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Translation of Synthetic and Endogenous Messenger Ribonucleic Acid In Vitro by Ribosomes and Polyribosomes from *Clostridium pasteurianum*

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Ribosomes and polyribosomes from *Clostridium pasteurianum* were isolated and their activities were compared with those of ribosomes from *Escherichia coli* in protein synthesis in vitro. *C. pasteurianum* ribosomes exhibited a high level of activity due to endogenous messenger ribonucleic acid (RNA). For translation of polyuridylic acid [poly(U)], *C. pasteurianum* ribosomes required a higher concentration of Mg^{2+} and a much higher level of poly(U) than did *E. coli* ribosomes. Phage f2 RNA added to the system with *C. pasteurianum* ribosomes gave no significant stimulation of protein synthesis in a homologous system or with *E. coli* initiation factors. The 30*S* and 50*S* subunits prepared from *C. pasteurianum* ribosomes reassociated less readily than subunits from *E. coli*. The ability of the *C. pasteurianum* subunits to reassociate was found to be dependent upon the presence of a reducing agent during preparation and during analysis of the reassociation products. In heterologous combinations, *E. coli* 30*S* subunits associated readily with *C. pasteurianum* 50*S* subunits to form 70*S* particles, but *C. pasteurianum* 30*S* subunits and *E. coli* 50*S* subunits did not associate. In poly(U) translation, *E. coli* 30*S* subunits were active in combination with 50*S* subunits from either *E. coli* or *C. pasteurianum*, but *C. pasteurianum* 30*S* subunits were not active in combination with either type of 50*S* subunits. Polyribosomes prepared from *C. pasteurianum* were very active in protein synthesis, and well-defined ribosomal aggregates as large as heptamers could be seen on sucrose gradients. An attempt was made to demonstrate synthesis in vitro of ferredoxin.

Numerous experimental approaches have been used to demonstrate the complete synthesis in vitro of a particular protein from its native messenger ribonucleic acid (mRNA; 13). In general, these have involved the use of: RNA isolated from purified RNA bacteriophages as a messenger; a source of mRNA isolated from bacterial cells which have been infected with deoxyribonucleic acid (DNA) bacteriophages; DNA from purified DNA bacteriophages to direct transcription and translation in vitro to produce viral proteins; the purified DNA of transducing bacteriophages which have incorporated specific bacterial genes to direct production in vitro of the bacterial gene product; mRNA isolated from highly differentiated eukaryotic cells, such as reticulocytes, which produce one type of protein in great abundance.

The demonstration of the complete synthesis in vitro of a protein from the native mRNA of a prokaryotic organism has been reported recently for the enzymes alkaline phosphatase (3) and tryptophanase (10). These experiments made use of cells which were induced for the synthesis of the desired enzyme as the source of polyribosomes or mRNA.

Another approach which appears to be promising involves the use of an organism which normally produces large amounts of a single protein. Because of its relative abundance in certain species of *Clostridium*, and because of its low molecular weight and other unique physical properties (17), ferredoxin

appears to be an ideal protein to study. We hope to develop a disrupted cell system from *Clostridium* which is capable of synthesizing ferredoxin and to isolate the messenger for this protein. Two other groups have reported the synthesis in vitro of ferredoxin by cell-free systems from *C. pasteurianum* (14, 25).

We hope that a study of this type will also extend our knowledge of bacterial protein synthesis, which has largely been restricted to aerobic organisms, to strict anaerobes. This report describes some characteristics of the cell-free amino acid-incorporating system of *C. pasteurianum* determined by use of both ribosomes and polysomes.

MATERIALS AND METHODS

Reagents. The following L-[^{14}C]amino acids were purchased from Schwarz/Mann: phenylalanine, valine, alanine, aspartic acid, glutamic acid, and leucine. L-[2,3-$^{3}H_2$]valine was obtained from Amersham-Searle. Polyuridylic acid [poly(U)] was obtained from Miles Chemicals. Phosphoenolpyruvate and pyruvate kinase were obtained from Boehringer Mannheim Corp. *Escherichia coli* B transfer RNA (tRNA; stripped) was obtained from General Biochemicals Corp. Lysozyme (crystalline, from egg white) and deoxyribonuclease I (from bovine pancreas, ribonuclease-free) were purchased from Worthington Biochemical Corp. Alumina A-305 was purchased from Alcoa. Ribonuclease-free sucrose was obtained from Mann Research Corp.

Growth of bacteria. *E. coli* A19, a nuclease-free mutant (6), was obtained from H. Fraenkel-Conrat (University of California, Berkeley). The lyophilized culture was inoculated into 300 ml of medium containing the following (per liter): KH_2PO_4, 2 g; K_2HPO_4, 11 g; $(NH_4)_2SO_4$, 4 g; Casamino Acids, 8 g; 1 M $MgSO_4$, 2 ml; $FeCl_3$ (1 mM in 0.1 N HCl), 1 ml; and glucose, 12 g. The culture was grown aerobically at 37 C. When the turbidity at 660 nm had reached 3.0 (usually 24 hr), the culture was transferred to 12 liters of the above medium in a New Brunswick jar fermentor. The culture was grown with stirring (400 rev/min) and forced aeration (11,000 cc/min) until it reached the desired optical density. The generation time was 30 to 40 min. Larger quantities of cells were grown in a New Brunswick 200-liter fermentor. The cells were harvested with a Sharples centrifuge and stored frozen at −90 C.

E. coli MRE600 (2) was kindly provided by M. Grunberg-Manago (Institut de Biologie Physico-chimique, Paris, France). The cells were grown to log phase (A_{660} = 2) in medium containing (per liter): KH_2PO_4, 13.6 g; $(NH_4)_2SO_4$, 2.0 g; KOH, 5.5 g; $MgSO_4$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; Difco Casamino Acids, 4.0 g; Difco yeast extract, 1.0 g; and dextrose (anhydrous), 10.0 g. Growth and harvest were carried out as for *E. coli* A19. The doubling time was 30 to 35 min.

C. pasteurianum ATCC 6013 was grown as described elsewhere (18). A 10-liter culture grown to log phase was used to inoculate 200 liters of medium that lacked $CaCO_3$ and was contained in a New Brunswick fermentor. During the growth in the fermentor at 30 C, concentrated NH_4OH was added automatically to maintain a constant *p*H of 7.0. Approximately 10 liters was required. *C. pasteurianum* produces acetic and butyric acids as end products of fermentation. If the *p*H is not adjusted, it falls to about *p*H 4 during growth. Preliminary results indicated that ribosomes prepared from cells grown without *p*H control were not very active in catalyzing amino acid incorporation. The growth of *C. pasteurianum* was followed by removing 2-ml samples, adding 0.4 ml of 6 N HCl (to solubilize $CaCO_3$ carried over with the inoculum and other insoluble salts), and reading the absorbance at 660 nm. A fully grown culture attains an A_{660} value of 11 to 15. To stop the growth in the fermentor at a particular time, the contents were chilled to 10 C within 15 min with a New Brunswick rapid chiller. The cells were harvested in a Sharples model A-16 centrifuge and stored at −90 C.

Preparation of ribosomes. Several different methods of preparing ribosomes were used to test the effects of different preparation techniques on the behavior of the ribosomes. The alumina grinding method of Nirenberg (16) was used in the preparation of *E. coli* A19 ribosomes and S-100 fractions. About 30 to 50 g of cells suspended in TMK buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride, *p*H 7.8; 0.014 M magnesium acetate; 0.06 M KCl; 0.006 M 2-mercaptoethanol] were used. The S-30 fraction was incubated for 30 min at 37 C, with no additions made to the extract. At the end of this time, the extract incorporated less than 10 pmoles of L-[^{14}C]phenylalanine per 15 min per mg of ribosomal protein in the absence of poly(U). The S-30 was then centrifuged at 104,000 × *g* for 2 hr. The top four-fifths of each tube were combined, dialyzed against TMK buffer, and stored at −90 C (supernatant fraction or S-100). The pellets were suspended in TMK buffer, centrifuged again, and suspended in approximately 15 ml of buffer. The ribosome solution was divided into 0.5-ml portions which were stored at −90 C.

E. coli MRE600 ribosomes were prepared by alumina grinding and pelleted from a nonpreincubated S-30 fraction. The ribosomes were washed twice by centrifugation through buffer containing 1 M NH_4Cl and were finally taken up in TMK buffer and frozen.

Ribosomes were also prepared from *C. pasteurianum* cells by several different procedures. *C. pasteurianum* ribosomes (I) were prepared by using lysozyme to break open the cells. The cells (about 50 g) were suspended in an equal volume of buffer; lysozyme (1 mg/g cells) was added, and the mixture was warmed to room temperature and then returned to an ice bath. The suspension became very viscous. Deoxyribonuclease (1 mg) was added, and incubation was continued for 20 min. The suspension was centrifuged at 30,000 × *g* twice, and the resulting S-30 fraction was treated as described for *E. coli* A19.

C. pasteurianum ribosomes (II) were prepared by sonic oscillation. The ribosomes were pelleted from a

nonpreincubated S-30 fraction and washed once by centrifugation through TMK buffer, before being suspended in TMK buffer and frozen.

C. pasteurianum ribosomes (III) were also prepared by sonic oscillation and were pelleted from a nonpreincubated S-30 fraction. The ribosomes were washed twice by centrifugation through buffer containing 2 M NH_4Cl, and were then preincubated in a complete amino acid incorporation assay (containing no mRNA or initiation factors); they were finally washed once by centrifugation through TMK buffer, before being suspended in TMK buffer and frozen. The preincubation reduced the endogenous activity of the ribosomes in amino acid incorporation assays by 60 to 70% without reducing their ability to respond to exogenous messengers. The exact details of this preparation procedure (III) will be published elsewhere (M. R. Stallcup and J. C. Rabinowitz, *in preparation*).

Crude initiation factors were prepared from the supernatant solution of the first NH_4Cl wash of the ribosomes; the initiation factors were concentrated by ammonium sulfate precipitation [5.2 g of solid $(NH_4)_2SO_4$ were added to each 10 ml of NH_4Cl wash] and then dissolved in and dialyzed against TMK buffer.

Preparation of 30S and 50S subunits. Ribosomes were concentrated by centrifuging for 2.5 hr at 48,000 rev/min in a Spinco no. 50 rotor and suspending the pellets in a small volume of TMK buffer so that the final A_{260} of the suspension was about 5,000. The ribosomes (0.25 ml) were added to 2.25 ml of buffer which lacked Mg^{2+} so that the final Mg^{2+} concentration was 1 mM. After 4 hr at 0 C, 0.8 ml was layered on each of three 60-ml 10 to 35% linear sucrose gradients in buffer containing 1 mM magnesium acetate. Centrifugation was for 16 hr at 23,000 rev/min in a Spinco SW25.2 rotor. The gradients were analyzed in a Gilford recording spectrophotometer with the use of a flow-through cuvette. The contents were forced through the cuvette with a 50% sucrose solution, and fractions of 2.0 ml were collected in a fraction collector. The fractions containing the appropriate subunit were combined with care to avoid contamination by the other subunit. The solutions were dialyzed for 3 hr against two changes of 2 liters of buffer. The subunits were recovered by centrifugation at 48,000 rev/min for 2.5 hr in a Spinco no. 50 rotor and were suspended in buffer containing 5 mM magnesium acetate to give a final A_{260} value of 60 for the 30*S* particles and 120 for the 50*S* particles.

Preparation of polysomes. In some experiments, the procedure used for the preparation of polysomes was a modification of that used by Nepokroeff and Aronson (14). The cells were grown at 30 C in a 500-ml flask, and during the growth the *p*H was maintained between 6.5 and 7.2 by the addition of 10 N KOH. The *p*H was measured every hour. When the A_{660} reached 3.5 to 4, the culture was poured over an equal volume of ice and centrifuged at 5,000 × *g* for 5 min. The cells were suspended in 25 ml of a 10% sucrose solution which contained 5 mM NaCl and 5 mM potassium phosphate, *p*H 7.8. A 25-mg amount of lysozyme was added, and the mixture was rapidly brought to 37 C by placing the solution (in a 50-ml Erlenmeyer flask) in a water bath at 55 C. The temperature was reached in 45 sec, and the suspension was then transferred to a bath at 37 C for an additional 2 min. At this time, 25 ml of a 10% sucrose solution containing 20 mM magnesium acetate was added, and the suspension was centrifuged at 12,000 × *g* for 5 min. The pellet, which contained many spheroplasts as well as damaged cells, was suspended in 25 ml of the TMK buffer; 75 μg of deoxyribonuclease was added, and the mixture was incubated at 0 C for 30 min and then centrifuged for 5 min at 5,000 × *g*. The supernatant fraction is referred to as the S-5. The S-5 fraction could be centrifuged at 30,000 × *g* for 30 min and the resulting pellet (P-30) could be suspended in 5 ml of TMK buffer.

At a later stage, two modifications of the procedure were introduced. One involved the addition of 0.5% Brij 58 and 0.5% sodium deoxycholate in the TMK buffer in which the lysozyme-treated cells were suspended. This procedure resulted in much better breakage of the cells, as evidenced by the formation of a very viscous solution which was solubilized by deoxyribonuclease and by a three- to four-fold increase in ribosomal and polysomal material in the S-5. The other modification involved the use of 10% sucrose containing 0.01 M Tris-chloride buffer, *p*H 7.8, and 0.06 M KCl instead of the sucrose-NaCl-KPO_4 buffer, and incubating the cells with lysozyme at 20 C for 5 min instead of at 37 C for 2 min. This latter change was made because it appeared to lead to more consistently good polysome preparations.

Analytical sucrose gradients. Linear sucrose gradients of 5 ml were prepared containing 10 to 35% (w/v) sucrose in 0.01 M Tris-chloride buffer (*p*H 7.8), 0.06 M KCl, and the desired concentration of magnesium acetate (0.014 M for undissociated ribosomes and polysomes). Centrifugation was carried out in a Spinco model L-2 ultracentrifuge in the SW-50.1 rotor (2 hr at 48,000 rev/min for ribosomes and 75 min at 35,000 rev/min for polysomes). Gradients were analyzed with a Gilford recording system by use of a flow-through cuvette and a Beckman fractionating system.

Preparation of f2 RNA. Phage f2 RNA was prepared by a slight modification of the procedure of Eisenstadt and Brawerman (4). A 5-ml amount of the virus solution (A_{260} = 250) in 0.1 M NaCl, 0.05 M Tris-chloride buffer, and 0.01 M ethylenediaminetetracetic acid, *p*H 7.6, was added to an equal volume of cold 80% redistilled phenol. The suspension was mixed on a Vortex mixer for 3 min at room temperature. The RNA was precipitated from the aqueous phase by the addition of 0.1 volume of 10% NaCl and 2.5 volumes of absolute ethanol. The precipitate was washed three times with cold 66% aqueous ethanol containing 0.3% NaCl. The RNA was dissolved in water to a final A_{260} of about 250 and was stored at −20 C.

Amino acid incorporation. The assay was similar to that described by Nirenberg (16) with a few modifications. The reaction mixture contained, in a final

volume of 0.25 ml: 0.1 M Tris-chloride buffer, pH 7.8; 0.05 M KCl; 1 mM adenosine triphosphate; 0.04 mM guanosine triphosphate; 6 mM 2-mercaptoethanol; 9 mM phosphoenolpyruvate; 10 mM magnesium acetate; 400 μg of *E. coli* B tRNA; 5 μg of pyruvate kinase; 0.25 mM each of 19 unlabeled amino acids; 0.04 mM of a labeled amino acid (^{14}C, 15 mCi/mmole; or ^{3}H, 83 mCi/mmole). Supernatant solution, ribosome fractions, and poly(U) or f2 RNA were added as indicated. A 1-mg amount of ribosomal protein was found to be equivalent to 30 A_{260} units of ribosomes. The reaction was incubated for 30 min at 37 C and stopped by the addition of 3.0 ml of cold 10% trichloroacetic acid. The mixtures were then heated at 90 C for 20 min and cooled to 0 C for 30 min. The precipitates were collected on glass-fiber filters (Whatman GF/A) and washed five times with 5-ml portions of cold 5% trichloroacetic acid. The filter papers containing the precipitates were then placed in scintillation vials containing 10 ml of Bray's solution (1) and counted in a Nuclear-Chicago Mark I scintillation counter.

Attempt at synthesizing ferredoxin with the polysome preparation. In a 10-fold scaled up reaction system, the P-30 fraction of polysome preparation (10.9 mg protein) was incubated with 4 mg of *E. coli* S-100, 10 μCi each of L-[^{14}C]alanine, L-[^{14}C]aspartate, and L-[^{14}C]glutamate (100 mCi/mmole), and the other components of the amino acid incorporation system in a final volume of 2.4 ml. After incubation for 30 min at 37 C, 530 mg of Tris base was added, and the pH was adjusted to 8.0 with acetic acid. The mixture was incubated again at 37 C for 30 min to strip the charged tRNA (20). The solution was passed through a Sephadex G-25 column (2.2 by 20 cm) to remove amino acids. The fractions other than those where amino acids eluted were combined and lyophilized. The total radioactivity in this fraction was 2×10^6 counts/min. To the dried product was added 2 mg of apoferredoxin$_{ox}$ (7), 0.5 ml of 1 M Tris-chloride buffer, pH 8.5, 2.4 g of urea, 25 μliters of 2-mercaptoethanol, a drop of antifoam, and water to make a final volume of 5 ml. The solution was bubbled with purified N_2 for 4 hr at room temperature, placed on the Sephadex G-25 column, and washed through with 0.1 M Tris-chloride buffer (pH 8.5)-0.05 M mercaptoethanol. The fractions containing material with absorbance at 280 nm were combined, giving a final volume of 13 ml. To this solution were added 80 μliters of 0.1 M $Fe(NH_4)_2(SO_4)_2$, 50 μliters of 0.17 M Na_2S, and 10 μliters of 2-mercaptoethanol. After 15 min at 37 C, the solution of reconstituted ferredoxin was applied to diethylaminoethyl (DEAE)-cellulose column (8 by 0.8 cm). The column was washed with 10 ml of 0.15 M Tris-chloride buffer (pH 7.4) and then with 20 ml of 0.005 M Tris-chloride buffer (pH 7.4) containing 0.23 M NaCl. The reconstituted ferredoxin was eluted with 0.005 M Tris-chloride buffer (pH 7.4)-0.58 M NaCl. The fractions that showed absorbance at 390 nm were combined and counted for radioactivity. Most of the counts were eluted with the preliminary wash solutions before the elution of the ferredoxin. The solution was diluted sixfold with water and chromatographed through another DEAE-cellulose column by the same procedure as before. The A_{390} was used as a measure of ferredoxin concentration, and the radioactivity in the ferredoxin fractions was determined. The A_{280}, corrected for the expected absorbance of ferredoxin at this wavelength, was used as a measure of contaminating protein.

RESULTS

Endogenous amino acid incorporation by ribosomes. *C. pasteurianum* (I, II, or III) ribosomes were capable of carrying out amino acid incorporation without the addition of mRNA. These preparations differed in their activities and ranged from 0.1 to 0.7 nmole of valine incorporated per mg of ribosomal protein. This variation depended on which method of preparation was used and also, in part, on the stage of growth at which the cells were harvested. Variation among individual preparations made by the same method was also observed. Apparently, the mRNA in *C. pasteurianum* is relatively stable when these methods of preparation of ribosomes are used. Sucrose gradient profiles of ribosomes from log-phase cells prepared by method I showed the presence of degraded polysomal material. After incubation in the incorporation system, the polysome region disappeared. Ribosomes obtained from cells harvested in stationary phase had about one-third the endogenous activity of ribosomes from cells harvested in log phase. Sucrose gradient profiles of ribosomes from stationary-phase cells showed less polysomal material and a larger proportion of 30*S* and 50*S* particles than the profiles of ribosomes from log-phase cells.

The endogenous activity of the ribosomes, as mentioned above, depended on the stage of growth at which the cells were harvested as well as on the method of preparation. The data in Table 1 show the activity of *C. pasteurianum* (I) and *E. coli* A19 ribosomes as a function of the time of harvest of the cells. *E. coli* ribosomes were essentially devoid of endogenous activity. However, the *E. coli* preparations were made with the alumina grinding procedure, whereas lysozyme was used in obtaining *C. pasteurianum* ribosomes. It was found that alumina grinding destroys the endogenous activity of the clostridial ribosomes as well.

The presence of polysomal material in *C. pasteurianum* ribosomes prepared by the lysozyme method and the ability of the ribosomes to catalyze amino acid incorporation in vitro without the addition of an mRNA source suggests that there is little degradation of poly-

somes by ribonuclease during the preparation. Assays for ribonuclease activity confirmed this. When procedure B of Neu and Heppel (15) was used, it was found that extracts of *C. pasteurianum* had about one-third to one-half the ribonuclease activity on a variety of RNA substrates that *E. coli* A19 extracts had (*E. coli* A19 is a ribonuclease I-free mutant).

Poly(U)-directed phenylalanine incorporation. *C. pasteurianum* ribosomes could use poly(U) as a messenger, but required a higher Mg^{2+} concentration for optimal activity (25 mM) than did *E. coli* ribosomes (15 mM), as shown in Fig. 1. Although the concentration of Mg^{2+} which gave optimal activity for *C. pasteurianum* ribosomes was consistently found to be 25 mM, the amount of stimulation by poly(U) at lower Mg^{2+} concentrations was variable and appeared to be dependent on the ribosome preparation as well as on the lot of poly(U).

Another difference between the *E. coli* and clostridial systems in response to poly(U) is shown in Fig. 2. The *E. coli* ribosomes were saturated at a much lower level of poly(U) than the *C. pasteurianum* ribosomes. With 10 μg of poly(U), which is a near-saturating amount for *E. coli* ribosomes, there was very little polyphenylalanine synthesis by *C. pasteurianum* ribosomes. At 10- to 100-fold higher poly(U) concentrations, the difference between *E. coli* and *C. pasteurianum* ribosomes was less pronounced.

Since it has been shown (5, 23) that at high Mg^{2+} concentrations synthetic messenger can stimulate leucine incorporation, it was thought that at these concentrations general amino acid incorporation was occurring. However, as shown in Table 2, only the incorporation of

TABLE 1. *Effect of time of harvest on endogenous activity*[a]

Organism	A_{660} at harvest	Stage of growth	Endogenous activity
E. coli A19	0.7	Early log	20
	2.0	Mid-log	20
	5.6	Stationary	20
C. pasteurianum (I)	3.0	Early log	720
	5.0	Mid-log	690
	10.0	Stationary	230

[a] The assays were performed as described in Materials and Methods. Each assay contained about 1 mg of ribosomal protein. The endogenous activity is expressed as picomoles of phenylalanine incorporated per milligram of ribosomal protein. No exogenous mRNA was added.

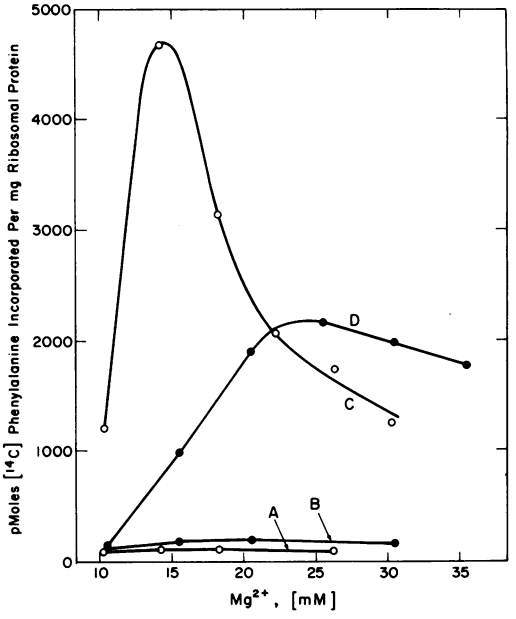

FIG. 1. *Effect of Mg^{2+} concentration on L-[^{14}C]-phenylalanine incorporation by E. coli MRE 600 ribosomes and C. pasteurianum ribosomes (III). The assays contained, in a volume of 0.125 ml, 0.1 mg of ribosomal protein and 0.5 mg of E. coli A19 S-100. The curves represent: A, E. coli ribosomes, endogenous activity; B, C. pasteurianum ribosomes, endogenous activity; C, E. coli ribosomes plus 30 μg of poly(U); D, C. pasteurianum ribosomes plus 50 μg of poly(U).*

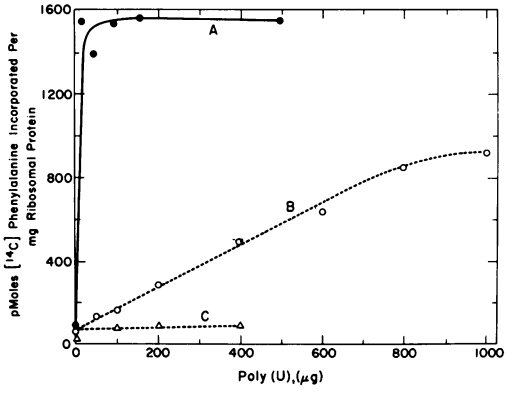

FIG. 2. *Effect of poly(U) concentration on L-[^{14}C]-phenylalanine incorporation by E. coli and C. pasteurianum ribosomes. The reaction was carried out in 0.25 ml as described in Materials and Methods. E. coli S-100 (0.3 mg of protein) was used. A, E. coli A19 ribosomes, 0.5 mg of protein, 14 mM Mg^{2+}; B, C. pasteurianum ribosomes (I), 0.4 mg of protein, 24 mM Mg^{2+}; C, C. pasteurianum ribosomes (I), 0.4 mg of protein, 14 mM Mg^{2+}.*

phenylalanine seemed to be responsive to the very high poly(U) concentration. In these studies with poly(U), *E. coli* A19 S-100 was used because higher activities were obtained than with *C. pasteurianum* S-100. Qualitatively identical results were obtained with clostridial S-100.

Phage f2 RNA-directed incorporation. Amino acid incorporation by *C. pasteurianum* ribosomes was not stimulated by f2 RNA. The effect of Mg^{2+} concentration on the incorporation of valine in the absence and presence of f2 RNA is shown in Fig. 3. Results identical to those shown in Fig. 3 were also obtained when the S-100 fraction of *C. pasteurianum* was used. All attempts to show stimulation by f2 RNA were unsuccessful. For example, the ribosomes (I) were preincubated at 37 C in the incorporating mixture, and were then centrifuged at 100,000 × *g* to separate them from endogenous mRNA. Sucrose gradient profiles showed the disappearance of the polysomal material. This ribosome preparation, however, was still unable to translate f2 RNA, although it was active with poly(U). By using an S-100 fraction which had been treated with DEAE-cellulose and precipitated with $(NH_4)_2SO_4$, it could be shown that incorporation by *E. coli* ribosomes and f2 RNA was stimulated fivefold by the addition of (*l*)-10-formyltetrahydrofolate and that [^{14}C]formate from (*l*)-10-[^{14}C]formyl-tetrahydrofolate was incorporated. In the case of the *C. pasteurianum* system, the formyl donor had no effect nor was ^{14}C incorporated into the product.

TABLE 2. *Effect of poly(U) on the incorporation of several amino acids*[a]

Poly(U) (μg)	Amino acid incorporated (pmoles/mg of ribosomal protein)			
	Phenyl-alanine	Leucine	Alanine	Valine
0	295	300	319	253
5	1,035	411	330	253
500	2,860	530	340	274

[a] The assays were performed as described in Materials and Methods with the use of *C. pasteurianum* ribosomes (I, 0.43 mg of ribosomal protein), *E. coli* A19 S-100 (0.4 mg protein), and 25 mM Mg^{2+}.

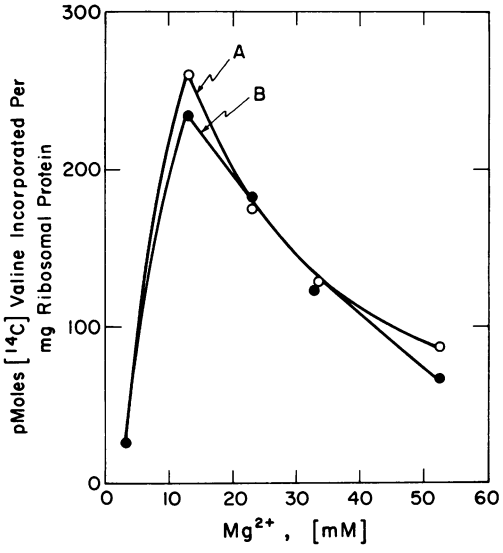

FIG. 3. *Incorporation of* L-[^{14}C]*valine in the presence of f2 RNA. The assay was performed as described except that* Mg^{2+} *was varied as shown and 0.6 mg of C. pasteurianum (I) ribosomal protein, 12.5* A_{260} *units of f2 RNA, and 0.3 mg of E. coli A19 S-100 were used. A, f2 RNA present; B, no f2 RNA present.*

The inability of clostridial ribosomes to use f2 RNA as a messenger is reflected by the lack of binding interaction between them. Binding of f2 RNA to ribosomes was examined by incubating [^{32}P]f2 RNA with S-30 fractions and analyzing the ribosome profile on sucrose gradients. The *E. coli* system showed the formation of particles heavier than 100*S* in the presence of f2 RNA. Radioactivity was associated with these particles. However, the profile of clostridial ribosomes showed no change in the presence of f2 RNA, and all radioactivity remained in the lighter region of the gradient.

The lack of translation of f2 RNA could possibly be due to a difference in specificity of *C. pasteurianum* initiation factors or to a lack of initiation factors on the ribosomes. The effect of *E. coli* initiation factors on f2 RNA translation was therefore determined. *E. coli* MRE600 salt-washed ribosomes and *C. pasteurianum* ribosomes (III) were used in these studies. Results of incorporation by these preparations are shown in Table 3. Although *E. coli* initiation factors were active with *E. coli* ribosomes, they were ineffective in stimulating f2 RNA-mediated valine incorporation by *C. pasteurianum* ribosomes.

It has been shown that a particular ratio of viral RNA to ribosomes must be used to achieve maximal incorporation of valine by *E. coli* ribosomes (22). No amino acid-incorporating activity was detected in the clostridial system when the f2 RNA to *C. pasteurianum* ribosome ratio, based on A_{260}, was varied from 0.25 to 1.7.

Reassociation of C. pasteurianum 30S and 50S subunits. *C. pasteurianum* ribosomes could be dissociated by incubation in 1 mM

Mg^{2+}, and the subunits could be separated on a sucrose gradient. Sucrose gradient analyses of the isolated subunits showed them to be free from contaminating subunits of the other size. When the subunits were combined, incubated at a high Mg^{2+} concentration, and recentrifuged through a sucrose gradient, there was no evidence of reassociation to 70S particles, although similar experiments done with *E. coli* particles showed extensive reassocation. Varying the Mg^{2+} concentration from 5 to 40 mM at 60 mM KCl, and varying the KCl concentration from 12 to 500 mM at 15 mM Mg^{2+} failed to bring about reassociation of the *C. pasteurianum* subunits. That a necessary soluble "factor" was not lost when the subunits were prepared was shown by adding the top portion of a sucrose gradient after centrifugation of dissociated ribosomes to an incubation mixture of 30S and 50S subunits. No reassociation could be detected under these conditions either.

However, it was found that when 2-mercaptoethanol was included in the sucrose gradients used to prepare the *C. pasteurianum* subunits, reassociation to 70S particles could be achieved at high Mg^{2+}. The *C. pasteurianum* 70S particles thus formed by reassociation were unstable on the sucrose gradients used for analysis unless 2-mercaptoethanol was included in the gradient. Figure 4a shows reassociation products analyzed on a gradient that did not contain mercaptoethanol; the 70S particles had mostly fallen apart during centrifugation, and gave a broad, diffuse band between the 50S and 70S regions. Figure 4b shows that the same *C. pasteurianum* reassociation products analyzed on a sucrose gradient containing mercaptoethanol gave a well-defined band at 70S.

The *C. pasteurianum* subunits prepared and analyzed in the presence of 2-mercaptoethanol still did not reassociate as readily as *E. coli* subunits. Figure 5 shows the results of reassociation experiments with homologous and heterologous combinations of *E. coli* and *C. pasteurianum* subunits, performed by incubation at 37 C for 10 min in 25 mM Mg^{2+}. The *C. pasteurianum* subunits were prepared in the presence of 2-mercaptoethanol. Under these conditions, *E. coli* subunits reassociated completely to 70S particles (Fig. 5a), whereas *C. pasteurianum* subunits gave substantial, but still incomplete, reassociation (Fig. 5b). *E. coli* 30S and *C. pasteurianum* 50S subunits gave almost complete reassociation to 70S particles (Fig. 5c), but *C. pasteurianum* 30S subunits did not reassociate with *E. coli* 50S subunits to a significant extent (Fig. 5d).

When the reassociation of ribosomal subunits was attempted in the presence of mRNA, different results were obtained. As shown in Table 4, the 50S subunit from *C. pasteurianum* when combined with the 30S subunit of *E. coli* could use poly(U) as a messenger, but the clostridial 30S subunit was unable to combine with the 50S subunit of either *E. coli* or *C. pasteurianum* to synthesize protein in response to the low level of poly(U) tested (20 μg).

TABLE 3. *Effect of E. coli initiation factors on amino acid incorporation by C. pasteurianum ribosomes in the presence of f2 RNA*[a]

Salt-washed ribosomes	Initiation factors	Valine incorporated (pmoles/mg of ribosomal protein	
		Endogenous activity	Plus f2 RNA
E. coli MRE 600	*E. coli*	460	6,790
	None	70	70
C. pasteurianum (III)	*C. pasteurianum*	460	480
	E. coli	270	290
	None	110	120

[a] The incorporation assays contained 0.1 mg of ribosomal protein and, where indicated, 0.17 mg of crude initiation factors and 1.1 A_{260} unit of f2 RNA. L-[2,3-$^3H_2$]valine was the labeled amino acid. The Mg^{2+} concentration used was 11 mM for *E. coli* ribosomes and 15 mM for *C. pasteurianum* ribosomes. The final assay volume was 0.125 ml.

Amino acid incorporation by C. pasteurianum polysomes. Polysomes prepared as described in Materials and Methods showed the presence of dimers to hexamers. The pattern of a typical S-5 preparation is shown in Fig. 6a. When the S-5 fraction was centrifuged at 30,000 × *g* for 30 min, the resulting pellet was enriched in the higher aggregates (Fig. 6b and 6c). The endogenous incorporation, expressed as nanomoles of valine incorporated per milligram of ribosomal protein, was 2 to 2.4 for the P-30 fraction. A typical ribosomal preparation had an activity of about 0.2. Figure 7 shows the transformation of the polysome preparation to 30S, 50S, and 70S particles after incubation in the amino acid-incorporating system. When the P-30 fraction was separated on a sucrose gradient and different regions of the gradient were tested for endogenous incorporating activity, the activity was found in the heavier polysomal region (Fig. 8).

The response of the polysomes to added poly(U) in L-[^{14}C]phenylalanine incorporation assays was similar to that of the *C. pasteurianum* ribosomes. Optimal activity was found

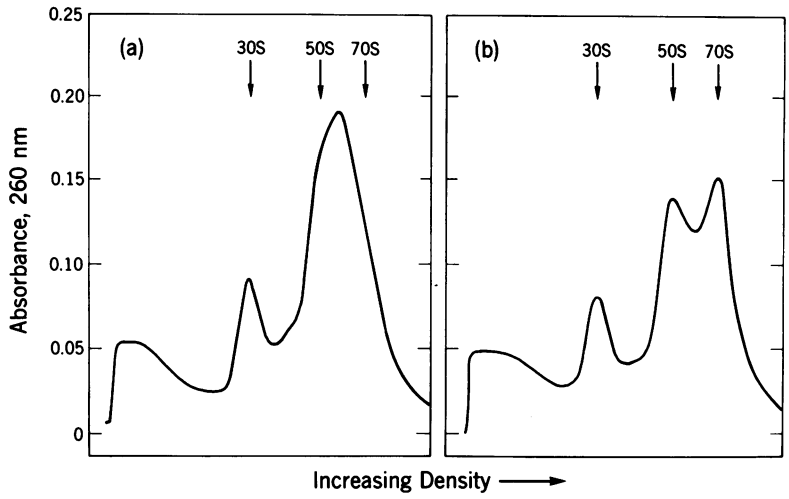

FIG. 4. *Stabilization of reassociated C. pasteurianum 70S particles by addition of 2-mercaptoethanol to the analytical sucrose gradients. C. pasteurianum 30S (0.4 A_{260} units) and 50S (0.8 A_{260} units) subunits were incubated for 10 min at 37 C in TMK buffer containing 25 mM Mg^{2+} and 10 mM 2-mercaptoethanol. The mixture was cooled and layered in a volume of 0.2 ml on a 5-ml sucrose gradient (10 to 35%) containing TMK buffer and 25 mM Mg^{2+}. The gradient in Fig. 4a contained no mercaptoethanol; the gradient in Fig. 4b contained 8 mM mercaptoethanol. Centrifugation was for 2 hr at 45,000 rev/min in a Spinco SW50.1 rotor.*

at 25 mM Mg^{2+}, and increased stimulation was obtained at high poly(U) concentrations.

Attempt to synthesize ferredoxin in vitro with the polysome preparation. The procedure followed for this experiment is listed in Materials and Methods, and the results are shown in Table 5. The recovery of ferredoxin from the second DEAE-cellulose column was essentially complete, as calculated from the A_{390} values. However, the radioactivity in the ferredoxin-containing fractions was reduced from 40,000 to 4,000 counts/min in this step, and there was a parallel reduction in the nonferredoxin protein from 10.3 to 1.3 mg (based on the assumption that 1 mg of contaminating protein/ml gives an A_{280} of 1.0). The ferredoxin from the final step is estimated to be only 50 to 60% pure, from the A_{390} and A_{280} values. All of these results make it difficult to conclude how much, if any, of the remaining 4,000 counts/min is present in the ferredoxin. The remaining 4,000 counts/min is only 0.2% of the total radioactivity (2×10^6 counts/min) which was eluted from the first G-25 column as the total polypeptide material synthesized in the assay. From the specific activity of the [^{14}C]amino acids used and from the known amino acid composition of *C. pasteurianum* ferredoxin (24), it was calculated that ferredoxin synthesized de novo in this experiment would have a specific activity of 3,000 counts per min per pmole.

DISCUSSION

In this report, we have presented some initial observations on the protein-synthesizing system of *C. pasteurianum* in vitro. Although the general characteristics of the system are similar to the well-studied system of *E. coli*, some differences were noted.

Ribosomes prepared from *C. pasteurianum* cells by several different methods exhibit a high degree of endogenous protein synthesis in amino acid incorporation assays containing no added mRNA. The exact level of this endogenous activity was found to be dependent on the time of harvest of the cells and the method of preparation of the ribosomes. It should be noted that essentially the same qualitative results were obtained for all of the experiments reported in this paper, no matter which of the listed methods for ribosome preparation was employed. Extracts from these cells exhibit very low levels of ribonuclease activity, a property that could account, in part, for the observed stability of the activity due to endogenous mRNA. The endogenous activity of salt-washed ribosomes from *C. pasteurianum* is dependent upon added initiation factors from the NH_4Cl washings, which indicates that some reinitiation is occurring on the endogenous mRNA during the assays (Table 3).

The response of *C. pasteurianum* ribosomes to poly(U) as a messenger in protein synthesis

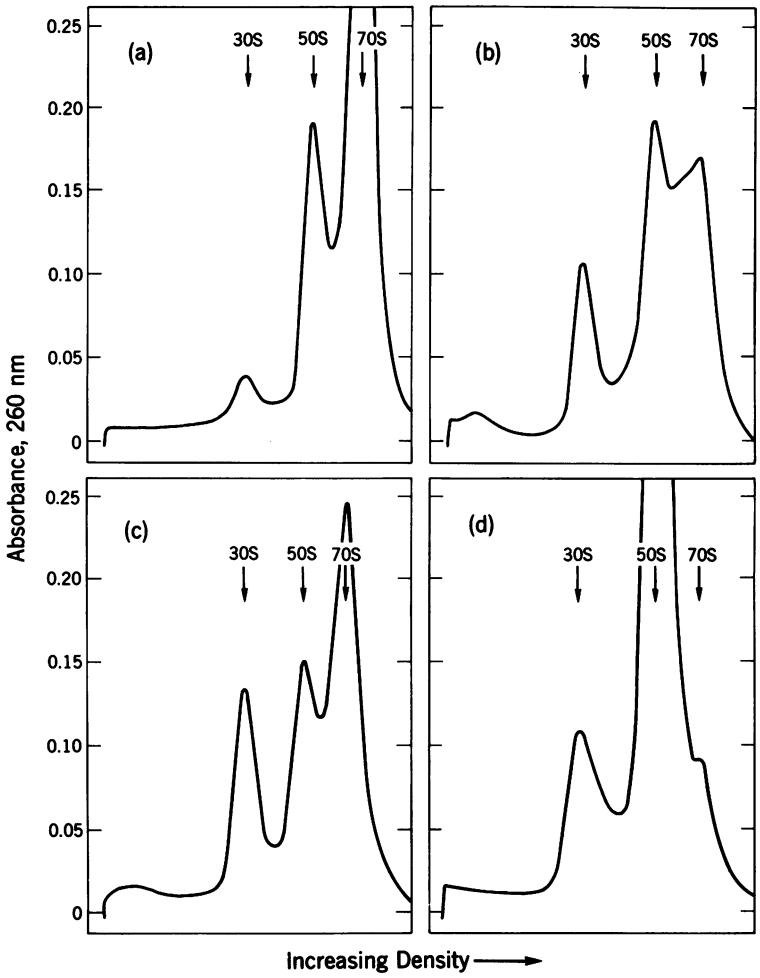

FIG. 5. *Reassociation of homologous and heterologous combinations of E. coli and C. pasteurianum subunits. The 30S subunits (0.6A$_{260}$ unit) and 50S subunits (1.2A$_{260}$ units) were incubated at 37 C for 10 min in a total volume of 0.2 ml of TMK buffer containing 25 mM Mg^{2+} and 10 mM 2-mercaptoethanol. The samples were analyzed on sucrose gradients as in Fig. 4b (plus mercaptoethanol). The combinations of subunits were: 5a, E. coli 30S and E. coli 50S; 5b, C. pasteurianum 30S and C. pasteurianum 50S; 5c, E. coli 30S and C. pasteurianum 50S; 5d, C. pasteurianum 30S and E. coli 50S.*

assays is dramatically different from the response exhibited by *E. coli* ribosomes. *C. pasteurianum* ribosomes require a much higher Mg^{2+} concentration for optimal translation of poly(U) than do *E. coli* ribosomes, and they require more than 10 times the concentration of poly(U) in order to approach the level of polyphenylalanine synthesis observed with *E. coli* ribosomes. These differences indicate that the *C. pasteurianum* ribosomes have greater difficulty in initiating protein synthesis with poly(U) as a messenger. Since the Mg^{2+} optimum is about 15 mM for the endogenous activity of *C. pasteurianum* ribosomes, the higher Mg^{2+} level needed for poly(U) translation must be involved in the initiation process. The need for higher Mg^{2+} in initiation with poly(U), but not in polypeptide chain elongation with poly(U), has also been suggested by others (19, 21). Moreover, since the rate of elongation should not be dependent on poly(U) concentration, the requirement for high poly(U) concentrations by the *C. pasteurianum* ribosomes can also be attributed to the initiation phase of the protein synthesis process.

C. pasteurianum ribosomes fail to show any synthesis of protein in response to f2 RNA, even when *E. coli* initiation factors are added

TABLE 4. *Poly(U)-mediated phenylalanine incorporation by ribosomal subunits*[a]

Ribosome subunits		Phenylalanine incorporated[b]
30S	50S	
E. coli		1,282
	E. coli	584
E. coli	*E. coli*	7,580
C. pasteurianum		62
	C. pasteurianum	688
C. pasteurianum	*C. pasteurianum*	88
E. coli	*C. pasteurianum*	4,940
C. pasteurianum	*E. coli*	332

[a] Incorporation assays were done as described in Materials and Methods with the exception that 25 mM Mg^{2+} and 20 μg of poly(U) were used. The complete mixture lacking poly(U) was incubated for 5 min at 37 C. Poly(U) was added and the incubation was continued for 30 min. The A_{260} values of the subunits used were: *E. coli* 30S, 2.5; 50S, 3.7; *C. pasteurianum* 30S, 5.8; 50S, 3.8 (30 A_{260} units are equivalent to 1 mg of ribosomal protein).

[b] Expressed as picomoles per milligram of ribosomal protein.

to the assay. Species specificity with respect to translation of natural mRNA has been reported before. Lodish (11) found that, whereas *E. coli* ribosomes and initiation factors could translate all three cistrons of f2 RNA, ribosomes from *Bacillus stearothermophilus* could translate only the maturation protein cistron. He determined that the 30S subunits were the components which determined this specificity, regardless of which type of 50S subunit or initiation factors were used (12). Szer and Brenowitz (22), working with ribosomes from *E. coli* and from two cryophilic prokaryotes, observed a difference in the ability of these organisms to translate MS2 RNA. The protein synthesis systems from the cryophilic organisms could translate the MS2 RNA but required a much higher amount of the messenger than did *E. coli* ribosomes. In contrast to Lodish's findings, Szer and Brenowitz found that the initiation factors, instead of the ribosomes, determined the affinity of the system for the MS2 RNA. The ability of the *E. coli* ribosomal system and the inability of the *C. pasteurianum* ribosomal system, as described in this paper, to translate f2 RNA was shown to be a property of the salt-washed ribosomes, regardless of whether the homologous or heterologous initiation factors were used (M. R. Stallcup and J. C. Rabinowitz, *in preparation*).

From the studies done with ribosomal subunits, it appears that the *C. pasteurianum* 30S subunit is somehow damaged during the low Mg^{2+} isolation procedure, because the *C. pasteurianum* 30S subunits, when combined with 50S subunits, are completely inactive in the translation of poly(U); levels of poly(U) 10 times higher than that used in the experiments shown in Table 4 also failed to stimulate protein synthesis by the *C. pasteurianum* 30S subunits with 50S subunits. The *C. pasteurianum* 50S subunit is active in combination

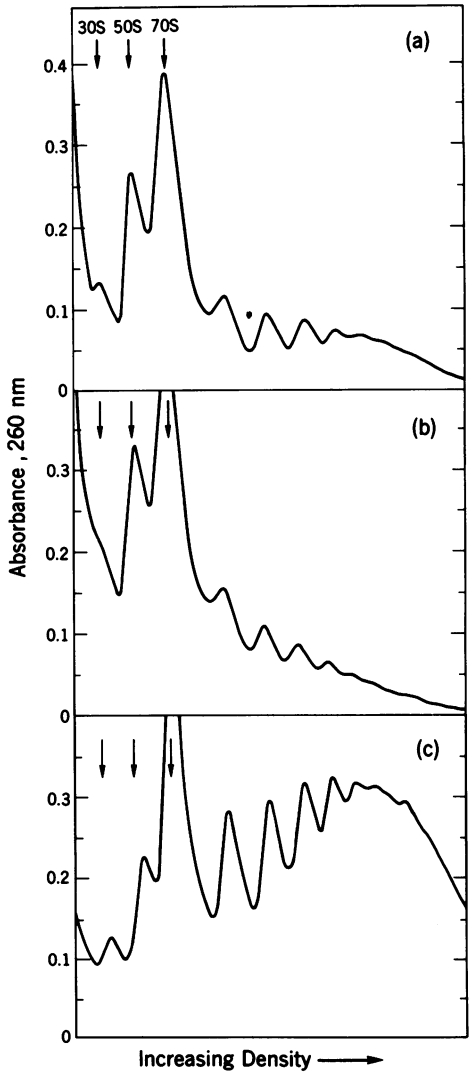

FIG. 6. *Sucrose gradient profiles of C. pasteurianum polysomes. Polysomes were prepared as described in Materials and Methods; 0.2-ml portions of the fractions were layered on 5-ml 10 to 35% sucrose gradients in TMK buffer and centrifuged for 75 min at 35,000 rev/min in an SW50.1 rotor. (a) S-5 fraction. (b) S-30 fraction. (c) P-30 fraction.*

with the *E. coli* 30*S* subunit. Moreover, it was shown that the ability of the *C. pasteurianum* subunits to reassociate to form a stable 70*S* particle is dependent on the presence of a reducing agent, 2-mercaptoethanol, in the sucrose gradients used to prepare the subunits and to analyze the reassociation products. The *C. pasteurianum* subunits, as prepared here, do not reassociate as readily as *E. coli* subunits at the same Mg^{2+} concentration.

Polysomes prepared from *C. pasteurianum* as a P-30 fraction showed well-defined ribosomal aggregates as large as heptamers on sucrose gradients. The amino acid-incorporating activity, expressed in terms of picomoles of valine incorporated per milligram of ribosomal protein, was at least 50 times that of a previously reported polysome fraction prepared from *C. pasteurianum* (14).

Since our work began, two reports of the cell-free synthesis of ferredoxin have appeared (14, 25). The criteria used by these groups as proof of ferredoxin synthesis in vitro included the examination of tryptic digests of the protein products for radioactively labeled peptides that resembled the peptides of unlabeled, purified apoferredoxin (14), and copurification of the radioactively labeled protein products with carrier ferredoxin and demonstration of a constant ratio of radioactivity to protein after successive crystallizations (25). However, no attempt was made in either report to quantitate the amount of ferredoxin synthesized.

In our experiment, the criteria applied to test for the synthesis of ferredoxin in vitro included (i) the ability of radioactively labeled protein product to be reconstituted with inorganic iron and sulfide to holoferredoxin along with added pure carrier apoferredoxin and (ii) the copurification of radioactive protein

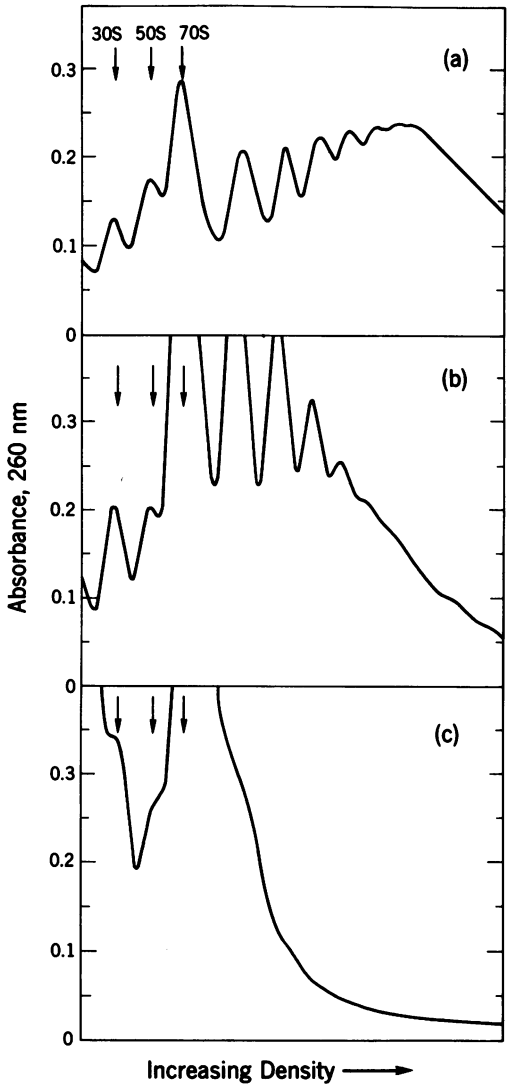

FIG. 7. *Effect of incubation on sucrose gradient profiles of polysomes. The P-30 fraction in TMK buffer containing 1.1 mg of protein per ml was incubated under the conditions described, and 0.2 ml was centrifuged in a sucrose gradient as described in Fig. 6. (a) Polysomes incubated at 0 C for 30 min. (b) Polysomes incubated at 37 C for 30 min. (c) Polysomes incubated in the presence of the incorporating assay at 37 C for 30 min.*

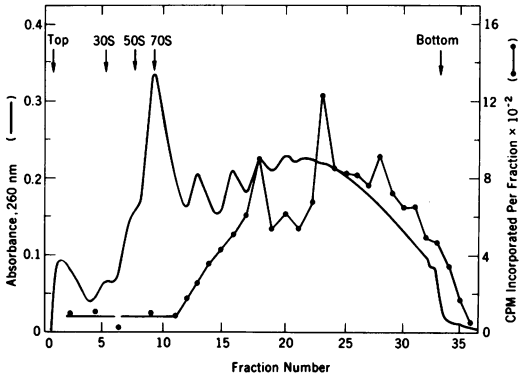

FIG. 8. *Incorporation by fractionated polysomes. Polysomes (0.8 mg of protein in 0.2 ml) were layered on a sucrose gradient and centrifuged as described in Fig. 6. The effluent from the flow-through cuvette was collected in 5-drop fractions (approximately 0.2 ml), and 0.1 ml of a solution containing the necessary components for an incorporation assay including* L-[^{14}C]-*valine,* L-[^{14}C]*leucine, and* L-[^{14}C]*alanine, and 0.4 mg of E. coli A19 S-100 was added. Incubation was for 30 min at 37 C.*

TABLE 5. *Copurification of radioactive protein products with carrier ferredoxin*

Purification step	Total radioactivity (counts/min)	Total A_{390} units	Apparent[a] total ferredoxin (mg)	A_{390}/A_{280}	Total A_{280} units	Apparent[b] total protein other than ferredoxin (mg)
Lyophilized protein product	2×10^6					
First DEAE-cellulose column	4×10^4	8.2	1.61	0.4	20.4	10.3
Second DEAE-cellulose column	4×10^3	8.0	1.57	0.71	11.2	1.3

[a] These numbers were calculated by use of a molar extinction coefficient of 30,600 at 390 nm (9) and a molecular weight of 6,000 for ferredoxin.

[b] These values were obtained as follows. The value 0.81 for the A_{390}/A_{280} ratio of pure *C. pasteurianum* ferredoxin (9) were used to calculate the "partial A_{280}" due to ferredoxin. This "partial A_{280}" value was subtracted from the "total A_{280}" value. The remainder was converted to "mg of protein other than ferredoxin" by assuming that 1 mg/ml gives an A_{280} value of 1.

product along with the reconstituted carrier ferredoxin by DEAE-cellulose column chromatography. Hong and Rabinowitz (8) have demonstrated that modifications of the N-terminus of ferredoxin or removal of the two C-terminal amino acids greatly reduces the stability of the reconstituted ferredoxin. Any ferredoxin that reverted back to apoferredoxin would be lost during the course of the purification. Therefore, the reconstitution step and the copurification, if carried to the stage of pure ferredoxin, would insure that any radioactivity remaining with the repurified carrier ferredoxin was indeed "active" (capable of being reconstituted with iron and sulfide), whole ferredoxin which had been synthesized in vitro. Finally, we have attempted to use precise calculations to determine from the radioactivity whether a significant amount of ferredoxin was synthesized in vitro.

As shown by the data in Table 5 and in the Results section, we have not been able to obtain convincing evidence for the synthesis of ferredoxin in vitro, with the use of rigorous criteria for proof. However, the large amino acid-incorporating activity of this polysome preparation suggests that further attempts are justified, perhaps with the use of ferredoxin-specific antibodies (9) as a means of identifying the protein.

ACKNOWLEDGMENTS

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