*POLYPEPTIDE CHAIN INITIATION IN E. COLI: ISOLATION OF HOMOGENEOUS INITIATION FACTOR F_2 AND ITS RELATION TO RIBOSOMAL PROTEINS**

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Abstract.—Previous work has shown that F_2, one of several ribosomal factors involved in polypeptide chain initiation, functions in the binding of formyl-methionyl-transfer RNA (fMet ~ $tRNA_f$) to a messenger RNA-ribosome complex. F_2 was isolated from 1.0 *M* ammonium chloride washes of *E. coli* Q13 ribosomes as a protein homogeneous on polyacrylamide gel electrophoresis at both pH 4.5 and 7.8. Its molecular weight is approximately 80,000. Comparison of electrophoretic patterns of ribosomal proteins from NH_4Cl-washed and unwashed ribosomes and F_2, at pH 4.5, shows that F_2 corresponds to the slowest-moving component of the proteins derived from unwashed ribosomes. This component is missing from the NH_4Cl-washed ribosomes. The activity of F_2 is stimulated by two additional factors, initiation factor F_1 and a factor(s) present in a narrow ammonium sulfate fraction of the ribosomal NH_4Cl wash. The nature of the latter is unknown.

The formation of a complex involving the 30*S* ribosomal subunit, fMet~ $tRNA_f$, and messenger RNA is an obligatory step in the initiation of protein synthesis in *E. coli*. Ribosomal initiation factors and GTP are required for this reaction.[1] Studies made to ascertain the specific role of individual initiation factors showed that F_3 functions in the binding of mRNA to the ribosomes and that F_2 functions in the binding of fMET~$tRNA_f$ to the mRNA-ribosome complex,[2] but the precise mechanism by which the initiation factors and GTP function in chain initiation is poorly understood. Recently we have found[3] that the activity of F_2, one of the initiation factors, is inhibited by SH-binding reagents. Interestingly, either GTP or 30*S* ribosomes can protect F_2 against inactivation by these agents, suggesting that F_2 has "essential" SH groups that become masked upon formation of a GTP-F_2 complex or upon binding to ribosomes. In the present paper we wish to report on the isolation of electrophoretically homogeneous F_2 and its relation to the ribosomal proteins. The involvement of SH groups in F_2 activity and the formation of a GTP-F_2 complex will be the subject of an ensuing communication.

Materials and Methods.—Ribosomes and ribosomal proteins: Purified 1.0 *M* NH_4Cl-washed *E. coli* Q13 ribosomes were prepared as previously described.[2] The ribosomal pellet was suspended in a buffer containing 250 mM NH_4Cl, 5 mM magnesium acetate, 20 mM Tris-HCl buffer (pH 7.8), 0.5 mM dithiothreitol (DTT), and 50% glycerol (v/v) at a concentration of approximately 1000 A_{260} units/ml and stored at −10°. Under these conditions, the ribosomes kept their activity for at least one month. "Unwashed" ribosomes were prepared by suspending the crude ribosomal pellet in a buffer containing 20 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl, 10 mM magnesium acetate, and 1 mM DTT, centrifuging at 105,000 × *g* for 4 hr at 2°, and repeating the buffer washing once more.

We are indebted to Miss Martha J. Miller for this preparation. Since ribosomes that have not been washed with 1.0 *M* NH_4Cl lose their activity rather rapidly (owing to presence of proteolytic enzymes?), they were used immediately after assay had shown high amino acid incorporation activity in the absence of added initiation factors and no stimulation by this addition.

Proteins were prepared for gel electrophoresis from purified and from "unwashed" ribosomes by extraction with 66% acetic acid (according to Waller[4]) followed by dialysis, for 4 hr at 4°, against β-alanine (0.35 *M*) -acetate buffer, pH 4.5, to remove excess acetic acid.

F_2 *assay:* The virtually absolute requirement of F_2 for the AUG-dependent binding of fMet~$tRNA_f$ to purified ribosomes at low Mg^{2+} concentrations was the basis for assay of F_2 activity. In the presence of F_2, binding is stimulated by the initiation factor F_1 and, as we found, is further stimulated by an ammonium sulfate fraction of the 1.0 *M* NH_4Cl ribosomal wash. This fraction will be referred to as the supplemental (S) fraction. It can be replaced by purified preparations of the initiation factor F_3, and it itself contains F_3 activity. Although it has previously been referred to as F_4,[1] it is better to designate it as fraction S until a clear-cut decision as to its nature can be made. F_1 and fraction S were always added in excess to F_2 assay samples. Unless otherwise stated, all assay samples contained, in a final volume of 0.05 ml, Tris-HCl buffer, pH 7.2, 50 mM; NH_4Cl, 150 mM; magnesium acetate, 3.5 mM; GTP, 0.2 mM; ApUpG (AUG), 0.04 A_{260} unit; fMet~tRNA labeled with ^{14}C-methionine, 20 μμmoles (4800 cpm); purified ribosomes (50*S* and 30*S* subunits), 2.5 A_{260} units; F_1 with 5.5 μg of protein; fraction S with 6 μg of protein; F_2, 0.0005–0.003 units; and glycerol, 2% v/v. Incubation was for 15 min at 25°; then binding was determined by the procedure of Nirenberg and Leder.[5] The Millipore filters were washed with a buffer containing 0.1 *M* Tris-HCl, pH 7.2, NH_4Cl (50 mM), and magnesium acetate (5 mM). They were then dried and inserted into scintillation vials. Ribosome-bound radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid contained 6 gm of 2,5-diphenyloxazole (PPO) and 100 mg of *p*bis 2-(5-phenyloxazolyl)benzene (POPOP) per liter of toluene. Net binding values were calculated by subtracting the small blanks obtained in the absence of F_2.

Under the above assay conditions, the amount of fMet~tRNA bound was proportional to the concentration of F_2 within a tenfold range of concentrations, as seen in Figure 1. One unit was defined as the amount of F_2 causing the binding of 1 mμmole/sample in 15 min. Specific activity is expressed in units/mg protein. Protein was determined spectrophotometrically.[6]

Assay components: (1) F_1 was prepared from the 1.0 *M* NH_4Cl ribosomal wash[2] with starting 100 gm of frozen *E. coli* Q13 cells. After the ribosomes had been stirred overnight in a buffer containing 1.0 *M* NH_4Cl, 20 mM Tris-HCl (pH 8.1), 2 mM magnesium acetate, and 10 mM mercaptoethanol, the suspension was centrifuged at 105,000 *g* for 4.5 hr. The supernatant was dialyzed for 6 hr against a buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 10 mM mercaptoethanol. The dialyzed solution was loaded onto a column (1.7 × 35 cm) of DEAE-cellulose previously equilibrated with the above buffer, and the column was then washed with this buffer at a rate of about 60 ml/hr. Fractions (about 1.8 ml) were collected. Those containing the peak of F_1 activity (#58 to 75), as assayed by stimulation of F_2 activity in the above assay (without fraction S), were pooled and stored at 4°.

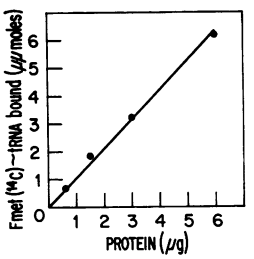

FIG. 1.—Ribosomal binding of fMet~tRNA as a function of the F_2 concentration. Standard assay with the use of step 2 F_2.

(2) Fraction S was an ammonium sulfate fraction (at 0.55–0.70 saturation) of the supernatan tremaining after ammonium sulfate precipitation of F_2 from the NH_4Cl ribosomal wash (see F_2 purification, step 2). The precipi-

tate was dissolved in a small volume of a buffer containing 20 mM Tris-HCl (pH 7.8), 20 mM NH_4Cl, 20 mM magnesium acetate, 2 mM DTT, and 50% glycerol (v/v), and stored at −10°. This fraction was suitably diluted before use in an identical buffer, except for a lower (5%) glycerol content.

(3) Crude fMet∼$tRNA_f$, labeled with ^{14}C in the methionine residue, was prepared as previously,[2] except that dialyzed *E. coli* Q13 supernatant was used as a source of Met∼tRNA synthetase and formyl transferase. When required, contaminating GTP was removed by gel filtration on Sephadex G-50.

(4) The trinucleoside diphosphate ApUpG (AUG) was a product of the Miles Laboratories, Inc.

Other assays: Purified F_2 was assayed for contamination with other activities as follows: Exonuclease was assayed by Miss Martha J. Miller with ^{14}C-poly A as substrate, as described by Salas *et al.*[7] Elongation factors T and G were kindly assayed[8, 9] by Dr. Julian Gordon, Rockefeller University. GTPase activity was assayed in the absence and presence of ribosomes, as described by Kolakofsky *et al.*,[10] with GTP labeled with ^{32}P in the γ phosphate. This was diluted with unlabeled GTP to a specific radioactivity of 355 cpm/$\mu\mu$mole.

Gel electrophoresis: Disc electrophoresis on polyacrylamide gels was run either at pH 4.5 or 7.8. Electrophoresis at pH 4.5 was carried out by the procedure of Reisfeld *et al.*[11] with some modifications. All stock solutions used for preparing the gels contained 8.0 *M* urea, deionized as described by Duesberg *et al.*[12] The final concentrations (w/v) of acrylamide and *bis*acrylamide in the separating gels were 7.5% and 0.3%, respectively. The corresponding concentrations in stacking gels were 3.5% and 0.42%. Electrophoresis was conducted for 4 hr at 25° and 90 v in β-alanine (0.35 *M*)-acetate buffer, pH 4.5.[11] The column length was 11 cm.

For electrophoresis at pH 7.8, the separating gel contained 0.375 *M* Tris-HCl buffer (pH 7.8), 0.06% (v/v) tetramethylethylenediamine (TEMED), 7.5% acrylamide, and 0.2% *bis*acrylamide (w/v). The stacking gel contained 0.046 *M* Tris-HCl buffer (pH 6.8), 0.04% TEMED, 3.5% acrylamide, and 0.2% *bis*acrylamide. The column length was 7 cm. Electrophoresis was conducted for 7 hr at 25° and 200 v in Tris (4.96 mM) -glycine (38.6 mM) buffer, pH 8.3.[13] After electrophoresis the gels were stained with amido black and then destained with 7% acetic acid.

Miscellaneous: Dithiothreitol (DTT) was obtained from Nutritional Biochemicals. Sephadex G-200 (Pharmacia, particle size 40–120 μ) was treated prior to use with 2 mM EDTA, and the EDTA was removed by a thorough washing with water. DEAE-SH cellulose (Serva, 0.83 meq/gm) was washed successively with 0.2 *N* NaOH, water, 0.2 *N* HCl, water, and suspended in a buffer containing 20 mM NH_4Cl, 20 mM Tris-HCl, (pH 7.8), 0.2 mM magnesium acetate, 2 mM DTT, and 5% glycerol (v/v). Hydroxylapatite (Hypatite C, lot #6338) was a product of the Clarkson Chemical Co. Other reagents were as used in previous work.[2]

Results and Discussion.—Purification of F_2: F_2 was purified from the 1.0 *M* NH_4Cl-wash of *E. coli* Q13 ribosomes. All operations were carried out at 0–4° unless otherwise stated.

Step 1. NH_4Cl ribosomal wash: About 600 gm of frozen cells were processed each day. The cells were thawed and evenly suspended in 600 ml of a buffer (buffer A) containing 20 mM Tris-HCl, (pH 7.8), 10 mM magnesium acetate, and 2 mM DTT. 100-ml portions of this suspension were sonicated for two minutes in a Benson model S-125 sonic oscillator. The temperature of the suspension after sonic disintegration never exceeded 9°C. The suspension was centrifuged for 20 minutes at 30,000 *g* in a refrigerated Serval RC 2-B centrifuge. The supernatant was stirred with DNase (3 μg/ml) in the cold for a few minutes and centrifuged as above (S30 extract). The S30 extract

was centrifuged for 2.5 hr at 160,000 g in the #50 rotor of the Spinco model L ultracentrifuge. After a thorough draining, the ribosomal pellets were suspended in 250 ml of a buffer (buffer B) containing 1.0 M NH_4Cl, 20 mM Tris-HCl buffer (pH 7.8), 2 mM magnesium acetate, and 2 mM DTT, and stirred overnight. The suspension was centrifugated at 160,000 g as outlined above, and the resulting supernatant (1.0 M NH_4Cl wash) was stored at 2–4°. A total of 4 kg of frozen cells was worked up in this manner, yielding 1.664 ml of solution with 12.4 mg of protein/ml.

Step 2. Ammonium sulfate fractionation: About one third of the 1.0 M NH_4Cl wash was processed each time. Finely powdered ammonium sulfate (19.4 gm/100 ml) was added slowly while the mixture was being stirred. After 20 to 30 minutes, the mixture was centrifuged for 10 to 15 minutes at 30,000 g in the refrigerated Sorvall centrifuge, and the precipitate was discarded. More ammonium sulfate 5.7 gm/100 ml of supernatant was added. The mixture was centrifuged as above. The precipitate (0.35–0.45 saturation) was dissolved in a minimal volume of a buffer (buffer C) containing 20 mM NH_4Cl, 20 mM Tris-HCl (pH 7.8), 0.2 mM magnesium acetate, 2 mM DTT, and 5 per cent glycerol (v/v), and was stored at $-10°$. Prior to step 3, the pooled solutions were diluted with buffer C to a protein concentration of 20 mg/ml, dialyzed for three hours against several changes of buffer C and then dialyzed for another hour against the same buffer, except that the pH of the Tris-HCl was 7.0. A precipitate that formed during dialysis was removed by centrifugation and discarded. This step yielded 149 ml of solution with 15.2 mg of protein/ml. The ammonium sulfate supernatant (0.45 saturation) was used for the preparation of fraction S as described in *Materials and Methods.*

Step 3. Adsorption and elution from calcium phosphate gel and ammonium sulfate fractionation: The solution from the previous step was diluted to a protein concentration of 5 mg/ml with buffer C (pH 7.0). Calcium phosphate gel (30 mg dry weight/ml) was added in the proportion of 1 mg gel/mg protein and the mixture was stirred for 30 minutes. The gel was centrifuged down, suspended in 180 ml of 0.01 M potassium phosphate buffer, pH 7.5 (containing 10 mM mercaptoethanol), with the aid of a glass homogenizer and stirred for 20 minutes. The suspension was then centrifuged. The precipitated gel was successively eluted, as described above, with 80 ml of 0.03, 0.2, and 0.3 M potassium phosphate buffer (pH 7.5) containing 10 mM mercaptoethanol. The eluates containing F_2 of highest specific activity (usually 0.03 and 0.2 M phosphate) were pooled. A 0.35–0.45 saturation ammonium sulfate fraction was obtained from the pooled eluates as described in step 2. The precipitate was dissolved in a minimal volume of a buffer (buffer D) containing 20 mM Tris-HCl (pH 7.8), 20 mM, NH_4Cl, 0.2 mM magnesium acetate, 2 mM DTT, and 50 per cent glycerol (v/v). This yielded 21 ml of solution with 22 mg of protein/ml. It was stored at $-10°$.

Step 4. Sephadex G-200 chromatography: The solution from the previous step was put on a Sephadex G-200 column (2.7 × 75 cm) previously equilibrated with buffer C. The column was then washed with the same buffer at a flow rate of about 14 ml/hr, and 3.0-ml fractions were collected. The fractions of

highest specific activity (82 through 100) were pooled, yielding 55.5 ml of solution with 1.8 mg of protein/ml.

Step. 5. DEAE-cellulose chromatography: The solution from the above step was immediately loaded onto a column (2.2 × 45 cm) of DEAE-cellulose previously equilibrated with buffer C. Elution was carried out at a flow rate of 16 ml/hr with a 0.02–0.3 *M* linear gradient of NH_4Cl in buffer C (300 ml of buffer C in the mixer and 300 ml of 0.3 *M* NH_4Cl in buffer C in the reservoir). Fractions (about 2.6 ml) were collected, and those containing F_2 of highest specific activity (150 through 160) were pooled and immediately dialyzed overnight against a solution (adjusted to pH 7.5) containing 50 mM Tris-HCl, 20 mM NH_4Cl, 2 mM magnesium acetate, 10 mM mercaptoethanol, and ammonium sulfate at 0.70 saturation (buffer E). The precipitated protein was collected by centrifugation, dissolved in a minimal volume of buffer D, and stored at −10°. This yielded 0.6 ml of solution with 17.5 mg of protein/ml.

Step 6. Hydroxylapatite chromatography: The solution from step 5 was diluted to 2.5 ml with buffer C and dialyzed for 2.5 hours against several changes of the same buffer. The dialyzed solution was transferred to a column (0.8 × 19 cm) of hydroxylapatite previously equilibrated with buffer C containing, in addition, 1.0 mM potassium phosphate buffer, pH 7.5. Elution was carried out at a flow rate of about 2.0 ml/hr, with a 0.02–0.2 *M* linear gradient of potassium phosphate in buffer C (50 ml of 0.02 *M* potassium phosphate, pH 7.5 in buffer C in the mixer and 50 ml of 0.2 *M* potassium phosphate, pH 7.5, in buffer C in the reservoir). Fractions (of 1.1 ml) were collected. Fractions 24 through 36 were pooled and immediately dialyzed overnight against buffer E. The precipitate was dissolved in a minimal amount of buffer D, yielding 0.5 ml of solution with 4.4 mg of protein/ml, and stored at −10°. A summary of the purification procedure is given in Table 1.

From the data in Table 1, it can be calculated that 220 mg (about 1%) of the protein (20,640 mg) in the ribosomal wash at step 1 is F_2 protein. The

TABLE 1. *Purification of F_2.**

Step	Volume (ml)	Protein (mg)	Units†	Specific activity‡	Yield (%)
1. NH_4Cl-wash of ribosomes	1,664	20,640	3,483	0.17	100
2. $(NH_4)_2SO_4$ fractionation	149	2,265	1,314	0.58	38
3. Adsorption and elution from $Ca_3(PO_4)_2$ gel	21	455	602	1.34	17
4. Sephadex G-200 chromatography	55	100	330	3.3	9.5
5. DEAE-cellulose chromatography	0.6	10.5	107	10.2	3.2
6. Hydroxylapatite chromatography	0.5	2.2	35.7	16.2	1.0

* From 4 kg of frozen *E. coli* Q13 cells.
† 1 unit = 1.0 mμmole of fMet(^{14}C)~tRNA bound under standard assay conditions (15 min, 25°).
‡ Units/mg protein.

reasons for the very low F_2 yields are not clear. A preparation obtained by a slightly different procedure gave similar yields.

Properties and purity of F_2: The A_{280}/A_{260} ratio of the step 6 preparation was 1.8 (A_{280}/A_{260} at step 1, about 0.6). Disc electrophoresis on polyacrylamide gel at pH 4.5 and 7.8 (Fig. 2) showed that step 6 F_2 is homogeneous. This was not the case at step 5.

Gel filtration data suggested a molecular weight of about 100,000. A preliminary determination of the molecular weight of F_2 was carried out by the meniscus depletion method of Yphantis,[14] which employs the Spinco UV scanning

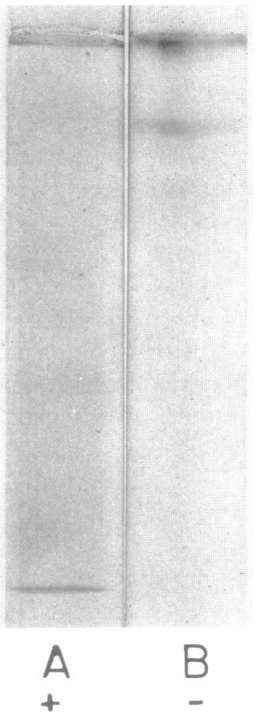

FIG. 2. — Polyacrylamide gel electrophoresis of F_2 (step 6). (*A*) pH 7.8; F_2, 20 μg. (*B*) pH 4.5; F_2, 8 μg. Origin at top in both cases. In (*A*) the anode, and in (*B*) the cathode, is at the bottom.

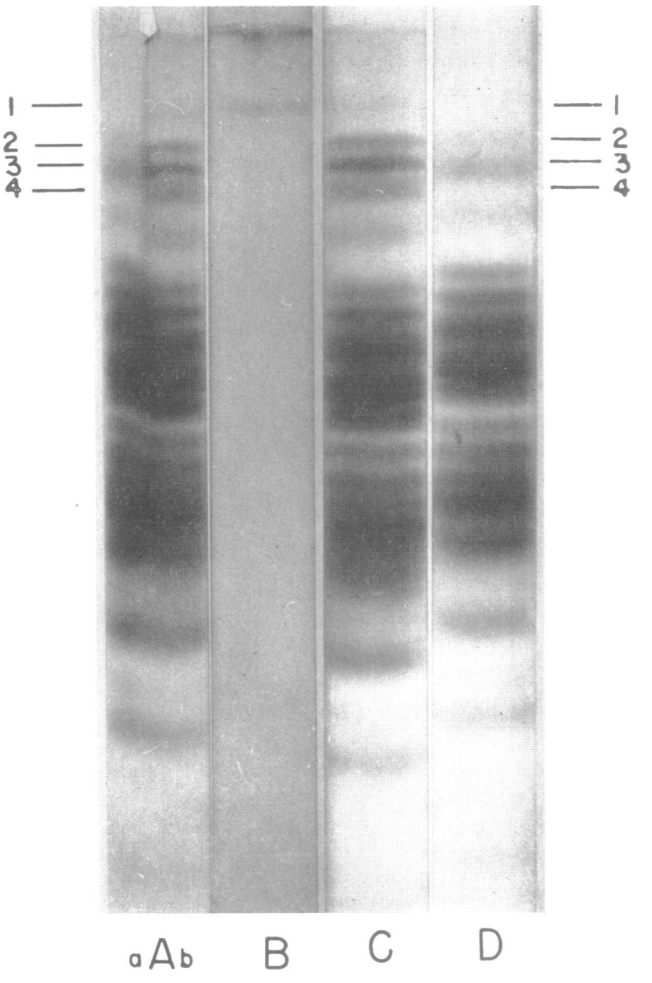

FIG. 3.—Polyacrylamide gel electrophoresis (pH, 4.5) of F_2 and of proteins from 1.0 *M* NH_4Cl-washed and unwashed 70*S* ribosomes. Origin at top, cathode at bottom. (*A*) Split gel with (*a*) protein (40 μg) from NH_4Cl-washed ribosomes or (*b*) protein (40 μg) from unwashed ribosomes. (*B*) F_2 (8 μg). (*C*) Protein (80 μg) from unwashed ribosomes. (*D*) Protein (80 μg) from NH_4Cl washed ribosomes. By numbering the first few protein bands from the top it is shown that in *A* (*a*) and *D*, band 1 (F_2) and band 4 are missing from the washed ribosomes, and bands 2 and 3 are fainter.

system at 280 mμ. A determination at one F_2 (step 6) concentration (0.27 mg/ml) gave a linear plot of log ΔA against the square of the radius and yielded a molecular weight of 80,000 daltons.

Despite physical homogeneity, F_2 (step 6) was found to have small amounts of other contaminating activities such as exonuclease and elongation factor G.

(1) Exonuclease assays showed that 1.6 μg of F_2 released 0.5 mμmole of ^{14}C-AMP from ^{14}C-poly A in 15 minutes at 37°, which corresponds to a specific nuclease activity of 1.25 μmoles/hr/mg protein. Assuming that the bulk of the contaminating nuclease is RNase II, it can be calculated from Singer's[15] data for the specific activity of pure *E. coli* RNase II (2120 μmoles/hr/mg protein, at 37°) that RNase II contamination of F_2 amounts to about 0.06 per cent.

(2) Specific assay[8] of F_2 for G factor activity, as measured by poly U-dependent polyphenylalanine synthesis from Phe(^{14}C)~tRNA in the presence of excess T factor, gave a specific activity of 2.4 mμmoles/10 min/mg, at 30°. The simultaneously measured specific activity of the purified G factor was 400. This represents a 0.6 per cent contamination. G factor activity in F_2 was also determined by measuring the ribosome-stimulated GTPase activity. The specific activity was 0.09 μmole/10 min/mg, at 30°. If the specific GTPase activity of purified G factor is taken to be 20,[8] contamination of F_2 with G factor, as measured by this assay, is 0.45 per cent. This value is in good agreement with the previous one.

(3) 1.9 μg of F_2 gave no detectable T factor activity as assayed[8] in the presence of excess G.

F_2 (step 6) is presumably free from initiation factor F_1 and has no F_3 activity. From the data given in this section, the purity of the preparation may be 99 per cent or higher.

Relation of F_2 to ribosomal proteins: A comparison of gel electrophoretic patterns, at pH 4.5 (8.0 *M* urea), of F_2 (step 6) and of the proteins from "unwashed" and 1.0 *M* NH_4Cl-washed 70 *S* ribosomes is shown in Figure 3. Four of the more slowly moving components seen in the electropherogram of "unwashed" ribosomes (columns *A*(*b*) and *C*) are either missing (bands 1 and 4) or reduced in amount (bands 2 and 3) in the NH_4Cl-washed ribosomes (columns *A*(*a*) and *D*). The most acidic component (band 1) corresponds to F_2 (cf. columns *A*(*b*), or *C* and *B*). Gel electrophoresis at pH 4.5 of proteins derived from the NH_4Cl ribosomal wash shows the presence of bands 1 through 4 along with other more basic proteins.

Effect of F_1 and fraction S on the activity of F_2: Partially purified preparations of F_2 (e.g., those described by Salas *et al.*),[16] as assayed by AUG-dependent ribosomal binding of fMet~$tRNA_f$, were always found to be active to a greater or lesser extent without the addition of other initiation factors. However, their activity was stimulated by F_1 and, as reported in this paper, by fraction S. It was therefore of interest to determine whether homogeneous F_2 was still active in the absence of further factor additions. As shown in Table 2, F_2 alone had 24 per cent of the activity obtained in the additional presence of F_1 and fraction S, and 37 or 33 per cent of the activity obtained upon the addition

TABLE 2. *Effect of F_1 and fraction S on activity of F_2.**

Factor additions	fMet(^{14}C)~tRNA bound ($\mu\mu$moles)	$b - a$
(*a*) None	0.40	1.00
(*b*) F_2	1.40	
(*a*) F_1	0.44	2.69
(*b*) $F_2 + F_1$	3.13	
(*a*) S	0.25	3.03
(*b*) F_2 + S	3.28	
(*a*) F_1 + S	0.27	4.12
(*b*) $F_2 + F_1$ + S	4.39	

* Conditions of standard assay. The amounts of factors/sample were as follows: F_2 (step 6), 0.3 μg; F_1, 5.5 μg protein; and fraction S, 4.5 μg protein. Fraction S was a preparation obtained by ammonium sulfate fractionation and DEAE-cellulose chromatography.

of F_1 or fraction S, respectively. Whether the activity of step 6 F_2 by itself in the standard assay means that it can function in the absence of additional initiation factors, or whether this activity is due to contamination of the ribosomes with other factors, remains an open question at present.

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