Biochemistry

Volume 5, Number 1 January 11, 1966

Synthesis of Pyridoxine Labeled with Tritium at Specific Positions*

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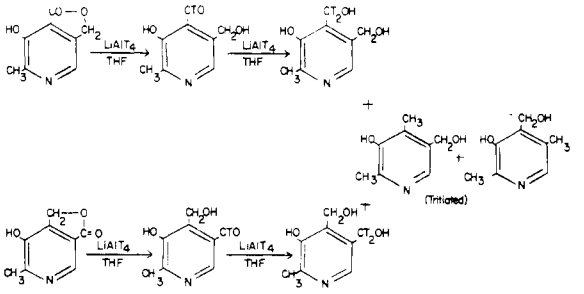
ABSTRACT: Commercially available pyridoxine labeled randomly with tritium was found to be radiochemically impure. The impurities were identified as 4- and 5-deoxypyridoxine, the well-known antimetabolites of vitamin B_6. In the present study pyridoxine labeled with tritium at the 4- or 5-hydroxymethyl group was synthesized by reducing the corresponding 4- or 5-pyridoxic acid lactone with tritiated lithium aluminum hydride in anhydrous tetrahydrofuran. The tritium-labeled pyridoxine was separated from the by-products (among them tritium-labeled pyridoxal or isopyridoxal and 4- and 5-deoxypyridoxine) through ion-exchange column chromatography. The chemical and radiochemical purity of the final product was shown by column chromatography, paper chromatography (three solvent systems), paper electrophoresis, and bioautography; yield 70–74% based on the lactones and 20–21% based on the radioactivity. Degradation studies on the pyridoxine labeled with tritium at the 4-hydroxymethyl group showed that more than 96% of the radioactivity was at the 4-hydroxymethyl group and less than 4% was probably at position 6 of the pyridine ring.

In order to obtain more information on the structure of the metabolites of vitamin B_6 in animals, pyridoxine labeled with tritium was needed in addition to the ^{14}C-labeled that has been synthesized in our laboratories (Argoudelis and Kummerow, 1964). Pyridoxine labeled randomly by the tritium gas exposure method (Wilzbach, 1957) is commercially available. However, a paper chromatogram of the commercial product revealed about 15% radioactive impurities. Although some investigators have used pyridoxine labeled randomly with tritium to study its metabolism in animals (Cox *et al.*, 1962; Booth and Brain, 1962; Brain *et al.*, 1963; Loo and Ritman, 1964; Johansson *et al.*, 1964; Greenberg and Peng, 1965;) and humans (Brain *et al.*, 1963; Brain and Booth, 1964; Johansson *et al.*, 1964), to our knowledge only one group (Booth and Brain, 1962) has reported the presence of a radioactive impurity in the tritium-labeled pyridoxine, without however identifying the nature of the impurity.

The introduction of the Wilzbach technique for labeling organic compounds with tritium has opened new vistas to the biochemist. However, the most important problem in application of this technique is that of radiochemical purification. The difficulties arise from the fact that the by-products may have properties similar to those of the compound exposed and, in the absence of added carrier, may have a much higher specific activity (Wilzbach, 1962). Some of the side reactions which have been observed in gas exposure labeling are fragmentation, addition of fragments to the compound exposed (Riesz and Wilzbach, 1958a), polymerization (Riesz and Wilzbach, 1958a), replacement of substituents (Dorfman and Wilzbach, 1959), addition of tritium at points of unsaturation (Dutton *et al.*, 1958), isomerization (Riesz and Wilzbach, 1958a), and racemization (Riesz and Wilzbach, 1958b). To this list one could add the side reaction "tritiumolysis" which apparently takes place when pyridoxine is exposed to tritium gas. The radioactive impurities from the pyridoxine that was labeled with tritium by the Wilzbach

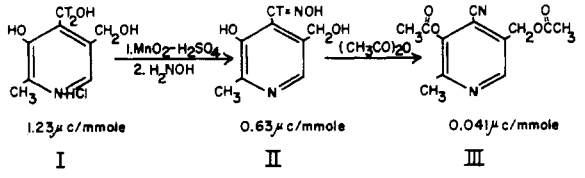
* From The Burnsides Research Laboratory, University of Illinois, Urbana, Ill. *Received May 27, 1965; revised September 27, 1965.* This investigation was supported by a U. S. Public Health Service grant (AM 00257). It was presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1965.



SCHEME I

technique have been identified as 4- and 5-deoxypyridoxine. This side reaction could be expected, as 4- and 5-deoxypyridoxine were formed during the catalytic hydrogenation of pyridoxine (Heyl *et al.*, 1953). Pyridoxal labeled with tritium by the Wilzbach technique (Pal and Christensen, 1961) might also contain these radioactive impurities. The fact that pyridoxine labeled with tritium by the Wilzbach technique contains as impurities the well-known antimetabolites of vitamin B_6 brings into question the results reported in the literature on the metabolism of vitamin B_6.

In order to gain further information on the structure of the metabolites it seemed more appropriate to use a tritium-labeled pyridoxine in which the position of the label was known rather than unknown as in the commercially available pyridoxine. For the known labeling of pyridoxine advantage was taken of the facile reduction of 4-pyridoxic acid lactone (Mowat and Webb, 1960) and of 5-pyridoxic acid lactone (Argoudelis and Kummerow, 1964) to pyridoxine by lithium aluminum hydride. Thus, in order to synthesize pyridoxine labeled with tritium at the 4- or 5-hydroxymethyl group, the corresponding 4- or 5-pyridoxic acid lactone was reduced with tritiated lithium aluminum hydride in anhydrous tetrahydrofuran. The previously mentioned side reaction tritiumolysis was also observed when either the 4- or the 5-pyridoxic acid lactone was reduced with tritiated lithium aluminum hydride. During this reduction both 4- and 5-tritiated deoxypyridoxines were formed, which is in accordance with the amide vinylogy principle (Gaylord, 1954). The 4-hydroxymethyl group of pyridoxine is a vinylog of the electron-donating phenolic hydroxy group while the 5-hydroxymethyl group is a vinylog of the heterocyclic nitrogen. When the lactones were reduced in tetrahydrofuran that was not freshly distilled over lithium aluminum hydride, the main product was the corresponding aldehyde, providing thus a method for the specific labeling with tritium of pyridoxal or isopyridoxal (Scheme I). The same effect (*i.e.*, reduction of the lactone till the aldehyde stage) was noticed when a moderate excess of lithium aluminum hydride was used for the reduction of the lactones. In order to determine the percentage of radioactivity at the expected position, pyridoxine labeled with tritium at the 4-hydroxymethyl group was transformed to compound III (Heyl, 1948) (Scheme II). The fact that about 3.3% of the radioactivity was in some other position than at the 4-hydroxymethyl group was explained as follows. It has been reported that pyridine is reduced by lithium aluminum hydride to form the unstable compound 1,2-dihydropyridine (Bohlmann, 1952). It thus seems probable that, at some stage of the reduction of 4-pyridoxic acid lactone to pyridoxine, the pyridine ring of some molecules was reduced by the tritiated lithium aluminum hydride to give finally tritiated 1,6-dihydropyridoxine which, being unstable, was oxidized to pyridoxine labeled with tritium also at position 6 during the subsequent manipulations. The fact that labeled compounds, one more acidic than the 4-pyridoxic acid lactone and another more basic than pyridoxine, were eluted from the ion exchange column favors this hypothesis.



SCHEME II

Experimental Section

Paper Chromatography (Table I). The solvent mixtures used for chromatography of pyridoxine and its derivatives had the following percentage composition (volume for volume): *t*-amyl alcohol–acetone–water–concentrated ammonium hydroxide (40:35:20:5) (solvent A); ethanol–water–concentrated ammonium hydroxide (80:16:4) (solvent B); 1-butanol saturated with water (solvent C). Chromatograms were run on Whatman No. 1 paper by the ascending technique from 15 to 18 hr, dried, examined under ultraviolet light, and sprayed with 2,6-dichloroquinonechlorimide (Rodwell *et al.*, 1958).

TABLE I: Detection of Pyridoxime and Related Compounds.

Compound	Paper Chromatography R_F Value in Solvent A	B	C	Paper Electrophoresis[a]
Pyridoxine	0.34	0.59	0.60	(−) 4 cm
4-Deoxypyridoxine	0.49	0.68	0.71	(−) 6 cm
5-Deoxypyridoxine	0.57	0.73	0.78	(−) 6 cm
Pyridoxal	0.59			
Isopyridoxal	0.23			

[a] pH 5.1; 120 v; 8 hr.

Radioisotopes and Counting Procedures. Pyridoxine

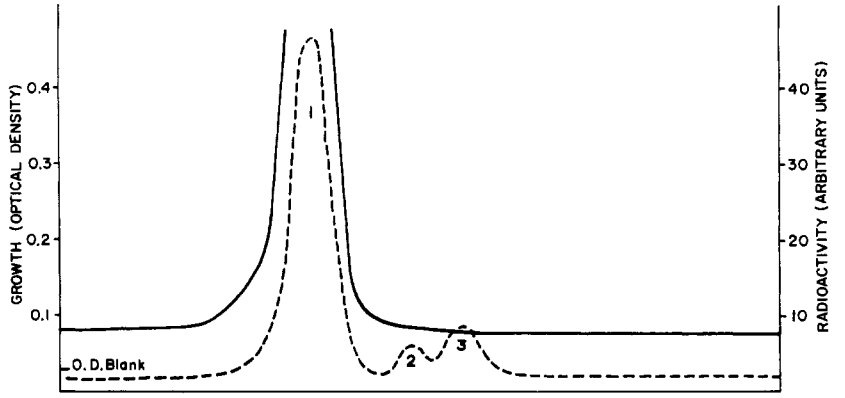


FIGURE 1: Strip counter (dotted line) and bioautographic (solid line) records of a paper chromatogram of pyridoxine labeled with tritium by the Wilzbach technique. The chromatogram was developed in solvent A. Peak 1 represents pyridoxine, peak 2, 4-deoxypyridoxine, and peak 3, 5-deoxypyridoxine.

labeled randomly with tritium (specific activity 331 mcuries/mmole) and tritiated lithium aluminum hydride (specific activity 250 mcuries/mmole) were purchased from Volk Radiochemical Co. The radioactive compounds on the paper chromatograms were located either with a Vanguard paper strip counter or the paper was cut into 0.5-cm sections which were placed diagonally in conventional 5-dram counting vials. To the vials was added 10 ml of scintillation solution (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene, dissolved in 1 l. of reagent grade toluene); a Model 314EX-2 Packard Tri-Carb liquid scintillation spectrometer was used for counting. To locate the radioactive fractions from the column chromatography effluent, one drop (disposable pipet) from each tube was placed in a counting vial and 10 ml of a solution consisting of eight parts of scintillation solution and two parts of absolute ethanol was added. The counting efficiency for tritium in this solution was 13%.

Bioautography. The paper chromatogram was cut into 0.5-cm sections which were placed in colorimeter tubes. To each tube 5 ml of distilled water was added and left at room temperature for 1 hr. The paper was removed from the water and drained on the lip of the tube, the tubes were plugged, steamed for 10; min, and assayed for pyridoxine (Toepfer and Lehmann, 1961) using as test organism *Saccharomyces carlsbergensis* (ATTC No. 9080). To examine the same paper chromatogram for radioactivity and growth the 0.5-cm sections of paper were first measured for radioactivity, removed from the scintillation solution, dried at room temperature, and then dipped into 5 ml of distilled water for the microbiological assay.

Ultraviolet absorption spectra were determined with a Model 11M Cary recording spectrophotometer.

Column Chromatography. To separate the pyridoxine from the compounds that were formed during the reduction of the lactones, Dowex 50 in the sodium form was found to be superior to Dowex 1-formate (Argoudelis and Kummerow, 1964). The use of Dowex 50 had the additional advantage of avoiding the necessity for removal of the aluminum ions from the reaction mixture prior to its application to the column. The ion-exchange resins were treated as follows: to Dowex 50W-X8 (200–400 mesh) resin in the hydrogen form was added excess 6 N sodium hydroxide until the supernatant liquid was blue to litmus paper. The resin was allowed to settle, the fines were decanted, and the resin was rinsed with distilled water repeatedly until the supernatant liquid was clear. The resin was suspended in 3 N hydrochloric acid and heated for 0.5 hr on a steam bath. The hydrochloric acid solution was decanted and the treatment with 3 N hydrochloric acid was repeated three more times. The resin was rinsed with distilled water until the rinsings had pH 4–5, at which time 6 N sodium hydroxide was added until the supernatant liquid was blue to litmus paper. The resin was rinsed with distilled water until the rinsings had a neutral pH. The Dowex 1-X8 (200–400 mesh) resin in the chloride form was poured into the column and washed with 3 N hydrochloric acid until the effluent from the column was free from ultraviolet-absorbing impurities. Burets (100 ml) wrapped with aluminum foil were used as columns. The volume of the resin in the column was 50 ml.

Identification of the Radioactive Impurities of Pyridoxine Labeled by the Wilzbach Technique. About 20 μcuries of the commercial tritium-labeled pyridoxine was spotted on Whatman No. 1 filter paper along side of a mixture of pyridoxine and 4- and 5-deoxypyridoxine. The paper chromatogram was developed in solvent A. Examination of the chromatogram under ultraviolet light revealed only one spot, that of pyridoxine. However, the paper strip counter disclosed three main peaks corresponding to pyridoxine, 4-, and 5-deoxypyridoxine, and a very small peak with a R_F value higher than that of 5-deoxypyridoxine and which could very well correspond to 2,4,5-trimethyl-3-hydroxypyridine. The section of the chromatogram that corresponded to 4-deoxypyridoxine was cut off and dipped for 2 hr into 0.1 N hydrochloric acid, the solution was filtered, and 10 mg of commercial 4-deoxypyridoxine was added. The solution was lyophilized and the residue recrystallized

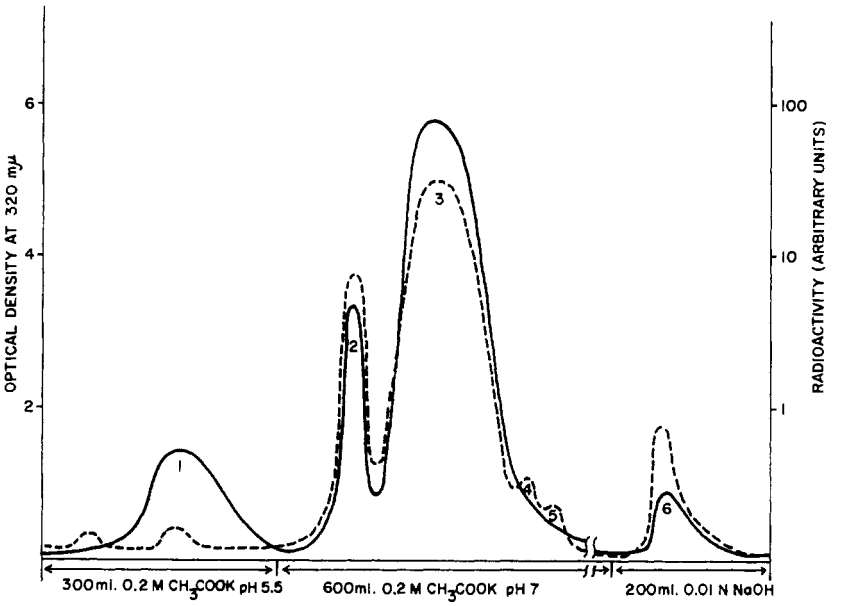


FIGURE 2: Chromatographic separation of the products of reduction of 4-pyridoxic acid lactone by lithium aluminum hydride (solid line, OD at 320 mμ). The dotted line indicates the radioactivity pattern of a similar reduction with tritiated lithium aluminum hydride. The separation was performed on a Dowex 50W-X8 (Na^+) column. Peak 1 represents 4-pyridoxic acid lactone, peak 2, pyridoxal, peak 3, pyridoxine, peak 4, 4-deoxypyridoxine; peak 5, 5-deoxypyridoxine, and peak 6, an unidentified compound.

from absolute ethanol–acetone mixture. The recrystallization was repeated three more times with no change in the specific activity of the compound. A sample from the last recrystallization was rechromatographed in solvent systems A, B, and C and on paper electrophoresis (Siliprandi *et al.*, 1954). In all cases the single radioactive spot which was obtained coincided with the ultraviolet fluorescence spot and the color spot given with 2,6-dichloroquinonechlorimide. 5-Deoxypyridoxine was identified following the same technique. It was not possible to separate by paper electrophoresis at pH 5.1 a mixture of 4- and 5-deoxypyridoxines. This fact could explain why only one radioactive impurity was reported (Booth and Brain, 1962) when commercial tritium-labeled pyridoxine was subjected to paper electrophoresis under the same conditions. The bioautographic pattern of a very small aliquot from the commercially available tritium-labeled pyridoxine revealed only one peak (solvent A) (Figure 1). It was not possible to demonstrate any antimetabolite activity in spite of the fact that each tube contained 2 mμg of pyridoxal. Apparently, this was due to an insufficient amount of the antimetabolites to exhibit antimetabolite activity even though the same sections of the chromatogram showed considerable radioactivity. It seems very probable that the specific activity of the impurities was much higher than that of pyridoxine. The percentage of the radioactive compounds was calculated from a radiochromatogram by the triangulation technique. Average values for 4- and 5-deoxypyridoxine were 6 and 8%, respectively; the remaining activity was due to pyridoxine.

Synthesis of Pyridoxine Labeled with Tritium at the 4-Hydroxymethyl Group. 4-Pyridoxic acid lactone was synthesized according to the procedure of Heyl (1948), and prior to its reduction was recrystallized three times from ethyl alcohol. The ampoule that contained the tritiated lithium aluminum hydride was opened in an atmosphere of dry nitrogen gas and immediately transferred into 16 ml of tetrahydrofuran (freshly distilled over lithium aluminum hydride) to which had been added 10 mg of unlabeled lithium aluminum hydride. One milliliter of this solution was removed in order to determine the specific activity of the tritiated lithium aluminum hydride by its ability to reduce a known amount of benzaldehyde.[1] The remaining 15 ml was added to 24 mg of 4-pyridoxic acid lactone in a 50-ml round-bottom flask equipped with a reflux condenser and a calcium chloride tube. The suspension was refluxed on a steam bath for 4 hr, and 10 ml of water and then glacial acetic acid were added until the solution became acidic. This solution was applied to a Dowex 50 (sodium form) column; the flask and the column were rinsed with 0.1 M acetic acid until 100 ml of effluent was collected. The column was eluted with 0.2 M potassium acetate buffer, pH 5.5, and 10-ml fractions were collected at a flow rate of about 50 ml/hr. The starting material was eluted with this treatment (Figure 2); total volume collected was 300 ml. The column was then eluted with 0.2 M potassium acetate, pH 7.0; 5-ml fractions were collected and the total volume of the effluent

[1] We wish to thank Dr. R. F. Nystrom for this suggestion.

was 600 ml. Finally, the column was eluted with 200 ml of 0.01 N sodium hydroxide. This effluent contained an unidentified compound and the aluminum ions. The fractions that contained the pyridoxine were combined, made alkaline by adding one pellet of sodium hydroxide, and applied to a Dowex 1 (chloride form) column. The column was drained to the glass wool and eluted with 300 ml of 0.01 N sodium hydroxide. The solvent was changed to 0.1 N hydrochloric acid and the column was eluted with 200 ml; 5-ml fractions were collected. The fractions that contained the pyridoxine (about 30 ml) were combined, a few drops of concentrated hydrochloric acid was added, and the solution was lyophilized. The residue was recrystallized from a water–acetone mixture; yield 21 mg (70% based on 4-pyridoxic acid lactone and 20% based on the radioactivity of the tritiated lithium aluminum hydride). The specific activity of the tritiated lithium aluminum hydride was found to be (calculated from the tritium-labeled benzyl alcohol) about twice as high as the specific activity of pyridoxine. Tritium-labeled 4- and 5-deoxypyridoxine were identified in the corresponding fractions (Figure 2) from column chromatography by a technique analogous to the one that was used for their identification in the commercial tritium-labeled pyridoxine. For the transformation of pyridoxine (I) (Scheme II) into 2-methyl-3-acetoxy-4-cyano-5-acetoxymethylpyridine (III) the procedure of Heyl (1948) was followed, using 5 g of labeled pyridoxine (specific activity 1.23 μcuries/mmole). The radioactivity of pyridoxal oxime (II) (specific activity 0.63 μcurie/mmole) was measured in an ethanol scintillation solution (2:8) while for compound III (specific activity 0.041 μcurie/mmole) plain scintillation solution was used. Internal standards were used to calculate the specific activities of the different compounds.

Synthesis of Pyridoxine Labeled with Tritium at the 5-Hydroxymethyl Group. 5-Pyridoxic acid lactone was prepared according to the procedure of Baddiley and Mathias (1952) and prior to its reduction was recrystallized three times from ethyl alcohol. The reduction of 5-pyridoxic acid lactone with tritiated lithium aluminum hydride as well as the separation of pyridoxine from the by-products through ion-exchange column chromatography were carried out exactly the same way as for the 4-pyridoxic acid lactone. The ultraviolet as well as the radioactivity chromatographic pattern of the effluent from the Dowex 50 column was similar to the one for 4-pyridoxic acid lactone. However, less starting material and isopyridoxal and more pyridoxine were eluted from the column. Starting from 24 mg of 5-pyridoxic acid lactone 22 mg of labeled pyridoxine was isolated (74% based on the 5-pyridoxic acid lactone and 21% based on the tritiated lithium aluminum hydride). In the reduction of 4- and 5-pyridoxic acid lactone, the first eluate (100 ml) from the Dowex 50 column contained a considerable amount of radioactivity. Part of this radioactivity was due to tritiated water and compounds that were removed during lyophilization; the rest was not identified. The identity of pyridoxine labeled with tritium at the 4- or 5-hydroxymethyl group was proven by ultraviolet absorption spectra, paper chromatography in solvents A, B, and C, and paper electrophoresis (Siliprandi *et al.*, 1954). In all cases the single radioactive spot which was obtained corresponded to authentic pyridoxine and coincided with the ultraviolet fluorescence spot and the color spot given with 2,6-dichloroquinonechlorimide; bioautography (solvent A) showed that the labeled pyridoxine was free from pyridoxal or isopyridoxal. Pyridoxal and isopyridoxal, which were formed during the reduction of 4- or 5-pyridoxic acid lactones, respectively, were identified by their characteristic ultraviolet spectra and paper chromatography in solvent A (Table I). In this work no attempt was made to study if there is any isotope effect during the ion-exchange column chromatography of the tritium-labeled compounds.

Acknowledgment

We wish to thank Miss Ruth Ann Noble and Mrs. Janet Mahannah for skillful technical assistance.

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Characteristics of *Clostridium pasteurianum* Ferredoxin in Oxidation–Reduction Reactions*

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ABSTRACT: Studies of some characteristics of pure *Clostridium pasteurianum* ferredoxin pertinent to its role in oxidation–reduction reactions were performed.

It was found that reduced ferredoxin when treated with mercurials liberates two more atoms of iron(II) per mole than does oxidized ferredoxin; that there are no detectable free sulfhydryl groups in either species; that two electrons are transferred per mole of ferredoxin undergoing oxidation or reduction in several different reactions; and that the oxidation–reduction potential of ferredoxin varies systematically with pH.

Early studies of pyruvate oxidation and of hydrogen evolution in anaerobic organisms suggested the existence of a low potential electron carrier (Wolfe and O'Kane, 1953; Peck and Gest, 1957). Recently, Mortenson and his associates (1962) successfully isolated such a carrier which they named ferredoxin. It was found to be a low molecular weight protein containing nonheme iron, and it was demonstrated to be a necessary component in the phosphoroclastic cleavage of pyruvate by *Clostridium pasteurianum* (Mortenson *et al.*, 1963). Valentine and Wolfe (1963) implicated ferredoxin as a necessary cofactor in a wide variety of reactions occurring in many different anaerobes.

In 1962 Tagawa and Arnon noted the similarity between bacterial ferredoxin and the soluble nonheme iron protein cofactor involved in the photosynthetic reduction of pyridine nucleotides by choroplasts which had been described by San Pietro and Lang (1958). Although the chemical composition of clostridial ferredoxin has been studied (Lovenberg *et al.*, 1963), quantitative aspects of electron transport mediated by the protein have not been resolved. The data to be presented in this communication suggest that: iron in *C. pasteurianum* ferredoxin undergoes reversible oxidation and reduction concomitantly with that of the protein itself; that reduction of the protein is not associated with the appearance of free sulfhydryl groups; that the oxidation–reduction potential of ferredoxin is a function of pH; and that ferredoxin functions as a two-electron carrier.

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Experimental Section

Materials

Clostridium pasteurianum was grown in a medium containing ammonium sulfate as described previously (Lovenberg *et al.*, 1963).

Hydrogenase. A DEAE-cellulose treated extract obtained from *C. pasteurianum* after the cells had been sonically disrupted for 5 min in a Biosonik 20 kc sonic oscillator at 0° (Buchanan *et al.*, 1963) was used as a source of hydrogenase. The extract contained approximately 5 mg of protein/ml. Its activity was demonstrated by the reduction of methyl viologen by hydrogen (Peck and Gest, 1956).

Ferredoxin. Pure ferredoxin was prepared as described (Mortenson, 1964). Purity was ascertained by measurement of the A_{390}/A_{280} ratio on a Beckman DU spectrophotometer. All ferredoxin used in these studies had a ratio greater than 0.79. Even minimal deterioration of the protein was found to markedly alter experimental results. A molar extinction coefficient (390 mμ) of 3.0×10^4 for pure ferredoxin was used. This value was based on the molecular weight determined by sedimentation equilibrium studies, the extinction coefficient per milligram of protein as determined by the phenol protein assay (Lowry *et al.*, 1951), and the observation that the value for dry weight of pure ferredoxin was only 70% of that measured in the colorimetric reaction (Lovenberg *et al.*, 1963).

Ferredoxin that has been maximally reduced by dithionite has an absorbancy at 415 mμ equal to 46% that of the oxidized protein. This value was used in all calculations concerning per cent reduction in the experiments to be described. Reduction by dithionite was completely reversible.