R^2= Et; M^+= m/e 626

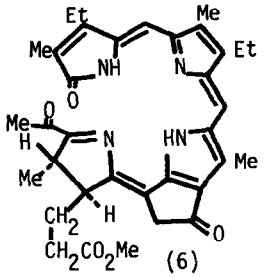
R^1=n-Pr, R^2= Et; M^+= m/e 640

R^1=i-Bu, R^2= Et; M^+= m/e 654



(5)

$h\nu/O_2$



(6)

Table 1: ^{18}O Incorporation into photobilins arising from natural (3) and synthetic (5) methyl pheophorbides of bacteriochlorophylls-c from *Chloropseudomonas ethylicum*.

m/e	M	M+2	M+4	F	F+2	F+4
PREDICTED *(Two-Molecule Mechanism)*	46.2	43.6	10.2	68.0	32.0	zero
PREDICTED *(One-Molecule Mechanism)*	68.0	zero	32.0	68.0	32.0	zero
OBSERVED [Natural photobilins (4)]	68.4	zero	31.6	69.4	30.4	0.2
OBSERVED [Synthetic photobilin (6)]	66.4	1.3	32.2	66.7	33.0	0.3

This is to be expected, since loss of $COCH_3$ involves cleavage of a labelled oxygen atom. Detailed analysis of the data at m/e 583 is shown in Table 1, and quantitatively confirms loss of a labelled oxygen atom from the parent molecular ion at m/e 626. Mass spectra for the synthetic photobilins showed, as expected, only one homolog to be present. Unlabelled, ions were observed at m/e 596 and 553 (- $COCH_3$). The spectrum of the labelled photobilin clearly shows incorporation of label at m/e M+4 and F+2, but not at m/e M+2 or F+4. Quantitative analysis of the data is also given in Table 1, from which a *One-Molecule Mechanism* is again obvious.

The present work establishes that photodegradation of these bacteriochlorophyll derivatives occurs by a *One-Molecule Mechanism*. These results might have been anticipated, but our endeavors are certainly justified by the fact that, prior to earlier work using the same technique, heme catabolism had incorrectly been assumed to proceed by way of a *One-Molecule Mechanism*. Our work provides information on the breakdown of a natural chlorophyll; the relevance of this to plant chlorophyll degradation during leaf senescence and during fruit and grain ripening is difficult to assess, but photo-oxidative degradation remains a good possibility.

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