Conversion of Light to Chemical Free Energy

I. PORPHYRIN-SENSITIZED PHOTOREDUCTION OF FERREDOXIN BY GLUTATHIONE*

(Received for publication, September 6, 1968)

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SUMMARY

Iron analysis and titration data show that each mole of ferredoxin from Clostridium pasteurianum reduces 2 moles of ferricyanide.

The midpoint reduction potentials of the first and second reducing equivalents of this bacterial ferredoxin are found to be $-0.367$ and $-0.398$ volt, respectively.

In aqueous solutions at pH 7.4 under helium, the oxidized ferredoxin is photoreduced by glutathione in the presence of hematoporphyrin. After the illumination, the reaction system slowly returns in the dark to its original condition in the absence of oxygen.

Calculations based on the present data and the literature value of the midpoint potential of glutathione show that 3.2 kcal of light energy are stored per mole of electrons transferred from glutathione to ferredoxin in the final reaction mixture.

The spectrometric and electron spin resonance data show that in this reaction the excited hematoporphyrin first captures an electron from glutathione to form a porphyrin radical, which then transfers its extra electron to ferredoxin.

EXPERIMENTAL PROCEDURE

Materials

C. pasteurianum ferredoxin was purchased from Worthington and recrystallized by the method of Mortenson (7). The ratio of absorbances of the oxidized ferredoxin at 390 and 280 nm, respectively, is $A_{390} : A_{280} \approx 0.78$. The ratios of the absorbance of the ferredoxin reduced with a slight excess of sodium dithionite to the absorbance of the oxidized ferredoxin are 0.454 and 0.920 at 425 nm and 600 nm, respectively.

Hematoporphyrin IX dihydrochloride (molar extinction coefficient at the Soret peak $e_{394} = 1.37 \times 10^5$ M$^{-1}$ cm$^{-1}$) was purchased from Sigma. Methyl viologen (N,N'-Dimethyl-4,4'-dipyridinium dichloride) and oxidized and reduced glutathione were purchased from Mann. The midpoint reduction potential of methyl viologen, determined at both pH 9 and 11 by reductive titration of sodium dithionite and by oxidative titration of the dithionite reduced solution with ferricyanide, was found at 23° to be $-0.444 \pm 0.002$ volt. The titrations were carried out in a Radiometer automatic titrator (type TTT11) designed for anaerobic titrations. The molar extinction coefficient of methyl viologen reduced with a slight excess of sodium dithionite in aqueous Tris buffer was found to be $(1.14 \pm 0.02) \times 10^4$ M$^{-1}$ cm$^{-1}$ at 600 nm. The ratio of the absorbance of reduced methyl viologen at 600 and 425 nm, respectively, was found to be $A_{600} : A_{425} = 0.141 \pm 0.002$.

To keep the ionic strength approximately constant, all 0.05 M KH$_2$PO$_4$-K$_2$HPO$_4$ buffer solutions and 0.01 M Tris-HCl buffer solutions were made 0.15 M in NaCl.

Methods

Iron Analysis—The concentration of total iron in a buffered ferredoxin solution of measured absorbance at 425 nm was determined by means of a Perkin-Elmer model 303 atomic absorption spectrophotometer, with freshly prepared potassium ferri-

* This work was supported in part by Research Grants GB-3631 and GB-7430X from the National Science Foundation.
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Titrations of Ferredoxin with Ferri cyanide—To determine the oxidation-reduction capacity of ferredoxin, the protein was reduced and then titrated in an inert atmosphere in the apparatus shown in Fig. 1 by the following procedure. A solution of clostridial ferredoxin in aqueous Tris buffer at pH 7.4 was reduced completely by an excess of granular zinc in the presence of a trace amount of methyl viologen as a mediator in the side tube, F. The "completely reduced" ferredoxin solution was decanted from the zinc in F into the optical cell, G, to have its absorption spectrum taken. A previously weighed amount of solid K$_3$Fe(CN)$_6$ was then added from the side tube D or E to the solution for partial oxidation of the ferredoxin. Final complete reoxidation of the ferredoxin was achieved by introducing oxygen through the stopcock, B. By using different previously weighed amounts of K$_3$Fe(CN)$_6$, and following each oxidation by measuring the absorbance of the solution at 425 nm, the oxidation-reduction capacity of the ferredoxin could be determined.

Measurement of Oxidation-Reduction Equilibrium between Ferredoxin and Methyl Viologen—A solution of ferredoxin and methyl viologen in Tris buffer at pH 7.4 was reduced stepwise in the apparatus of Fig. 1 as follows. The solution was initially placed in cell G under helium to have its absorption spectrum taken. It was then poured into the side tube F, shaken with an excess of granular zinc in F for a few seconds, and decanted back into cell G for spectrometric measurement. The absorbances of the solution at 425 nm and 600 nm give, after corrections for the absorbance of ferredoxin at 600 nm and for the absorbance of methyl viologen at 425 nm, a quantitative measure of the ratios of the reduced to oxidized forms of ferredoxin and methyl viologen, respectively. The solution in G was then transferred again into F, further reduced with zinc, and decanted back into G for spectrometric measurement. After this procedure was repeated a number of times, the process was terminated at about 75% reduction in order to avoid over-reduction of methyl viologen by zinc, and air was let in through B to reoxidize the solution in G. The absorption spectrum of the reoxidized solution was found to be the same as that of the original solution. The absorbance at 600 nm of totally reduced methyl viologen was determined by reduction of the final solution with a slight excess of dithionite.

Photoreduction of Ferredoxin by Glutathione—The photoreduction of clostridial ferredoxin by glutathione in the presence of hematoporphyrin in buffered aqueous solution was also carried out in the apparatus shown in Fig. 1. In those experiments in which the thermal reversal of the photoreduction in the dark was followed, the reaction system was sealed off under helium below the O ring C and above the side tube D with a torch. A solution of ferredoxin, glutathione, and hematoporphyrin was kept in the optical cell G. An oxygen-scavenging solution of methyl viologen, ferredoxin, and granular zinc was kept separately in the side tube D. That the deoxygenation procedure was adequate and that air did not leak into the sample cell were shown separately by the observation of an aqueous solution of protoheme, prepared by the partial reduction of hemin with sodium dithionite and added after the deoxygenation step, to remain in the same reduced state for at least 1 day. This demonstration is essential for establishing the thermal reversal of the observed photoreduction in the dark.

Illumination was accomplished by means of a Sylvania Sun Gun, with the reaction system immersed in a cooling water bath. Both white light and the light filtered by Corning No. 5-62 narrow band filter, 375 nm < λ < 415 nm, were effective. Ferredoxin reduction was measured at 425 nm by means of a Cary model 11 spectrophotometer against a reference cell which contained all of the reactants except ferredoxin and was kept in the dark. At 425 nm the absorption peak of ferredoxin has minimal overlap with the Soret peak of hematoporphyrin. Reoxidation of the ferredoxin by oxygen, tolubene blue, or K$_3$Fe(CN)$_6$ was found to be rapid and complete.

ESR Measurements—A Varian E-3 electron spin resonance spectrometer with temperature control attachment was used for ESR measurements. An ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (g = 2.00354) was used as the reference for g value determinations (8). For measurements at −170°, a cylindrical quartz ESR cell attached to a glass manifold similar to that in Fig. 1 was used. For measurements at room temperatures, a flat glass ESR cell was used.

Reactions with DPPH as a Monitor—The procedure for carrying out such reactions in the absence of oxygen was similar to that described above for the photoreduction of ferredoxin. In order to minimize the absorption of light by DPPH, a low intensity Inco light source was used. The abbreviations used are: ESR, electron spin resonance; DPPH, 2,2-diphenyl-1-picrylhydrazyl.
Titration of 100% reduced clostridial ferredoxin with potassium ferricyanide at pH 7.4

<table>
<thead>
<tr>
<th>$A_O$</th>
<th>$A_R$</th>
<th>$A$</th>
<th>$Y_{obs}$</th>
<th>$[\text{Fe(CN)}_4]^{3-}$</th>
<th>$[\text{Fe}_{\text{Fd}}]$</th>
<th>$m/n^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.002</td>
<td>0.457</td>
<td>0.545</td>
<td>0.963</td>
<td>75.1</td>
<td>279</td>
<td>3.44</td>
</tr>
<tr>
<td>1.562</td>
<td>0.717</td>
<td>1.280</td>
<td>0.956</td>
<td>118.3</td>
<td>437</td>
<td>3.54</td>
</tr>
<tr>
<td>1.110</td>
<td>0.506</td>
<td>1.026</td>
<td>0.862</td>
<td>74.7</td>
<td>307</td>
<td>3.54</td>
</tr>
<tr>
<td>0.620</td>
<td>0.280</td>
<td>0.350</td>
<td>0.535</td>
<td>24.6</td>
<td>173</td>
<td>3.54</td>
</tr>
<tr>
<td>0.820</td>
<td>0.374</td>
<td>0.486</td>
<td>0.485</td>
<td>33.2</td>
<td>229</td>
<td>3.43</td>
</tr>
<tr>
<td>0.650</td>
<td>0.280</td>
<td>0.358</td>
<td>0.220</td>
<td>11.3</td>
<td>173</td>
<td>3.50</td>
</tr>
</tbody>
</table>

* Average 3.50 ± 0.05.

Consequently, if the assumption implied in Equation 2 is correct, the right-hand side of Equation 3 should remain constant over a wide range of experimental values of $Y$. The experimentally measured values of $[\text{Fe(CN)}_4]^{3-}$, $[\text{Fe}_{\text{Fd}}]$ and $Y$ in six separate titration experiments are listed in Table I together with the values of $m/n$ calculated according to Equation 3.

Table I shows that the value of $m/n$ calculated from the experimental data according to Equation 3 remains constant over a 5-fold variation of $Y$. The average value of $m/n$, 3.50 ± 0.05, is within experimental uncertainties equal to the simple fraction $\frac{7}{2}$. Since Lovenberg, Buchanan, and Rabinowitz (9) already showed that the value of $m$ should be in the neighborhood of 7, and since both $n$ and $m$ have to be integers, the present data enable us to conclude unequivocally that $m = 7$ and $n = 2$; i.e., each clostridial ferredoxin molecule contains 7 iron atoms and has a total oxidation-reduction capacity of 2 electrons.

Furthermore, the constancy of $m/n$ in Table I establishes the validity of Equation 2. From Equation 2, the following general relationship between the molar extinction coefficients $e_0$, $e_{n-1}$, $e_n$, ..., $e_1$, $e_0$ of the molecular species of ferredoxin with $n$, $n - 1$, $n - 2$, ..., 1, 0 iron atom, respectively, in the oxidized form can be deduced:

$$\frac{e_n - e_0}{n} = \frac{e_{n-1} - e_0}{n-1} = \frac{e_{n-2} - e_0}{n-2} = \ldots = \frac{-e_1 - e_0}{1}$$

which is generally applicable. It can be readily shown that whenever the fraction of the component reacted as determined spectrometrically according to Equation 2 is equal to the corresponding fraction determined chemically over a wide range of the reaction, the set of extinction coefficients given by Equation 4 is the only set possible. A familiar example is hemoglobin, with $n = 4$.

Knowing the number of iron atoms per ferredoxin molecule, we computed the following molar extinction coefficients at 425 μm of ferredoxin from the linear absorbance against iron concentration plot or from the values of $A_O$ and $[\text{Fe}_{\text{Fd}}]$ in Table 1:

$$e_0 = 1.37 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$
$$e_2 = 2.51 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$
$$e_4 = 1.14 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$

Since for $n = 2$, Equation 4 gives $(e_2 - e_0)/2 = (e_1 - e_0)/1$, we have

$$e_1 = \frac{e_2 + e_0}{2} = 1.83 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$

**Midpoint Reduction Potentials of Ferredoxin**

In order to determine whether ferredoxin undergoes 1- or 2-electron oxidation-reduction steps and to determine its midpoint reduction potentials, a buffered solution of clostridial ferredoxin and methyl violoen (MV++) was reduced stepwise with granular zinc under helium by the procedure described above. Since the midpoint reduction potential of methyl violoen in Tris and in phosphate buffers at pH 7.4 was already determined to be $-0.444 \pm 0.002$ volt by potentiometric titration under helium, the potential, $E_{\text{obs}}$, of the mixture at each stage of the reduction could be readily computed from the spectrometrically measured ratio of the reduced to oxidized violoen, $\beta = [\text{MV}^+]/[\text{MV}++]$.

The results of these experiments are summarized in Table II as well as in Fig. 2, and discussed in the following four sections.
1. Let us first assume that clostridial ferredoxin can only undergo 2-electron oxidation-reduction steps, and write the chemical reaction as

$$R + 2MV^{++} \rightarrow O + 2MV^+ \quad \text{(Case I)}$$

where $R$ and $O$ represent the reduced and oxidized forms, respectively, of the ferredoxin. At equilibrium

$$\frac{[MV^+]_O}{[MV^{++}]_R} = K$$

and hence the fraction of ferredoxin reduced $\alpha = 1 - Y$ satisfies the equation

$$\frac{\alpha}{1 - \alpha} = \frac{[R]}{[O]} = \frac{1}{K} \left( \frac{[MV^+]_1}{[MV^{++}]_1} \right)^2 = \frac{\beta^2}{K}$$

or

$$\log \frac{\alpha}{1 - \alpha} = 2 \log \beta - \log K \quad \text{(5)}$$

Equation 5 predicts that the plot of $\log [\alpha/(1 - \alpha)]$ against $\log \beta$ should be a straight line with a slope equal to 2. With the use of the experimental values in Table II, it was found that the plot deviates only slightly from a straight line, but that the slope of this approximately linear plot is $1.1 \pm 0.1$ instead of 2. Consequently, we conclude that this assumption that clostridial ferredoxin can only undergo 2-electron oxidation-reduction steps is not valid.

2. Let us next assume that the ferredoxin can undergo 1-electron oxidation-reduction steps; i.e. $O$ first gains 1 electron to become an intermediate $I$ which is then further reduced to $R$. The reactions may in this case be represented by

$$R + MV^{++} \rightarrow I + MV^+ \quad \text{(Case II)}$$

$$I + MV^{++} \rightarrow O + MV^+$$

At equilibrium, we have

$$K_2 = \frac{[MV^+]_I}{[MV^{++}]_R} = \beta \frac{[I]}{[R]}$$

$$K_1 = \frac{[MV^+]_O}{[MV^{++}]_I} = \beta \frac{[O]}{[I]}$$

hence

$$\frac{\alpha}{1 - \alpha} = \frac{2[R] + [I]}{2[O] + [I]} = \frac{\frac{2\beta}{K_2} + 1}{2K_1 \beta ^{-1} 1}$$

i.e.

$$\alpha = \frac{\frac{\beta}{K_2} + 1}{\frac{\beta}{K_1} + \frac{\beta}{K_2} + 1} \quad \text{(6)}$$

Since the validity of Equation 2 has already been established by the data in Table I, we have

$$\alpha = 1 - Y = 1 - \frac{A_1}{A_0} = \frac{A_0 - A_R}{A_0 - A_R}$$

Therefore, $\alpha$ can be calculated from the observed absorbances at 425 nm (after a correction is made for the absorbance of $MV^+$ at this wave length) as listed under $\alpha_{obs}$ in Table II.

To determine $K_1$ and $K_2$, a computer program was set up to find their values which minimize the expression

$$\sum_i \frac{[\alpha_{calc} - \alpha_{obs}]^2}{\Delta \alpha_{calc}^2}$$

where $(\alpha_{calc})_i$ is the fraction of ferredoxin reduced which is
calculated from the observed value of \( \beta \) in the \( i \)th experiment of Table II according to Equation 6, with a particular pair of assumed values of \( K_2 \) and \( K_1 \), \( (\Delta \alpha_{\text{obs}})_i \) is the estimated experimental error in \( (\alpha_{\text{obs}})_i \), and the summation is over all of the experiments listed in Table II. The values of \( K_1 \) and \( K_2 \) which minimize the above summation are \( K_1 = 0.048 \) and \( K_2 = 0.162 \). Values of \( \alpha_{\text{calc}} \) computed from these values of \( K_1 \) and \( K_2 \) and the experimental values of \( \beta \) according to Equation 6, are listed in the last column of Table II. The agreement between \( \alpha_{\text{obs}} \) and \( \alpha_{\text{calc}} \) is generally excellent.

The two midpoint reduction potentials, \( E_{\text{IO}'} \) and \( E_{\text{IO}''} \), of clstridial ferredoxin at 23°C and pH 7.4 can now be readily computed as follows.

\[
E_{\text{IO}'} = E_{\text{MV}'} - \frac{RT}{F} \ln K_1 = -0.444 - 0.0587 \log (0.048) = -0.367 \pm 0.003 \text{ volt}
\]

\[
E_{\text{IO}''} = E_{\text{MV}'} - \frac{RT}{F} \ln K_2 = -0.398 \pm 0.006 \text{ volt}
\]

The uncertainties in \( E_{\text{IO}'} \) and \( E_{\text{IO}''} \) are computed from the data in Table II.

Tagawa and Arnon (10) recently made an extensive study of the oxidation-reduction properties of both spinach and clstridial ferredoxins. They concluded that the clstridial ferredoxin has an oxidation-reduction capacity of only 1 electron per molecule with a midpoint reduction potential of \(-0.39 \text{ volt}\) which is independent of pH in the range pH 6.13 to 7.4. On the other hand, Sobel and Lovenberg (11) concluded from their experimental work that the fully oxidized clstridial ferredoxin molecule is reduced by a 2-electron transfer step with a single midpoint reduction potential which varies linearly with pH in the range pH 5.5 to 9.0. Our result is only in partial agreement with either of these earlier conclusions.

3. To explore the physical significance of the approximate linearity of the plot in Fig. 2, let us consider a macromolecule with \( n \) identical groups which can undergo oxidation-reduction reaction independently. For such a molecule, it is easy to show that (a) the plot of \( (RT/F) \ln \alpha/(1 - \alpha) \) against \( E_{\text{obs}} \) should be linear with the slope equal to \(-1\), (b) the midpoint reduction potentials, \( E_{\text{io}'} \), for successive reducing equivalents are all different. Let us first prove these two statements for the general case, and then apply the result to the particular case with \( n = 2 \) for comparison with the present data.

When such a macromolecular component is at equilibrium with titrant molecules, e.g. \( MV^{++} \) and \( MV^+ \), the mole fractions of various species of the macromolecular component are proportional to their respective statistical probabilities. Denoting by \( X_n, X_{n-1}, \cdots X_1 \) the mole fractions of total ferredoxin for the molecular species with \( n, n - 1, \cdots 0 \) iron atom, respectively, in the oxidized state, we have

\[
\frac{X_n}{C_{\text{IO}'}^{n+2}} = \frac{X_1}{C_{\text{IO}'}^{n+2}(1 - \alpha)} = \frac{X_2}{C_{\text{IO}'}^{n+2}(1 - \alpha)^2} = \cdots = \frac{X_n}{C_{\text{IO}'}^{n+2}(1 - \alpha)^n}
\]

where

\[
C_i = \frac{n!}{i!(n - i)!}
\]

represent the binomial coefficients.

\[
E_{\text{obs}} = E_{\text{MV}^{++}} - \frac{RT}{F} \ln \beta
\]

\[
= E_{\text{io}'} - \frac{RT}{F} \ln \frac{X_0}{X_1}
\]

\[
= E_{\text{io}'} - \frac{RT}{F} \ln \frac{\alpha}{1 - \alpha} - \frac{RT}{F} \ln \left( \frac{C_{\text{io}'}^{n+2}}{C_i} \right)
\]

which proves Statement a.

Likewise, the general equation is

\[
E_{\text{obs}} = E_{\text{MV}^+} - \frac{RT}{F} \ln \frac{X_{n-1}}{X_1}
\]

\[
= E_{\text{io}'} - \frac{RT}{F} \ln \frac{\alpha}{1 - \alpha} - \frac{RT}{F} \ln \left( \frac{C_{\text{io}'}^{n+2}}{C_i} \right)
\]

The intrinsic midpoint reduction potential \( E_{\text{io}'} \) of the reducible group is obtained by putting \( n = 1 \) in Equation 8 or 9; hence

\[
E_{\text{io}'} = E_{\text{obs}} + \frac{RT}{F} \ln \frac{\alpha}{1 - \alpha}
\]

Consequently, the approximate agreement between the theoretical straight line and the experimental data in Fig. 2 indicates that the 2 reducible iron atoms in each clstridial ferredoxin molecule can indeed undergo oxidation-reduction reactions almost independently.

Elimination of \( E_{\text{obs}} \) between Equations 9 and 10 gives

\[
E_{\text{io}'} - E_1 = \frac{RT}{F} \ln \frac{C_{\text{IO}'}^{n+2}}{C_i}
\]

which proves Statement b.

4. For the particular case with \( n = 2 \), we obtain from Equation 11.

\[
E_2 - E_1 = \frac{RT}{F} \left[ \ln \left( \frac{C_2}{C_i} \right) - \ln \left( \frac{C_1}{C_i} \right) \right] = - \frac{2RT}{F} \ln 2
\]

\[
= -0.0356 \text{ volt}
\]

The above observed values give

\[
E_2 = E_1 = -0.031 \pm 0.003 \text{ volt}
\]

Thus the difference between the observed and statistical values of \( E_{\text{io}'} - E_{\text{io}''} \) is

\[
0.005 \pm 0.002 \text{ volt}
\]

or

\[
0.11 \pm 0.07 \text{ kcal per mole}
\]

Since this difference is of the same order of magnitude as the experimental uncertainties and considerably smaller than \( kT = 0.6 \text{ kcal per mole} \), we conclude that the 2 reducible iron atoms in each clstridial ferredoxin molecule do not interact with each other appreciably. Consequently, they cannot be covalently
bonded to each other, but must be separated by a distance greater than the van der Waals diameter of the iron atoms.

Having quantitatively characterized the oxidation-reduction properties of clostridial ferredoxin, we can now use this information to study its photoreduction by glutathione.

**Hematoporphyrin-sensitized Photoreduction of Ferredoxin by Glutathione**

1. When an aqueous solution of clostridial ferredoxin ($2.2 \times 10^{-4}$ M), hematoporphyrin ($6.4 \times 10^{-4}$ M), and glutathione ($2 \times 10^{-4}$ M) in phosphate buffer at pH 7.35 was illuminated at room temperature, the ferredoxin was partially reduced, but there was no change in the absorption spectrum of the porphyrin if at the end of the illumination period there was still an appreciable fraction of the ferredoxin in the oxidized form. The photoreduced ferredoxin can be immediately and quantitatively reoxidized to its original form by $O_2$, $K_3Fe(CN)_6$, or toluylene blue. The absorption spectra of the solution at various stages of the photoreduction experiment are shown in Fig. 3.

2. No reaction occurs in the dark in this model system. Even under illumination, no reaction occurs between ferredoxin and glutathione in the absence of hematoporphyrin. Without glutathione, ferredoxin is not reduced by hematoporphyrin when the solution is illuminated. However, a slight irreversible degradation of the ferredoxin chromatophore did occur in all of the experiments in which a solution of hematoporphyrin and ferredoxin was illuminated without a suitable electron donor.

3. When solutions of hematoporphyrin and glutathione were illuminated in the absence of ferredoxin, the hematoporphyrin was reduced to products with absorption spectra very similar to those of the reduced forms of uroporphyrin reported by Mauzerall (6). Hematoporphyrin can be photoreduced by glutathione at pH 7.4 first to dihydrohematoporphyrin, also called hematophlorin, with absorption maxima at 485 and 725 m$\mu$, respectively, then to tetrahydrohematoporphyrin with absorption maximum at 500 m$\mu$, and finally to the colorless hematophlorinogen. At pH 8.6 hematophlorin shows a new absorption maximum at 650 m$\mu$, similar to that reported for uroporphyrin (6). In the dark, hematophlorin is relatively stable at 0°, but dismutates at a measurable rate to hematoporphyrin and tetrahydrohematoporphyrin at room temperature.

4. Since no reduced hematoporphyrin was observed in the above-described photoreduction of ferredoxin by glutathione if at the end of the experiment an appreciable fraction of the ferredoxin was still in the oxidized form, we would like to know whether this absence of hematophlorin was due to a rapid dark reaction in which the reduced porphyrin was reoxidized by ferredoxin. With this aim, the following experiment was carried out. A buffered solution of hematoporphyrin was photoreduced with glutathione to produce a solution containing $6 \times 10^{-4}$ M of hematoporphyrin, estimated from the extinction coefficient of uroporphyrin (6), in a sealed vessel. Ferredoxin was then added from a side tube in the dark. It was found that the hematophlorin peak at 735 m$\mu$ did not decrease any faster in the presence of the oxidized form of ferredoxin than it usually does by the dismutation reaction. Furthermore, after air was let in, there was no increase in the absorbance characteristic of the reoxidation of reduced ferredoxin. Consequently, we concluded that hematophlorin does not rapidly reduce ferredoxin in the dark.

These four types of observations show that for the present model reaction light is required, that the net chemical change is the reduction of ferredoxin and the oxidation of glutathione, and that the ferredoxin is reduced directly not by phlorin but through 1 electron reduction steps by excited porphyrin molecules or porphyrin radicals similar to those postulated for chlorophyll from experiments with systems in vivo.

**Elucidation of Mechanism**

The following two simple mechanisms are consistent with the above observations.

**Mechanism I**

\[
P + \hbar \rightarrow P^* \rightarrow P + \text{thermal energy} \\
P^* + \text{GSH} \rightarrow P_{\text{ox}} + \text{Gox} \\
P_{\text{ox}} + \text{Fdox} \rightarrow P + \text{Fdox} \\
2\text{Gox} \rightarrow \text{GSSG}
\]

where $P$ and $P^*$ represent the pigment in its ground and excited states, GSH and GSSG represent the reduced and oxidized forms of glutathione, $P^*$ represents a radical derivative of the pigment, and the subscripts indicate the oxidation-reduction states.

**Mechanism II**

\[
P + \hbar \rightarrow P^* \rightarrow P + \text{thermal energy} \\
P^* + \text{Fdox} \rightarrow P_{\text{ox}} + \text{Fdox} \\
P_{\text{ox}} + \text{GSH} \rightarrow P + \text{Gox} \\
2\text{Gox} \rightarrow \text{GSSG}
\]

The difference between these two mechanisms lies in the role of the porphyrin. Mechanism I assumes that the excited pigment molecule is first reduced by glutathione to the free radical $P_{\text{Re}}$, whereas Mechanism II assumes that it is first oxidized by ferredoxin to the free radical $P_{\text{ox}}$. To decide between these two mechanisms, it is necessary to determine which of the two por-
wise with DPPH, a solution of hematoporphyrin + ferredoxin + slight reduction of DPPH. When the reactants were taken pair- upon illumination, while a solution of GSH + DPPH showed DPPH showed no significant reduction or oxidation of DPPH

solutions of hematoporphyrin + DPPH and of ferredoxin +

troscopy and by ESR measurements.

reaction. The reactions of DPPH in the dark and upon illumi-

and as a reducer, it was chosen as a diagnostic tool to determine

the kinetically favored one.

DPPH—Since the relatively stable free radical 2,2-diphenyl-l-

picryl hydrazyl can react with other radicals both as an oxidizer

and as a reducer, it was chosen as a diagnostic tool to determine

the kinetically favored path of this photooxidation-reduction

reaction. The reactions of DPPH in the dark and upon illumin-

ation with each reactant in the model system, taken singly as

and pairwise, was followed both by optical absorption spec-
troscopy and by ESR measurements.

In experiments with each reactant taken singly with DPPH, solutions of hematoporphyrin + DPPH and of ferredoxin + DPPH showed no significant reduction or oxidation of DPPH upon illumination, while a solution of GSH + DPPH showed slight reduction of DPPH. When the reactants were taken pair-

wise with DPPH, a solution of hematoporphyrin + ferredoxin +

DPPH showed ~10% decrease of DPPH concentration upon illumination for a length of time which caused ~70% decrease of DPPH concentration in a solution of hematoporphyrin + GSH + DPPH.

The results of ESR measurements are summarized in Fig. 4. For successive measurements on the same sample or on samples with the same line shape and width, the peak to peak height was found to be proportional to the number of unpaired spins and hence may be used as a measure of DPPH molecules under constant illumination. Fig. 4 shows a 68% decrease in the ESR signal of DPPH in Solution A (GSH + hematoporphyrin + DPPH) after 18 min of illumination, but only a 10% decrease in Solution B (ferredoxin + hematoporphyrin + DPPH) under the same experimental conditions. The control experiments with Solutions C (GSH + DPPH) and D (hematoporphyrin + DPPH) showed that the observed decrease in DPPH signal in Solutions A and B cannot be due to direct photooxidation-reduction reactions of DPPH with GSH and hematoporphyrin, respectively. The 5.8% decrease in the ESR signal of DPPH in Solution C is presumably due to the reduction of excited DPPH molecules by GSH since, even with the band filter, there was appreciable absorption of the actinic light by DPPH. Some decrease of the ESR signal in Solution B is expected, because, as described above, illumination of a ferredoxin + hematoporphyrin solution in the absence of a suitable electron donor always caused slight irreversible degradation of the ferredoxin, and presumably some of the degradation products may act as sulfhydryl reagents.

These experimental results show that Mechanism I is kineti-
cally preferred to Mechanism II.

Production and Characterization of Radical Intermediate—To test the validity of Mechanism I, the ESR spectrum of the radical produced by the photo-reduction of hematoporphyrin by glutathione was taken and compared with that of the reduced hematoporphyrin radical produced by the chemical reduction of hematoporphyrin with zinc in the dark and by the photo-redox of hematoporphyrin with hematohemin, respectively. It was found that hematoporphyrin can be cleanly reduced by granular zinc with no detectable incorporation of zinc ion into the porphyrin if the reduction is carried out in phosphate buffer at ice bath temperature. Zn(II)-hematoporphyrin, which can be easily detected because its Soret peak at 405 mp has higher absorbance than that of the Soret peak of hematoporphyrin at 394 mp (12), was not formed under these experimental conditions. The production of reduced hematoporphyrin radical through the photoreduction of hematoporphyrin by hematoporphyrin, $P + PH_2 \xrightarrow{h\nu} 2P_{\text{lep}}$, was carried out in the same way as that described by Mauzerall and Feher for uroporphyrin and uroporphyrin (13).

The ESR spectra of reduced hematoporphyrin radicals in frozen samples prepared by these three different methods are summarized in Table III. The values in Table III show that the same reduced hematoporphyrin radical was produced in all three generating reactions. This result establishes the validity of the second step of Mechanism I discussed above.

Reduction of Ferredoxin by Radical Intermediate in Dark—When a concentrated solution of hematoporphyrin in phosphate buffer was shaken briefly with granular zinc and rapidly de-
canted onto ferredoxin in the dark, up to 10% reduction of the ferredoxin was observed. However, if the porphyrin solution
was shaken with zinc long enough to reduce most of the porphyrin to the phlorin before it was decanted onto the ferredoxin, no reduction of ferredoxin was observed.

In addition, when a solution of hematoporphyrin, hematophlorin, and ferredoxin was illuminated with light at the porphyrin absorption peak, ferredoxin was 100% reduced. However, a similar reaction mixture which was kept in the dark for 3 hours showed no ferredoxin reduction.

These observations show that hematoporphyrin cannot reduce ferredoxin in the dark, but the 1 electron reduced porphyrin, i.e. the reduced hematoporphyrin radical, does reduce ferredoxin rapidly in the dark.

Finally, by illuminating a mixture of hematoporphyrin, hematophlorin, and some further reduced forms of the porphyrin a solution of reduced hematoporphyrin radical was prepared. The ESR signal of the frozen mixture was recorded and was shown to survive the melting of the solution, shaking for 3 min, and refreezing. When this solution was again melted and mixed with ferredoxin from a side tube in the dark and quickly refrozen, the ESR signal disappeared completely with concomitant reduction of 75% of the ferredoxin.

These observations are all consistent with the second step of Mechanism I. The results described above enable us to conclude that Mechanism I correctly represents the principal reaction path of the present model system. In other words, the excited hematoporphyrin molecule is first reduced by glutathione to form a reduced hematoporphyrin radical, which subsequently reduces the ferredoxin.

### Demonstration of Energy Storage

The conversion of light to chemical free energy in the present model system can be shown either by the observed spontaneous reversal of the photooxidation-reduction reaction in the dark or by quantitative electrochemical considerations.

- **Spontaneous Reversal of Photoreduction of Ferredoxin in Dark**
  - The hematoporphyrin-sensitized photoreduction of ferredoxin by glutathione was reversed slowly when the light was turned off. The data of a typical experiment are summarized in Table IV. In this experiment the ferredoxin was photoreduced and then allowed to recover completely. This procedure was repeated through three cycles. The reaction system was completely sealed in glass under helium. To terminate the experiment, the solution was illuminated briefly and then oxygen was let in by breaking the glass seal. The reoxidation of ferredoxin by oxygen was immediate and complete.

Since the observed spontaneous reversal of ferredoxin reduction in the dark must be driven by a decrease in the total free energy of the system, the preceding photooxidation-reduction reaction in the opposite direction must have resulted in an increase of total free energy; i.e. part of the absorbed light must have been converted into chemical free energy by the model system.

### Electrochemical Considerations

The amount of chemical free energy in the present model system can be shown either by the observed spontaneous reversal of the photooxidation-reduction reaction in the dark or by quantitative electrochemical considerations.

### Table III

<table>
<thead>
<tr>
<th>Generating reaction</th>
<th>g value</th>
<th>Line width</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP + GSH → hp</td>
<td>2.0025</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>HP + Zn → dark</td>
<td>2.0024</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>HP + HP + GSSG → hp</td>
<td>2.0025</td>
<td>7.2 ± 0.3</td>
</tr>
</tbody>
</table>

* The Varian E-3 EPR spectrometer was calibrated by taking the g value of DPPH in moderately concentrated ethanol solutions to be 2.0034 (13).

* Field difference between the maximum and the minimum of the derivative curve.

* HP, hematoporphyrin; HPhlorin, hematophlorin.

### Table IV

**Spontaneous reversal of photoreduction of ferredoxin in dark**

<table>
<thead>
<tr>
<th>Time</th>
<th>Lighting control</th>
<th>ΔG/n</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 sec</td>
<td>In dark</td>
<td>0.791</td>
<td>Original 100% oxidized ferredoxin</td>
</tr>
<tr>
<td>20 sec</td>
<td>In dark</td>
<td>0.677</td>
<td>Reduction = 18%</td>
</tr>
<tr>
<td>40 sec</td>
<td>In dark</td>
<td>0.713</td>
<td>Reduction = 13%</td>
</tr>
<tr>
<td>60 sec</td>
<td>In dark</td>
<td>0.748</td>
<td>Reduction = 15%</td>
</tr>
<tr>
<td>180 sec</td>
<td>In dark</td>
<td>0.688</td>
<td>Reduction = 5%</td>
</tr>
<tr>
<td>90 sec</td>
<td>In dark</td>
<td>0.713</td>
<td>Reduction = 5%</td>
</tr>
<tr>
<td>240 sec</td>
<td>In dark</td>
<td>0.731</td>
<td>Reduction = 5%</td>
</tr>
<tr>
<td>480 sec</td>
<td>In dark</td>
<td>0.747</td>
<td>Reduction = 5%</td>
</tr>
<tr>
<td>600 sec</td>
<td>In dark</td>
<td>0.749</td>
<td>Reduction = 5%</td>
</tr>
<tr>
<td>600 sec</td>
<td>in dark</td>
<td>0.749</td>
<td>Reduction = 5%</td>
</tr>
<tr>
<td>0 sec</td>
<td>O2 let in</td>
<td>0.750</td>
<td>Reoxidation was immediately complete</td>
</tr>
</tbody>
</table>

With the solution composition at the end of the first illumination period in Table IV as an example, we may calculate \( E_{Fd} \) and \( E_{GSSG} \) as follows.

\[
E_{Fd} = -0.345 \text{ volt}
\]

With \( E'_{GSSG} = -0.25 \text{ at pH 7.35 and 23° (14)} \) and neglecting the small changes from the initial values of \([GSSG]\) and \([GSH]\) due to the reaction, we obtain

\[
E_{GSSG} = -0.25 - 0.0587 \log \frac{8 \times 10^{-4}}{(3.2 \times 10^{-3})^{1/2}} = -0.20 \text{ volt}
\]

Therefore Equation 12 gives

\[
\Delta G/n = -F(-0.345 + 0.20) = 3.3 \text{ kcal per faraday}
\]
Acknowledgment—We thank Dr. Kunpo Huang for his expert assistance in the ESR measurements.

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