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OXIDATION–REDUCTION POTENTIALS AND STOICHIOMETRY OF ELECTRON TRANSFER IN FERREDOXINS

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SUMMARY

The electron-carrier properties of a plant (*Spinacia olarecea*) and a bacterial (*Clostridium pasteurianum*) ferredoxin were compared. The oxidation–reduction potentials, measured at different partial pressures of H_2 and at different pH values, were found to be: $E'_0 = -0.42$ V for spinach ferredoxin and $E'_0 = -0.39$ V for clostridial ferredoxin. The oxidation–reduction potentials of spinach and clostridial ferredoxin were independent of pH in the ranges investigated: pH 6.67–8.19 for spinach ferredoxin and pH 6.13–7.41 for clostridial ferredoxin. The oxidation–reduction of clostridial ferredoxin, like that of spinach ferredoxin, was found to involve the transfer of 1 electron per molecule. These results are consistent with the view that, despite their diverse origin and function and their chemical differences, plant and bacterial ferredoxins have similar characteristics as electron carriers.

INTRODUCTION

Ferredoxin, a name given in 1962 by MORTENSON, VALENTINE AND CARNAHAN[1] to an iron-containing protein which they isolated from *Clostridium pasteurianum*, is now applied to a family of plant and bacterial proteins (including those isolated before 1962 under different names) that have a low molecular weight, a strongly negative oxidation–reduction potential and contain, in equimolar amounts, non-heme iron and labile sulfur (literature cited in ref. 2). Ferredoxins act as electron carriers in photosynthesis[2–4] and in certain phases of anaerobic metabolism[5]; they are found in all photosynthetic cells[2,4,6] but only in certain non-photosynthetic obligate anaerobes[5]. Based on their characteristic absorption spectra, ferredoxins have been divided[2] into two types that also differ in several other aspects at the molecular level: a bacterial type, which includes ferredoxins from all bacteria, whether photosynthetic or not, and a chloroplast or plant type, which includes ferredoxins from leaves and algae.

A notable feature, revealed by the early studies of plant and bacterial ferredoxins, was the similarity of their properties as electron carriers. The preliminary

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PCMB, *p*-chloromercuribenzoate.

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measurements of the oxidation–reduction potentials of bacterial and plant ferredoxin gave values close to those of the hydrogen electrode and were, within the narrow range investigated, relatively independent of pH (ref. 4). The same number of electrons appeared to be transferred during oxidation–reduction of plant and bacterial ferredoxins. TAGAWA AND ARNON[4] found that the oxidation–reduction of *C. pasteurianum* ferredoxin involves a transfer of 1 electron. Likewise, WHATLEY, TAGAWA AND ARNON[8] found that the oxidation–reduction of spinach ferredoxin involved a transfer of 1 electron.

The similarity of plant and bacterial ferredoxins as electron carriers has recently been questioned by SOBEL AND LOVENBERG[9], who reported that *C. pasteurianum* ferredoxin is a 2-electron carrier and that its oxidation–reduction potential is a function of pH. A difference between plant and bacterial ferredoxins in the number of electrons transferred during oxidation–reduction would, if confirmed, be of considerable theoretical interest both from the standpoint of the mechanism of hydrogenase action and of photosynthesis. Plant and bacterial ferredoxins are each reduced either by H_2 and hydrogenase or, without the mediation of any known enzyme or intermediate, by the chlorophyll-containing organelles of plants and photosynthetic bacteria[4,7]. In the photosynthetic reactions, plant and bacterial ferredoxins are to a considerable extent interchangeable[4].

The aim of this investigation was to compare, by the use of the same methods, the electron-carrier properties of bacterial (*C. pasteurianum*) and plant (spinach) ferredoxin. In determining the number of electrons transferred during oxidation–reduction, spinach ferredoxin was used as a standard since the transfer of 1 electron during its oxidation–reduction[8] was confirmed[10] and is consistent with the valence changes of its iron[11] and its magnetic resonance properties[12].

The present results indicate that, similar to spinach ferredoxin, the oxidation–reduction of clostridial ferredoxin involves a transfer of 1 electron per molecule. The oxidation–reduction potentials of both spinach and clostridial ferredoxin were found to be independent of pH in the region near neutrality. These results are consistent with the view that, despite their chemical differences, diverse origin and function, plant and bacterial ferredoxins have similar characteristics as electron carriers.

METHODS

Preparation of ferredoxins

Our method for preparing crystalline spinach ferredoxin is described in the review by LOSADA AND ARNON[13]. Crystalline spinach ferredoxin had a $A_{420\,m\mu}/A_{276\,m\mu}$ ratio = 0.49. The preparations used in most of the present experiments had a $A_{420\,m\mu}/A_{276\,m\mu}$ ratio of 0.47–0.48.

Clostridial ferredoxin

Crude ferredoxin was extracted from frozen *C. pasteurianum* cells with acetone according to MORTENSON[14] and purified by DEAE-cellulose chromatography in essentially the same way as described for spinach ferredoxin. The preparations used had a $A_{390\,m\mu}/A_{280\,m\mu}$ ratio not less than 0.79.

The degree of reduction of clostridial ferredoxin was measured by the decrease in $A_{425\,m\mu}$, using the molar extinction coefficient (425 mμ) of $26.6 \cdot 10^3$. The molar

extinction coefficient (425 mμ) was calculated by applying to our pure preparation of ferredoxin the extinction coefficient (390 mμ) of $30 \cdot 10^3$ used by SOBEL AND LOVENBERG[9]. Our clostridial ferredoxin, when maximally reduced by dithionite, had a $A_{425\,m\mu}$ equal to 48% that of oxidized ferredoxin. Hence a molar extinction coefficient (425 mμ) of $13.9 \cdot 10^3$ was used in measuring the degree of reduction of ferredoxin.

Hydrogenase

A crude hydrogenase preparation, free of ferredoxin, was prepared from *C. pasteurianum*. Five g frozen cells were suspended in 7.5 ml of water for 15 min. The suspension was sonicated for 10 min and centrifuged at 38000 × *g* for 5 min. The supernatant fluid was put over a 1 cm × 4 cm DEAE-cellulose column that was equilibrated with 1 M phosphate buffer (pH 6.5) and washed with 40 ml of water. The passed solution was heated at 60° for 10 min under H_2 and centrifuged at 39000 × *g* for 5 min. The resulting supernatant fluid was used as the hydrogenase preparation.

Reduction of ferredoxin by H_2

Reduction of ferredoxins by the H_2–hydrogenase system was carried out at 25° in Thunberg-type cuvettes, at different H^+ concentrations, and at different partial pressures of H_2. The H_2 used was freed from traces of O_2 and saturated with water vapor (at 25°) by being passed successively (as fine bubbles) through two bottles of alkaline pyrogallol and two bottles of water. The Thunberg-type cuvettes were first evacuated and then filled with H_2—a procedure repeated 4 times. The final partial pressure of H_2 was measured with the aid of a mercury column, a correction being made for the partial pressure of water vapor[15]. The reaction was started by adding hydrogenase from the side arm. The reduction of ferredoxin was allowed to equilibrate for 15 min in a constant-temperature water bath. The degree of ferredoxin reduction was measured spectrophotometrically with a Cary Model 14 spectrophotometer.

Photoreduction and dark reoxidation of ferredoxin

Aside from reduction by the H_2–hydrogenase system, ferredoxin was also reduced photochemically with the aid of spinach chloroplasts under anaerobic conditions[8]. The chloroplasts had been preheated at 55° for 5 min to destroy their capacity to use water as the electron donor and evolve O_2. The substitute electron donors supplied to the chloroplasts were either the couple ascorbate–dichlorophenolindophenol (DCIP) or cystein. Ascorbate–DCIP was the electron donor when $NADP^+$ was subsequently used to reoxidize in the dark the photoreduced ferredoxin. Cystein was the electron donor when benzyl viologen was subsequently used to reoxidize in the dark the photoreduced ferredoxin; ascorbate–DCIP could not be used here since reduced benzyl viologen was rapidly reoxidized (probably by dehydroascorbate) even under anaerobic conditions. A millimolar extinction coefficient was determined for benzyl viologen by reduction with sodium dithionite under anaerobic conditions. The values obtained (at 600 mμ) ranged from 6.55 to 7.05; the value used here was $\varepsilon_{mM} = 7.0\ mM^{-1} \cdot cm^{-1}$.

Other chemical determinations

Iron was determined by the *o*-phenanthroline method after wet combustion of the organic material[16]. The sulfhydryl groups were titrated with mercurials as

described by BOYER[17] and by LOVENBERG, BUCHANAN AND RABINOWITZ[18]. Chlorophyll was measured as described by ARNON[19].

RESULTS AND DISCUSSION

Molecular weight of spinach ferredoxin

Crystals of spinach ferredoxin are shown in Fig. 1.

Before the stoichiometry of spinach ferredoxin could be measured, it was necessary to establish its molecular extinction coefficients, which in turn required an accurate determination of molecular weight. The molecular weight previously assigned to spinach ferredoxin was 17000 (ref. 20) and 14000 (ref. 8). A new determination of molecular weight was made from the amino acid composition and amino- and carboxyl-terminal sequences on an aliquot of a preparation of spinach ferredoxin used in this investigation. These analyses, made by MATSUBARA, SASAKI AND CHAIN[21], who later established the complete amino acid sequence of spinach ferredoxin, gave a molecular weight of 11580 on an aliquot of a preparation that was dried *in vacuo* at 60°.

The above determination probably excluded labile sulfur in spinach ferredoxin[22], which might have been lost during drying. The number of labile sulfur groups was obtained by titration of the sulfhydryl groups of spinach ferredoxin with *p*-chloromercuribenzoate (PCMB), sodium mersalyl and $HgCl_2$. As shown in Table I, 9 moles of PCMB (in good agreement with the results of FRY AND SAN PIETRO[22]), 9 moles of sodium mersalyl or 4.5 moles of $HgCl_2$ were required to titrate 1 mole of spinach ferredoxin.

The half-cystein residues per molecule of spinach ferredoxin, first reported as

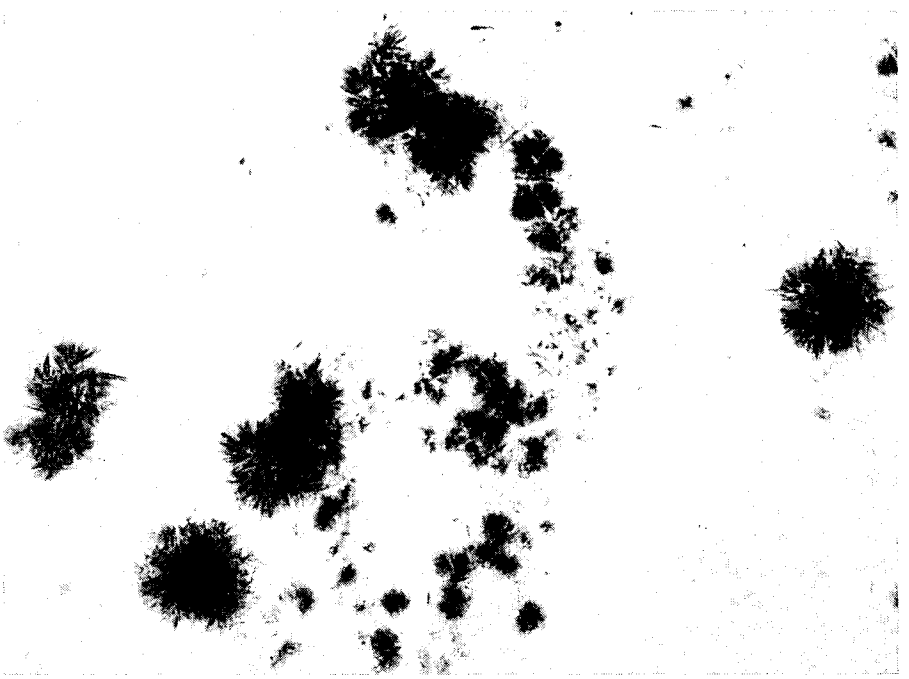

Fig. 1. Microphotograph of crystalline spinach ferredoxin. Magnification 275 ×.

6 (ref. 23), were found by MATSUBARA, SASAKI AND CHAIN[21] to be 5. The 5 sulfhydryl groups would bind 2.5 moles of $HgCl_2$, 5 moles of PCMB or 5 moles of sodium mersalyl. The remaining 2 moles of $HgCl_2$, 4 moles of PCMB and 4 moles of sodium mersalyl would each combine with 2 labile sulfur (sulfide) groups. Thus, the results in Table I are consistent with the 5 sulfhydryl groups and 2 labile sulfur (sulfide) groups that are present in a molecule of spinach ferredoxin.

TABLE I

TITRATION OF SULFHYDRYL AND LABILE SULFUR GROUPS OF SPINACH FERREDOXIN WITH MERCURY COMPOUNDS

Three ml ferredoxin solution of 0.15 M Tris–HCl buffer (pH 7.8) were titrated stepwise by 5 μl of mercury compound until no change in absorbance was observed. All titrations were designed to be completed within 50 μl of mercury compound. The end of titration was determined in all cases by the decrease in $A_{420\,m\mu}$ and $A_{465\,m\mu}$ and, in addition, by the increase in $A_{250\,m\mu}$ in the titration with PCMB.

Mercury compound	*μmoles of mercury compound used per μmole ferredoxin based on absorbance change at*		
	250 mμ	*420 mμ*	*465 mμ*
PCMB	9.3	9.0	9.2
Sodium mersalyl	—	9.1	9.1
$HgCl_2$	—	4.6	4.6

TABLE II

IRON CONTENT OF SPINACH FERREDOXIN

The top two analyses were made after wet ashing with H_2SO_4 and the bottom two analyses after acidification with 1.2 M HCl.

Ferredoxin used (mg)	*Iron found (μg)*	*Iron in ferredoxin (%)*	*Minimum mol. wt. of ferredoxin based on iron content*	*Number of Fe atoms per molecule of ferredoxin**
6.68	64.4	0.97	5760	2
3.34	33.3	1.00	5590	2
0.84	8.2	0.98	5700	2
1.68	17.5	1.04	5380	2

* Based on molecular weight of 11590.

By adding 2 atoms of sulfur to 11580 (obtained from amino acid analysis) the molecular weight of spinach ferredoxin becomes 11640. Table II shows that this value for the molecular weight is in very good agreement with the iron analysis that gave an iron content of about 1%, which in turn corresponds to 2 iron atoms per molecule of spinach ferredoxin.

An independent, direct determination of iron and labile sulfur by different methods[18] was made by Dr. B. B. BUCHANAN. The results showed 2 labile sulfur and 2 iron atoms per molecule of spinach ferredoxin.

The new molecular weight for spinach ferredoxin, although considerably lower than the 17000 value of APELLA AND SAN PIETRO[20] and the 19000 value of DAVENPORT

AND HILL[24] for leaf ferredoxin from other species, is in good agreement with the molecular weight of 12000 (and 2 iron atoms) reported by BENDALL, GREGORY AND HILL[25] for parsley ferredoxin and in very good agreement with the molecular weight of 11500 (and 2 iron and labile sulfur atoms) reported by KERESZTES-NAGY AND MARGOLIASH[26] for alfalfa ferredoxin. These close chemical similarities, which extend also to amino acid composition, strongly support the idea that only one species of chloroplast ferredoxin is present in leaves of green plants and that its molecular weight is within the range 11500–12000.

Extinction coefficients of spinach ferredoxin

The extinction coefficients for spinach ferredoxin used previously were provisional, based on a molecular weight of 14000 (ref. 8). Table III gives extinction coefficients (for oxidized and reduced forms of ferredoxin) obtained from a solution of 0.65 mg of spinach ferredoxin per ml, which, according to the new molecular weight (11650), corresponds to 0.056 μmole.

Despite the discrepancy of about 20 % between the old and the new molecular weights, the molar extinction coefficients for spinach ferredoxin given in Table III are in good agreement with the provisional ones[8]. This agreement is a fortuitous result of two compensating errors in the earlier determination: the sample of ferredoxin used earlier was imperfectly dried and its water content offset the higher molecular weight used in the calculations of the provisional extinction coefficients. Thus, the earlier finding that 1 electron per molecule is transferred during oxidation–reduction of spinach ferredoxin[8] is not invalidated by the more accurate determination of extinction coefficients given in Table III.

Oxidation–reduction potentials of clostridial and spinach ferredoxin

The oxidation–reduction potentials of clostridial and spinach ferredoxin (Fd) were determined by measuring their per cent reduction when at equilibrium with the H_2–hydrogenase system (Eqn. 1).

$$H_2 + Fd_{oxidized} \overset{\text{hydrogenase}}{\rightleftharpoons} Fd_{reduced} + 2\,H^+ \qquad (1)$$

The per cent reduction of ferredoxin was determined at different pH's and at different partial pressures of H_2. The oxidation–reduction potential of ferredoxin was computed from Eqns. 2 and 3 (ref. 15).

$$E_{h(Fd)} = E'_0 + 2.303\frac{RT}{nF}\log\frac{[Fd_{oxidized}]}{[Fd_{reduced}]} \qquad (2)$$

$$E_{h(H_2)} = 2.303\frac{RT}{F}\log[H^+] - 2.303\frac{RT}{2F}\log p_{H_2} \qquad (3)$$

At equilibrium $E_{h(Fd)} = E_{h(H_2)}$. Thus,

$$E'_0 + 2.303\frac{RT}{nF}\log\frac{[Fd_{oxidized}]}{[Fd_{reduced}]} = -2.303\frac{RT}{F}\,pH - 2.303\frac{RT}{2F}\log p_{H_2} \qquad (4)$$

and

$$E'_0 = -2.303\frac{RT}{F}\,pH - 2.303\frac{RT}{2F}\log p_{H_2} - 2.303\frac{RT}{nF}\log\frac{[Fd_{oxidized}]}{[Fd_{reduced}]} \qquad (5)$$

TABLE III

EXTINCTION COEFFICIENTS OF SPINACH FERREDOXIN

	276 mμ	*325 mμ*	*420 mμ*	*465 mμ*
Absorbance	1.135	0.720	0.545	0.495
E_{ox}, $(mg/ml)^{-1} \cdot cm^{-1}$	1.739	1.103	0.835	0.759
E_{ox}, $mM^{-1} \cdot cm^{-1}$	20.16	12.78	9.68	8.80
E_{red}, $mM^{-1} \cdot cm^{-1}$	*	*	4.55	3.70
$\Delta E_{(ox-red)}$	*	*	5.13	5.10

* Because of $A_{276\,m\mu}$ and $A_{325\,m\mu}$ by the reducing agent (dithionite) the absorbance of reduced ferredoxin at these wavelengths was not measured.

TABLE IV

OXIDATION–REDUCTION POTENTIALS AND PER CENT REDUCTION OF SPINACH FERREDOXIN AT VARIOUS PARTIAL PRESSURES OF H_2

Reaction mixture contained in a final volume of 3 ml, 0.289 μmole of ferredoxin, 500 μmoles sodium phosphate (pH 7.56) and 30 μl of the hydrogenase preparation. Temp., 25°; pH, 7.56; other conditions are given in METHODS.

p_{H_2} (*atm*)	E_h (*V*)	*Reduction of ferredoxin* (%)	E'_0 (*V*)
0.026	−0.397	30.0	−0.419
0.087	−0.416	52.5	−0.415
0.326	−0.433	70.0	−0.411
0.968	−0.447	79.5	−0.412

TABLE V

OXIDATION–REDUCTION POTENTIALS OF CLOSTRIDIAL FERREDOXIN AT DIFFERENT PARTIAL PRESSURES OF H_2

Reaction mixture contained in a final volume of 3 ml, 0.08 μmole of ferredoxin, 500 μmoles sodium phosphate (pH 7.16) and 30 μl of the hydrogenase preparation. Temp., 25°; pH, 7.16; other conditions are given in METHODS.

p_{H_2}	E_h (*V*)	*Reduction of ferredoxin* (%)	E'_0 (*V*)
0.029	−0.379	38.3	−0.391
0.087	−0.393	54.0	−0.389
0.326	−0.409	70.4	−0.386
0.968	−0.424	78.8	−0.389

Measurements at different partial pressures of H_2 and a constant pH (7.56 or 7.16) gave for spinach ferredoxin $E'_0 = -0.42$ V and for clostridial ferredoxin $E'_0 = -0.39$ V (Tables IV and V). These values are in very good agreement with the E'_0 values for spinach ferredoxin (Table VI) and clostridial ferredoxin (Table VII) that were obtained by measurements at a constant partial pressure of H_2 but at different pH's.

TABLE VI

OXIDATION–REDUCTION POTENTIALS OF SPINACH FERREDOXIN AT 0.968 atm H_2 AND AT DIFFERENT pH's

Experimental conditions were the same as described in Table IV except that at pH's higher than 7.66, 1000 μmoles Tris–HCl buffer replaced the sodium phosphate buffer.

pH	E_h (*V*)	*Reduction* (%)	$log \frac{Fd_{ox}}{Fd_{red}}$	E'_0 (*V*)
6.67	−0.394	34.7	0.267	−0.410
7.03	−0.416	48.4	0.029	−0.418
7.16	−0.423	56.1	−0.105	−0.417
7.66	−0.453	84.5	−0.732	−0.410
7.72	−0.456	84.7	−0.731	−0.413
7.88	−0.466	89.3	−0.902	−0.413
8.19	−0.484	93.0	−1.111	−0.418

TABLE VII

OXIDATION–REDUCTION POTENTIALS OF CLOSTRIDIAL FERREDOXIN AT DIFFERENT pH VALUES

Experimental conditions were the same as described in Table V except that in all treatments the H_2 pressure (including the partial pressure of water vapor) was 1 atm. Sodium phosphate buffer (500 μmoles in 3 ml) was used to maintain the different pH's.

pH	E_h (*V*)	*Reduction of ferredoxin* (%)	$log \frac{Fd_{ox}}{Fd_{red}}$	E'_0 (*V*)
6.13	−0.363	29.2	0.385	−0.386
6.55	−0.388	53.6	−0.063	−0.385
7.02	−0.415	76.6	−0.515	−0.385
7.41	−0.438	88.0	−0.865	−0.387

The present E'_0 values for clostridial and (to a lesser extent) spinach ferredoxin are slightly less electronegative than the preliminary values reported[4]. The difference may have been caused by an imperfect equilibration between ferredoxin and H_2 that resulted from a partial inactivation of the present hydrogenase preparation. In any case, the present E'_0 values for spinach and clostridial ferredoxin do not alter the earlier conclusion that the reducing power for plant and bacterial ferredoxins is close to that of H_2 and greater than that of any other class of cellular electron carriers[4].

Number of equivalents of electrons (n) transferred during oxidation–reduction of 1 mole of ferredoxin

The oxidation–reduction potentials given in Tables VI and VII were calculated on the basis of $n = 1$, both for the spinach and the clostridial ferredoxin. On that basis the E'_0 of spinach ferredoxin was independent of pH between pH 6.13 and 7.41. Above pH 7.5 clostridial ferredoxin was over 90 % reduced and too unstable to give reliable measurements.

A calculation from our data of the oxidation–reduction potentials of clostridial ferredoxin on the basis of $n = 2$ would show, in agreement with SOBEL AND LOVEN-

BERG[9], a variation of E'_0 with pH. However, $n = 2$ for clostridial ferredoxin is deemed unlikely on the basis of the following analysis of the preceding data.

By rearranging Eqn. 4 we obtain

$$2.303 \frac{RT}{nF} \log \frac{[\mathrm{Fd_{oxidized}}]}{[\mathrm{Fd_{reduced}}]} = -2.303 \frac{RT}{F} \mathrm{pH} - 2.303 \frac{RT}{2F} \log p_{\mathrm{H_2}} - E'_0 \quad (6)$$

or

$$\log \frac{[\mathrm{Fd_{oxidized}}]}{[\mathrm{Fd_{reduced}}]} = -n \cdot \mathrm{pH} - \frac{n}{2} \log p_{\mathrm{H_2}} - \frac{nFE'_0}{2.303\ RT} \quad (7)$$

At a constant pH, E'_0 is also constant and a part of Eqn. 7 may be represented by a constant,

$$K = -n \cdot \mathrm{pH} - \frac{nFE'_0}{2.303\ RT}$$

Eqn. 7 may then be written as

$$\log \frac{[\mathrm{Fd_{oxidized}}]}{[\mathrm{Fd_{reduced}}]} = -\frac{n}{2} \log p_{\mathrm{H_2}} + K$$

Thus, at a constant pH, $\log [\mathrm{Fd_{oxidized}}]/[\mathrm{Fd_{reduced}}]$ becomes a function of $\log p_{\mathrm{H_2}}$. By plotting the experimentally obtained values for the per cent reduction of ferredoxin at different partial pressures of H_2, the value of n is obtained graphically. If $n = 1$, the plot should give a straight line with a slope of $-1/2$. Fig. 2 shows that this is the case for clostridial ferredoxin and Fig. 3 shows that this is also the case for spinach ferredoxin. Thus, this analysis of the data supports the conclusion that $n = 1$ for clostridial as well as for spinach ferredoxin.

Stoichiometry of NADP$^+$ and benzyl viologen reduction by ferredoxin

The value of n for clostridial ferredoxin was also determined directly, by determining the stoichiometry of its oxidation–reduction. First, clostridial ferredoxin was photoreduced anaerobically (see METHODS) and then reoxidized in the dark by NADP$^+$ or benzyl viologen.

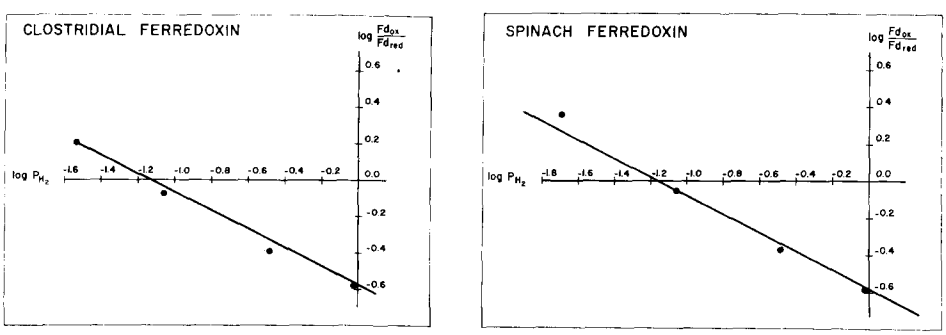

Fig. 2. Plot of the logarithm of the ratio of oxidized to reduced clostridial ferredoxin *versus* the logarithm of the partial pressure of H_2.

Fig. 3. Plot of the logarithm of the ratio of oxidized to reduced spinach ferredoxin *versus* the logarithm of the partial pressure of H_2.

A source of error was encountered in measuring the stoichiometry of $NADP^+$ reduction by reduced clostridial ferredoxin that was not encountered with reduced spinach ferredoxin[8]. Clostridial ferredoxin, both in the oxidized and reduced states, has an appreciable $A_{340\,m\mu}$ and, when undergoing oxidation–reduction, shows marked changes in absorption at that wavelength. Direct measurements of NADPH at 340 $m\mu$ gave erratic values because of the steepness of the absorption curve of clostridial ferredoxin near that wavelength. The value 2.1 obtained by this method for the stoichiometry of $NADP^+$ reduction by ferredoxin (bottom of Column 2 in Table VIII) came closest to the value of 2.0 that was consistently obtained by the improved method described below.

To obtain accurate measurements of NADPH at 340 $m\mu$ it was necessary to use a method that would cancel out the changes in ferredoxin absorption at that wavelength. Accordingly, as shown in Table VIII, ferredoxin was photoreduced in two parallel cuvettes, a and b. In Cuvette a, reduced ferredoxin was reoxidized by the addition of $NADP^+$ and in Cuvette b by the admission of air. By subtracting $A_{340\,m\mu}$ of Cuvette b (due to ferredoxin) from $A_{340\,m\mu}$ of Cuvette a (due to both NADPH and ferredoxin), an accurate measurement of the NADPH formed was obtained.

The results in Table VIII show that 2 moles of reduced clostridial ferredoxin were used up in reducing 1 mole of $NADP^+$. Since the reduction of $NADP^+$ involves the transfer of 2 electrons, the value of n from these experiments is 1.

One of the methods used by SOBEL AND LOVENBERG[9] to determine the value of n was to measure the stoichiometry of ferredoxin reduction by NADPH. In our hands, however, this method proved to be unreliable. At the relatively high concentrations of NADPH that are used in this method, the measuring 340 $m\mu$ beam pro-

TABLE VIII

STOICHIOMETRY OF $NADP^+$ REDUCTION BY REDUCED CLOSTRIDIAL FERREDOXIN

The measurements were made with three Thunberg-type cuvettes (a, b, c) each of which contained, in a final volume of 3 ml: heated spinach chloroplasts (equivalent to 0.025 mg chlorophyll); 500 μmoles sodium phosphate buffer (pH 7.1); 1 μmole ascorbate and 0.02 μmole of DCIP. No further additions were made to Cuvette c but each of Cuvettes a and b also contained 0.072 μmole clostridial ferredoxin ($A_{390\,m\mu} = 0.720$). In addition, Cuvette a had 1 μmole $NADP^+$ in the side arm. The photoreduced ferredoxin in Cuvette b was reoxidized by the admission of air whereas the photoreduced ferredoxin in Cuvette a was reoxidized by tipping in $NADP^+$. The absorbance measurements in Columns 1 and 2 were made on Cuvette a using Cuvette c as a reference. The absorbance measurements in Column 3 were made on Cuvette a, using Cuvette b (after air was admitted) as a reference.

	$A_{425\,m\mu}$	$A_{340\,m\mu}$	$A_{340\,m\mu}$
Oxidized ferredoxin (1)	0.663	0.747	−0.002
Photoreduced ferredoxin (2)	0.343	0.717	—
Photoreduced ferredoxin reoxidized by $NADP^+$ (3)	0.654	0.810	0.068
ΔA due to reoxidized ferredoxin*	0.311	—	—
ΔA due to NADPH**	—	0.063	0.070
Ferredoxin reoxidized (μmole)	0.064	—	—
NADPH formed (μmole)	—	0.030	0.032
Ferredoxin/NADPH ratio		2.1	2.0

* (3) − (2).
** (3) − (1).

vided sufficient excitation energy to give rise to the native fluorescence of NADPH (ref. 27). The fluorescence of NADPH made it impossible to measure accurately absorption changes at 340 mμ.

Other evidence that $n = 1$ for clostridial ferredoxin is given in Table IX. In these experiments reduced ferredoxin was reoxidized by benzyl viologen, the oxidation–reduction of which involves a transfer of 1 electron[15]. The use of benzyl viologen for measuring the stoichiometry of ferredoxin oxidation–reduction had the advantage that the reduced form of this dye absorbs at 600 mμ—a wavelength at which the absorption by reduced or oxidized ferredoxin is very small. Table IX shows that the coupled oxidation–reduction reaction between ferredoxin and benzyl viologen gave a molar ratio of 1.

The stoichiometry of oxidation by $NADP^+$ and benzyl viologen, as well as the slope of the plot log $[Fd_{oxidized}]/[Fd_{reduced}]$ *vs.* log p_{H_2} are consistent in indicating that the oxidation–reduction of clostridial ferredoxin, like that of spinach ferredoxin, involves a transfer of 1 electron per molecule.

TABLE IX

STOICHIOMETRY OF BENZYL VIOLOGEN REDUCTION BY REDUCED CLOSTRIDIAL FERREDOXIN

The reaction mixture was the same as described in Table VIII except that 10 μmoles cystein replaced ascorbate and 0.3 μmole benzyl viologen replaced $NADP^+$. The absorption measurements were made by the difference between the sample cuvette containing the complete reaction mixture and the reference cuvette from which ferredoxin was omitted. Oxidized ferredoxin was measured at 415 mμ to minimize interference by benzyl viologen.

	$A_{415\ m\mu}$	$A_{600\ m\mu}$
Oxidized ferredoxin (1)	0.673	0.153
Photoreduced ferredoxin (2)	0.420	0.126
Photoreduced ferredoxin reoxidized by benzyl viologen (3)	0.690	0.270
ΔA due to reduction of ferredoxin*	0.253	—
ΔA due to reduction of benzyl viologen**	—	0.117
Ferredoxin reduced (μmole)		0.051
Benzyl viologen reduced (μmole)		0.050
Ferredoxin/benzyl viologen ratio		1.02

* (1) − (2).
** (3) − (1).

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