Sodium borohydride is a well known reducing agent. In methanol at room temperature formyl and keto groups are readily reduced to alcohol groups, while ester and carboxyl groups, carbon-carbon and carbon-nitrogen double bonds are only reduced with difficulty (2, 17). Of the groups present in chlorophyll b the C1 formyl and the C6 keto groups should be amenable to reduction, while the C3 vinyl and the C5 and C6 ester groups should be unaffected. Similar considerations apply to chlorophyll a and other derivatives containing formyl and keto groups.

Since previous methods of reducing phaeophyllides a and b had caused either reduction of the C3 vinyl group (6, 8) or had proceeded poorly (10), it was of interest to investigate the action of sodium borohydride upon these compounds and their magnesium salts (phyllins). Furthermore, there remained the possibility that reduction of the C6 keto group might involve formation of an intermediate similar to the pink product of the "Krasnovsky reaction" (the reversible photobleaching of chlorophyll a by ascorbic acid in pyridine) (23).

This communication describes the preparation and the properties of the products obtained by treating methyl chlorophyllides a and b and other chlorophyll derivatives with sodium borohydride. HCl numbers, wave lengths and relative absorbancies of visible absorption maxima and minima, useful for identification purposes, are included.

**Materials and Methods**

Solvents were reagent grade and were used without further purification. Sodium borohydride was obtained from Metal Hydrides Inc., Beverly, Mass. and was about 99% pure.

Methyl chlorophyllides a and b, chlorophylls a and b, and pheophorphyrin as and phylloerythrin were obtained pure by standard procedures (13, 15, 19, 20, 22, 25). "Meso" derivatives were prepared by catalytic hydrogenation of the parent compounds in acetone containing palladium black as the catalyst (14). Free acids were obtained by hydrolysis of the C5 ester in strong hydrochloric acid for 30 minutes at room temperature (12). For the phase test, 0.1 ml of methanolic magnesium methoxide (18 mg Mg/ml) was added to 3 ml of dimethylformamide solution of pigment (18). Acid numbers were determined by the method of Willstätter and Stoll (25). Methoxy determinations were made according to Clark (3).

Visible absorption spectra were measured by a Cary recording spectrophotometer, model 11M. Infra-red absorption spectra were measured with a Perkin-Elmer model 21 double-beam recording spectrophotometer.

The names and structural formulae of the compounds and products are those given by Fischer et al (10, 12), and are indicated in figure 1.

**Reduction of Methyl Chlorophyllide b**

Preliminary experiments showed that dilute methanolic solutions of chlorophyll b were readily converted by milligram amounts of sodium borohydride into a product whose color in methanol was blue-green, and whose "red" absorption maximum was at about 665 mμ. Larger amounts of sodium borohydride converted the blue-green product into one whose color was purple and whose "red" absorption maximum was at about 635 mμ.

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1 Received revised manuscript April 1, 1959.
2 Contribution from the Division of Applied Biology, National Research Council, Ottawa. Issued as N.R.C. No. 5201.
HOLT—REDUCTION OF CHLOROPHYLL DERIVATIVES

A typical preparation of the blue-green product was as follows: One hundred ml of fresh methanolic sodium borohydride (10 mg/100 ml) were added to 36 mg of methyl chlorophyllide b dissolved in 1 ml of pyridine. After 15 minutes the pigment was transferred into ethyl ether by washing with 5 % (v/v) aqueous acid phosphate and with distilled water. It was dried and dissolved in 200 ml of 30 % (v/v) CHCl₃ in petroleum ether (b.p. 30 to 60° C) and then chromatographed on a sugar column using mixtures (v/v) containing 4 % CHCl₃ plus 1.0 or 1.4 or 1.6 % isopropanol in petroleum ether. Pigment from the main band was isolated by removing the sugar column from the glass column and by carefully cutting out the few minor contaminant bands which were present. This pigment crystallized readily from aqueous acetone.

Identification of the product as Mg-methyl pheophorbide-b-3-methanol: The C₇ ester, the C₆ hydro-

![Figure 2](image2.png)

Fig. 2. Visible absorption spectra of methyl chlorophyllide b-3-methanol, and methyl pheophorbide b-3-methanol in ethyl ether.

![Figure 3](image3.png)

Fig. 3. Infra-red absorption spectra of 1) Methyl pheophorbide b, 2) Methyl pheophorbide b-3-methanol and 3) Methyl pheophorbide a. Solvent: CHCl₃. Concentration: 5 to 6 % (w/v).

![Figure 4](image4.png)

Fig. 4. Visible absorption spectra of Mg-9-oxy-desoxo-methyl pheophorbide a and 9-oxy-desoxo-methyl pheophorbide a in ethyl ether.

![Figure 5](image5.png)

Fig. 5. Infra-red absorption spectra of pheophytin b-3-methanol (upper curve) and Mg-pheophytin b-3-methanol in CCl₃.
(c) C6 vinyl group: Hydrogenation in acetone containing palladium black (14) caused a shift of the "red" absorption maximum in ether from 654 to 645 m\(\mu\). The spectrum was identical with that obtained by treating meso-methyl chlorophyllide b with sodium borohydride, in which case the absorption maximum shifted from 632 to 645 m\(\mu\). The positive phase test was retained in both products. The wave lengths of the absorption maxima of the magnesium-free product agreed well with those given earlier (8) for mesopyropheophorbide-b-3-methanol. Such agreement was to be expected since replacement of the C6-carbomethoxy group by a hydrogen atom has little or no effect on the visible spectrum.

The above findings eliminated all but the C6 formyl group as sites of reduction. The evidence for its reduction was as follows: (a) The visible absorption spectrum, given in figure 2, was very similar to that of chlorophyll a. The wave lengths of the absorption maxima of the magnesium-free product (table I) were identical for all practical purposes with those given earlier for methyl pheophorbide b-3-methanol, obtained using aluminum isoproxide (10). (b) The infrared absorption spectrum, given in figure 3, showed that the formyl group was absent. The same spectrum showed that the C6 keto group and at least 1 ester group were present (21).

Reduction of Methyl Chlorophyllide a: Two hundred and fifty ml of fresh methanolic sodium borohydride (0.3 g/250 ml) were added to 60 mg of methyl chlorophyllide a dissolved in 1 ml of pyridine. After 15 minutes at room temperature the pigment was transferred into ethyl ether and then dried. It was adsorbed on a sucrose column from 15% CHCl3 in petroleum ether. This removed 2 minor bands from the main band, and left 4 minor bands close to the top of the column. Pigment in the main band was rechromatographed using 0.8% isopropanol in petroleum ether which removed a small amount of yellow-green pigment. The spectra of the reduced products of methyl chlorophyllide a and methyl pheophorbide a are given in figure 4.

Identification of the product as Mg-9-oxy-desoxo-methyl pheophorbide a: By the use of some of the tests given in 1A, it was shown that the magnesium-free derivative of the reduction product contained the C6 ester and C6 vinyl groups. The presence of the C6 carbomethoxy group was shown by a) the infra-red absorption spectrum of the free acid of the reduced product contained an ester carbonyl band (21); b) the methoxyl content of the free acid was 4.8% (calculated: 5.2%).

The above evidence eliminated all but the C6 hydroxyl, the C6 keto group and the C6-C6 bond as sites.

### Table I

**WAVE LENGTHS AND RELATIVE ABSORBANCES OF ABSORPTION MAXIMA AND MINIMA OF CHLOROPHYLL DERIVATIVES**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maxima</th>
<th>Minima</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M\mu)</td>
<td>R</td>
</tr>
<tr>
<td>Mg-methyl pheophorbide b-3-methanol</td>
<td>430 1.00</td>
<td>503 (plateau)</td>
</tr>
<tr>
<td>Mg-methyl pheophorbide a (methyl chlorophyllide a)</td>
<td>429 1.00</td>
<td>497</td>
</tr>
<tr>
<td>Methyl pheophorbide b-3-methanol</td>
<td>411 1.00</td>
<td>475</td>
</tr>
<tr>
<td>Methyl pheophorbide a</td>
<td>408 1.00</td>
<td>470</td>
</tr>
<tr>
<td>Mg-9-oxy-desoxo-methyl pheophorbide b-3-methanol</td>
<td>415 1.00</td>
<td>517</td>
</tr>
<tr>
<td>9-Oxy-desoxo-methyl-pheophorbide b-3-methanol</td>
<td>401 1.00</td>
<td>504</td>
</tr>
<tr>
<td>Mg-9-oxy-desoxo-methyl pheophorbide a</td>
<td>408 1.00</td>
<td>515</td>
</tr>
<tr>
<td>9-Oxy-desoxo-methyl pheophorbide a</td>
<td>396 1.00</td>
<td>500</td>
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</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>(M\mu)</th>
<th>R</th>
<th>(M\mu)</th>
<th>R</th>
<th>(M\mu)</th>
<th>R</th>
<th>(M\mu)</th>
<th>R</th>
<th>(M\mu)</th>
<th>R</th>
<th>(M\mu)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg-methyl pheophorbide b-3-methanol</td>
<td>474</td>
<td>80.0</td>
<td>503</td>
<td>47.6</td>
<td>548</td>
<td>33.8</td>
<td>586</td>
<td>19.1</td>
<td>630</td>
<td>11.1</td>
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<td>Mg-methyl pheophorbide a (plateau)</td>
<td>473</td>
<td>88.0</td>
<td>507</td>
<td>52.7</td>
<td>548</td>
<td>36.4</td>
<td>588</td>
<td>20.7</td>
<td>633</td>
<td>13.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl pheophorbide b-3-methanol</td>
<td>458</td>
<td>33.4</td>
<td>486</td>
<td>28.6</td>
<td>524</td>
<td>24.8</td>
<td>550</td>
<td>35.0</td>
<td>578</td>
<td>55.8</td>
<td>626</td>
<td>28.2</td>
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<tr>
<td>Methyl pheophorbide a</td>
<td>452</td>
<td>39.8</td>
<td>481</td>
<td>30.8</td>
<td>522</td>
<td>26.2</td>
<td>546</td>
<td>56.0</td>
<td>580</td>
<td>66.5</td>
<td>630</td>
<td>27.5</td>
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<tr>
<td>Mg-9-oxy-desoxo-methyl pheophorbide b-3-methanol</td>
<td>467</td>
<td>169.0</td>
<td>558</td>
<td>97.0</td>
<td>597</td>
<td>46.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-Oxy-desoxo-methyl pheophorbide b-3-methanol</td>
<td>450</td>
<td>46.7</td>
<td>539</td>
<td>72.0</td>
<td>572</td>
<td>117</td>
<td>612</td>
<td>57.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg-9-oxy-desoxo-methyl pheophorbide a (broad)</td>
<td>460</td>
<td>157.0</td>
<td>561</td>
<td>100</td>
<td>601</td>
<td>37.3</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-Oxy-desoxo-methyl pheophorbide a</td>
<td>465</td>
<td>42.6</td>
<td>539</td>
<td>96.8</td>
<td>571</td>
<td>140</td>
<td>615</td>
<td>57.2</td>
<td></td>
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</tbody>
</table>

R = Ratio of absorbance at "blue" maximum divided by the absorbance at wave length indicated.

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of reaction. The presence of the C5 secondary alcohol group, the C6 hydrogen and the intact C5—C6 bond was shown by a) the infra-red absorption spectrum indicated that the C5 keto group was absent; b) the phase test was negative; c) oxidation by chromic acid regenerated pheophorbide a (see section 4); d) the maxima of the visible absorption spectrum matched well those given earlier for 9-oxy-desoxo-methyl pheophorbide a (10).

Reduction of Porphyrins: Porphyrin as (10 mg) was dissolved in a small volume of pyridine and added to 10 ml of sodium borohydride in methanol (5 mg/ml). After 5 minutes the pigment was transferred intoethyl ether. The reduction product was readily removed from the starting material by extraction of the ether mixture with 2% HCl. Gradual neutralization of the dilute acid solution with NaHCO₃ facilitated the return of the reduction product from the aqueous layer into the ether layer. Attempts to prepare the reduced derivative of porphyrin as from 9-oxy-desoxo-pheophorbide a by treatment with HI in warm glacial acetic acid (15, 15) were not successful.

The spectrum of the reduction product differed from those given earlier for 9-oxy-desoxo-pheophyrin as (5, 6) by possessing an extra weak band at about 646 μm. The impurity responsible for this band was removed by crystallization from acetone or by extracting the ether solution with 0.2% (w/w) aqueous HCl. The wave lengths and relative intensities of the absorption maxima of the purified product agreed well with those given by Fischer and Grassi (5) which differ by several millimicrons from the earlier data of Fischer and Hasenkamp (6). Further confirmation that the reduction product was 9-oxy-desoxo-pheophyrin as was shown by its re-oxidation to pheophyrin as (see section 4).

Phylloerythrin was likewise readily reduced to the 9-oxy-desoxo derivative. The spectrum of the recrystallized product was identical with that produced by the catalytic hydrogenation of phylloerythrin in formic acid containing palladium black (6, 11).

Re-oxidation of the 9-Oxy-desoxo-derivatives of Porphyrin as and Porphophyrin a: Five-tenths ml of 0.5% (w/v) chromic acid in glacial acetic acid were added to 50 ml of a benzene solution containing 3 to 5 mg of these compounds. After 15 minutes 0.5 ml more of the acid solution was added, and after 30 minutes the solution was washed well with aqueous bicarbonate and with distilled water. The benzene was removed under vacuum and the pigment was dissolved in ethyl ether. In the case of the porphyrin 2% HCl removed the reduced products, and 10% HCl removed pheophyrin as. With pheophorbide the acid concentrations were 8 and 12%. The formation of pheophyrin as and of pheophorbide a was demonstrated conclusively by their absorption spectra, by a positive phase test, by methanolysis to yield the expected open ring derivatives (4, 7, 12, 18, 24), and by the fact that borohydride reduced them back to the 9-oxy-desoxo derivatives.

Discussion

Mg-methyl pheophorbide b-3-methanol is a compound whose properties could be predicted to be very similar to those of methyl chlorophyllide a. In the red region of the spectrum it absorbs maximally in ether at 654 μm, which distinguishes it from methyl chlorophyllide a since this absorbs maximally at 659 to 660 μm (see table I and figure 2). The HCl number, 14 (table II), is almost identical with that of methyl pheophorbide a, 15, and is much lower than that of methyl pheophorbide b, 21 (25). A significant similarity between the 3-methanol derivative and methyl chlorophyllide a is seen in their infra-red absorption spectra between 1600 and 1800 cm⁻¹. An earlier study of the infra-red spectrum of chlorophyll a in non-polar solvents had shown the presence of an intense band at about 1650 cm⁻¹, which was absent from the spectra of chlorophyll b and of phyllophytin a. The occurrence of this band is shown in figure 5 and supports the earlier hypothesis that the oxygen of the C5 formyl group of chlorophyll b or methyl chlorophyllide b reduces the ability of magnesium to promote enolization of the β-keto ester of ring V (21).

The shift of the “red” absorption maximum from 660 to about 630 μm upon reduction of the C5 keto group of methyl chlorophyllide a was reminiscent of the strong shift to about 642 μm which occurred upon methanolysis to yield Mg-chlorin α-trimethyl ester (18). This strong shift has bearing upon a recent postulate that a hydrated form of ring V cycles between the enol- and keto-form during photosynthesis (1, 16). If such a hydrated form exists its spectrum in vivo should be shifted from about 685 μm to about 655 μm, unless the binding to a specific protein changes the spectrum and differentiates it entirely from the predominant chlorophyll α-protein complex. This follows from the fact that replacing the C5 hydrogen atom of 9-oxy-desoxo-chlorophyll a by a hydroxyl group can have at the most only a small effect upon the parent spectrum. This is especially observable in porphyrins, e.g., the spectra of desoxo-phylloerythrin and 9-oxy-desoxo-phylloerythrin do not differ significantly (12).

It was observed (but not studied quantitatively) that the C5 keto group is far more resistant to reduction than are formyl or keto groups at the β positions of the pyrrole nuclei. One explanation of this might be that under the conditions of the reduction, ring V existed chiefly as the enol. The addition of a small
amount of borohydride immediately converted the phase test intermediate of chlorophyll b (generated in pyridine and methanolic magnesium methoxide, 1 : 1) into one resembling that of chlorophyll a.

**Summary**

By means of dilute methanolic solutions of sodium borohydride a selective reduction of the C₆ formyl group of the b series of derivatives, without reduction of the C₃ keto group or other groups in the molecule can be effected. Mg-methyl phorphorbide b-3-methanol, the reduction product of methyl chlorophyllide b, has a visible absorption spectrum characteristic of the a series of pigments, but has its "red" absorption maximum shifted 5 to 6 µm toward the "blue" relative to that of methyl chlorophyllide a. It gives a positive phase test, and has the acid number, 14. The infrared absorption spectrum of this product in non-polar solvents has a strong absorption band at about 1650 cm⁻¹ just as does that of methyl chlorophyllide a. This band disappears when magnesium is removed and is not present in the spectrum of methyl chlorophyllide b. In much higher concentrations sodium borohydride effects reduction of the C₆ keto group of methyl chlorophyllide a and of the 3-methanol derivative of methyl chlorophyllide b. The reduced products have their "red" absorption maxima shifted from the 660 to the 630 µm region. Consequently hydration of the C₆ keto group must effect a similar "blue" shift. Phorphorpyrin and phylloerythrin are also reduced to their 9-oxy-desoxy derivatives. 9-Oxy-desoxy-phorphorpyrin a is oxidized to phorphorpyrin b by dilute solutions of chromic acid in benzene. At no time during the reduction of the C₆ keto group by sodium borohydride in methanol at room temperature is an intermediate reduction product observed.

The author is very grateful for helpful discussions with Dr. S. F. MacDonald of the Division of Pure Chemistry, National Research Council. Infra-red absorption spectra were measured by Mr. F. Rollin. Technical assistance was provided by Mr. W. Meath.

**Literature Cited**


