Pyropheophytin a Accompanies Pheophytin a in Darkened Light Grown Cells of *Euglena* [1]

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Z. Naturforsch. **36 c**, 827–833 (1981); received June 19, 1981

Euglena, Chlorophyll Degradation, Pyropheophytin, Photosynthesis, High Performance Chromatography

Light-grown non-dividing cells of *Euglena gracilis* Klebs var. *bacillaris* Cori form pheophytin a like pigments from chlorophyll a without loss of viability when they are allowed to incubate in darkness without shaking. This is accompanied by the loss of long-wavelength components in the red absorption band of intact cells. After extraction of these cells with acetone, transfer of the pigments to ether and treatment of the ether solution with dilute acid, two pigments can be separated by high performance liquid chromatography on reverse phase silica gel (RP-8) using methanol: water = 95:5 (v/v) as the eluting solvent: In addition to pheophytin a, the eluate contains an unknown pigment. With increasing times of incubation of the cells in darkness, the proportion of pheophytin a decreases and the proportion of the unknown increases suggesting, that the unknown is formed from pheophytin a. This pigment has been identified as pyropheophytin a. It has the same absorption spectrum as pyropheophytin a (prepared by pyridine pyrolysis of pheophytin a) and contains phytol as the longchain esterifying alcohol. On conversion to the methyl ester, the resulting methyl phorbide is identical with authentic pyromethylpheophorbide a by tlc, hplc, absorption, absorption difference and mass spectroscopy. This is the first report of pyropheophytin a or any pyrochlorophyll derivative from plants or oxygenic plant-like microorganisms where it may serve as an intermediate in chlorophyll degradation.

Introduction

The biosynthesis [1–4] and breakdown [5, 6] of porphyrin complexes (hemes) is well established, but for the magnesium porphyrins (chlorophylls and bacteriochlorophylls) only the biosynthetic pathway is known in some detail [1, 2, 7]. Particularly obscure, is the pathway of chlorophyll degradation in cells and the nature of the chemical intermediates in this process [8, 9]. Light-grown cells of *Euglena gracilis* var. *bacillaris* placed in darkness without shaking convert a large proportion of their chlorophyll a to a pheophytin (Phe)-like pigment [10]. This appeared to be a promising system in which to look for other possible intermediates in chlorophyll degradation. In this paper we provide further information concerning pheophytin formation in *Euglena* and show that pyropheophytin a (Pyrophe) (a derivative of Phe lacking the carboxymethyl group on the isocyclic ring [11]) is also formed.

Materials and Methods

All commercial chemicals used in this study were reagent grade or better and all solvents used in the chemical characterization of pigments were distilled prior to use. *Euglena gracilis* Klebs var. *bacillaris* Cori was grown in one liter of the pH 3,5 medium of Hutner at 26° with shaking as described previously [10, 12]. Illumination was supplied by mixed white and red fluorescent lamps providing a light intensity of 150 ft c ($180\ \mu W \cdot cm^{-2}$) at the surface of the culture [12]. When the cells reached about 2×10^6 cells/ml (three days of growth) the

[1] Supported by grants from the National Institutes of Health (GM 14595), the Deutsche Forschungsgemeinschaft and the Department of Energy.
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Abbreviations: Chl = chlorophyll a, Phe = pheophytin a, Phyrophe = pyropheophytin a, HPLC = high performance liquid chromatography, TLC = thin layer chromatography.

0341-0382/81/0900-0827 $ 01.00/0

culture was centrifuged at 500 × *g* for 1 min at room temperature [13]. The pellet was resuspended in the same volume of resting medium (1.0% (w/v) with respect to mannitol, 0.01 M with respect to $MgCl_2$, 0.01 M with respect to KH_2PO_4 and 0.25% (w/v) with respect to *cis, cis, cis, cis*-1,2,3,4-cyclopentanetetracarboxylic acid (Aldrich) adjusted to pH 3.5 with KOH [14]). All of these manipulations were done aseptically. The cell suspension so obtained was shaken at 26° under illumination as before, for three more days. Aliquots were then placed in the dark at 26° without shaking, or in some cases in the light, with shaking for various times. Dim green safelights [15] were used when necessary. Absorption spectra of these cells were measured as previously described [13]. Cell viability was measured as colony formation after plating the cells on pH 3.5 medium as described previously [16].

Each sample was then centrifuged at 800 × *g* for 5 min at room temperature and the cell pellet was resuspended in 100 ml of 0.1 M potassium phosphate buffer, pH 7.0, at room temperature and was centrifuged. The following steps were peformed under dim laboratory lighting. The pellet was resuspended in 100 ml of cold acetone and was centrifuged at 8000 × *g* for 5 min at 4 °C. The extraction was repeated three times to yield a total of about 350 ml of cold extract. The pigments were then transferred into fresh cold peroxide-free diethyl ether [17], and the ether was acidified by the addition of 10% (v/v) HCl (about 5 ml of acid for each 100 ml extract), washed neutral with distilled water, dried overnight over anhydrous sodium sulfate, and evaporated *in vacuo* at about 25°. The dry pigments were stored at − 20° under nitrogen in darkness.

For some experiments, part of this crude pigment fraction was further separated by chromatography on silica gel thin layer plates (silicagel 60, Merck), using carbon tetrachloride/acetone = 9/1 (v/v) as the solvent [18]. The rapidly moving pheophytin a region was eluted with acetone, the eluate was taken to dryness *in vacuo* and the pigment was stored under nitrogen at − 20°.

To determine the nature of the esterifying alcohol, the pigment was saponified with methanolic KOH as described previously [21]. The alcohols obtained were identified by gas chromatography using previously described methods [19]. Analytical high performance liquid chromatography (HPLC) [19] was carried out with a Laboratory Data Control (LDC) constametric II pump, using either an LDC Model 1202 variable wavelength double beam absorbance detector set at 667 nm, or a Schöffel model FS 970 L.C. fluorescence detector ($\lambda_{exc} = 408$ nm, $\lambda_{em} \geq 580$ nm). The separation of the pigments solubilized in acetone was performed using a stainless steel column (15 × 0.5 cm) packed with LiChrosorb RP-8/5 µm (Merck). Methanol/water (95/5 v/v) was used as eluant at a flow-rate of 1.0 ml/min. Preparative HPLC employed previously published procedures [20]. Absorption and derivative spectra in the visible and ultraviolet regions were recorded on a model 320 spectrophotometer (Perkin-Elmer). Coupled HPLC-absorption spectroscopy was performed in a homebuilt system using a model II double beam variable wavelength detector (LDC-Latek) combined with a model 8000 intelligent recorder (Bryans) for scanning and data manipulation. Details of the method will be published separately. Mass spectra were recorded on a model CH 7 mass spectrometer (Varian) in the EI-mode (70 KV) with direct inlet.

Pyropheophytin a

An authentic sample of Phe was obtained from crude pheophytin "Sandoz". It was converted to Phyrophe by refluxing in pyridine for 12 h under nitrogen [11, 18].

Pyromethylpheophorbide a

For transesterification (replacement of the phytyl group by methyl on the propionic acid side chain) the unknown pigment was refluxed for 1 h in chloroform methanol (1:1 v/v) containing 2.5% (v/v) H_2SO_4. The products were purified by standard procedures, involving TLC on 20 × 20 cm glass plates coated with 0.75 mm silica "H" (Merck) using 4% (v/v) acetone in carbon tetrachloride as the developing solvent [18].

Authentic pyromethylpheophorbide was prepared from Phe by transesterification and subsequent pyrolysis to remove the carbomethoxy group [18]. Analytical TLC was done with commercial plates (HPTLC, Merck, 10 × 10 cm). Either a silica coating developed with 6% (v/v) acetone in carbon tetrachloride or a C-18 reverse phase coating with methanol as solvent was used.

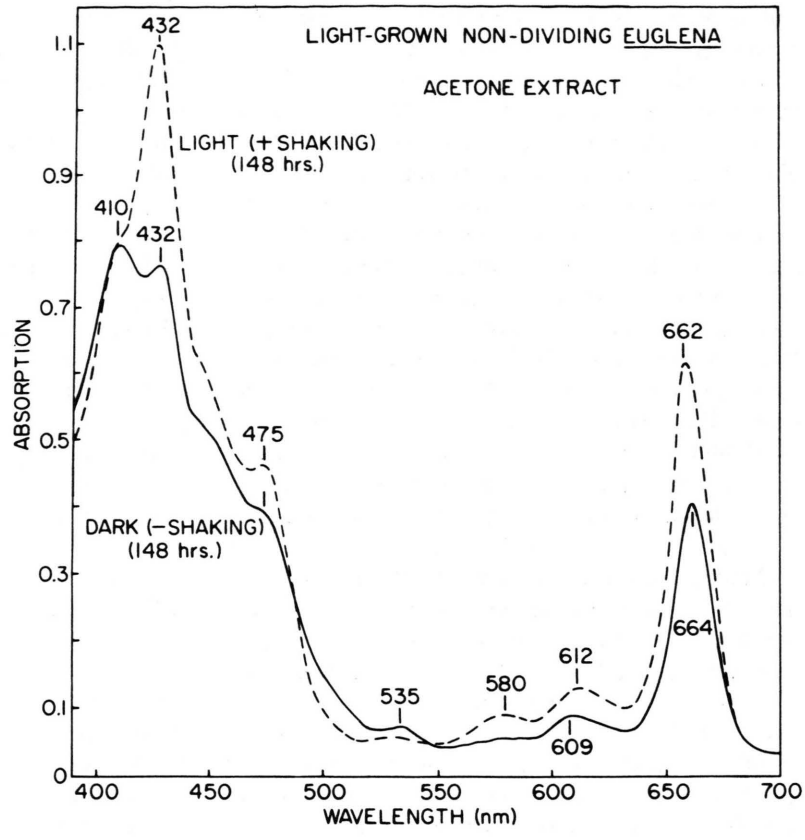

Fig. 1. Absorption spectra of acetone extracts of light-grown non-dividing cells of *Euglena* maintained in the light with shaking or in darkness without shaking for 148 h.

Results and Discussion

Acetone extracts of normal light-grown non-dividing cells of *Euglena* shaking in the light show absorption peaks due to the presence of Chl (432 nm and 662 nm) and carotenoids (Fig. 1). Extracts of the same cells incubated in darkness without shaking show additional absorption peaks at 410 nm and 535 nm attributable to Phe-like pigment(s) [10]. In either culture, there is no significant loss of cell viability during the experimental period as determined by plating (data not shown).

Evidence of pheophytin a formation is also seen in the absorption spectrum of intact cells maintained in darkness without shaking (Fig. 2). There is an increased absorption at 413 nm expected for the Soret band of Phe. Also evident is a loss of absorption on the long wavelength side of the red absorption region. A similar selective loss of chlorophyll a components absorbing at longer wavelengths has been observed during Phe formation in *Euglena* cells growing in the presence of 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) in the light [22]

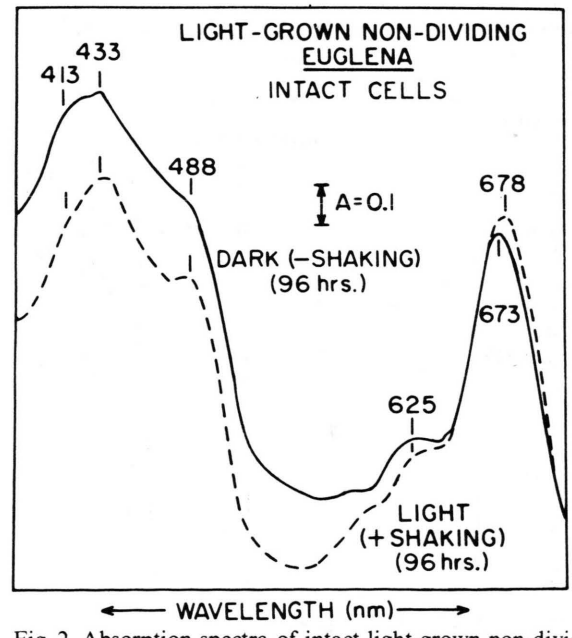

Fig. 2. Absorption spectra of intact light-grown non-dividing cells of *Euglena* maintained in the light with shaking or in darkness without shaking for 96 h.

and is attributed to a selective loss of the light-harvesting systems of photosystem I of photosynthesis. Perhaps inhibition of normal light-driven hydrogen ion pumping by darkness or DCMU allows accumulation of H^+ near the chlorophyll of photosystem I leading to pheophytin formation.

To elucidate the structure of the pigment(s) formed from Chl, they were extracted from the cells and the extracts were acidified to convert all remaining chlorophyll(s) to pheophytin(s). The crude mixture was then subjected to chromatography. Whereas only a single band is obtained on silica tlc-plates, two components can be separated by reverse-phase HPLC (Fig. 3). One peak is chromatographically identical with Phe ($r = 16.2$ min) as expected since any remaining Chl was converted to Phe by acidification during the preparative procedure. It is accompanied by a second peak eluting later ($r = 23.2$ min). During incubation of the cells in darkness, the unknown compound increases at the expense of Phe suggesting that the latter is progressively converted to the unknown compound (Fig. 3, inset). *In situ* spectroscopy of the HPLC eluate at the two maxima (Fig. 3) gave identical absorption spectra indicating either the presence of a different esterifying alcohol or a chromophore modified at a position not severely affecting the π-electron system. To test these possibilities larger amounts of the unknown pigment were isolated using preparative HPLC. After saponification the only long chain alcohol found by gas chromatography is phytol, excluding the first possibility. The amount of phytol found is greater than expected using the known extinction coefficient for Phe to estimate the amount of pigment saponified. This indicates that the extinction coefficient of the unknown pigment is lower than that of Phe.

These properties of the unknown pigment suggested that it might be a pheophytin carrying different substitutents at C-13² than pheophytin a. In particular the absorption spectrum of the unknown pigment is essentially the same as that of pyropheophytin a, a derivative lacking the 13²-carbomethoxy group [11, 18, 23]. This suggestion was substantiated by the chemical correlations shown in Fig. 4. Heating of pheophytin a with pyridine yielded pyropheophytin a, which is identical with the unknown pigment ($r = 23.2$ min) by HPLC, TLC and uv-vis spectroscopy. Also, the unknown remained unchanged after refluxing in pyridine under identical conditions.

Conversely, the unknown pigment was converted to its methyl ester and compared to an authentic sample of pyromethylpheophorbide a, obtained from pheophytin a as shown in Fig. 4 (right side).

The absorption spectra, difference spectrum (Fig. 5) of the two methyl phorbides and their 2nd and 4th derivatives (data not shown) are identical.

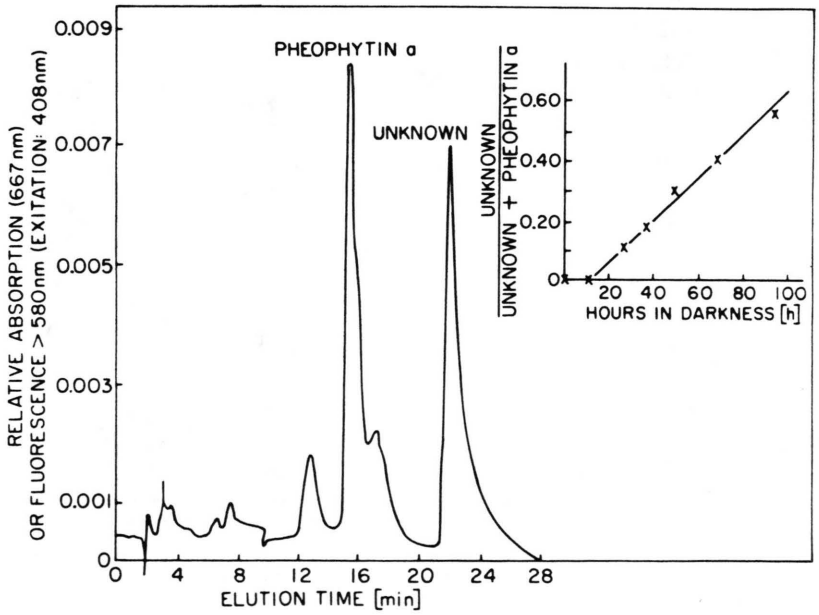

Fig. 3. Elution pattern of pigments extracted from light-grown non-dividing cells of *Euglena* incubated in darkness for 96 h. and subjected to high performance liquid chromatography after acidification. The inset shows the ratios of unknown pigment to unknown plus pheophytin a in cells incubated in darkness for various times, which have been calculated from the relative intensities of the HPLC peaks after correction for the different extinction coefficients ($\varepsilon^{667}_{\text{Pyrophe}} = 49.01 \cdot g^{-1} \cdot cm^{-1}$, $\varepsilon^{667}_{\text{Phe}} = 63.71 \cdot g^{-1} \cdot cm^{-1}$).

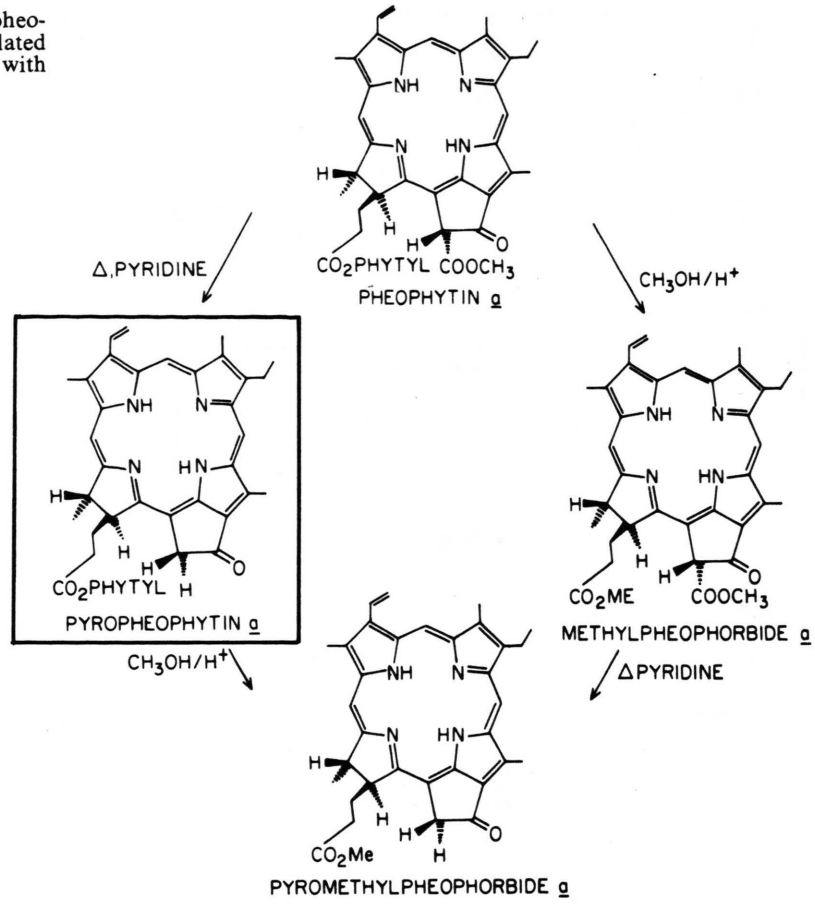

Fig. 4. Chemical correlation of pyropheophytin a, the "unknown pigment" isolated from dark-adapted *Euglena* (in box), with authentic derivatives of chlorophyll a.

The two pigments also have the same R_f values on "normal" and "reverse phase" thin layer chromatography (Fig. 6) and HPLC. Finally, the mass spectra of the two methyl phorbides are also in excellent agreement (Table I). Taken together, the data show that the unknown pigment is pyropheophytin a (boxed in Fig. 4).

Pyropheophytin a is found along with pheophytin a in light-grown non-dividing cells of *Euglena* incubated in darkness without shaking. Since the proportion of pyropheophytin increases with time in darkness while the propertion of pheophytin decreases, pyropheophytin may be formed from pheophytin. However, since all pigments were converted to their magnesium-free forms with acid before analysis, the formation of pyrochlorophyll a as an intermediate is not excluded.

To our knowledge this is the first report of a pyrochlorophyll or pyropheophytin in an oxygenic photosynthetic organism. Derivatives of pyrochlorophyll are major antenna pigments in greeen sulfur bacteria [24, 25]; pyromethylpheophorbide a and its derivatives have been isolated from the excrements of various herbivores [26]. Little is known of the steps involved in chlorophyll degradation in photosyn-

Table I. Mass spectrum (relative intensities in %) of the methylpheophorbide from the "Unknown Pigment" compared with authentic pyromethylpheophorbide a.

MS-peak (*m/e*)	Pyromethyl-pheophorbide a	Methylpheophorbide of "Unknown Compound"
548	100	100
533	6	5
471	40	31
445	3	3
433	18	12
412	4	3
403	5	3
274	3	5
237	26	26

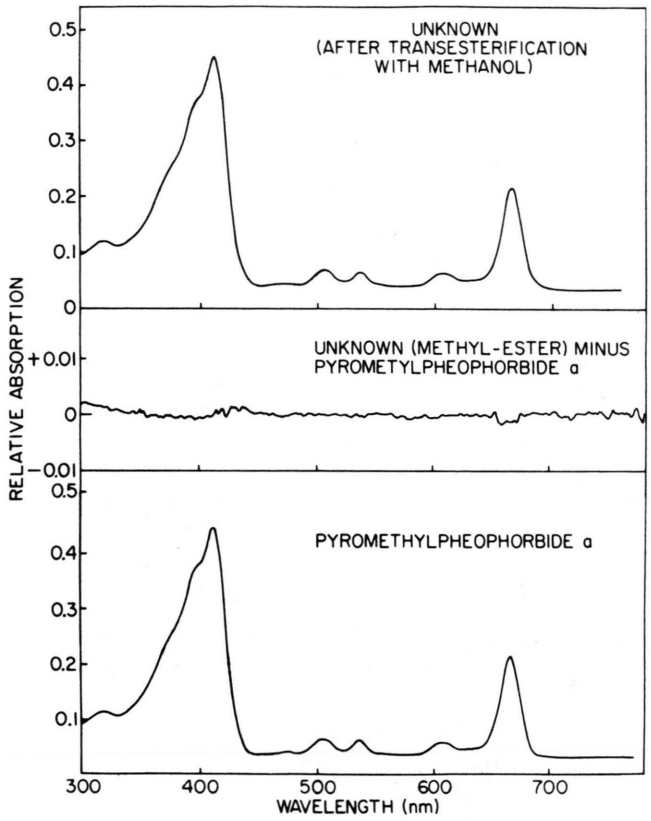

Fig. 5. Absorption spectra (in methanol) of pyromethylpheophorbide a and the methylpheophorbide prepared from the unknown pigment, as well as their difference spectrum (expanded absorption scale).

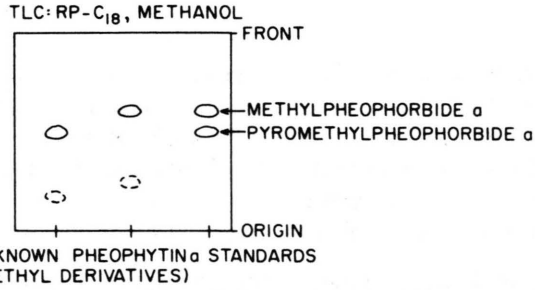

Fig. 6. A comparison of the methyl phorbide prepared from the unknown pigment with methyl pheophorbide a and pyromethylpheophorbide a on a thin layer chromatogram (C-18 reverse phase in silica, methanol as eluent).

thetic organisms [8, 9] and it is entirely possible that pheophytin a and pyropheophytin a (or pyrochlorophyll a) serve as intermediates in such a pathway. It might be noted that the methine bridges adjacent to the reduced ring IV in chlorins are susceptible to oxidative ring opening [27] especially if they bear an alkyl substituent [28–30]. One way of creating such a situation may be the conversion of pheophytin a (bearing a $CHCOOCH_3$-substituent at the C-15 methine bridge) to pyropheophytin a bearing a CH_2-substituent instead.

Note added in proof: We are aware or the work of O. Shimomura (FEBS Lett. **116,** 203 (1981)), who recently suggested a bile-pigment structure derived from pyrochlorophyll b for "compound F" involved in bioluminescence of an euphausid shrimp.

Acknowledgements

We thank Dr. W. Schäfer (München-Martinsried) for recording the mass spectra. The skillful technical assistance (in Munich) of I. Blos (GC) and H. Wieschhoff (preparative HPLC) is acknowledged. We are indebted to Prof. H. H. Inhoffen for a generous gift of pheophytin "Sandoz". This work was supported by the National Institutes of Health (J.A.S.) the Deutsche Forschungsgemeinschaft (S.S.) and the US Department of Energy (H.W.S.).

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