In the 28-day simulated use test, the remaining Product C tablets had the same original average content and content range as the starting tablets. Both Product A and B tablets experienced some potency loss as well as an increasing variability.

In conclusion, the compressed tablet formulation was as active as two other widely used nitroglycerin tablet formulations. However, the uniformity and stability significantly exceeded those of the two popularly used molded tablet formulations, particularly under the stress conditions likely to be encountered in a patient usage regimen.

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Electrochemistry of Drug Action I: Electroreduction of Ferredoxins

YIE W. CHIEN

Abstract □ Ferredoxin serves as an electron carrier in the oxidation–reduction system in anaerobic microorganisms, transferring electrons from a low potential donor to electron-accepting biochemicals. The anaerobicidal activity of some drugs may be due to their interference with the electron transport function of ferredoxin. Two types of ferredoxins (isolated from *Clostridium pasteurianum* and spinach) were studied, and their electrochemical reduction and biochemical properties were analyzed using a sensitive ac polarographic technique. The reduction potential of both ferredoxins was linearly related to pH. The mechanisms of electron transport in ferredoxin molecules were found to be related to their sulfur–iron bonds. The dissociation of the sulfur–iron bonds resulted in the formation of a free sulfhydryl group and the interruption of the electroactivity of ferredoxin. This sulfur–iron dissociation process was found to be pH dependent. The electroreduction of ferredoxins was an energy-requiring, pH-dependent process.

Keyphrases □ Electrochemistry—of drug action, electrochemical reduction and biochemical properties of ferredoxins studied using ac polarography □ Ferredoxins—electrochemical reduction and biochemical properties studied using ac polarography □ Electroreduction—ferredoxins, studied using ac polarography, effect of pH □ Polarography, ac—study of electroreduction and biochemical properties of ferredoxins □ Oxidation–reduction systems—ferredoxins studied using ac polarography, effect of pH

Ferredoxin, a nonheme iron-containing protein, was first isolated from *Clostridium pasteurianum*, an anaerobe, by Mortenson *et al.* (1), and crystalline preparations were obtained later from several other anaerobic bacteria (2, 3).

Much research interest has been generated since regarding the biochemical significance of ferredoxins (4) and their relationship to the anaerobicidal activity of several drugs (5–7). These studies indicate that ferredoxin serves as an oxidation–reduction enzyme in anaerobes by transferring electrons from a low-potential donor, *e.g.*, pyruvic acid, to electron-accepting biochemicals, *e.g.*, pyridine nucleotides, or to H^+ to produce H_2, a terminal electron acceptor characteristic of anaerobes. The interruption of this vital pyruvate phosphoroclastic reaction may result in selective toxicity to the anaerobes.

Recently, dc polarography was used to measure the redox potentials of some antimicrobial agents; their antianaerobic activity was related to their redox potentials relative to the redox potential (−470 mv) of ferredoxin (5). Studies of the complexation of metronidazole, an agent effective against anaerobes, with cupric ion indicated that ac polarography has several advantages over dc polarography in terms of sensitivity and reproducibility of electrochemical measurements (8). These advantages should be beneficial to the mechanistic analysis of ferredoxin–drug interactions.

In this study, the electrochemical reduction of two types of ferredoxins was examined, using an ac polarographic technique, to gain a better understanding of the electrochemistry of their electron transport mechanisms.

EXPERIMENTAL

Materials—Clostridial and spinach ferredoxins are available as frozen solutions in neutral tromethamine buffer[1] and were used as obtained. Triple-distilled, instrument-grade mercury[2] was applied in a dropping mercury electrode. Freshly deionized, triple-distilled

[1] Trizma, Sigma Chemical Co., St. Louis, MO 63178

[2] Bethlehem Apparatus Co., Hellertown, Pa.

Table I—Characterization of the ac Polarographic Peaks for Ferredoxins

Electroactive Species[a]	ac Peak Potential, v	
	Peak 1	Peak 2
Ferric ion[b]	−0.166,	—
Cystine	—	−0.400
Ferredoxin Type I	−0.170	−0.533
Ferredoxin Type III	−0.170	−0.515

[a] pH 6.01, 37°. [b] Ferric chloride solution, 8×10^{-5} M.

water was utilized to prepare the McIlvaine buffer (5). The McIlvaine buffer at various pH's was then used as a supporting electrolyte solution for preparing all ferredoxin solutions just prior to a polarographic measurement.

Procedure—Six milliliters of McIlvaine buffer solution, alone or containing various concentrations of ferredoxins, *N*-ethylmaleimide[3], cystine[4], or ferric chlorides[4], was deaerated with pure nitrogen gas for 5 min in a thermostated (37°) three-electrode microcell system prior to measurement. A nitrogen atmosphere was maintained over the tested solution during electrochemical analysis[5] to prevent oxygen contamination.

For the ac polarogram, the controls were preset as follows: modulation amplitude, 10 mv; frequency, 100 Hz; phase, 178.6°; scan rate, 2 mv/sec; drop time, 0.5 sec; and sample duration, 10 msec. For the dc polarogram, the scan rate was 2 mv/sec, the drop time was 0.5 sec, and the time constant was 1 sec. The ac polarographic measurement of *N*-ethylmaleimide, without or with cystine or ferredoxins, was conducted under the same conditions. The current height of the *N*-ethylmaleimide peak at −0.769 v was measured to calculate the concentration of electroactive *N*-ethylmaleimide.

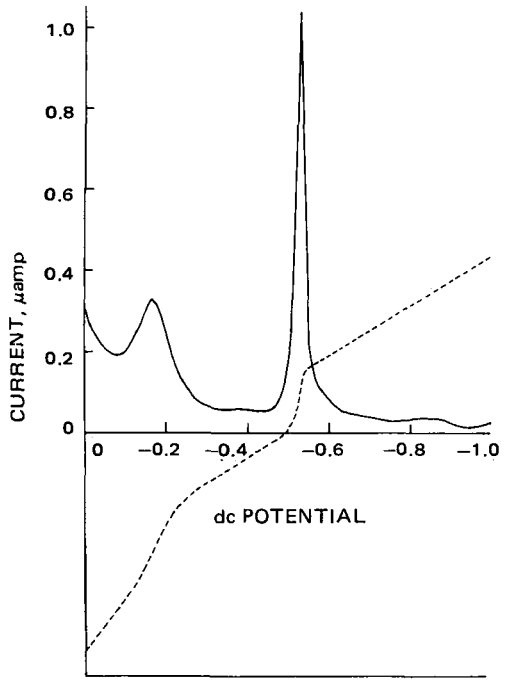


Figure 1—*The ac (solid line) and dc (dotted line) polarograms of ferredoxin (Type I, 80 μg/ml) at pH 6.01 and 37°. Two ac peaks were observed at* E_p (i_{ac}) *of −0.170 v (0.184 μamp) and −0.533 v (0.946 μamp). The second dc peak at* $E_{1/2}$ *of −0.527 v and* i_d *of 0.125 μamp is more obvious than the first dc peak.*

[3] Aldrich Chemical Co., Milwaukee, WI 53233
[4] Matheson, Coleman & Bell, Norwood, OH 45212
[5] The ac and dc polarographic analyses were performed on a PAR electrochemistry system (model 170, Princeton Applied Research Corp., Princeton, N.J.) with an automated mercury drop timer (model 172A, Princeton Applied Research Corp.).

Table II—Agreement of ac Peak Potential (E_p) with dc Half-Wave Potential ($E_{1/2}$) for Ferredoxin Type I at Various pH Values

pH Profile	E_p, v	$E_{1/2}$, v
4.51	−0.445	−0.430
6.01	−0.533	−0.527
7.52	−0.585	−0.570

RESULTS AND DISCUSSION

Electrochemical Reduction of Ferredoxins—To act as an electron transport enzyme, the ferredoxin molecule has to be active electrochemically. Using the differences in absorption spectra between the oxidized and reduced forms of ferredoxins, Tagawa and Arnon (2) estimated that the ferredoxins from *Cl. pasteurianum* and spinach have redox potentials at −0.417 and −0.432 v, respectively, at pH 7.55. However, a redox potential of −0.470 v recently was reported for clostridial ferredoxin at pH 6.0 (5).

In this investigation, both Type I (clostridial) and Type III (spinach) ferredoxins were examined by polarographic analysis, and their electrochemical activity was confirmed. One typical polarogram is illustrated in Fig. 1. In this illustration, the ac polarogram of ferredoxin Type I is compared to its dc polarogram at pH 6.01. The ac polarography gave more distinct reduction peaks for ferredoxin than did the conventional dc polarography. The distinct ac peak at a reduction potential of −0.533 v agreed well with the half-wave potential ($E_{1/2}$) of −0.527 v measured by dc polarography. In addition, the ac peak at −0.170 v was defined much more clearly than the dc polarographic curve.

This ac reduction peak (−0.170 v) was observed to superimpose on the single ac reduction peak (−0.166 v) resulting from the electrochemical reduction of ferric ion to ferrous ion under the same condition (Table I). Therefore, this reduction peak may be assigned to the electroreduction of an uncoordinated ferric ion in the ferredoxin solution tested. The uncoordinated ferric ion may have resulted from the breakdown of a small fraction of ferredoxin molecules after their isolation and purification.

The electroactivity of cystine was also examined to characterize the reduction peak at −0.533 v. The result (Table I) indicates that the electrochemical reduction of the disulfide bond in the cystine molecule is much less negative (−0.400 v) and cannot account for the functional group undergoing reduction at −0.533 v (at pH 6.01) in the ferredoxin molecule. This observation suggests that there is no disulfide linkage in the ferredoxin molecule and that the more negative reduction peak (−0.533 v) may be related to the reduction of a bond whose bond strength is stronger than that of disulfide linkage, *e.g.*, the coordinated sulfur–iron bond in the active centers of a ferredoxin molecule. The characteristics of this sulfur–iron bond will be analyzed later.

When the activities of the oxidized and reduced species of an electroactive compound at the immediate surface of an electrode are equal, an ac peak potential (E_p) should be observed in an ac polarogram and a half-wave potential ($E_{1/2}$) should be seen in a dc polaro-

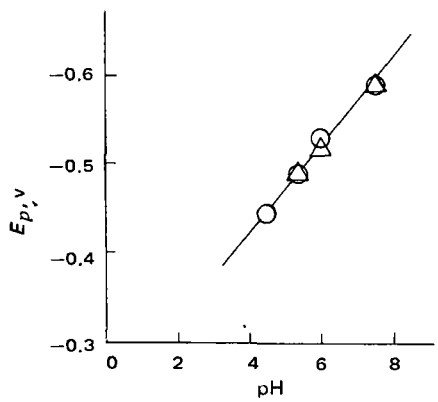


Figure 2—*Linear relationship of ac peak potential (*$-E_p$*) with pH as expected from Eq. 2. Key:* ○, *ferredoxin Type I; and* △, *ferredoxin Type III. From the slope of the linearity,* m/n *was calculated to be 0.77.*

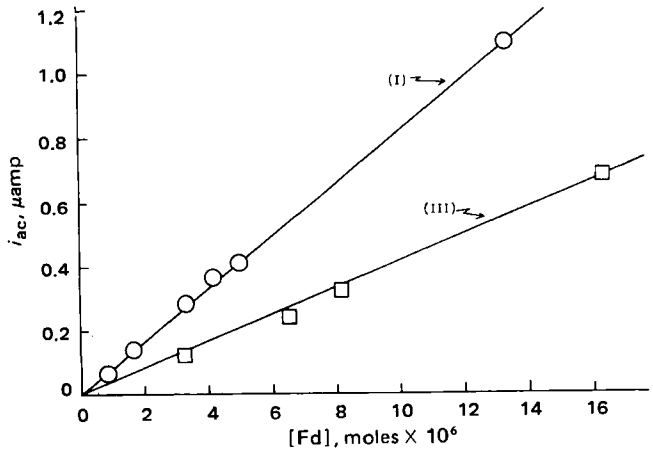


Figure 3—*Linear relationship between ac current, i_{ac}, and the molar concentration of ferredoxins, [Fd]. The slopes (i_{ac}/mole) of the linearities are 82.02 × 10^3 and 41.56 × 10^3 μamp/mole for Types I and III, respectively.*

gram. These two potentials should have the same magnitude according to electrochemical thermodynamics. As demonstrated in Table II, the values of E_p agreed with the magnitude of $E_{1/2}$ at the various pH conditions studied. This agreement permits the use of the more sensitive ac polarographic technique to study the electroreduction process of ferredoxins.

If the electrochemical reduction of ferredoxin is an electrodic reaction (Scheme I):

$$\text{ferredoxin (oxidized)} + m\text{H}^+ + ne \rightarrow \text{ferredoxin (reduced)}$$

Scheme I

then the equilibrium electrode potential, E_e, is defined by the Nernst equation (9):

$$E_e = E^\circ + \frac{2.303RT}{nF}\log\frac{[\text{oxidized}]}{[\text{reduced}]} - \frac{2.303RT}{nF}m\text{pH} \quad \text{(Eq. 1)}$$

where E° is the standard redox potential; n and m are the number of electrons transferred and the number of protons consumed, respectively; F is the Faraday constant; and R and T are the gas constant and absolute temperature, respectively.

At the peak of an ac reduction polarogram, it is reasonable to assume that [oxidized] ≃ [reduced] in view of the minor difference in the diffusivity between the oxidized and reduced species of ferredoxin molecules. So, at 37°:

$$-E_p = -E_e = -E^\circ + \frac{m}{n}0.061\ \text{pH} \quad \text{(Eq. 2)}$$

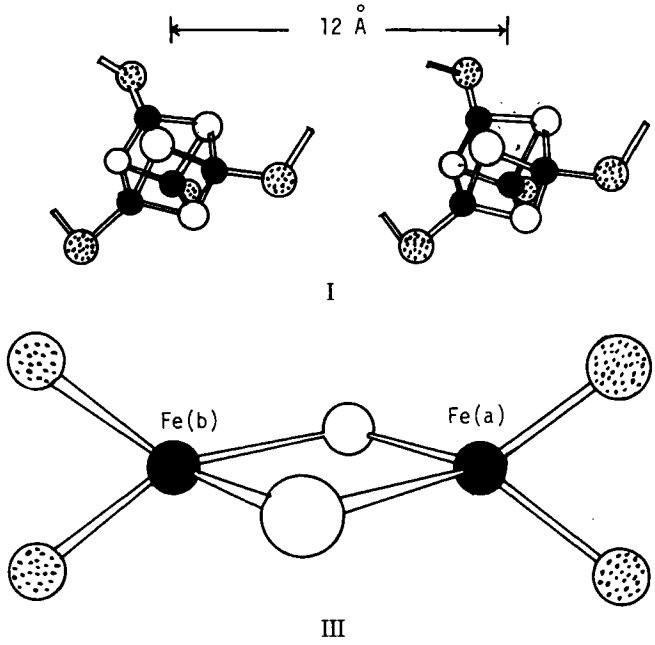
That is, a linear relationship should exist between the ac peak potential ($-E_p$) and the pH profile of the ferredoxin solution (Fig. 2). Both ferredoxins (Types I and III) followed this relationship very well. From the slope, the ratio of m/n was 0.77. This value of m/n was slightly lower than the expected value of unity for one- or two-electron reduction, indicating that the availability of proton (H^+) to ferredoxin molecules may be the rate-limiting (slower) step in the electrochemical reduction of ferredoxins.

In addition to the agreement observed between the ac peak potential and the dc half-wave potential (Table II), the data in Table III reveal that the measurement sensitivity was improved eightfold by using ac polarography. The current heights for the electrochemical reduction of ferredoxins were substantially amplified from 0.125 to 0.946 μamp for Type I ferredoxin and from 0.037 to 0.242 μamp for Type III ferredoxin.

Table III—Comparison of ac Polarography with dc Polarography for Ferredoxins[a]

	dc Polarography		ac Polarography	
Ferredoxin	$E_{1/2}$, v	i_d, μamp	E_p, v	i_{ac}, μamp
Type I	−0.527	0.125	−0.533	0.946
Type III	−0.511	0.037	−0.515	0.242

[a] Concentration of ferredoxin = 80 μg/ml, pH 6.01, 37°.

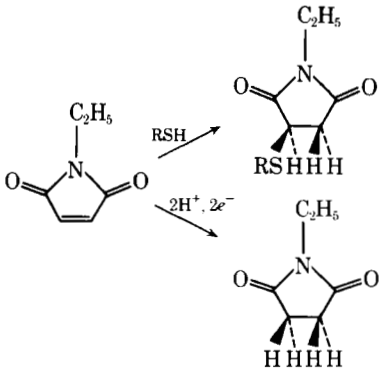


As expected from the laws of diffusion, the current height (i_{ac}) of the ac reduction peak at −0.533 v was linearly proportional to the molar concentration of ferredoxins Types I and III in solution (Fig. 3). From the slopes, the reduction current per mole, i_{ac}/mole, of ferredoxin was calculated to be 82.02 × 10^3 and 41.56 × 10^3 μamp/mole for Types I and III, respectively. The magnitude of the molar reduction current for Type I ferredoxin (82.02 × 10^3 μamp/mole) was twice that for Type III ferredoxin (41.56 × 10^3 μamp/mole). This observation supports an earlier report (10) that Type I ferredoxin functions as a two-electron carrier and that Type III ferredoxin behaves as a one-electron carrier in reduction–oxidation processes. This agreement suggests that ac polarographic measurement provides a sensitive, direct approach toward the mechanistic analysis of the electroactivity of ferredoxins.

Sulfur–Iron Coordination in Ferredoxins—A representation (11) of the current understanding of the configuration of an active site in a two(iron–sulfur) protein (ferredoxin Type III) is reproduced in Structure III. Ferric, cysteine sulfur, and inorganic sulfur atoms are represented by solid, dotted, and open circles, respectively (reproduced from Ref. 11). Each molecule of ferredoxin Type III contains one active center. The formal description of this active center is two iron atoms, two inorganic sulfur atoms, and two cysteine sulfur atoms from the peptide chain of 97 amino acid residues.

On the other hand, X-ray crystallography of an eight(iron–sulfur) ferredoxin Type I (11) revealed that there are two active centers separated by 12 Å (Structure I). The X-ray data also showed that the four iron atoms in each of the two active centers are arranged in a tetrahedron geometry, with an average iron–iron separation of 3.1 Å. The iron atoms are coordinated to the polypeptide chain of 55 amino acid residues *via* the cysteine sulfurs. The labile sulfides are located outside each face of the tetrahedron so that each iron atom is coordinated to four sulfur atoms, three inorganic and one mercaptide, which are also tetrahedrally disposed with respect to the iron atom. The mean bond lengths are 2.35 and 2.01 Å for the iron–sulfur inorganic and iron–sulfur cysteine bonds, respectively. The difference in bond length indicates that the inorganic sulfur atom binds with the iron atom less tightly than does the cysteine sulfur atom.

It was established (11) that an oxidized ferredoxin Type I molecule contained two ferrous ions and two ferric ions in each of its two active centers. Upon reduction, the ratio of ferrous to ferric ions was shifted from 2:2 to 3:1. Although the complete reduction of ferredoxin Type I required the addition of two electrons (12, 13), the oxidation–reduction behavior conformed well to the Nernst expression (Eq. 1) for n = 1, *i.e.*, with the electrons being transferred one at a time (14). It was reported that the redox potentials of these two active centers do not differ by more than a statistical factor. In the present investigation, only one electrochemical reduction peak was detected for each



Scheme II—Electrochemical reduction and complexation of N-*ethylmaleimide with thiol group (RSH) in a protein molecule,* e.g., *ferredoxin.*

of the two types of ferredoxins examined (Table III). These observations led to the suggestion that these two redox active sites are equivalent and do not interact.

The linkage between the inorganic sulfur atom and the iron atom was reported to be labile to acid. The breakdown of this sulfur–iron inorganic bonding was related to the disappearance of the brown color and to the formation of hydrogen sulfide gas in a ferredoxin (oxidized) solution upon acidification. Experiments were designed to establish the effects of acidification on the electroactivity of ferredoxin molecules and also to characterize the relationship of the reduction peak at −0.533 v (at pH 6.01) to the sulfur–iron coordination.

It was observed that ferredoxin Type I gradually lost its electroactivity at pH 3.51; ferredoxin Type III was even more sensitive to acidity and completely lost its electroactivity after short exposure at pH 4.51. Accompanying the diminished electrochemical activity, the brown color of ferredoxin solutions faded and a white precipitate shortly formed. Upon neutralization of these acidic ferredoxin solutions to pH 6.06, the white precipitate redissolved and the reduction peak at −0.170 v was observed. However, the brown color and the reduction peak at −0.533 v could not be restored. These observations are in perfect agreement with the results reported earlier (15) and suggest that the reduction peak at −0.533 v may be related to an electroactive sulfur–iron coordination. Upon acidification, the breakdown of this electroactive sulfur–iron bond results in the loss of electroactivity of ferredoxins at the peak of −0.533 v. The following experiments with *N*-ethylmaleimide provided additional evidence concerning the correlation of the reduction peak at −0.533 v to the sulfur–iron bonding.

N-Ethylmaleimide has been used to detect thiols and thiol groups in protein molecules. The electrochemical reduction of the double bond in the *N*-ethylmaleimide molecule (Scheme II) gave a dc polarogram with an $E_{1/2}$ value of −0.775 v (16). This $E_{1/2}$ value was very close to the E_p (−0.769 v) observed in the present study. The interaction of a free sulfhydryl group with *N*-ethylmaleimide resulted in the saturation of its double bond and a quantitative reduction in the magnitude of its current height (sulfhydryl groups cannot be electrochemically reduced). This provided a sensitive procedure for detecting free sulfhydryl groups in ferredoxin molecules.

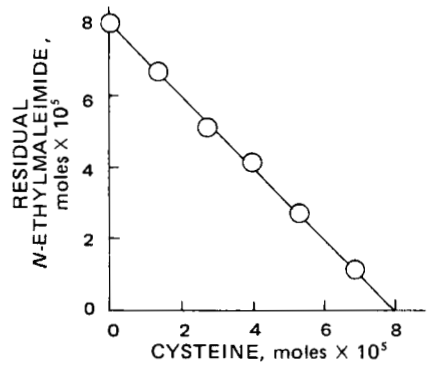


Figure 4—*Decrease in the concentration of* N-*ethylmaleimide in the presence of various concentrations of cysteine. Overall,* 8×10^{-5} *mole of cysteine is needed to complex with* 8×10^{-5} *mole of* N-*ethylmaleimide.*

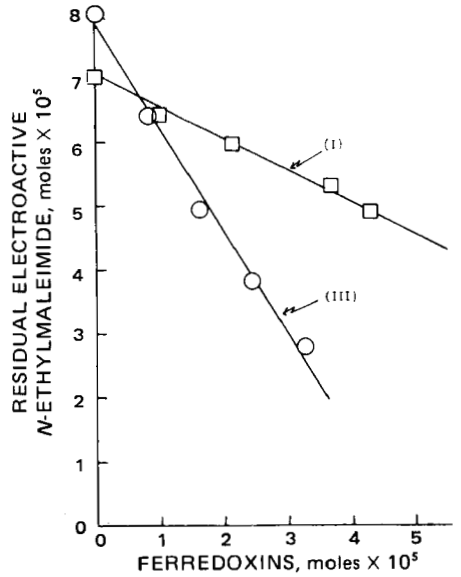


Figure 5—*Complexation of* N-*ethylmaleimide with ferredoxin Types I and III at pH 4.51 and 37°. From the slope of the linear relationships, the ratio of* N-*ethylmaleimide to ferredoxin was calculated to be 0.48 and 1.62 for Types I and III, respectively.*

As reported previously (16), the interaction of *N*-ethylmaleimide with cysteine was also measured by an ac polarographic technique to check its feasibility for detecting the quantitative interaction between *N*-ethylmaleimide and sulfhydryl groups. The result (Fig. 4) indicated that the concentration of electroactive *N*-ethylmaleimide decreased linearly as the concentration of cysteine in the mixture was increased. A quantitative 1:1 complexation between *N*-ethylmaleimide and the sulfhydryl group was observed (Fig. 4). The same results were obtained for pH values of 6.01, 4.51, and 3.51.

If the ferredoxin molecule contains any sulfhydryl groups existing in an uncoordinated state, the addition of ferredoxin to an *N*-ethylmaleimide solution also should result in a reduction in the concentration of the electroactive *N*-ethylmaleimide, as demonstrated earlier for cysteine (Fig. 4). The results shown in Fig. 5 indicated that, at pH 4.51 and 37°, ferredoxin Types I and III contain 0.48 and 1.62 uncoordinated sulfhydryl groups, respectively. The higher value of the free sulfhydryl group observed for ferredoxin Type III than for Type I suggested that Type III is less stable at pH 4.51 than Type I.

The stability of ferredoxin Type I was further studied at a lower

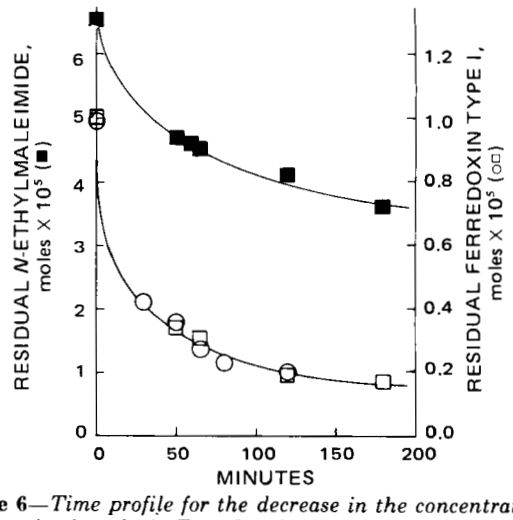


Figure 6—*Time profile for the decrease in the concentration of electroactive ferredoxin Type I and* N-*ethylmaleimide at pH 3.51 and 37°. Key:* ○, *ferredoxin alone;* □, *ferredoxin in the presence of* N-*ethylmaleimide; and* ■, N-*ethylmaleimide in the presence of ferredoxin.*

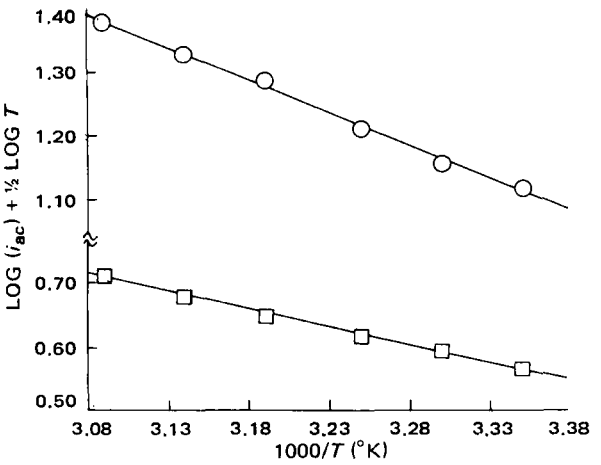


Figure 7—*Linear relationship between log (i_{ac}) + ½ log T and the reciprocal of temperature (Eq. 7) at pH 6.01. From the slope, the ΔG was calculated to be 9.64 kcal/mole for ferredoxin Type I (○) and 4.82 kcal/mole for ferredoxin Type III (□).*

pH (3.51) in the absence or presence of *N*-ethylmaleimide to characterize the relationship of its electroactivity to sulfur–iron bonds. The concentration of electroactive ferredoxin diminished with time at this pH (independent of the presence of *N*-ethylmaleimide) (Fig. 6). Meanwhile, the concentration of electroactive *N*-ethylmaleimide, which complexed with any sulfhydryl group formed, concomitantly decreased. These results (Fig. 6) indicated that more sulfhydryl groups were exposed as time went on and complexed with *N*-ethylmaleimide. The diminishing of the electroactive ferredoxin concentration followed the same pattern as the decrease in the electroactive *N*-ethylmaleimide concentration. Overall, a value of 3.6 was determined for the number of free sulfhydryl groups uncoordinated from each molecule of ferredoxin Type I after 180 min of acidic hydrolysis. Thus, the ac peak at −0.533 v (Table II and Fig. 1) is related to the electroactivity of the sulfur–iron coordination in ferredoxin.

The acidic cleavage of sulfur–iron coordination may increase the iron–iron distance and thus interrupt the antiferromagnetic interaction between iron atoms in its active center, resulting in a loss of electroactivity. The acidic conditions applied here characterize the relationship between the electroactivity of ferredoxins and their sulfur–iron coordinations. In the neutral pH range, both the electroactivity and sulfur–iron coordination were stable when some precautions, *e.g.*, the elimination of oxygen, were taken.

Temperature-Dependent Electroreduction of Ferredoxins—The effects of temperature on the electroreduction of ferredoxins were examined at pH 5.4–7.5, where the stability of ferredoxins was ascertained. In all cases, the magnitude of the ac currents, i_{ac}, for the sulfur–iron reduction peak increased as the temperature increased from 20 to 50°, while the magnitude of peak potentials (E_p) was essentially constant. On the other hand, the current height for the reduction of uncoordinated ferric ion was temperature independent.

The current height (i_{ac}) at the peak of an ac reduction wave, resulting from the application of an alternating potential (E_{ac}) at a limiting low sinusoidal frequency (W), is given by (17):

$$i_{ac} = \frac{n^2F^2AE_{ac}CW^{1/2}D_o^{1/2}}{4RT} \qquad \text{(Eq. 3)}$$

where n is the number of electrons involved, F is the Faraday constant, A is the surface area of the electrode, C is the concentration of the electroactive species, D_o is the diffusivity of the oxidized form of the electroactive species, R is the gas constant, and T is the absolute temperature.

Taking the logarithm on both sides of Eq. 3 results in:

$$\log (i_{ac}) = \log \left(\frac{n^2F^2AE_{ac}CW^{1/2}}{4R}\right) + \tfrac{1}{2}\log D_o - \log T \qquad \text{(Eq. 4)}$$

Also, it is known (18) that the diffusion is an energy-activated process, and the diffusivity of the oxidized species is thus defined by:

$$D_o = l^2\frac{RT}{Nh}e^{-\Delta G/RT} \qquad \text{(Eq. 5)}$$

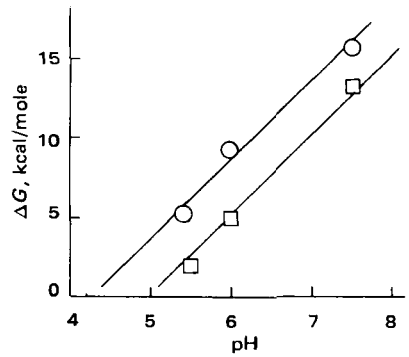


Figure 8—*Relationship between ΔG, activation free energy, and pH profile of ferredoxin solutions. Key: ○, ferredoxin Type I; and □, ferredoxin Type III.*

where l is a mean jump distance, N is Avogadro's number, h is the thickness of diffusion path, and ΔG is activation free energy. Taking the logarithm on both sides of Eq. 5 gives:

$$\log (D_o) = \log \left(l^2\frac{R}{Nh}\right) + \log T - \frac{\Delta G}{2.303R}\frac{1}{T} \qquad \text{(Eq. 6)}$$

Substituting Eq. 6 for the log D_o term in Eq. 4 results in:

$$\log (i_{ac}) + \tfrac{1}{2}\log T = \text{constant} - \frac{\Delta G}{4.606R}\frac{1}{T} \qquad \text{(Eq. 7)}$$

Equation 7 suggests that a linear relationship exists between log (i_{ac}) + ½ log T and the reciprocal of temperature (T^{-1}). A typical plot is shown in Fig. 7 for ferredoxin Types I and III at pH 6.01. The variation in the value of ½ log T was only 1.7% as the temperature was raised from 20 to 50°. From the slope of the linearity, the activation free energy (ΔG) was calculated to be 9.64 kcal/mole for ferredoxin Type I and 4.82 kcal/mole for ferredoxin Type III. The twofold higher ΔG value observed for Type I than for Type III may be related to differences in the complexity of their active centers.

The resultant ΔG values were observed to be pH dependent and increased as the pH of ferredoxin solutions increased (Fig. 8). Both ferredoxin Types I and III showed the same linear ΔG *versus* pH relationship. This observation implied that the conformation of ferredoxin molecule and, hence, its diffusivity were sensitive to the variation in proton (H^+) concentration in the solution. As the concentration of the proton decreased (as pH increased), the conformation of ferredoxin molecule was changed so that its molecular diffusion required a higher value of activation free energy (ΔG).

In summary, the results demonstrated that ac polarography is the technique of choice for studying the electroreduction of ferredoxins. The reduction peak at −0.533 v (at pH 6.01) was attributed to an electroactive sulfur–iron coordination in ferredoxin molecules. The acidic disruption of this sulfur–iron coordination led to interruption of the electroactivity of ferredoxins at low pH. The electroreduction of ferredoxins required energy and was pH dependent.

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Clofibrate Microcapsules: Preparation and Release Rate Studies

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Abstract □ Microencapsulation of clofibrate and dissolution characteristics of clofibrate microcapsules were investigated. Spherical droplets of clofibrate, prepared by a capillary jet method, were encapsulated in gelatin by simple coacervation, using sodium sulfate as the coacervating agent. The microcapsules, which were hardened up to 8 hr with formaldehyde, were recovered as discrete, free-flowing particles. Dissolution of clofibrate from the microcapsules was not adequately described by either square root of time or Langenbucher kinetics but followed predominantly zero-order release patterns at all hardening times. A linear correlation was found between the hardening time and the $t_{50\%}$ release time.

Keyphrases □ Clofibrate—microcapsules prepared, release rate studied □ Microcapsules—clofibrate, preparation described, release rate studied □ Dosage forms—microcapsules, clofibrate, preparation described, release rate studied □ Release rate—clofibrate from microcapsules □ Hypocholesterolemic agents—clofibrate, microcapsules, preparation described, release rate studied

Clofibrate USP, a liquid hypocholesterolemic agent with an unpleasant odor and taste, is administered at rather frequent time intervals (1). Because of these properties, microencapsulation of the drug may result in a more acceptable and effective dosage form.

Simple coacervation with gelatin has been known for many years (2) and has been studied as a means of encapsulation for various pharmaceuticals and chemicals (3). Only a few reports of the dissolution characteristics of such microcapsules are available (4–7), possibly because of the difficulty of obtaining discrete, free-flowing, and reproducible microcapsules.

This study reports the microencapsulation of clofibrate by simple coacervation with gelatin and the effect of hardening time on the dissolution of the microcapsules.

EXPERIMENTAL

Materials—All materials were of USP or reagent grade and were used without further purification. The gelatin[1] used had the following specifications as provided by the manufacturer: type, B-lime treated; bloom, 275; viscosity, 63.9 mpoises; pH (of solution of gelatin), 5.70; moisture, 10.5%; and isoionic point, 4.9.

Production of Monodisperse Spheres—The method employed for the production of monodisperse spheres of clofibrate[2] liquid was similar to that reported earlier (8). The apparatus is shown in Fig. 1. A fine capillary tube, C, was attached to an aspirator bottle, R, which served as the reservoir for the liquid to be encapsulated. The internal diameter of the capillary tube was such that air pressure had to be applied to force the liquid through the capillary. A filter, F, was fitted between the capillary and the aspirator bottle to prevent obstruction by particulate matter.

By varying the internal diameter of the capillary and/or air pressure, the diameter of the droplets produced could be changed. To attain uniformity of droplet production, all experiments were conducted under identical conditions, using the same capillary tube and forcing clofibrate from the capillary tube under identical air pressure.

The droplets leaving the capillary tube were allowed to fall into the gelatin solution. This solution was continuously stirred to prevent coalescence of clofibrate droplets.

Microencapsulation Procedure—Simple coacervation was used to achieve microencapsulation (2), and all experiments were conducted under identical conditions with the same or similar equipment. Coacervation was carried out at 40 ± 1°. The gelatin solution was prepared by soaking 10 g of gelatin in 100 ml of distilled water, allowing it to hydrate for about 12 hr, and then warming to 40° to effect solution. Then 40 ml of clofibrate in the form of monodisperse spheres was added to the gelatin solution, and the mixture was stirred continuously at 30 rpm to prevent coalescence of clofibrate droplets.

After stirring for about 5 min, a 20% (w/w) solution of sodium sulfate, also at 40°, was added to the mixture. Stirring was continued for 15 min more to ensure complete encapsulation, and the formation of coacervate-coated spheres was verified microscopically. The product was then poured into 500 ml of a 7% (w/w) sodium sulfate solution at about 4° to gel the liquid shell of the microcapsules. The mixture was maintained at less than 10° and stirred continuously for 30 min to complete the gelling process.

Recovery of Microcapsules—For this investigation, it was essential to obtain microcapsules in the form of a free-flowing powder. The method of Madan *et al.* (8) was modified slightly and, instead of 70% 2-propanol at room temperature, an equal volume of chilled 2-propanol was added to the product to dehydrate and to flocculate the coacervated droplets. The microcapsules were allowed to settle,

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[2] Ayerst Laboratories, Rouses Point, N.Y.