*PTERIDINES AND THE FUNCTION OF THE PHOTOSYNTHETIC REACTION CENTER**

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Communicated by William Arnold, June 2, 1969

Abstract.—The photoreduction and interaction with the photosynthetic "reaction center" of 2-amino,4-hydroxy-6-substituted pteridine indicates that these low-potential (~ -0.7 v), naturally occurring compounds play a primary role in photosynthetic electron transport. These unconjugated pteridines, which occur in association with the photosynthetic apparatus of green plants and photosynthetic bacteria, can be reduced by light in the presence of a bacterial chromatophore fraction from the dihydro form to the tetrahydro form. 6,7-Dimethyl-tetrahydropteridine readily reduces spinach ferredoxin. This compound also specifically interacts with reaction-center chlorophyll and bacteriochlorophyll and produces spectral shifts similar to those produced by light. It is proposed that the electron produced by excited-state chlorophyll is captured and separated by a pteridine at -0.67 v at the photosynthetic reaction center.

It has been 38 years since van Niel, in his classic studies of the comparative biochemistry of photosynthesis, first proposed that the primary photochemical act involved the initial separation of an oxidant and a reductant, mediated by light.[1] Since that time it has become clear that in bacterial photosynthesis a single photochemical act, producing a strong reductant, is the initial photochemical step. In higher plants the primary photochemistry involves two separate photochemical events: system I, using reaction-center chlorophyll P 700, which produces a strong reductant similar to the bacterial system; and system II, which produces a strong oxidant and is coupled with the evolution of molecular oxygen.[2] Comparative studies indicate that system I of green plant photosynthesis and the primary photochemical act of the bacteria may be similar in nature.

The actual compounds involved in stabilizing the primary electron on the reduction side of photosynthesis have not been identified. It has been clear for many years that the nonheme iron compound, ferredoxin, plays a role in the electron transport on the reducing side of photosynthesis.[3,4] However, in photosynthetic bacteria, the role of this compound on the reduction side of photosynthesis has not been clear. It is known to play a role in nitrogen fixation and hydrogenase activity, but its involvement in the primary photochemistry of bacterial photosynthesis has been open to serious question.[5,6]

For primarily theoretical reasons, Calvin has proposed that the primary photochemical act of photosynthesis should yield a stabilized reductant with a potential $E_0{}^1$ of about -1.0 v.[7] More recent work of Zweig and Avron,[8] Black,[9] and Kok *et al.*[10] has indicated that isolated photochemical systems involving photo system I in higher plants and algae can reduce viologen dyes with known chemical

potentials of less than −0.7 v, a full 0.3 v below the potential of ferredoxin, a proposed primary acceptor in system I of higher plants and bacteria.[11]

For physical-chemical considerations it thus seemed clear, in view of the occurrence of a low-potential reductant, that a primary electron acceptor and stabilizer of system I of higher plants and in bacterial photosynthesis should be closer to the potentials reached by these photochemical systems in the light. Such a class of compounds with lower potentials has been known to exist in many plant and animal forms.

The unconjugated, 6-substituted pteridines similar to biopterin occur in a wide variety of forms in nature. The substitution of a side chain at the 6 position on the ring is apparently essential to biological activity, and this substitution is manifested by a variety of short carbon chains. Rembold[12] has measured the potentials of several of these unconjugated pteridines by polarographic methods. The compounds listed below, taken from the experiments of Rembold, are examples of the low potentials of these naturally occurring compounds, all of which contain the pteridine ring, with the substitution in the 2 position of an amino group; in the 4 position, of a hydroxy group; and in the 6 position, of a side chain of varying lengths (see Fig. 5).

Substitution on pteridine ring[12]	E_0 (v)
2—NH_2—4—OH—6—$(CHOH)_2 \cdot CH_3$ (biopterin)	−0.696
2—NH_2—4—OH—6—$(CHOH)_2 \cdot CH_2OH$ (neopterin)	−0.690
2—NH_2—4—OH—6,7—CH_3 (dimethyl-tetrahydropteridine)	−0.732

The presence of unconjugated pteridines in anaerobic systems was first shown in the obligately anaerobic photosynthetic bacteria, which was shown to contain large quantities of unconjugated pteridines.[13] McLean *et al.*[14] have shown that compounds related to biopterin could stimulate electron transport in photosynthesis, and work has also been presented to show that these unconjugated pteridines are associated with the photochemical apparatus in anaerobic photosynthetic bacteria, blue-green algae, and in chloroplasts of higher plants.[15]

We have previously shown that photosynthetic phosphorylation by isolated chromatophore fractions from *Chromatium* strain D, as well as CO_2 fixation by whole cells of *Chromatium*, can be inhibited by specific pteridine inhibitors such as 4-phenoxy-2,6-diamino pyridine which indeed inhibits the development of the photosynthetic apparatus in dark, aerobically grown, pigmentless *Rhodospirillum rubrum*. This inhibition can be reversed by biopterin.[16] That photochemical electron transport is involved in these inhibition reactions has been demonstrated: 4-phenoxy-2,6-diamino pyridine specifically inhibits light-induced reduction of flavin mononucleotide in a chromatophore system from *Rhodospirillum rubrum*.[17]

The work described below will suggest that these low-potential unconjugated pteridines are involved in the primary photochemical reduction in photosynthesis and may indeed be the primary site at which the reducing power of photosynthesis is stabilized in both system I of higher plants and in the single primary photochemical act of bacterial photosynthesis.

Materials and Methods.—Ferredoxin reduction: Spinach ferredoxin (Sigma Chemical Co., St. Louis) was placed in a cuvette with 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8 tetrahydropteridine hydrochloride (Aldrich Chemical Co., Milwaukee), and reduction was monitored with a Cary 14R spectrophotometer.

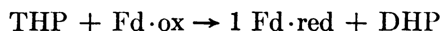
Pteridine photoreduction: Biopterin (obtained through the courtesy of Dr. H. Rembold) was placed in a cuvette under argon with chromatophores from either *R. rubrum* or *Chromatium* prepared as previously described.[18] Prior to illumination, the biopterin was reduced to a dihydro level with $Na_2S_2O_4$. Reduction was monitored with a Farrand recording spectrofluorometer as a decrease in fluorescence yield at 435 mm. Internal quenching was minimized by using suspensions containing microgram amounts of chlorophyll.

Preparation of chromatophore subfraction: Chromatophores in a 40,000 × *g* supernatant of an *R. rubrum* cell extract were treated with 0.4% (v/v) Triton X-100 in 0.01 *M* Tris-HCl (pH 7.8). This supernatant was then passed through Sephadex G-150 or Sepharose 2B. The pigment-protein fraction was collected and the interaction with tetrahydropteridine was examined by difference spectroscopy.

Preparation of Euglena chloroplast subfraction: Euglena gracilis strain Z, cultured autotrophically as described earlier,[19] was prepared by breaking the cells at 5,000 psi in the French press. The broken cell suspension was centrifuged at low speed to remove unbroken cells, and the green supernatant was then treated with detergent as for *R. rubrum* chromatophores. The detergent-treated chloroplasts were then put through the French pressure cell at 10,000 psi and the resulting homogenate was centrifuged at 40,000 × *g*. The green supernatant was then put through Sephadex or Sepharose columns as had been done with the chromatophores.

Results.—Although polarographic measurements have indicated that the pteridines have an extremely low potential, ranging from −0.5 to −1.0 v, the true chemical or biological potential was difficult to measure in either chemical or enzymatic reactions. It was also difficult to work with compounds in the completely reduced form in any chemical or biological system, since autoxidation was almost instantaneous. The substitution of a methyl group in the 7 position of the pteridine ring allows for the preservation of this pteridine in the tetrahydro-reduced form over a short period of time. This compound, 6,7-dimethyl-tetrahydropteridine, the formula for which is indicated in Figure 5 (III), is known to substitute for the cofactor in the enzymatic and chemical reactions for the hydroxylation of phenylalanine to form tyrosine.[20, 21] However, no chemical or biological potential of pteridines have been measured directly in biological or chemical systems.

Reduction of spinach ferredoxin by tetrahydropteridine (THP): Spinach ferredoxin, with a known measured potential of −0.42 v, was placed in a cuvette in the spectrophotometer and mixed with 6,7-dimethyl-tetrahydropteridine. An immediate and stoichiometric reduction of ferredoxin was observed, as shown in Figure 1. This experiment clearly indicates that the active chemical potential of the tetrahydropteridines is much below that of ferredoxin. The reaction involves the reduction of ferredoxin (Fd) at the expense of tetrahydropteridine (THP), with the concomitant formation of reduced ferredoxin and dihydropteridine (DHP), as indicated in the following reaction mechanism:



$$\text{THP} + \text{Fd}\cdot\text{ox} \rightarrow 1\ \text{Fd}\cdot\text{red} + \text{DHP}$$

Photoreduction of pteridine by light in a photosynthetic system: The ability of light energy, in association with an isolated photochemical system from either green plants or photosynthetic bacteria, to reduce oxidized pteridine is shown in Figure 2. In this case the dihydropteridine, reduced chemically by dithionite, is placed with a chromatophore preparation from either *R. rubrum* or *Chromatium* strain D, and the rapid photoreduction of pteridine, as measured by the quench-

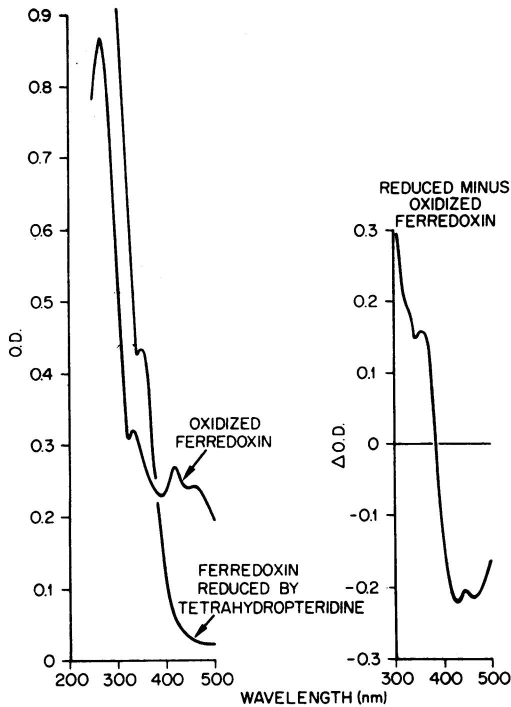


FIG. 1.—Reduction of spinach ferredoxin (0.42 v) by tetrahydropteridine. As indicated in the left-hand spectrum, spinach ferredoxin in its oxidized form has characteristic peaks of 275, 330, 420, and 455 nm. Upon addition of tetrahydropteridine, a typical spectrum of reduced ferredoxin concomitant with the loss of the blue peaks is observed. The figure on the right is the difference spectrum of oxidized ferredoxin vs. tetrahydropteridine-reduced ferredoxin.

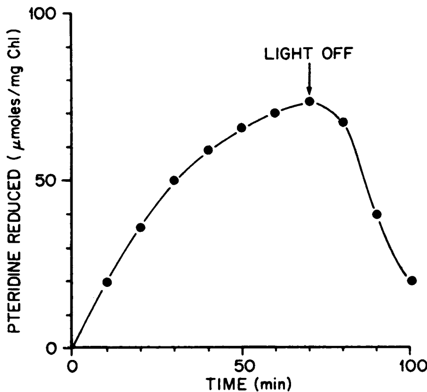


FIG. 2.—Pteridine (dihydrobiopterin) reduction by light in the presence of a bacterial chromatophore fraction containing 0.4 μg/ml bacteriochlorophyll. Dihydrobiopterin (3×10^{-8} moles per ml) was added and the cuvette illuminated from above. Photoreduction was monitored by loss of fluorescence at 435 nm. At the point indicated, the light was turned off and reoxidation occurred.

ing of fluorescence, is observed. This reaction is carried out under an argon atmosphere. When the light is shut off, a rapid reoxidation and concomitant regaining of fluorescence from the oxidized pteridine material is observed. Therefore, it is quite clear that the photochemical systems isolated from bacteria have the ability to photoreduce pteridines and produce a photoreductant at a much lower biological potential than ferredoxin.

Interaction of pteridines with the "reaction center" of photosynthetic tissues: Removal of pteridines and other soluble small molecules from a chromatophore fraction of *R. rubrum* was accomplished by repeated washings (using both centrifugation and passage through either Sephadex or Sepharose columns) with basic

Tris or carbonate buffers. This treatment removes the majority of pteridines from these pigmented protein complexes. These chromatophore fractions are apparently unaltered chemically, as far as the protein-pigment complex is concerned, since the absorption spectrum of bacteriochlorophyll is essentially unaltered from the spectrum *in vivo*. Clayton has shown that unwashed chromatophore preparations or whole cells when illuminated show a difference spectrum, light versus dark, concomitant with changes proposed at the "reaction center." There is a bleaching in *R. spheroides* at 870 nm, assumed to be the reaction-center chlorophyll.[22] In *R. rubrum* this shift is at 890 nm. Other changes in the spectrum, indicating a change in the oxidation-reduction state of the chlorophyll

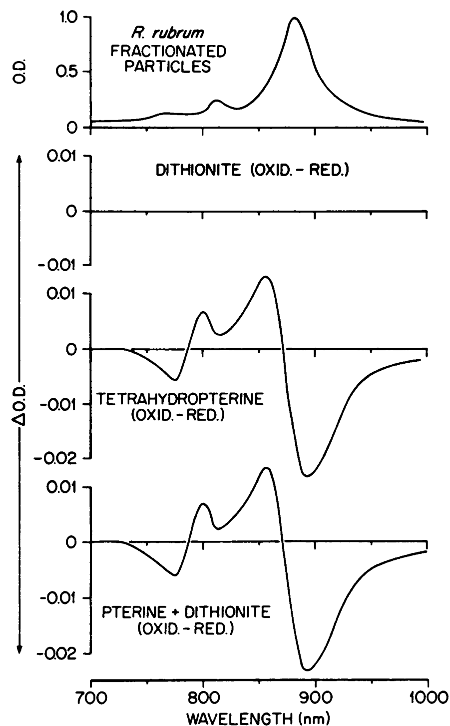


FIG. 3.—Interaction of reduced pteridine with reaction-center chlorophyll. The top figure is the far-red absorption spectrum of the purified chromatophore fraction from *R. rubrum*. The second figure is a difference spectrum with the addition of dithionite to one cuvette. There are no apparent changes. The third figure shows the difference effect upon the addition of tetrahydropteridine. The major shift occurs at 890 nm. As shown in the lower figure, although oxidized pteridine has no direct effect, the addition of dithionite and oxidized pteridine shows differences identical to that observed after adding tetrahydropteridine.

protein complex of the reaction center, also occur.[23] If chromatophore preparations that have had the pteridine removed are exposed to the strong reductant dithionite, no differences in the absorption spectrum at the reaction center are noted. However, if 6,7-dimethyl-tetrahydropteridine is added in the dark to such a preparation, a difference spectrum is observed which is strikingly similar to the light-induced difference spectrum reflecting a change at the reaction center in whole chromatophore preparations. As shown in Figure 3, this reaction is specific for reduced pteridines, in that neither dithionite alone nor oxidized pteridines cause any change in the absorption spectrum. However, if the oxidized pteridine is added to the preparation and dithionite is subsequently added, virtually identical shifts of the absorption spectra are obtained as when adding the reduced pteridine directly. This clearly indicates that the *in situ* presence of

a reduced pteridine in the reaction center causes a similar shift in the spectrum to that induced by the light in whole chromatophores.

If similar experiments are performed with subchloroplast fractions from *Euglena*, it can be shown that the absorption spectrum of the chlorophyll protein complex, a combination of systems I and II, is clearly unaltered by the washing procedures (Fig. 4). However, in these preparations an oxidation-reduction difference spectrum can be demonstrated when dithionite is added. If tetrahydropteridine is added to this preparation already reduced by dithionite, a further change occurs. As shown in Figure 4, the pteridine-induced change is primarily at P 700, considered to be the reaction center of system I in green plants and algal chloroplasts. The initial dithionite response may well reflect a change in light system II.

Again, this strongly indicates a specific interaction of tetrahydropteridine with reaction-center molecules in photosynthetic bacteria and system I from chloroplasts of the green alga, *Euglena*. The total change in chlorophyll in the difference spectra reflects a change of about 1 per cent of the total chlorophyll.

Discussion.—It thus seems clear that the 2-amino,4-hydroxy,6-substituted pteridines fulfill the biological, chemical, and physicochemical requirements of a primary photochemical electron acceptor in photosynthesis, at a potential of approximately −0.7 v. It is clear that this compound occurs in association with the pigment-protein complex characteristic of the reaction center of chromatophore fractions and chloroplasts. That these compounds show a specific complex with the reaction center, yielding an oxidized versus reduced-difference spectrum similar in many ways to the light versus dark difference spectrum, has been clearly demonstrated. The occurrence of low-potential pteridines has been observed in many forms but has not been related with any potential electron transport system prior to these observations.

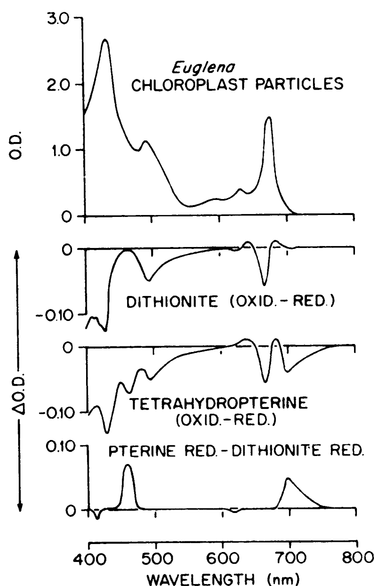


FIG. 4.—Effect of tetrahydropteridine on a *Euglena* chloroplast fraction. Top figure is the absorption spectrum of the purified fraction. The second figure is a difference spectrum obtained by adding dithionite to one cuvette. The third figure indicates the change observed after adding tetrahydropteridine. The bottom figure is simply a subtraction of the second and third, showing the net effect of tetrahydropteridine at 700 nm.

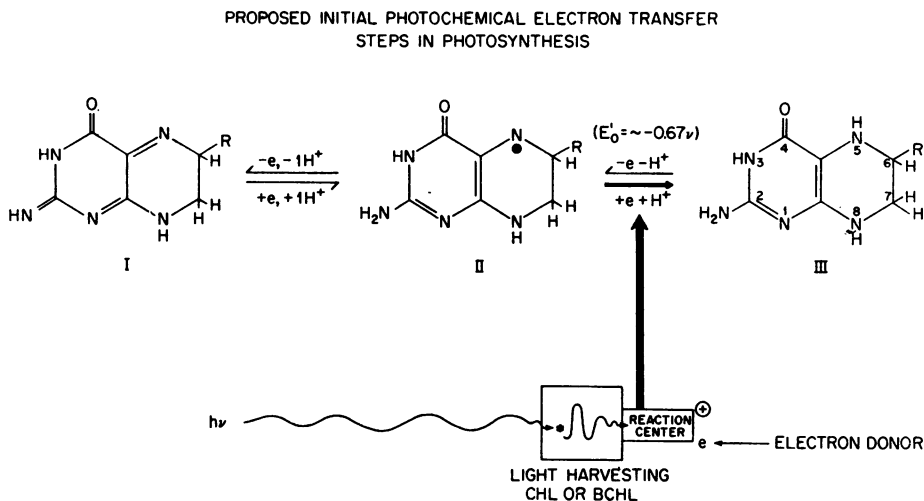


FIG. 5.—Oxidation-reduction scheme of pteridines, indicating a single-electron step induced by light energy from the reaction center.

The mechanism of the acceptance and consequent stabilization of the primary electron produced in the photochemical act is still not completely clear. Theoretical and observed considerations require that a single electron transfer is involved in the production of a primary photochemical reductant. The conversion of oxidized pteridine to tetrahydropteridine is known to occur by a series of reactions, and the pathway for these reactions involves a series of single electron steps and the occurrence of quinone intermediates. The evidence has been demonstrated for the presence of a quinoid intermediate form and the single electron stepwise oxidation or concomitant reduction of pteridine.[24, 25] A scheme for the photoreduction steps in photosynthesis via known reactions of pteridines is shown in Figure 5. It therefore seems possible that the primary photochemical act in photosynthesis may involve the capture of a single electron by a pteridine semiquinone for the direct reduction to the tetrahydro form (III) at a potential level of about -0.67 v.

We wish to thank Drs. G. W. Kidder and V. C. Dewey of Amherst College for collaboration during much of this work, and Drs. William Arnold of Oak Ridge National Laboratory and John Totter of the Atomic Energy Commission for helpful and stimulating discussion of these results.

* This research was partially supported by grant GM 15595 from the National Institute of General Medical Sciences, U.S. Public Health Service, to the University of Tennessee, and partially by the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

1 van Niel, C. B., *Arch. Mikrobiol.*, **3**, 1 (1931).

2 San Pietro, A., and C. C. Black, *Ann. Rev. Plant Physiol.*, **16**, 155 (1965).

3 Chance, B., and A. San Pietro, these PROCEEDINGS, **49**, 633 (1963).

4 Arnon, D. I., in *Photosynthetic Mechanisms of Green Plants* (Washington, D. C.: National Academy of Sciences, 1963), NAS-NRC publ. 1145, p. 195.

5 Bennett, R., and R. C. Fuller, *Biochem. Biophys. Res. Commun.*, **16**, 300 (1964).

6 Bennett, R., N. Rigopoulos, and R. C. Fuller, these PROCEEDINGS, **52**, 762 (1964).

[7] Calvin, M., and G. M. Androes, in *La Photosynthèse* (Paris: Centre National de la Recherche Scientifique, 1962), p. 21.
[8] Zweig, G., and M. Avron, *Biochem. Biophys. Res. Commun.*, **19**, 397 (1965).
[9] Black, C. C., *Biochim. Biophys. Acta*, **120**, 332 (1966).
[10] Kok, B., H. S. Rurainski, and O. V. H. Owens, *Biochim. Biophys. Acta*, **109**, 347 (1965).
[11] Tagawa, K., and D. I. Arnon, *Nature*, **195**, 537 (1962).
[12] Rembold, H., in *Pteridine Chemistry*, ed. W. Pfleiderer and E. C. Taylor (London: Pergamon Press, 1964), p. 465.
[13] Fuller, R. C., N. Nugent, G. W. Kidder, and V. C. Dewey, *Federation Proc.*, **25**, 205 (1966).
[14] McLean, F. I., Y. Fugita, H. S. Forrest, and J. Myers, *Science*, **149**, 636 (1965).
[15] Nugent, N., N. Rigopoulos, and R. C. Fuller, in *Plant Physiology, Proceedings of the Annual Meeting* (College Park, Maryland: University of Maryland Press, 1966), p. xxiii.
[16] Fuller, R. C., N. A. Nugent, G. W. Kidder, V. C. Dewey, and N. Rigopoulos, manuscript in preparation.
[17] Nugent, N. A., and R. C. Fuller, manuscript in preparation.
[18] Fuller, R. C., S. F. Conti, and D. B. Mellin, in *Bacterial Photosynthesis*, ed. H. Gest, A. San Pietro, and L. P. Vernon (Yellow Springs, Ohio: The Antioch Press, 1963), p. 71.
[19] Fuller, R. C., and M. Gibbs, *Plant Physiol.*, **34**, 324 (1959).
[20] Kaufman, S., *Ann. Rev. Biochem.*, **36**, 171 (1967).
[21] Bobst, A., and M. Viscontini, *Helv. Chim. Acta*, **49**, 884 (1966).
[22] Clayton, R. K., in *Molecular Physics in Photosynthesis* (New York: Blaisdell Publishing Company, 1965), p. 24.
[23] Sybesma, C., *Biochim. Biophys. Acta*, **172**, 177 (1969).
[24] Bobst, A., *Helv. Chim. Acta*, **50**, 2222 (1967).
[25] *Ibid.*, **51**, 607 (1968).