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## Abstract

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## STUDY OF THE NATURE OF THE PRIMARY PHOTOREDUCED FORM OF CHLOROPHYLL AND ITS ANALOGS USING $D_2O$

*(Presented by Academician A. N. Terenin, 2 III 1957)*

The data presented in previous communications (1,2) testify in favor of the fact that the primary photoreduced form of chlorophyll and its analogs (1b) that we have discovered, which is the first stage of photoreduction of these pigments (3,4), is by its nature a free radical—an ionized semiquinone, formed by transfer of an electron from the reductant to the long-lived excited biradical (5) form of the pigment.

However, the question of whether the formation of the primary reduced form is indeed the result only of electron transfer to the pigment, or whether a proton also participates in this process, still could not be considered definitively resolved.

Deliberately simplifying the scheme, one may imagine the following two series of reactions in the photoreduction of chlorophyll and its analogs, which include the formation of the primary reduction product with the properties found in previous studies:

$$\mathrm{Chl}^{\vee} + \mathrm{RH}_2 \to \cdot\mathrm{Chl}^{-} + \mathrm{RH}_2^{+} \to \cdot\mathrm{ChlH} + \cdot\mathrm{RH}, \tag{1}$$

$$\mathrm{Chl}^{\vee} + \mathrm{RH}_2 \to \mathrm{ChlH}^{-} + \mathrm{RH}_2^{+} \to \mathrm{ChlH}_2 + \mathrm{R}, \tag{2}$$

where $\mathrm{Chl}^{\vee}$ is chlorophyll in the biradical state, $\mathrm{RH}_2$ is the reductant; the underlined species are the primary and secondary reduced forms.

For a final decision on the question of which of these series of reactions should be preferred, at least with respect to the formation of the primary reduced form, we decided to apply the well-known kinetic method (6), already used in our laboratory (7), based on replacing labile hydrogen (H) in the reductant by deuterium (D).

As is known (6), in the case of reactions involving hydrogen transfer, such replacement usually leads to a slowing of the reaction, since the activation energy

Fig. 1

Figure 1: Fig. 1

is then greater; apparent exceptions are explained by particular features, especially the multistep character of the given reaction (6). Therefore, experiments on the influence of replacing H by D in one or several components of a reaction on the rate of this reaction can usually give an answer to the question of whether hydrogen participates in the given reaction. In particular, in this way the participation of hydrogen in photosynthesis (8), the Hill reaction (7,9), and the photoreduction of chlorophyll (7) was confirmed.

Thus, the task before us was to determine how replacement of H by D in the reductant would affect the rate of formation of the primary reduced form of the pigments. As in a number of previous studies—

studies (1), pheophytin proved to be the most convenient pigment for these experiments; its primary reduced form is the most stable (1) and makes it possible to carry out experiments that are impossible with the primary reduced form of chlorophyll because of the very high rate of its reverse reactions.

The work was carried out under vacuum conditions in a pyridine solution with the addition of 10% $H_2O$ or $D_2O$. Pyridine is convenient in this case because it contains no labile H atoms capable of being replaced by D. Therefore, the exchange of hydrogen for deuterium in dissolved ascorbic acid is determined entirely by the ratio of the concentrations of the added $D_2O$ and of this acid. In our experiments the concentration of $D_2O$ (500 mg per 5 ml of solution) was 100 times greater than the concentration of ascorbic acid (5 mg per 5 ml); therefore it may be assumed that, in experiments with $D_2O$, the equilibrium was almost completely shifted toward the formation of ascorbic acid with labile D atoms, and that almost exclusively these atoms could participate in the reduction reaction.

**Fig. 1.** Effect of replacing H by D in ascorbic acid on the rate of formation of the primary (*I*) and secondary (*II*) reduced forms of pheophytin a. **1** –$H_2O$, **2** –$D_2O$

Figure 1 shows the effect of replacing H by D in ascorbic acid on the rate of formation of the primary and secondary reduced forms of pheophytin a. The formation of the primary reduced form was followed from the change in the value of the extinction coefficient of the solution at 470 mμ (1), illuminated with red light at −40° (1). The rate of formation of the secondary reduced form was measured from the change in the maximum at 525 mμ at room temperature.

As follows from the figure, replacement of H by D in the reducing agent does not slow the formation of the primary reduced form; on the contrary—even after some time the curve in the presence of $D_2O$ begins to run slightly higher than in the presence of $H_2O$, which may be explained by more rapid secondary changes

Fig. 2. Effect of replacing H by D in ascorbic acid on the rate of transition of the primary reduced form of pheophytin into the secondary one. 1—$H_2O$, 2—$D_2O$

Figure 2: Fig. 2. Effect of replacing H by D in ascorbic acid on the rate of transition of the primary reduced form of pheophytin into the secondary one. 1—$H_2O$, 2—$D_2O$

of the primary form in the presence of $H_2O$, limiting the further rise of the curve.

In contrast to this, replacement of $H_2O$ by $D_2O$ considerably decreases the rate of appearance of the red reduced form (525 mµ). The slowing of chlorophyll reduction upon such replacement had already been shown earlier (7), and at that time the course of the reaction was judged from the decrease of the red maximum of chlorophyll under the corresponding conditions. The effect of $D_2O$ on the reduction of chlorophyll was again confirmed by us by measuring the rate of increase of the maximum at 525 mµ.

The decrease in the rate of transition of the primary reduced product of pheophytin a into the secondary form was shown by us in a direct experiment, the results of which are shown in Fig. 2. The test solution of the above composition was illuminated at −40°, as a result of which almost complete reduction of pheophytin a occurred, with formation of the primary reduced form (1). Then the reaction tube (1) was inserted into the spectrophotometer, and the extinction coefficient at 525 mµ (1) was measured. As the solution warmed in the dark, transformation of the primary reduced···

form into the secondary one. Fig. 2 shows that this transformation is greatly slowed when $H_2O$ is replaced by $D_2O$.

It is interesting to note that when pheophytin a is used, in contrast to chlorophyll (7), the rate of decrease of the red maximum (670 m$\mu$) under illumination differs little in the $D_2O$ and $H_2O$ variants, not only at low temperature but also at room temperature. However, the reduced solution obtained immediately after illumination at room temperature differs considerably in its composition in the two cases. If, in the presence of $H_2O$, the solution contains almost exclusively the secondary reduced form and accordingly has a red-violet coloration, then in the presence of $D_2O$ the color of the solution is brownish-brown, and it contains a large amount of the primary form. This once again shows the substantially greater stability of the primary reduced form of pheophytin, as compared with that of chlorophyll. The use of $D_2O$ may therefore serve as an additional methodological device for delaying the transition of this form into the secondary one.

**Fig. 2.** Effect of replacing H by D in ascorbic acid on the rate of transition of the primary reduced form of pheophytin into the secondary one. 1—$H_2O$, 2—$D_2O$

Thus, the results of experiments using $D_2O$ quite definitely indicate that the proton does not participate in the formation of the primary reduced form of the pigments, unlike in the formation of the secondary reduced form. This form is produced as a result of the primary photochemical transfer of an electron from the reducing agent to the pigment, which is in a long-lived excited state (see equation (1)). Electron addition apparently occurs at some point in the system of conjugated bonds, as a result of which the absorption spectrum of the pigment changes sharply and becomes diffuse (1v). The secondary reduced form is apparently the result of subsequent proton addition. The absorption spectrum of the reduced pigment becomes more discrete, although it differs from the spectrum of the original pigment (1,2).

The difference in the properties of the primary reduced forms of various pigments of the chlorophyll group, in particular these forms of chlorophyll and pheophytin, is apparently explained by the different effect on the system of conjugated bonds of the positive atoms ($2H^{++}$ or $Mg^{++}$) located at the center of the molecule. In the presence of $2H^+$ (pheophytin), the extraneous electron is accepted more readily and is held more strongly than in the presence of a divalent metal, in particular $Mg^{++}$ (chlorophyll). This accounts for the considerable difference in the stability of the electronic reduced forms of these pigments (1,2).

Additional data on the influence of $D_2O$ on the photoreduction reaction of chlorophyll were obtained by the potentiometric method.

Fig. 3 presents curves of the change in potential of a platinum electrode immersed in a solution of chlorophyll or pheophytin in pyridine with the addition of $H_2O$ or $D_2O$ in the presence of a reducing agent under illumination. In accordance with what was already shown earlier (1a,10), upon illumination in the presence of ordinary ascorbic acid (experiment with $H_2O$), the potential-change curve, after first decreasing, rapidly bends upward as a result of further changes in the electrode-active primary reduced form, and above all owing to its transition into the electrode-inactive secondary reduced form. Replacement of H by D in ascorbic acid (experiment

with $D_2O$, slowing this transition (see Fig. 2), at once changes the shape of the curve. The drop continues further and the minimum is reached much later. These data once again confirm that it is precisely the primary reduced form that possesses electrode activity.

A pattern of the influence of $D_2O$ on the formation of the primary and secondary reduced forms of the pigments, similar to that described above, was observed by us in several other solvents incapable of rapid exchange of their hydrogen for deuterium, in particular in acetone (with addition of pyridine). In contrast, addition of $D_2O$ instead of $H_2O$ to the methanol solution did not lead to a change in the rate of photoreduction of chlorophyll or pheophytin, in accordance with the presence of labile hydrogen in the solvent molecules, capable of being replaced by D. In this solvent, upon addition of a small amount of $D_2O$, ordinary ascorbic acid was converted into the deuterated form only to a small extent,

Fig. 3. Curves of photopotential change of pyridine solutions of pheophytin a ( ) and chlorophyll a + b ( ) in the presence of $H_2O$ (1) and $D_2O$ (2)

Figure 3: Fig. 3. Curves of photopotential change of pyridine solutions of pheophytin a ( ) and chlorophyll a + b ( ) in the presence of $H_2O$ (1) and $D_2O$ (2)

insufficient to affect the reaction rate.

**Fig. 3.** Curves of photopotential change of pyridine solutions of pheophytin a ( ) and chlorophyll a + b ( ) in the presence of $H_2O$ (1) and $D_2O$ (2)

The use of $D_2O$ also made it possible once again to confirm that the red-violet reduced compound formed during photoreduction of magnesium phthalocyanine ([1d,2]), despite its instability, is the secondary reduced form, since the rate of its formation, and especially of the reverse reaction, decreases when H is replaced by D in the reducing agent.

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