Stryer, L., Kendrew, J. C., and Watson, H. C. (1964), *J. Mol. Biol. 8*, 96.
Taylor, D. S. (1939), *J. Am. Chem. Soc. 61*, 2150.
Teale, F. W. J. (1957), *Biochim. Biophys. Acta 35*, 543.
Theorell, H., and Ehrenberg, A. (1951), *Acta Chem. Scand. 5*, 823.
Walker, L. R., Wertheim, G. K., and Jaccarino, V. (1961), *Phys. Rev. Letters 6*, 98.
Warburg, O., and Negelein, E. (1932), *Biochem. Z. 244*, 9.
Watson, R. E., and Freeman, A. J. (1961), *Phys. Rev. 123*, 2027.
Wertheim, G. K. (1964), Mössbauer Effect: Principles and Applications, New York, N. Y., Academic.
Wertheim, G. K., and Remeika, J. P. (1964), *Phys. Rev Letters 10*, 14.

Additional Observations on the Chemistry of Clostridial Ferredoxin*

Richard Malkin† and Jesse C. Rabinowitz

ABSTRACT: Apoferredoxins were prepared from clostridial ferredoxin by three different methods: treatment with the mercurial, sodium mersalyl at pH 7.4, and treatment with the iron chelating agent, α,α'-dipyridyl, at both pH 5.4 and 7.4. The apoferredoxin samples showed no enzymatic activity and were free of iron and labile sulfide. Their amino acid analyses and total half-cystine contents were identical. None of the apoferredoxins could be reconverted to ferredoxin by the addition of ferrous iron. A quantitative and simultaneous release of both iron and sulfide occurs when ferredoxin is treated with α,α'-dipyridyl in the presence as well as in the absence of sodium hydrosulfite at pH 5.4. Only a slow release occurs in the absence of the iron chelating agent when hydrosulfite is present. No H_2S is released from oxidized ferredoxin in the absence of α,α'-dipyridyl during the 3-hr period of these experiments. If the evolved H_2S originated from cysteine residues in ferredoxin, a quantitative conversion of cysteine to dehydroalanine should occur. No significant amounts of dehydroalanine were detected in the samples of apoferredoxin examined. The model compounds, L-cysteine methyl ester hydrochloride and reduced glutathione, were tested for the evolution of H_2S under a variety of conditions, but no H_2S was evolved under any of these conditions. These results indicate that the inorganic sulfide of clostridial ferredoxin does not arise from the cysteine residues of the peptide chain.

The nature of the iron linkage and the origin of inorganic or labile sulfide in bacterial ferredoxin have not yet been determined. Previous work from this laboratory (Lovenberg *et al.*, 1963) demonstrated that all clostridial ferredoxins examined contain equivalent amounts of iron, inorganic sulfide, and cysteine residues. A close association of iron and inorganic sulfide in the native protein was indicated since removal of one of the two components was accompanied by the simultaneous loss of the second component.

A recent report by Bayer *et al.* (1965) postulated a structure for clostridial ferredoxin in which the iron is bound to the protein exclusively through the sulfur atoms of the cysteine residues in the peptide chain. The release of inorganic sulfide is postulated to occur through a β-elimination reaction from the cysteine residues. The main evidence supporting this model was the reported reconstitution of ferredoxin by the addition of ferrous ammonium sulfate alone to an apoferredoxin prepared by treatment of reduced ferredoxin with α,α'-dipyridyl. No source of inorganic sulfide was required for reconstitution of ferredoxin from this material.

The purpose of this paper is to report our investigations on the model proposed by Bayer *et al.* (1965). An examination of the properties of apoferredoxins prepared by three methods has been made and we have attempted to evaluate the model from these data. Evidence is presented which indicates that the H_2S evolved from native ferredoxin does not arise from the cysteine residues in the protein.

Experimental Procedures

Materials. The following reagents were purchased from commercial sources: L-cysteine methyl ester hydrochloride, reduced glutathione and sodium *o*-[(3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxy-

* From the Department of Biochemistry, University of California, Berkeley, California. *Received November 24, 1965.* This study was supported in part by a research grant from the U. S. Public Health Service (AM-02109).

† Predoctoral trainee of the U. S. Public Health Service.

acetate (sodium mersalyl) (Mann Research Laboratories); 2,2′-bipyridine (α,α'-dipyridyl) (Matheson Coleman and Bell); DTNB[1] (Aldrich Chemical Co.); *o*-phenanthroline (G. F. Smith Chemical Co.); acetyldehydroalanine (K and K Laboratories); DEAE-cellulose (Carl Schleicher and Schuell Co.); DPNH (Sigma); rabbit muscle lactic dehydrogenase (Calbiochem); and Antifoam A spray (Dow Corning Corp.).

Growth of Organisms. Clostridium pasteurianum was grown on sucrose and ammonium sulfate (Lovenberg *et al.*, 1963). *C. acidi-urici* was grown on uric acid as described by Rabinowitz (1963).

Preparation of Ferredoxin. The ferredoxin from *C. acidi-urici* was isolated and crystallized as described by Buchanan *et al.* (1963), and the ferredoxin from *C. pasteurianum* was isolated and crystallized according to the procedure of Lovenberg and Sobel (1965).

Preparation of Apoferredoxins. MERSALYL APOFERREDOXIN. In a usual preparation, 10 mg of crystalline ferredoxin in 2 ml of 0.1 M Tris–HCl, pH 7.4, was treated with 2.0 ml of 0.1 M sodium mersalyl for 15 min at room temperature. The solution was then placed on a DEAE-cellulose column (1.2 × 10 cm) which had been equilibrated with 1 M potassium phosphate, pH 6.5, and had been washed with distilled water before use. After applying the protein solution, the column was washed with 10 volumes of distilled water and the protein–mersalyl complex was eluted with 0.5 M NaCl. The eluate was treated with 0.040 ml of 14.1 M mercaptoethanol for 15 min at room temperature. The solution was then dialyzed overnight at 4° to remove the mersalyl. The dialyzed solution was lyophilized to dryness and redissolved in distilled water. The yield was 8 mg.

α,α'-DIPYRIDYL APOFERREDOXIN, pH 5.4. The procedure described by Bayer *et al.* (1965) was followed as closely as possible. Ferredoxin, 7 mg, was dissolved in 2 ml of 0.1 M potassium acetate, pH 5.4, and 0.25 ml of a freshly prepared solution of 0.24 M $Na_2S_2O_4$ in 0.1 M Tris–HCl, pH 7.4, was added, followed by 2.0 ml of 0.5% α,α'-dipyridyl. The solution was allowed to stand for 1 hr at 4° and then dialyzed for 3 weeks against 6 l. of distilled water at 4° using a magnetic stirring motor for agitation. The water was changed every 1–2 days during this period. The protein solution was centrifuged after this period to remove some insoluble material and the supernatant colorless solution was lyophilized to dryness. The yield was 6 mg.

α,α'-DIPYRIDYL APOFERREDOXIN, pH 7.4. Ferredoxin, 9 mg, was dissolved in 2 ml of 0.1 M Tris–HCl, pH 7.4, in a serum-capped tube which was flushed with nitrogen. A small amount of Antifoam A was added. Then, 0.50 ml of a freshly prepared solution of 0.24 M $Na_2S_2O_4$ in 0.1 M Tris–HCl, pH 7.4, was added through the serum cap with a syringe and the tube flushed with nitrogen for 20 min at 25°; after the mixture had remained at room temperature for 2 hr, 2.0 ml of deoxygenated 0.5% α,α'-dipyridyl was added through the serum cap and the anaerobic solution was allowed to stand at room temperature for 24 hr. The solution was placed on a DEAE-cellulose column (1.2 × 10 cm) which had been equilibrated with 1 M potassium phosphate, pH 6.5, and had been washed with distilled water before use. This procedure separated the protein from the red iron–dipyridyl complex. After applying the solution, the column was washed with water until the eluate was colorless, and then with 5 column volumes of 0.05 M potassium phosphate, pH 6.5. The protein was eluted with 0.5 M NaCl, dialyzed overnight at 4° to remove the salt, and lyophilized. The yield was 7 mg.

Protein Determination. Protein was determined by a modification of the Lowry phenol reagent method (Rabinowitz and Pricer, 1962). The dry weight of all the apoferredoxin samples was found to be 80% of that determined by the colorimetric method using crystalline bovine serum albumin as a standard while that of native ferredoxin was 70% of that determined by the colorimetric method. All values reported in the paper were determined by the colorimetric method but were corrected by the factors and are expressed as dry weight of protein.

Enzymic Assay for Ferredoxin. Ferredoxin was assayed using the phosphoroclastic reaction as previously described by Lovenberg *et al.* (1963).

Iron Determination. Iron was determined using either *o*-phenanthroline (Lovenberg *et al.*, 1963) or α,α'-dipyridyl. In the latter procedure, ferredoxin was incubated anaerobically with 0.25 ml of 1 M potassium acetate, pH 5.4, and 1.0 ml of 0.5% α,α'-dipyridyl in a total volume of 2.5 ml. After standing 4 hr at room temperature, the absorbancy at 520 mμ was measured. In the studies on the effect of dipyridyl on ferredoxin (see below), the absorbancy was read immediately. It was found that 1 μmole of ferrous iron gave an absorbancy of 9 under these conditions, when a freshly prepared and standardized solution of ferrous ammonium sulfate was used.

Inorganic Sulfide Determination. Inorganic sulfide was determined as described by Lovenberg *et al.* (1963).

Amino Acid Analysis. Ferredoxin and apoferredoxin samples were hydrolyzed in 6 N HCl at 110° for 20 hr in evacuated, sealed tubes. Although the evacuations were done with great care (Crestfield *et al.*, 1963) in an attempt to improve the recovery of half-cystine residues the values were still lower than those found by the sulfhydryl analysis described below. The amino acid compositions were determined in the Beckman-Spinco amino acid analyzer. All values reported are mole ratios normalized to the assumed mol wt 5244 for the neutral and acidic amino acids of the iron and inorganic sulfide free protein.

Sulfhydryl Analysis. The free sulfhydryl groups of ferredoxin and the apoferredoxins were estimated by reaction with DTNB (Ellman, 1959). The samples were treated with 0.020 ml of 0.4% DTNB in 0.1 M Tris–HCl, pH 7.4, in a final volume of 1.0 ml. After 2 min at room temperature, the absorbancy at 412 mμ was determined. The sulfhydryl content was calulated on the

[1] Abbreviations used in this work: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DPNH, reduced diphosphopyridine nucleotide.

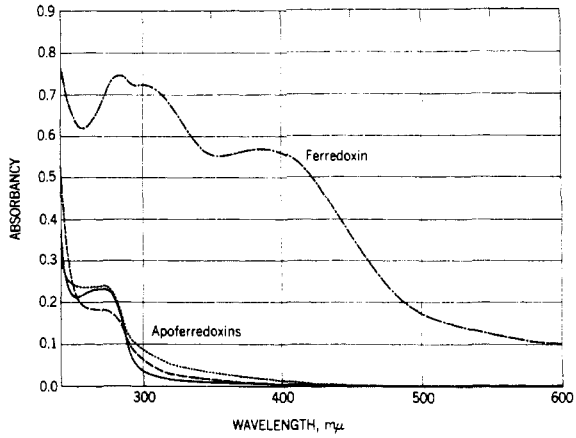

FIGURE 1: Absorption spectra of crystalline *C. acidi-urici* ferredoxin and apoferredoxins. Spectra were measured in 0.1 M Tris–HCl, pH 7.4, with a Cary Model 14 spectrophotometer. The following concentrations of protein, as determined by the Lowry phenol reagent method, were present in 1 ml: — · — *C. acidi-urici* ferredoxin, 127 μg; . . . . α,α′-dipyridyl apoferredoxin, pH 5.4, 120 μg; ——— α,α′-dipyridyl apoferredoxin, pH 7.4, 116 μg; — — — mersalyl apoferredoxin, 120 μg.

basis of the molar extinction coefficient of 13,600 at this wavelength.

Total half-cystine residues were determined after anaerobic reduction of the protein with sodium borohydride according to the procedure of Ferdinand *et al.* (1965). The reduction was carried out by incubating 0.5–2 mg of protein for 90 min at 40° in a solution containing $NaBH_4$ (1% w/v), urea (8 M), KCl (2 M), sodium borate pH 9.41 (78 mM), and EDTA (10 mM), and a small amount of Antifoam A in a final volume of 1.0 ml. The anaerobic system was flushed with prepurified nitrogen which was passed through Fieser's solution (Fieser, 1924) to remove traces of oxygen. The flushing was continued for the first 20 min of the incubation at 40°. At the end of the incubation, the tube was cooled to room temperature and the sodium borohydride was destroyed by the addition of 0.1 ml of deoxygenated 50% acetic acid. After all foaming had stopped, samples were withdrawn with a syringe and injected into the DTNB reaction mixture described above. Protein was determined by the Lowry phenol reagent method as described above.

Determination of Dehydroalanine. Protein samples were hydrolyzed under conditions in which the conversion of dehydroalanine to pyruvate occurs in high yield (Patchornik and Sokolovsky, 1964). From 0.5 to 1.0 mg of protein was heated in 3 N HCl in a final volume of 1.0 ml in a boiling water bath for 1 hr. A marble was placed on top of the tube to prevent evaporation. The solution was cooled and insoluble material removed by centrifugation. The pH of the supernatant solution was adjusted to 7.5 with NaOH and the volume adjusted to exactly 2.0 ml with 0.1 M potassium phosphate, pH 7.5. The solution was then assayed for pyruvate with lactic dehydrogenase and DPNH. Under the assay conditions employed, 1 μg or 0.0091 μmole of pyruvate gave a total absorbancy change at 340 mμ of 0.058.

Results

Analysis and Attempted Reconstitution of Apoferredoxins. The spectra of the three apoferredoxins prepared as described in Experimental Procedures and of native *C. acidi-urici* ferredoxin are shown in Figure 1. The results of the analyses of the samples for enzymatic activity and iron and sulfide content are shown in Table I.

TABLE I: Properties of Native Ferredoxin and Apoferredoxins.[a]

Protein	Enzymic Activity (u/mg)	Iron (μmole/mg of protein)	Inorganic Sulfide (μmole/mg of protein)
Native *C. acidi-urici* ferredoxin	45	1.11	0.96
Mersalyl apoferredoxin	0	0.10	0.01
α,α′-Dipyridyl apoferredoxin, pH 5.4	0	0.05	0.00
α,α′-Dipyridyl apoferredoxin, pH 7.4	0	0.07	0.03

[a] Specific activity, iron, and inorganic sulfide were determined as described in Experimental Procedures. Assays for enzymic activity were done with 0.8 μg of native ferredoxin and 24 μg of each of the apoferredoxins. Assays for iron and labile sulfide were done with 16 and 4 μg of native ferredoxin, respectively, and with 80 μg of the apoferredoxins in each assay.

The three apoferredoxin samples appear to be very similar, if not identical, in their properties. The variation in the iron content is not considered to be significant in relation to the method of analysis used.

The amino acid composition of the samples was determined as described in Experimental Procedures. Because of the small quantities of the apoferredoxins available, only the acidic and neutral amino acids were determined. However, the acidic and neutral amino acids represent most of the amino acids in *C. acidi-urici* ferredoxin since this ferredoxin contains only one basic residue (arginine) and no tryptophan. The results of these analyses are shown in Table II. The half-cystine content, as well as the neutral and acidic amino acid content of all the samples—ferredoxin, as well as the three apoferredoxins—were essentially identical.

Because of the low recovery of half-cystine residues in previous work, and in the amino acid analysis of the

TABLE II: Amino Acid Composition of *C. acidi-urici* Ferredoxin and Apoferredoxins.[a]

Amino Acid	Residues per mole			
	Ferredoxin from *C. acidi-urici*	Mersalyl Apoferredoxin	α,α'-Dipyridyl Apoferredoxin, pH 5.4	α,α'-Dipyridyl Apoferredoxin, pH 7.4
Aspartic acid	8.2	8.2	8.2	8.4
Threonine	1.0	0.6	1.0	1.2
Serine	2.4	2.1	2.6	2.7
Glutamic acid	4.5	4.4	4.1	4.1
Proline	4.2	4.4	4.2	4.0
Glycine	4.3	4.2	4.2	3.9
Alanine	8.4	8.9	9.0	8.3
Half-cystine	6.1	6.4	6.1	6.4
Valine	5.4	5.9	5.5	5.1
Isoleucine	4.1	4.5	4.3	4.7
Tyrosine	2.0	1.5	1.6	1.7

[a] Amino acids were determined as described in Experimental Procedures. Values are calculated on the basis of a molecular weight of 5400. Half-cystine residues were determined by amino acid analysis but, because of the lability of cysteine to acid hydrolysis, the values reported here are believed to be low.

TABLE III: Half-Cystine Content of *C. acidi-urici* Ferredoxin and Apoferredoxins.[a]

Protein	Moles of Half-cystine per Mole of Protein	
	Amino Acid Analysis	Reduction with $NaBH_4$
C. acidi-urici ferredoxin	6.1	7.3
Mersalyl apoferredoxin	6.4	6.9
α,α'-Dipyridyl apoferredoxin, pH 5.4	6.1	7.5
α,α'-Dipyridyl apoferredoxin, pH 7.4	6.4	7.9

[a] Half-cystine residues were determined after reduction with $NaBH_4$ as described in Experimental Procedures. In the case of ferredoxin, it was necessary to flush the solution with nitrogen for 15 min at room temperature after addition of the 50% acetic acid to ensure complete removal of the labile sulfide. Protein was determined by the Lowry phenol reagent assay and was corrected for the observed discrepancy between the dry weight determination and the colorimetric assay, as described in the Experimental Procedures. The molecular weight of the apoferredoxins was assumed to be 5400. This is the value obtained after elimination of 7 moles of iron and inorganic sulfide from native ferredoxin.

acid hydrolysate reported here (Table II and Table III), an independent method was used for half-cystine analysis. The half-cystine content of all the samples was determined with DTNB after reduction of the protein with sodium borohydride as described in Experimental Procedures. These results are summarized in Table III. No free sulfhydryl groups could be detected in any of the apoferredoxins before reduction by the DTNB procedure. However, after reduction, the half-cystine residues could be detected in the reaction with DTNB. This indicates that the cysteine residues were present as the disulfides in the protein. The total half-cystine content of the apoferredoxins was 7–8 moles/mole of protein. The value found for ferredoxin was also 7–8 by this method.

Attempts were made to reconstitute the α,α'-dipyridyl apoferredoxins by the addition of ferrous ammonium sulfate under the identical conditions reported by Bayer *et al.* (1965). The protein was incubated with ferrous ammonium sulfate under aerobic conditions, but the reisolated protein had a spectrum identical with that of the original apoferredoxin and showed no significant incorporation of iron, and no inorganic sulfide could be detected.

Effect of α,α'-Dipyridyl on Ferredoxin. While preparing apoferredoxin by the procedure described by Bayer *et al.* (1965) by treatment of ferredoxin with α,α'-dipyridyl, the evolution of H_2S was noted. The kinetics and the stoichiometry of the release of iron and inorganic sulfide from *C. pasteurianum* ferredoxin following the addition of sodium hydrosulfite and treatment with α,α'-dipyridyl are shown in Figure 2. The rate of release of iron and sulfide is almost identical, and after a reaction time of 3 hr, over 90% of the labile sulfide can be recovered in the zinc acetate trap and over 90% of the iron of ferredoxin can be accounted for as the red iron–dipyridyl complex. There is also a slow release of iron and sulfide when the protein is reduced even in the absence of dipyridyl; this can be contrasted with the failure of oxidized ferredoxin to release any sulfide or iron in the absence of dipyridyl.

A comparison of the rate of release of iron and sulfide from ferredoxin when treated with dipyridyl in the absence and presence of the reducing agent is shown in Figure 3. Although there is a slight lag when the reducing agent is omitted, the time course of the reaction is identical in both cases. The recoveries of iron and sulfide were again over 90% in these experiments.

Attempt to Demonstrate Dehydroalanine in the Protein. The data in Figures 2 and 3 show that the rate of release of inorganic sulfide and iron from ferredoxin is identical and quantitative under the conditions used by Bayer for the conversion of ferredoxin to apoferredoxin. If the inorganic sulfide liberated arose by the β-elimination

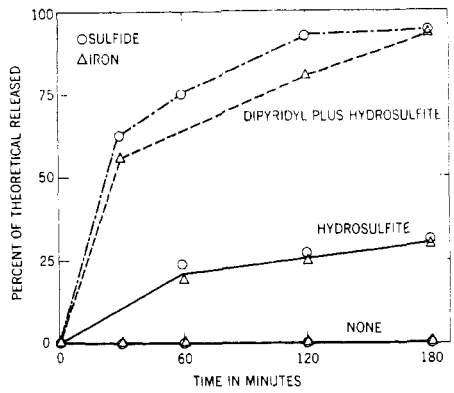

FIGURE 2: The reaction of α,α'-dipyridyl with *C. pasteurianum* ferredoxin; 3.2 mg of ferredoxin was dissolved in 0.20 ml of 1.0 M potassium acetate, pH 5.4, in an 18-mm test tube and a small amount of Antifoam A. When present, 0.40 ml of a 0.57 M $Na_2S_2O_4$ solution and 0.80 ml of a 0.5% α,α'-dipyridyl solution were quickly added. The final volume was 2.0 ml. Prepurified nitrogen was slowly flushed through the reaction vessel attached to a trapping tube in an all-glass bubbling train. The H_2S evolved was trapped in a receiver containing 4 ml of 1% zinc acetate. The reactions were carried out at room temperature and a reaction vessel and trap were used for each point. At the indicated times, each individual reaction mixture was removed from the train and immediately assayed for iron by the dipyridyl method. In reaction mixtures in which dipyridyl was present, iron released was determined from the absorbancy of the sample diluted in a known volume of 0.1 M potassium acetate, pH 5.4. The traps were vigorously stirred to resuspend any precipitated sulfide and aliquots were assayed for sulfide. Duplicate determinations were carried out for each trap. The theoretical values were calculated from the iron and sulfide content of the ferredoxin samples as determined by direct analysis. The sulfide values are corrected for the recovery of a standard solution of sodium sulfide under identical conditions. There was no release of H_2S in control experiments without ferredoxin.

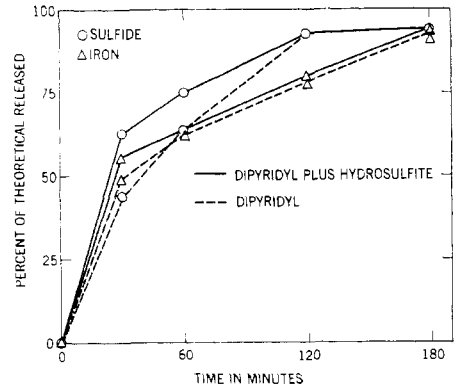

FIGURE 3: The reaction of *C. pasteurianum* ferredoxin with α,α'-dipyridyl. Conditions were identical with those in Figure 2.

TABLE IV: Dehydroalanine Analysis of Ferredoxin and Apoferredoxin.[a]

Sample	Amount (μmoles)	Pyruvate Formed (μmoles)
C. acidi-urici ferredoxin	0.10	0.0
Mersalyl apoferredoxin	0.11	0.0
α,α'-Dipyridyl apoferredoxin, pH 5.4	0.11	0.0
Acetyl dehydroalanine	1.64	1.28
Acetyl dehydroalanine + α,α'-dipyridyl apoferredoxin, pH 5.4	1.64 0.11	1.18
Sodium pyruvate	0.91	0.72
Sodium pyruvate + α,α'-dipyridyl apoferredoxin, pH 5.4	0.91 0.11	0.68

[a] The indicated amounts of material were hydrolyzed under the conditions described in Experimental Procedures. Aliquots were then assayed for pyruvate with lactic dehydrogenase. The concentration of acetyl dehydroalanine was based on the extinction coefficient of 6050 in 80% ethanol, as determined by Eiger and Greenstein (1948).

of H_2S from cysteine residues in the peptide chain as suggested by Bayer *et al.* (1965), the eight cysteine residues would have been converted to eight dehydroalanine residues. Hydrolysates of the apoferredoxins were therefore examined for the presence of pyruvic acid, the hydrolysis product of dehydroalanine.

The method used to hydrolyze the protein is described in Experimental Procedures. A quantitative conversion of the dehydroalanine residues in a peptide chain to pyruvate occurs under the conditions used (Table IV). The pyruvate formed is then assayed with lactic dehydrogenase and DPNH.

The results of the analyses of ferredoxin and two apoferredoxin samples are shown in Table IV. From the data summarized in this table, it is evident that the apoferredoxin samples do not contain a significant amount of dehydroalanine.

Ferredoxin itself was subjected to hydrolysis under these conditions. Although H_2S can be recognized by smell when the sample is acidified for hydrolysis, no pyruvate could be detected in the hydrolysate. This result suggests that the H_2S formed by acidification of ferredoxin does not arise by the β-elimination of H_2S from its cysteine residues.

Sulfide Elimination from Model Compounds. Bayer *et al.* (1965) have reported the evolution of H_2S from

cysteine and some of its derivatives in the presence and absence of reduced iron. Since the exact experimental conditions leading to evolution of H_2S from these compounds were not given, we subjected the model compounds to a variety of conditions and analyzed the samples for the evolution of H_2S.

Glutathione alone and glutathione incubated with an equimolar concentration of ferrous ammonium sulfate failed to release any detectable H_2S. Glutathione was heated at 37° for 15 min in 12% NaOH–1% zinc acetate, and for 30 min at 75° in water and in the NaOH–zinc acetate mixture of the sulfide assay. Under none of these conditions was any H_2S evolved.

L-Cysteine methyl ester hydrochloride was also tested for H_2S evolution under the standard assay conditions and in the presence of an equimolar concentration of ferrous ammonium sulfate. In neither case was there any detectable H_2S evolved. The concentrations of glutathione and L-cysteine methyl ester hydrochloride in the experiments described were sufficient to produce a final absorbancy of 0.40 if there was a stoichiometric release of H_2S from these compounds.

The sulfhydryl content of both these compounds was determined using DTNB and found to be 90% of the theoretical amount expected in the case of the reduced glutathione and 89% in the case of the cysteine derivative.

Discussion

The report of Bayer *et al.* (1965) concerning the structure of the iron–sulfur linkage in ferredoxin and the postulated source of the inorganic sulfide has led us to reinvestigate the chemical nature of ferredoxin.

Bayer and co-workers have proposed a structure for ferredoxin in which the iron atoms are bound exclusively to the sulfur atoms in cysteine. These workers conclude from their experiments that the inorganic or labile sulfide which appears upon acidification of the protein is due to the loss of H_2S from the cysteine residues in a β-elimination type reaction which is facilitated by the presence of the iron atoms. They presented the following evidence in support of this model: (1) cysteine methyl ester yields H_2S in the presence of a ferrous salt, and (2) the apoferredoxin obtained by treatment of ferredoxin with sodium hydrosulfite and the iron-chelating agent α,α'-dipyridyl could be reconverted to ferredoxin by the addition of ferrous ammonium sulfate alone. The authors argue that the failure to reconstitute the isolated apoferredoxin prepared by treatment with the mercurial, sodium mersalyl (Lovenberg *et al.*, 1963), was due to decomposition of the protein under the conditions of treatment because of the nonspecificity of the mercurial reagent.

Our experiments failed to confirm either of these observations. L-Cysteine methyl ester hydrochloride alone or in the presence of reduced iron failed to eliminate H_2S. Glutathione, tested either by itself or in the presence of reduced iron salts, and heated under various conditions, also failed to liberate H_2S. To date, among the compounds tested, the only ones found which give a positive test in the inorganic sulfide assay in our laboratory are Na_2S and ferredoxin.

Apoferredoxins were prepared in three ways. The mersalyl apoferredoxin was prepared as previously described (Lovenberg *et al.*, 1963). The α,α'-dipyridyl apoferredoxin, pH 5.4, was prepared using a procedure as similar to that described by Bayer *et al.* (1965) as possible from their description. The third apoferredoxin was prepared in a similar but somewhat modified manner. The reaction with dipyridyl was carried out at pH 7.4 and the protein was isolated by chromatography on DEAE-cellulose. This technique of isolation eliminated the need of dialysis for a 3-week period to remove the ferrous–dipyridyl complex as described by Bayer *et al.*

None of the apoferredoxins contains significant amounts of iron or inorganic sulfide, and none has biological activity. The amino acid content and total half-cystine content of all the samples are identical, within the error of the analyses. Since there were no free sulfhydryl groups in the proteins, it appears the cysteine residues have become oxidized to four disulfides, possibly during the isolation of the samples. Although there are some small differences in the ultraviolet absorption spectra of the three samples, they appear to be identical by the other properties examined.

The two apoferredoxins which were prepared by treatment of ferredoxin with α,α'-dipyridyl at pH 5.4 or pH 7.4 failed to regenerate native ferredoxin when incubated with ferrous ammonium sulfate under the conditions described by Bayer *et al.* (1965). The reconstituted material and the starting apoferredoxin were indistinguishable with respect to their spectra and lack of enzymic activity, labile sulfide, and iron.

The odor of H_2S was noted during the preparation of apoferredoxin with α,α'-dipyridyl at pH 5.4. This observation suggested the experiment summarized in Figure 2 showing identical rates of release of iron and sulfide when ferredoxin is treated with α,α'-dipyridyl under the conditions specified by Bayer *et al.* (1965) for the conversion of ferredoxin to the apoferredoxin. The recovery of both products at the end of the 3-hr incubation was over 90%. This result explains our failure to reconstitute the apoferredoxin by the addition of ferrous ammonium sulfate.

An attempt was also made to demonstrate the presence of dehydroalanine in the apoferredoxin peptide chain. Dehydroalanine would be the expected product formed from the cysteine residues if the latter were the source of the H_2S in a β-elimination reaction as proposed by Bayer *et al.* We were unable to identify any pyruvate in the acid hydrolysates of ferredoxin or the apoferredoxins under conditions known to convert peptide-bound dehydroalanine to pyruvate. Since amino acid analysis and total sulfhydryl analyses have indicated 7–8 moles of cysteine per mole of apoferredoxin, it can be concluded that the labile sulfide did not arise from cysteine sulfur through a β-elimination type reaction.

Reduced ferredoxin shows a slow release of iron and sulfide in the absence of any chelating agent whereas

oxidized ferredoxin shows no loss over the 3-hr incubation period under the conditions used. This observation of the lability of iron in reduced ferredoxin agrees with optical rotatory dispersion studies on ferredoxin and rubredoxin (Gillard *et al.*, 1965).

The reaction of ferredoxin with α,α'-dipyridyl in the absence or presence of the added reducing agent, sodium hydrosulfite, was almost identical. This result may be explained by assuming that there is some reduced iron in the protein which reacts with dipyridyl. This initial reaction of the reduced iron leads to the exposure of additional reducing groups in the protein which in turn can reduce any oxidized iron so that it will further react with dipyridyl. Determination of the oxidation state of the iron as it is released from the protein using *o*-phenanthroline indicates that most of the iron is reduced. This is thought to be due to a reduction of the iron by the protein, presumably by the liberated sulfhydryl groups (Lovenberg *et al.*, 1963).

Alternatively, it may be that the acidic conditions cause conformational changes in the protein leading to the observed results. Although it has been reported (Gillard *et al.*, 1965) that oxidized ferredoxin does not react with dipyridyl at neutral pH, an iron–dipyridyl complex is formed in the presence of 8 M urea. The effect of exposing ferredoxin to a medium at pH 5.4 may be analogous to the effect of 8 M urea, and the changes in the secondary or tertiary structure of the molecule due to unfolding may labilize the iron so that it can be chelated by the dipyridyl.

Acknowledgments

The authors wish to thank Dr. R. D. Cole and Mrs. Joan Hafeez for help with the amino acid analyses reported in this work, and Dr. D. E. Koshland, Jr. for the details on the dehydroalanine assay.

References

Bayer, E., Parr, W., and Kazamaier, B. (1965), *Arch. Pharm. 298*, 196.

Buchanan, B. B., Lovenberg, W., and Rabinowitz, J. C. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 345.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Eiger, I. Z., and Greenstein, J. P. (1948), *Arch. Biochem. 19*, 467.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Ferdinard, W., Stein, W. H., and Moore, S. (1965), *J. Biol. Chem. 240*, 1150.

Fieser, L. F. (1924), *J. Am. Chem. Soc. 46*, 2639.

Gillard, R. D., McKenzie, E. D., Mason, R., Mayhew, S. G., Peel, J. L., and Stangroom, J. E. (1965), *Nature 208*, 769.

Lovenberg, W., Buchanan, B. B., and Rabinowitz, J. C. (1963), *J. Biol. Chem. 238*, 3899.

Lovenberg, W., and Sobel, B. E. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 193.

Patchornik, A., and Sokolovsky, M. (1964), *J. Am. Chem. Soc. 86*, 1206.

Rabinowitz, J. C. (1963), *in* Methods in Enzymology, Vol. VI, Colowick, S. P., and Kaplan, N. O., Ed., New York, N. Y. Academic, p 703.

Rabinowitz, J. C., and Pricer, W. E., Jr. (1962), *J. Biol. Chem. 237*, 2898.