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Cell-Free Synthesis of Ferredoxin in Clostridial Extracts*

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ABSTRACT: Amino acids are incorporated into a specific protein, ferredoxin, in a cell-free system from *Clostridium pasteurianum*. Preparations most active in cell-free synthesis were prepared from osmotically shocked protoplasts. Evidence that ferredoxin was synthesized *in vitro* was provided by cochromatography of radioactive tryptic peptides with carrier peptides of ferredoxin. Comigration of radioactivity with carrier peptide was also demonstrated by paper electrophoresis. Only low levels of radioactivity were associated with the N-terminal tripeptide. Incorporation of amino acids into ferredoxin appears therefore, to be due largely to completion of preexisting peptide chains. The effects of variation in the growth condition on *in vitro* ferredoxin synthesis were also examined.

Studies, *in vitro*, of peptide synthesis have been most important for defining the biochemistry of protein synthesis. In order to relate the *in vitro* events to those occurring in the cell, however, it is necessary to study the synthesis of well-defined proteins. This is especially important for an understanding of possible controls at the level of translation or for elucidating the function of other cellular components such as membranes in protein synthesis.

There are now a number of reports of the *in vitro* synthesis of proteins. These studies include a variety of cellular proteins (Bishop *et al.*, 1960; von Ehrenstein and Lipmann, 1961; Bonner, 1965; Ganoza *et al.*, 1965; Nisman *et al.*, 1961; Lederman and Zubay, 1968) as well as those coded by viral RNAs (Nathans *et al.*, 1962; Nathans, 1965; Clark *et al.*, 1965; von Ravenswaay Classen *et al.*, 1967) or the T-4 phage genome (Salser *et al.*, 1967). The evidence provided has ranged from cochromatography of tryptic digests of the *in vitro* product with carrier peptides to increases of enzyme activity or of precipitate formed upon addition of a specific antibody. Aside from the generally recognized problem of intact cell (or protoplast) contamination (Tonomura and Rabinowitz, 1967), there are dangers of increased enzyme activity being due to activation (Rogers, 1965) or of fortuitous binding in immunological studies.

While these pitfalls have been more or less controlled, we felt they could be bypassed by studying the synthesis of a protein which can be readily isolated and characterized. For this reason, we have undertaken a study of the *in vitro* synthesis of a native bacterial protein, ferredoxin.[1] Many of the properties of Fd appeared ideal for the study of its *in vitro* synthesis. Since the initial characterization of Fd from *Clostridium pasteurianum* by Mortenson *et al.* (1962), the chemistry of this protein has been extensively documented. Fd is a low molecular weight protein which constitutes about 1% of the clostridial protein. Its amino acid composition is unusual in that leucine, arginine, methionine, histidine, and tryptophan are absent. The amino acid sequence of clostridial Fd has been determined (Tanaka *et al.*, 1964a). Because of its unique chemical properties, Fd can be readily isolated and purified by solvent fractionation and anion-exchange chromatography (Mortenson, 1964a). It seemed reasonable therefore, to expect that sufficient radioactive material could be obtained from an *in vitro* amino acid incorporation system to permit a definitive characterization of the products. In this communication, we report the development of a bacterial cell-free system from *Clostridium pasteurianum*, which can synthesize Fd.

Materials and Methods

Cultivation and Harvest. C. pasteurianum was grown at 30° according to the method of Carnahan *et al.* (1960) with sucrose and N_2 as the carbon and nitrogen sources, respectively (generation time 2–3 hr), unless stated otherwise. To prepare cell-free extracts for amino acid incorporation, cultures were harvested during exponential growth (3–5 × 10^8 cells/ml).

Escherichia coli (strain KB) was grown in C medium (Roberts *et al.*, 1957) with 0.4% glucose and harvested during exponential growth. To prepare cell-free extracts cells were harvested by centrifugation, washed three times with TKMM buffer, and finally resuspended in TKMM and passed through a French pressure cell at 8000 psi.

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[1] Abbreviations used are: Fd, ferredoxin; TKMM buffer, 0.01 M Tris–0.01 M KCl–0.01 M MgOAc–0.006 M 2-mercaptoethanol (pH 7.8).

Preparation of Extracts. Three methods for disrupting clostridial cells were employed. Dried cells (Carnahan *et al.*, 1960) were autolyzed by suspending cells in TKMM (ratio of 1 g of cells/10 ml of buffer). After stirring for 1 hr at 4°, DNase was added (2 μg/ml) and the lysate was centrifuged at 30,000*g* to obtain an S-30 extract (Matthaei and Nirenberg, 1961). Cells suspended in TKMM were also broken by passage through a French press at 8000–9000 psi, DNase added to the exudate, and an S-30 extract prepared. The supernatant from a 105,000*g* centrifugation of this S-30 extract then served as a source of activating enzyme.

The third method of extract preparation involved lysis of clostridial protoplasts. Exponentially, growing cells were suspended in 10% sucrose containing 5 μmoles/ml of NaCl, 5 μmoles/ml of potassium phosphate, pH 7.8, and 500 μg/ml of lysozyme and were incubated at 37°. Conversion into protoplasts (75–85% as determined microscopically) took 10–15 min. The reaction was terminated by the addition of cold 10% sucrose containing 40 μmoles/ml of magnesium acetate and the suspension was centrifuged at 15,000*g* for 5 min. The protoplast–cell pellet was washed twice in cold 10% sucrose containing TKMM buffer and finally resuspended in 1–2 times its volume of cold TKMM to lyse the protoplasts. DNase (5 μg/ml) was added in the cold and after 30 min, the extract was centrifuged at 8700*g* for 10 min and the pellet was discarded. This cycle was repeated twice and the final supernatant is designated S-8.7. An S-30 preparation was also prepared from this S-8.7 extract. Extracts were not dialyzed unless stated otherwise. Protein was determined by the method of Lowry *et al.* (1951).

Gradient Analysis. An S-8.7 extract (0.2 ml) was layered on 4.4 ml of a 10–30% sucrose gradient (in TKMM buffer) and centrifuged at 35,000 rpm for 45 min in an SW39 rotor. Fractions were collected by needle puncture, and diluted to 2 ml, and the $A_{260\,m\mu}$ was determined.

Activating Enzyme Fraction, tRNA, Labeled AA-tRNA. Cell-free extracts of *Clostridium* or of *E. coli* were centrifuged at 105,000*g* for 90 min, the upper two-thirds of the supernatant decanted and processed by the method of Nathans and Lipmann (1961) to obtain the "amino acid activating fraction." *E. coli* B or clostridial tRNA was prepared from the 105,000*g* supernatant and acylated or deacylated by the method of von Ehrenstein and Lipmann (1961). The tRNA was charged with either [^{3}H]- or [^{14}C]aspartic, alanine, isoleucine, and valine, as well as with the other amino acids (nonradioactive) present in Fd.

Incubation Conditions. The complete incorporation system was modeled after Matthaei and Nirenberg (1961) and contained the following per milliliter: 100 μmoles of Tris, pH 7.8; 50 μmoles of KCl; 10 μmoles of MgOAc; 5 μmoles of 2-mercaptoethanol; 2 μmoles of ATP; 1 μmole of GTP; 10 μmoles of phosphoenolpyruvate; 40 μg of pyruvate kinase; 0.05 μmole of each of the unlabeled amino acids in Fd; labeled amino acids ^{3}H or ^{14}C or labeled AA-tRNA (with the ^{3}H- or ^{14}C-labeled amino acids), specific activities as indicated; carrier amino acids (0.05 μmole of each) for incorporation with labeled AA-tRNA; NH_4Cl as noted and 5–29 mg of protein as S-8.7 or S-30 extracts. Reaction mixtures were incubated at 30° for 15–60 min as indicated, samples removed for kinetic studies, and the reaction terminated by addition of NaOH to 0.1 N containing carrier amino acids. The samples were then precipitated with trichloroacetic acid to 5%, heated to boiling for 15 min, and redissolved in alkali. This cycle was repeated twice and the samples counted in a Nuclear-Chicago low-background gas-flow counter (20% efficiency for ^{14}C). Samples containing ^{14}C and ^{3}H were precipitated and collected on B-6 Millipore filters after heating and washing as described above. The filters were monitored in a Packard Tri-Carb scintillation counter.

Extraction and Preparation of in vitro Synthesized Product. The large-scale incorporation mixtures contained 20–34 mg of protein/ml in a volume of 10 ml and were incubated at 30° for 10–15 min. An equal volume of acetone (−15°) was added and the mixture shaken (Mortenson, 1964a). The acetone-soluble fraction, which contains Fd, was obtained by centrifugation at 12,000*g* for 10 min. The acetone-precipitable fraction was reextracted with cold 50% acetone and the acetone-soluble fractions were pooled and concentrated by evaporation at room temperature. The residue was dissolved in 10^{-3} M NaOH, carrier Fd (6–25 mg) was added and then trichloroacetic acid to give a final concentration at 10%. The precipitate was incubated in trichloroacetic acid for 12–16 hr at room temperature and then heated to boiling for 15 min. The precipitate was collected, washed, oxidized with performic acid (Tanaka *et al.*, 1964b), and finally resuspended in distilled water.

The initial acetone extraction provides about 100-fold purification of Fd (Mortenson, 1964a). Only about 25% of the acetone-soluble fraction is Fd, however (see Figures 3 and 4) since there are peptides and other proteins soluble in acetone. Much of this contaminating material is soluble in trichloroacetic acid (L. Mortenson, personal communication) so that the final material is between 250- and 300-fold purified. The level of contamination of the Fd is thus small and somewhat variable.

For trypsin digestion, the pH was adjusted to 8.3–8.6 with dilute NaOH and the suspension incubated at 30° for 18–24 hr with 1 mg of trypsin/50 mg of substrate. The reaction was terminated by the addition of acetic acid (to 2 N) and the suspension concentrated by evaporation at room temperature. The dried tryptic digest was dissolved in either the electrophoresis buffer or the peptide column buffer.

Preparation of Aminoethylated Fd. The procedure used was provided by Dr. W. Lovenberg (NIH; modified from Raftery and Cole, 1963). Acetone-soluble fraction plus carrier Fd (15 mg) was dissolved in 1 ml of 2 M Tris-HCl, pH 8.5. The following were then added: 20 mg of *O*-phenanthroline, 0.07 ml of 2-mercaptoethanol, urea to 8 M, and water to bring the volume to 2 ml. Three, 0.2-ml portions of ethylenimine (Eastman-Kodak) were added at 10-min intervals. The mixture was dialyzed against two changes of 2 l. each of distilled water (1 hr) and then overnight against 4 l. of distilled water. The protein was concentrated to 2–3 ml by evaporation in a desiccator. Glacial acetic acid was added to 1 N and the solution passed over a G-25 Sephadex column (1 × 20 cm) equilibrated with 1 N acetic acid. The protein was lyophilized and dissolved in water for tryptic digestion.

In experiments where extracts had been incubated with [^{3}H]leucine to label non-Fd proteins and [^{14}C]amino acids to label all proteins, the purification of the Fd during aminoethylation could be monitored. In such cases, the Fd was purified 300–350-fold overall (3–3.5 times over the initial acetone extraction).

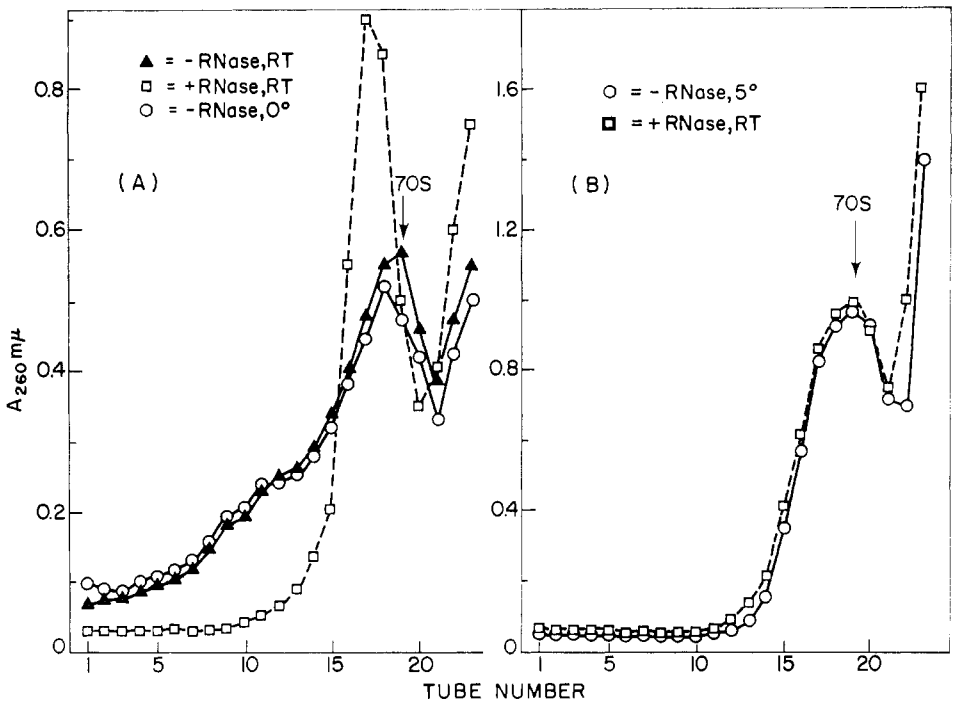

FIGURE 1: Sucrose gradient profiles of S-8.7 extracts: (A) prepared from osmotically lysed protoplasts; (B) prepared from frozen dried cells; centrifugation through 10–30% sucrose gradients in TKMM buffer in an SW39 rotor, 35,000 rpm for 45 min. All incubations were with RNase, 5 μg/ml, for 10 min at room temperature (RT).

Column Chromatography of Radioactive Product. The tryptic digest was analyzed by Dowex 50W-X2 column chromatography (Bio-Rad Lab., Calif.) according to the procedure of Tanaka *et al.* (1964b, 1966) with pyridine–acetic acid buffers (Margoliash and Smith, 1962). Fractions (3 ml) were collected and the peptides were detected by ninhydrin reaction (Moore and Stein, 1954). Samples from the fractions were also evaporated in scintillation vials, the residue dissolved in 10^{-3} M NH_4OH, and radioactivity monitored in Bray's solution (Bray, 1960).

Analysis of Paper Electrophoresis. Tryptic digests were also analyzed by paper electrophoresis using a pressure plate electrophoresis apparatus (E-C Company) with the pyridine–acetic acid buffer system of Ingram (1963). The sample (6–8 mg of digest) was applied to dry Whatman No. 3MM paper (15 × 45 cm). The paper was wetted with buffer and electrophoresis was carried out at 1000 V (current 12–18 mA) for 2 hr. Location of carrier peptides was determined by reaction with 0.2% ninhydrin in acetone (90°/15 min or overnight at room temperature). The distribution of radioactivity on paper was analyzed by counting sections of paper in a toluene base scintillation fluid. Recovery of radioactivity from paper was between 80 and 90% for both ^3H and ^{14}C.

Preparation of Carrier Fd. Fd was extracted from suspensions of autolyzed cells using the acetone method and was purified by DEAE-cellulose chromatography and ammonium sulfate precipitation (Mortenson, 1964a). Preparations of Fd had an $A_{390}:A_{285}$ ratio of 0.78 or greater indicating a purity of greater than 90% (Tanaka *et al.*, 1964b). Purity of carrier Fd was also established by amino acid analysis and by peptide analysis (two characteristic tryptic peptides, see Figure 3).

Reagents and Radiochemicals. Chemicals used were commercial products of the purest grade available. The sodium salts of ATP, CTP, GTP, UTP, and phosphoenolpyruvate, as well as pyruvic kinase were purchased from California Corp. for Biochemical Research. DNase, lysozyme, RNase, and trypsin were obtained from Worthington Biochemical Corp. Uniformly labeled [^{14}C]- and [^3H]amino acids of high specific activity (200–250 μCi/μmole) were obtained from New England Nuclear Corp. and Schwarz Bioresearch, Inc. *E. coli* B tRNA was purchased from General Biochemical Corp.

TABLE I: Incorporation of Amino Acids by Various Cell-Free Extracts of *C. pasteurianum.*[a]

Method of Preparation	cpm/mg of Protein
Lysis of protoplasts	450–575
French press	41–220
Autolysis of dried cells	30–88

[a] All extracts were S-30 preparations and were dialyzed for 3 to 4 hr at 4° against two changes of 200 volumes of TKMM buffer. Reaction mixtures were incubated at 30° for 30 to 40 min, with one of five uniformly labeled L-[^{14}C]amino acids; alanine, aspartic, isoleucine, leucine, or valine (specific activity 160–200 mCi/mmole).

Results

Characteristics of the Cell-Free System. In the initial studies, cell-free extracts were prepared from dried cells but these preparations exhibited low activity. To obtain

TABLE II: Effect of Chloramphenicol and RNase on Incorporation in a Clostridial S-30 Extract.[a]

Addition	Total Radioactivity,[b] cpm/mg of Protein	Per Cent Inhibition	Acetone-Soluble Radioactivity,[b] cpm/mg of Protein	Per Cent Inhibition
None	225		290	
CAL (133 μg/ml)	63	72	58	80
RNase (6.6 μg/ml)	23	90	41	86

[a] Conditions of incorporation: S-30 extracts (8.7 mg of protein/ml) were prepared from cells disrupted in the French press and dialyzed as in Table I. Incorporation mixtures were incubated at 37° for 60 min with 1 μCi of uniformly labeled L-[^{14}C]isoleucine (200 mCi/mmole). [b] Total radioactivity is the total hot trichloroacetic acid precipitable radioactivity; acetone-soluble radioactivity is the radioactivity which is soluble in 50% acetone and is also hot trichloroacetic acid precipitable.

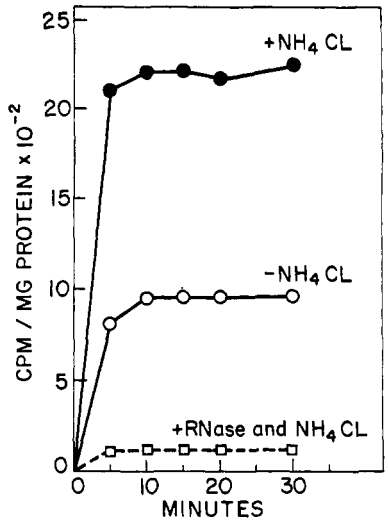

FIGURE 2: Relative levels of incorporation of [^{14}C]amino acids into an S-8.7 extract (2 mg of protein/ml) prepared from a protoplast lysate: 1.3 × 10^5 cpm was added as mixture of [^{14}C]AA-tRNAs (see Material and Methods); NH_4Cl, 160 μmoles/ml; RNase, 5 μg/ml. Incubation at 30°.

more active preparations, cell-free extracts were prepared from bacteria disrupted in the French press and by osmotic shock of protoplasts. In Table I, the incorporating activity of the three different cell-free preparations is compared. Extracts prepared from lysed protoplasts were more active than those prepared by the French press or from autolyzed dried cells.

This difference in incorporating efficiency could be due to variations in the amount of polyribosomes. Extracts from lysed protoplasts and from dried cells were centrifuged through sucrose gradients (Figure 1A,B). The protoplast lysate (Figure 1A) exhibits a ribonuclease sensitive polyribosomal region. The polyribosomal region in the extract from dried cells is negligible, however, and RNase had no observable effect on the sedimentation profile (Figure 1B).

The stimulatory effect of NH_4Cl as well as the inhibitory effect of RNase on amino acid incorporating activity is shown in Figure 2. At a concentration of 160 μmoles/ml of NH_4Cl, a twofold enhancement of incorporation is found.

The inhibitory effects of chloramphenicol and RNase on incorporation are summarized in Table II. Since Fd is soluble in acetone while most clostridial proteins are precipitated (Mortenson, 1964a), the acetone-soluble fraction serves as a measure of Fd biosynthesis. The fact that both RNase and chloramphenicol inhibit total protein synthesis and incorporation into the acetone-soluble fraction to approximately the same extent, suggest that Fd biosynthesis involves a mechanism similar to that of other proteins.

To further eliminate the possibility that intact protoplast contamination was responsible for the observed *in vitro* amino acid incorporation, the S-8.7 extracts were treated with sodium deoxycholate (0.3%) to lyse any residual protoplasts. The incorporating activity of extracts after treatment was essentially the same as before deoxycholate treatment (*i.e.*, 7.4 and 8.2 μμmoles of amino acid incorporated per mg of protein for treated and untreated extracts, respectively). In addition, preparation of an S-30 extract from the S-8.7 extract resulted in a more efficient amino acid incorporating system (Nepokroeff, 1967).

In order to obtain radioactive product for identification large quantities of active tRNA were needed. Commercial sources of *E. coli* tRNA were available and therefore tested in the clostridial system. Table III shows that AA-tRNA from *E. coli* B was as active as the clostridial tRNA species in the clostridial cell-free system. Other successful AA-tRNA heterologous systems have been reported (von Ehrenstein and Lipmann, 1961; Marshall *et al.*, 1967). On the basis of a universal genetic code, it is probably valid to assume that the amino acid incorporation which occurs in the clostridial system, employing *E. coli* AA-tRNA, results in the same polypeptide products as when tRNA from clostridium is used.

Peptide Analysis of Radioactive Product. In order to obtain sufficient radioactive product from a cell-free incorporating system, large-scale reaction mixtures (10 ml) of S-8.7 extract

TABLE III: Transfer Activity of Clostridial and *E. coli* B AA-tRNA in a Clostridial Cell-Free Incorporating System.[a]

Source of AA-tRNA	cpm/mg of Protein
Clostridial AA-tRNA	200
E. coli B AA-tRNA	211

[a] Conditions of incorporation: undialyzed clostridial cell-free S-30 extracts (3.2 mg of protein/ml), prepared from lysed protoplasts, were incubated with approximately 33,000 cpm of either the *E. coli* B or the clostridial [^{14}C]AA-tRNA species (0.5 mg and 0.6 mg of RNA, respectively) at 30° for 20 min. See Methods for [^{14}C]amino acids used.

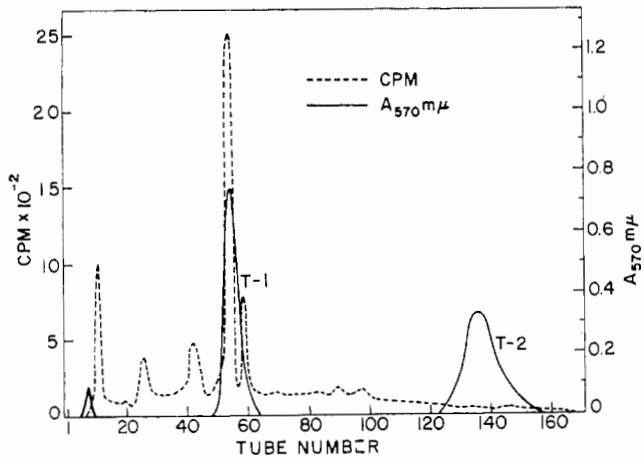

FIGURE 3: Peptide elution profile of radioactive and carrier peptides. An S-8.7 extract (25 mg of protein/ml) was incubated with 1.5×10^6 counts as [^{14}C]AA-tRNA (charged with [^{14}C]alanine, aspartic, isoleucine, and valine) plus 0.5 μmole of each of these four amino acids as [^{12}C]carrier, at 30° for 15 min. Incorporation into hot trichloroacetic acid precipitable protein was 13.4 $\mu\mu$M amino acid/mg of protein (2.3×10^3 cpm/mg of protein). The total acetone-soluble cpm was 1.6×10^4 while 4100 cpm were T-1 peptide. Recovery of total radioactivity was greater than 95%. Samples for radioactivity and colorimetric analysis were taken from each fraction except fractions 72 to 118 where every other fraction was analyzed.

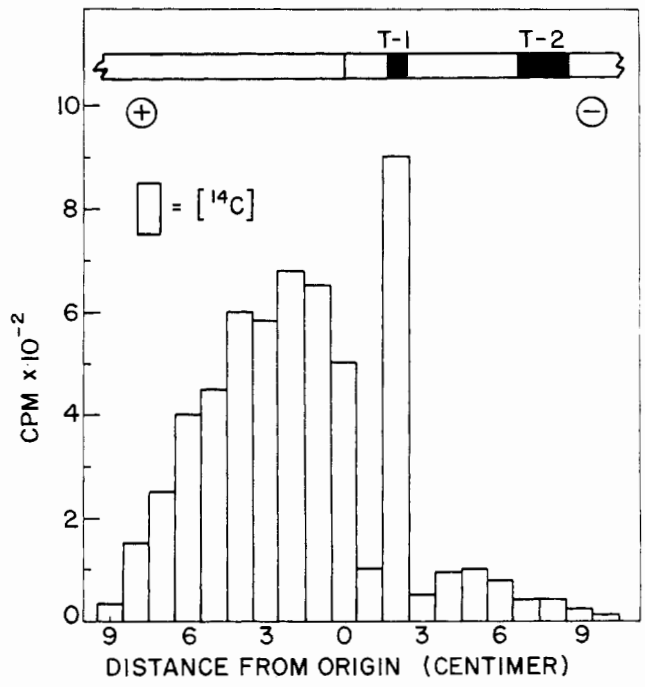

FIGURE 4: Electrophoresis of a tryptic digest of acetone-soluble fraction plus carrier Fd. An S-8.7 extract (19.8 mg of protein/ml) prepared from lysed protoplasts was incubated for 30 min at 30° with 5 μCi of each of the following [U-^{14}C]amino acids: alanine, aspartic acid, isoleucine, and valine. Total cpm incorporated was 4.9×10^5 and greater than 95% of this was recovered after electrophoresis. Electrophoresis conditions are described in Materials and Methods. Darkened areas on the band above the graph represent results of staining with ninhydrin and locate the T-1 and T-2 peptides.

prepared from protoplasts were used. tRNAs charged with labeled amino acids (only some of those present in Fd) were added to the reaction along with carrier amino acids. After incubation, the acetone-soluble fraction was prepared. This acetone-soluble fraction plus added carrier Fd was digested with trypsin and the total digest was chromatographed on a column of Dowex 50W-X2 resin (Figure 3). Trypsin hydrolyses Fd to form two peptides: the N-terminal tripeptide, T-2, and the large C-terminal polypeptide, T-1 (Tanaka *et al.*, 1964b). As seen in Figure 3, there is a radioactive peak coincident with the T-1 peptide. There is no radioactive peak associated with the N-terminal, T-2, peptide. A small amount of undigested Fd elutes early. This is a minor component and it has been estimated that greater than 92% of the Fd was hydrolyzed.

Besides the one radioactive Fd-peptide, there are at least four other radioactive peptides of non-Fd origin. The radioactive T-1 peptide contained 4100 cpm which corresponds to 25% of the acetone-soluble fraction radioactivity, and to 0.72% of the total radioactive product formed by the cell-free system.

Paper Electrophoresis. Peptide analysis of the radioactive products formed by the clostridial cell-free system was also accomplished by paper electrophoresis. Carrier Fd peptides were identified by ninhydrin treatment of a narrow guide strip of paper. It was possible to separate the T-1 and T-2 peptides in a relatively short time (2 hr) and the radioactivity was monitored by counting the paper directly in scintillation fluid (Figure 4). The N-terminal, tripeptide, T-2, has a positive charge and migrates approximately 8–9 cm. The large C-terminal peptide (T-1) is less positively charged and remains closer to the origin. Approximately 70% of the radioactive material from the acetone-soluble fraction migrated opposite that of the Fd peptides and was not further characterized. A peak of radioactivity was found to comigrate with carrier T-1 peptide, however, and confirms the synthesis of this peptide as determined by column chromatography (Figures 3 and 5). Examination of the T-2 peptide region revealed no peak of radioactivity and agrees with the low values found by column analysis.

Double-Labeling Experiment. The results of these experiments demonstrate that clostridial cell-free extracts are capable of incorporating amino acids into an Fd peptide, (T-1). Since the incorporation proceeded from AA-tRNA, further evidence is provided for true *in vitro* synthesis. Additional evidence to support the Fd nature of the radioactive peptide which cochromatographs with carrier T-1 peptide was obtained by a double-labeling technique. In this experiment, four ^{14}C-labeled amino acids that are present in Fd, plus [^{3}H]leucine, an amino acid not present in Fd, were added to a clostridial cell-free system. The acetone-soluble fraction was prepared, digested with trypsin, and fractionated on a column (Figure 5). The cell-free system incorporated the ^{14}C-labeled amino acids into the T-1 peptide as determined by cochromatography of carrier peptide with radioactive peptide (designated F in Figure 5). Little, if any, of the non-Fd amino acid leucine was incorporated into this peptide. Adjacent to and eluting later than the T-1 peptide is a non-Fd peptide region (designated NF), identified because of the cochromatography of both isotopic species.

It should also be noted that associated with the T-2, N-

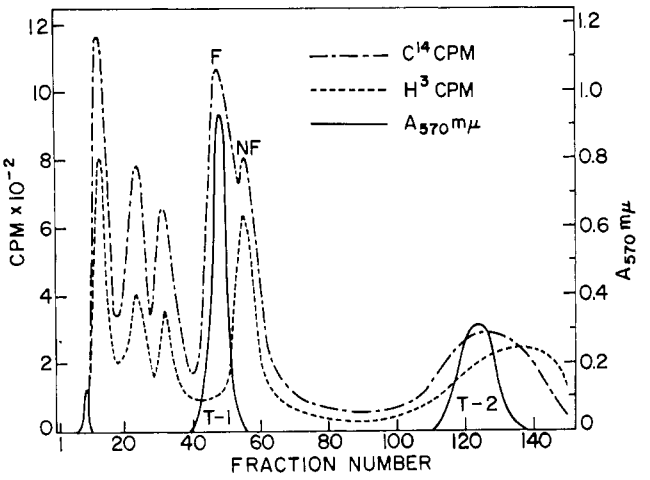

FIGURE 5: Peptide elution profile of a tryptic digest of double-labeled acetone-soluble fraction. An S-8.7 extract (22.5 mg of protein/ml) was incubated at 30° for 30 min with 10 μCi of each of the following L-[^{14}C]amino acids: alanine, aspartic, isoleucine, and valine, plus the other 11 unlabeled amino acids of Fd, and 100 μCi of DL-[^{3}H]leucine. The total cpm incorporated was 3.5×10^5 for ^{14}C and 0.5×10^5 for ^{3}H. Recovery of radioactivity was greater than 95%. Samples were taken for radioactivity and colorimetric analysis from each tube except tubes 62 to 100 where every other tube was sampled. F indicates the T-1 Fd peptide and, NF indicates a peptide of non-Fd origin.

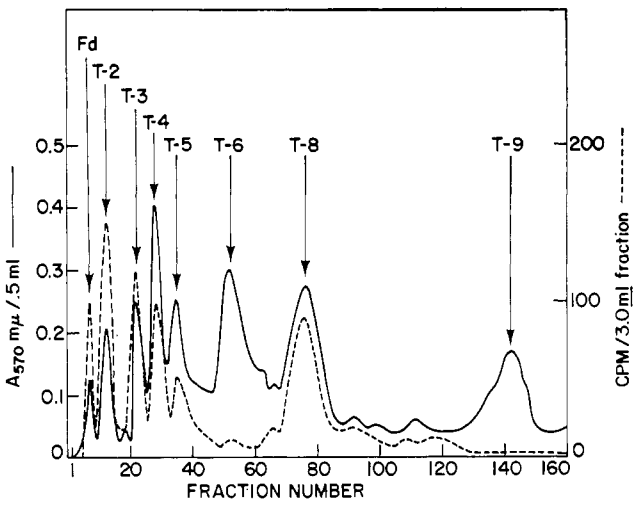

FIGURE 6: Dowex 50 elution profile of a tryptic digest of aminoethylated Fd. Incorporation conditions as in Figure 3. 10^5 cpm incorporated into trichloroacetic acid insoluble material (21.5% transfer from tRNA); acetone-soluble fraction: 2500 cpm. Aminoethylation and chromatographic procedures are described in Materials and Methods; recovery 2700 cpm. Peptides labeled as per Tanaka *et al.* (1964a, 1966) with the following order from the amino to carboxyl end: T-9, T-5, T-6, T-4, T-3, T-8, T-2.

terminal peptide, is a broad peak of ^{14}C radioactivity as well as ^{3}H activity that elutes after the T-2 carrier peptide. Although the ^{14}C-labeling pattern is consistent with that expected if *de novo* Fd synthesis had occurred, these non-Fd peptides within the T-2 region may comprise the major, if not the entire source of radioactive material.

Tryptic Digestion of Aminoethylated Fd. Further proof for incorporation of amino acids into Fd was provided by tryptic digestion of aminoethylated Fd (Figure 6). Aminoethylation of the cysteine residues prior to digestion with trypsin results in 7–9 peptides separable on Dowex 50 (Tanaka *et al.*, 1966). Radioactivity incorporated from [^{14}C]AA-tRNAs can be seen with four of the peptides and probably one more (T-5). Neither T-9 (N terminal) nor T-6 (close to N-terminal end) were labeled. All of the peptides were subjected to amino acid analysis (two-dimensional chromatography, Roberts *et al.*, 1957) to confirm their identification. T-3, T-4, T-5, T-8, and T-9 had the expected composition. The results for T-2 and T-6 were not conclusive.

The data are again generally consistent with chain completion *in vitro*, *i.e.*, very high activity in the C-terminal peptide (T-2) and those close to the carboxyl end (T-3, T-8). T-4 from the middle of the chain is relatively highly radioactive because it contains a high proportion of residues added as [^{14}C]AA-tRNA. About 80–90% of the non-Fd-labeled peptides normally present in the acetone-soluble fraction (Figures 3 and 5) were removed during the aminoethylation procedure (during the extensive dialysis and purification on Sephadex) as judged by the change in ratio of [^{3}H]non-Fd proteins to [^{14}C]Fd.

Variation of Growth Conditions. Since Fd participates in nitrogen fixation (Mortenson, 1964b), it seemed reasonable to expect that non-N_2 fixing clostridial cells would have less Fd than cells fixing nitrogen. It was assumed that by growing cells under non-N_2 fixing conditions the level of Fd mRNA *in vivo* might be reduced. As a result, there should be a reduction in the incorporation of amino acids into Fd (specifically the T-1 peptide). Cell-free extracts were prepared from lysed protoplasts using non-N_2 fixing cells (grown with $(NH_4)_2SO_4$) as well as from cells grown under N_2 fixing conditions. After incubation of each extract, the acetone-soluble fractions were analyzed separately for Fd peptides. The radioactivity data are summarized in Table IV. In a cell-free system from non-N_2 fixing cells, only 10% of the acetone-soluble fraction radioactivity chromatographed with T-1 peptide as compared

TABLE IV: Effect of Growth Conditions on Synthesis of T-1 Peptide of Ferredoxin.[a]

Source of Cells for S-30 Extract	Radioactivity Incorporated: Acetone-Soluble Fraction (% of total radioactivity)	Radioactivity Incorporated: T-1 Peptide (% of acetone-soluble fraction)
N_2 fixing	2.8	25
Non-N_2 fixing	3.5	10

[a] Conditions of incorporation: S-8.7 extracts from either non-N_2 fixing cells or from N_2 fixing cells (20–25 mg/ml, respectively) were incubated at 30° for 30 min with 10 μCi of each of the following uniformly labeled L-[^{14}C]amino acids: alanine, aspartic, isoleucine, and valine, plus the other [^{12}C]amino acids of Fd. Acetone-soluble fractions were prepared from each and separate peptide analyses were performed as in Figure 3.

with 25% for extracts prepared from N_2 fixing cells. This difference in the *in vitro* labeling of the T-1 peptide may reflect a higher level of Fd-mRNA in the N_2 fixing cells.

Discussion

The elution from columns of *in vitro* labeled peptides with carrier peptides and the comigration of the peptides in an electrophoretic separation provide evidence for the *in vitro* synthesis of Fd. The mechanism of synthesis of Fd is probably analogous to that of other proteins since incorporation of amino acids into T-1 peptide occurred when either free amino acids or AA-tRNA were used (Figures 3 and 5). In addition, both chloramphenicol and RNase inhibit the labeling of the acetone-soluble fraction to the same extent as total protein. Although only a fourth of the acetone-soluble fraction may be Fd (Table IV, Figure 3), the results of these inhibitor studies are at least consistent with a mechanism of Fd biosynthesis similar to that of other proteins.

Critical to all these incorporation studies is the authenticity of the cell-free protein synthesis. The conclusion that this system is free of cell or protoplast contamination is based on the following: (1) the clostridial system is sensitive to RNase, a potent inhibitor of cell-free protein synthesis (Figure 2), (2) the same or greater efficiency of total incorporation was obtained with extracts prepared by high-speed centrifugation as with those prepared by low speed (S-8.7) (Nepokroeff, 1967), (3) incorporation could be demonstrated in extracts when AA-tRNA served as the donor of amino acids for polypeptide synthesis (Table III and Figures 2 and 3), and (4) extracts which were prepared from protoplasts and treated with sodium deoxycholate, to disrupt any unlysed protoplasts, were as active as untreated extracts. On the basis of this evidence, the possibility of whole cell or intact protoplast contamination has been considered negligible.

No direct evidence was obtained for the labeling of the N-terminal peptide (T-2) of Fd. Conditions of incorporation would permit only the labeling of the terminal alanine residue in this tripeptide. The amount of radioactivity in this peptide would therefore be low even though appreciable *de novo* synthesis had occurred. The limited amount of radioactivity which was always associated with the T-2 peptide discouraged further investigation of the synthesis of the N-terminal tripeptide. In addition analysis of a tryptic digest of aminoethylated Fd showed no labeling of the N-terminal peptide and little radioactivity associated with penultimate peptide (Figure 6). On the basis of this labeling data, incorporation of amino acids into Fd by the clostridial cell-free system appears to be primarily the result of completion of preexisting peptide chains. Perhaps there was a limitation in these extracts of *N*-formylmethionyl-tRNA or other initiation factors.

The *in vitro* studies comparing extracts from nitrogen-grown and ammonia-grown cells indicate a difference in the biosynthesis of Fd based on the per cent of acetone-soluble fraction radioactivity associated with the T-1 peptide. Since Fd participates in nitrogen fixation (Mortenson, 1964b), it is reasonable to expect that nitrogen-fixing cells would have a greater concentration of Fd as a result of a relatively higher content of Fd mRNA. The observed increase in the labeling of T-1 peptide *in vitro* may reflect the increase *in vivo* of Fd mRNA. It is of interest that in addition to Fd, two other proteins which are involved in nitrogen fixation have also been shown to be present only in cells grown under N_2 fixing conditions (Mortenson *et al.*, 1967; Taylor, 1969).

Since very active extracts can be readily prepared from *C. pasteurianum*, this system affords the opportunity to study the biochemical events required for the synthesis of a well-characterized protein. Because of the unique properties of Fd, it is possible that the relevant mRNA has unusual physical and chemical characteristics (assuming it is monocistronic). It may be feasible, therefore, to isolate and characterize a specific, "native," mRNA.

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Studies on the Nature of the Type I and Type II Spectral Changes in Liver Microsomes*

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ABSTRACT: The spectral changes which occur when substrates (type I) and basic amines (type II) interact with the terminal oxidase (cytochrome P-450) of the hepatic microsomal mixed-function oxidase have been reexamined. The type I spectral change which has been called the spectral manifestation of the enzyme–substrate complex, was also found to be present in the type II spectral change, and to be the cause of the asymmetrical shape of the trough in difference spectrum. When correction is made for the type I component in the type II spectral change, the resultant spectral change becomes both symmetrical and greatly enlarged, as well as shifted slightly toward the blue end of the spectrum. In 3,4-benzpyrene-treated rats, the lack of type I spectral change when type I substrates are added is not due to absence of the type I binding site; rather, it is caused by an inability of type I substrate to interact. That the type I binding site is present in liver microsomes of 3,4-benzpyrene-treated rats was shown by the presence of types I and II composite spectral change.

Hepatic microsomal cytochrome P-450 has been implicated as the oxygen-activating terminal oxidase of a number of drug oxidase (Cooper *et al.*, 1965), steroid oxidase (Conney *et al.*, 1968), and polycyclic hydrocarbon oxidase (Silverman and Talalay, 1967) reactions. Because of the tremendous variation in size, shape, and functional groups (organic chemical classification) of the substrate molecules, it is difficult to consider the mixed-function oxidase as a single enzyme, although the terminal oxidase moiety, cytochrome P-450, has, until recently, been thought of as a single hemoprotein and different substrates have been shown to competitively inhibit the metabolism of one another (Rubin *et al.*, 1964).

Studies by a number of investigators (Imai and Sato, 1966; Sladek and Mannering, 1966) have suggested, on the basis of the double Soret-banded ethyl isocyanide complex of cytochrome P-450, that two forms of the hemoprotein exist in liver microsomes. Sladek and Mannering (1969) have suggested that rather than two forms, two different species exist, based upon an apparent selective induction of one form with polycyclic hydrocarbons (Alvares *et al.*, 1967; Hildebrandt *et al.*, 1968), and the selective elevation of substrate-metabolizing activity (Sladek and Mannering, 1969; Conney, 1967). Xenobiotics interacting with the hepatic microsomal mixed-function oxidase can be categorized into two groups with respect to their affect on the spectral properties of microsomal suspensions. One group, composed only of known substrates of the mixed-function oxidase, cause what has been termed the type I spectral change (Schenkman *et al.*, 1967a) when added to liver microsomal suspensions. It is characterized by the appearance in difference spectrum of an absorption peak at 388 nm and the disappearance of an absorption band at 420–22 nm. The second group of compounds is composed of basic amines, of which only aniline is a known substrate. These form the type II spectral change (Schenkman *et al.*, 1967a), which is characterized by the appearance of an absorption peak in the Soret region, and the disappearance of absorption at 390 nm; the position of the absorption peak is a characteristic of the compound added, and has been suggested as being due to the formation of a ferrihemochrome with cytochrome P-450 (Schenkman *et al.*, 1967a). However, the type II spectral changes differ from most basic amine ferrihemochromes by having an asymmetrical trough in difference spectrum.

The type I spectral change has been implicated as the spectral manifestation of a complex between the substrate and the mixed-function oxidase (Schenkman *et al.*, 1967a), and shown to parallel enzyme activity (Schenkman *et al.*, 1967b). Indeed, after pretreatment of rats with the inducer phenobarbital, which elevates drug metabolism, the type I spectral change is increased in parallel. A disturbing finding was the observation that the polycyclic hydrocarbon inducer 3,4-benzpyrene, which elevates the level of cytochrome P-450 in liver microsomes did not increase the magnitude of the type

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