The Irreversible Step in Formation of Initiation Complexes of *Escherichia coli*†

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ABSTRACT: At some stage during initiation the ribosomal subunits of *Escherichia coli* must become irreversibly coupled, since polysomal ribosomes, in contrast to free ribosomes, are not dissociated by initiation factor IF-3. To determine when irreversibility develops we have compared the response to IF-3 of mature, puromycin-reactive initiation complexes, made with GTP, and of intermediate, puromycin-unreactive complexes, made with GMPPCP. The latter complexes initially appeared to be dissociated by the factor, but this effect was found to be due to artificial loss of the ligands at the Mg^{2+} concentration customary in the test for dissociation. At a slightly higher Mg^{2+} concentration (4 m*M*), sufficient to retain the ligands, the GMPPCP complexes were not significantly dissociated by IF-3, at concentrations that caused complete dissociation of free ribosomes. It thus appears that the intermediate 70S initiation complex, though less stable to ionic dissociation than the mature complex, is in effect irreversible under physiological conditions.

The free 70S ribosomes produced by polysome runoff in bacteria (Kohler et al., 1968; Subramanian and Davis, 1973) reinitiate with the aid of three initiation factors.[1] In this process IF-3 causes net dissociation of free ribosomes into subunits (Subramanian and Davis, 1970) by reversibly forming 30S–IF-3 complexes (Beller et al., 1974; Sabol et al., 1973). Addition of IF-1 and IF-2 yields native 30S subunits, and these become 30S initiation complexes by addition of fMet-tRNA, GTP, and mRNA, accompanied by release of the IF-3 (Mazumder, 1972; Dubnoff et al., 1972; Vermeer et al., 1973). A 50S subunit is then added, to yield a presumed intermediate 70S complex; and hydrolysis of the GTP, accompanied by release of the IF-1 and IF-2, results in a mature 70S initiation complex. When the nonhydrolyzable analog GMPPCP is used instead of GTP the sequence stops at a stable, model intermediate 70S initiation complex, which retains the IF-2 and GMPPCP (Thach and Thach, 1971; Dubnoff et al., 1972; Benne and Voorma, 1972; Benne et al., 1973a); in contrast to the mature complex, the intermediate cannot transfer its fMet to puromycin (Hershey and Thach, 1967).

On theoretical grounds, supported by the observed failure of IF-3 to dissociate polysomal ribosomes (Subramanian et al., 1969), it seems clear that some step in the initiation sequence must be irreversible. Since IF-3 can cause the 30S initiation complex to release its fMet-tRNA (Dubnoff et al., 1972), it appears that some later step is the first irreversible one. The present results indicate that the complex made with GMPPCP is not dissociated by IF-3, thus suggesting that irreversibility is achieved with formation of the intermediate complex, before hydrolysis of GTP.

Materials and Methods

Materials. Trinucleotide AUG, random poly(A,U,G), and GMPPCP were obtained from Miles Laboratories; poly(U,G) (3:1) was from Schwarz Bioresearch; and GTP was from Sigma. Unfractionated stripped tRNA, from Nutritional Biochemicals, was charged with [^{3}H-methyl]methionine (4.0 Ci/mmol, New England Nuclear), in the absence of the other amino acids, under formylating conditions (Hershey and Thach, 1967). The specific activity was 2500 cpm/pmol of f[^{3}H]Met-tRNA. Phage R17 RNA was prepared by the method of Gesteland and Boedtker (1964). Cells of *Escherichia coli* strain Q13 were purchased from General Biochemicals. The buffers used were filtration buffer (10 m*M* Tris-HCl (pH 7.6), 60 m*M* NH_4Cl, and 10 m*M* $Mg(OAc)_2$), TKD (10 m*M* Tris-HCl (pH 7.6), 50 m*M* KCl, and 1 m*M* dithiothreitol), TKM_xD (TKD plus *x* m*M* $Mg(OAc)_2$), and TAM_xD_y (50 m*M* Tris-HCl (pH 7.6), 100 m*M* NH_4Cl, *x* m*M* $Mg(OAc)_2$, and *y* m*M* dithiothreitol).

Salt-Washed Ribosomes. All operations were done at 0–4° unless otherwise noted. S-30 extracts of *E. coli* MRE600 were prepared according to Modolell and Davis (1968), except that the alumina-ground cells were extracted with $TKM_{10}D$ buffer (1.5 ml/g). After sedimentation the ribosomes were resuspended in TKM_5D, one-third volume of 4 *M* NH_4Cl was added, and the solution was allowed to stand overnight at 0°. The ribosomes were then pelleted by centrifugation for 2 hr at 150,000*g*, resuspended in $TKM_{10}D$ with 10% glycerol to an A_{260} of 500, and stored in small portions at −76°.

Initiation Factors. To prepare crude IF the supernate from the salt-washed ribosomes was 70% saturated with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 20,000*g*, redissolved in TKD at 10–20 mg of protein/ml, and dialyzed against the same buffer for 1–2 hr.

IF-1, IF-2, and IF-3 were further purified from the 1 *M* NH_4Cl ribosome wash of *E. coli* strain Q13, obtained as described above except that the cells were broken by sonication. The factors, separated by the DEAE-cellulose chromatography step in the procedure of Dubnoff and Maitra (1971), proved to be cleanly fractionated from one another. Each factor was precipitated by $(NH_4)_2SO_4$, dissolved in TKD, and dialyzed against this buffer. IF-3 was further purified according to Dubnoff and Maitra (1971) to a single

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[1] Abbreviations used are: IF, initiation factor; GMPPCP, 5′-guanylylmethylene diphosphonate.

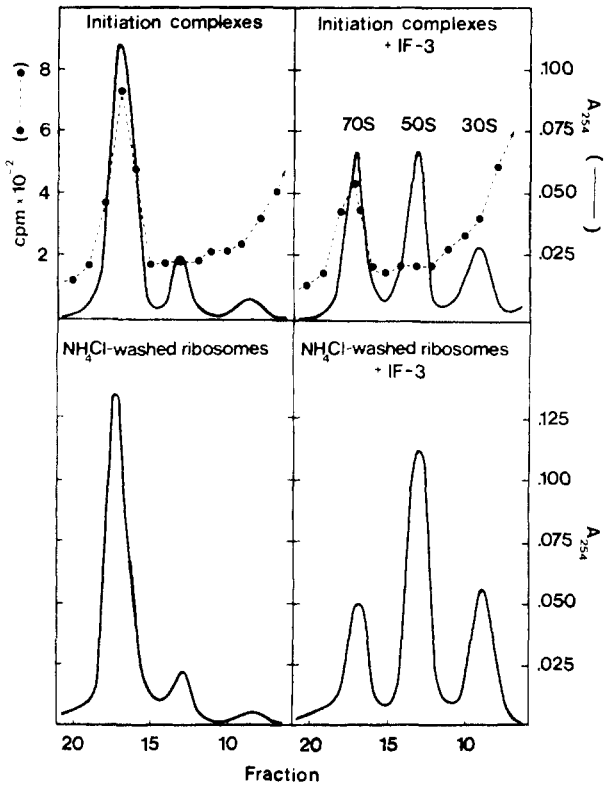

FIGURE 1: Effect of IF-3 on uncentrifuged GTP initiation complexes and on free ribosomes. Initiation complexes were made with triplet AUG, GTP, and f[^{3}H]Met-tRNA, in the presence of 5 mM Mg^{2+}, as described under Materials and Methods. Portions containing 0.4 A_{260} unit of ribosomes were removed and diluted fivefold in $TAM_{2.4}D$ (final Mg^{2+} = 3 mM), and to one portion pure IF-3 (3 μg) was added. NH_4Cl-washed ribosomes at the same concentration were similarly incubated with and without IF-3. After incubation for 3 min at 37° the reaction mixtures were chilled to 0°, layered on sucrose gradients in TKM_5, and analyzed for A_{254} and for radioactivity as described.

band on acrylamide gel electrophoresis, concentrated by dialysis against 20 mM NH_4HCO_3 (pH 7.0), and lyophilized. The IF-3 was dissolved in TKD before use.

Initiation Complexes. Reaction mixtures, in TAM_5D_2 with 1 mM GTP or GMPPCP (as indicated), contained per milliliter 20 A_{260} units of NH_4Cl-washed ribosomes; 350 pmol of f[^{3}H]Met-tRNA; either 1 mg of R17 RNA, or 0.25 mg of AUG or poly(A,U,G), or 0.05 mg of poly(U,G); approximately 0.5 mg of crude IF or 0.2 mg each of the DEAE-cellulose IF-1, IF-2, and IF-3 fractions. (The concentrations of the individual IF's in the crude preparation are difficult to measure, but the IF-2 would have to be at least equimolar with the ribosomes in order to form stable complexes with GMPPCP (Benne et al., 1973b).) Binding of f[^{3}H]Met-tRNA to ribosomes was assayed by filtration (Nirenberg and Leder, 1964), after incubation with synthetic messenger for 15 min at 22° or with phage RNA for 10 min at 37°. Values obtained in parallel mixtures without messenger were subtracted.

To separate the initiation complexes (together with unreacted ribosomes) from the supernate the incubation mixtures described above (usually 1 or 2 ml) were diluted with excess cold filtration buffer plus 1 mM dithiothreitol and were centrifuged at 150,000g for 1 hr. Pellets were dissolved in the same buffer with 10% glycerol at 100–200 A_{260}/ml and were stored in small portions at −76°.

The release of f[^{3}H]Met from initiation complexes by puromycin was measured by incubation for 3 min at 37° in TAM_5D_1, with or without the antibiotic (200 μg/ml); residual complexed radioactivity was determined by filter assay.

Dissociation by IF-3. Assays were performed as described (Gottlieb et al., 1974): approximately 0.5 A_{260} of initiation complexes, in a final volume of 100 μl of TKM_3D (or other medium as specified), was incubated with indicated amounts of IF-3 for 3 min at 37°. With uncentrifuged initiation complexes the reaction mixtures were diluted to yield the desired [Mg^{2+}]. The controls (NH_4Cl-washed ribosomes) were incubated under identical conditions but without mRNA and f[^{3}H]Met-tRNA.

Gradient Analyses. Reaction mixtures (containing 0.5 A_{260} unit of ribosomes) were layered on 4.8 ml of 10–30% linear sucrose gradients in TKM_5 (unless otherwise indicated) and were centrifuged at 45,000 rpm for 70 min in a Spinco SW50.1 rotor. A_{254} was analyzed with a recording ISCO gradient fractionator, followed by cutting out and weighing tracings of the appropriate peaks. Five-drop fractions were collected and counted in Bray's scintillation fluid after addition of 0.9 ml of water.

Results

Conditions Required for the Stability of Mature Initiation Complexes. Mature initiation complexes were prepared from NH_4Cl-washed ribosomes with GTP, f[^{3}H]Met-tRNA, and viral RNA or a synthetic polynucleotide, as described under Materials and Methods. In the products about 50% of the ribosomes were complexed with f[^{3}H]Met-tRNA (as shown by filtration analysis), 30% were free, and 20% were unlabeled but resistant to dissociation (i.e., fragmented polysomes surviving in the washed ribosome preparation, or damaged ribosomes). The nondissociable (labeled or unlabeled) ribosomes were distinguished from free ribosomes by their failure to be dissociated by pure IF-3 (see Figure 2), or by sedimentation (data not shown) at appropriate concentrations of Na^+ or K^+ (Beller and Davis, 1971; Beller and Lubsen, 1972).

Attempts to simulate intracellular conditions by the use of crude IF were unsuccessful, for the preparation caused extensive dissociation of mature initiation complexes, just as with free ribosomes. The cause appeared to be contamination by a deacylase or a nuclease, for on incubation of f[^{3}H]Met-tRNA with the crude IF, in the absence of ribosomes, the radioactivity became cold-acid-soluble. Accordingly, pure IF-3 was used in all subsequent experiments.

When incubated with pure IF-3 the mature initiation complexes still exhibited some breakdown, as shown by the loss of some radioactivity (but less than the loss of A_{254}) from the 70S region (Figure 1). This partial loss was apparently due to the action of enzymes retained in the incubation mixture used to make the complexes, for when the ribosomal particles were separated from that mixture by centrifugation and then were incubated with IF-3, at a concentration beyond that required for maximal dissociation of NH_4Cl-washed ribosomes, the initiation complexes were fully stable, i.e., no f[^{3}H]Met-tRNA was lost from the 70S region of the gradient (Figure 2). The uncomplexed ribosomes in the centrifuged preparation provided an internal control, their dissociation by IF-3 being shown by a decrease in the uv-absorbing material in the 70S region.

Apparent Stability of Centrifuged GMPPCP Complexes. Intermediate-stage complexes, formed with GMPPCP instead of GTP and then separated from the reaction mixture by centrifugation, initially appeared to be irreversible, for in the usual assay they were completely stable to IF-3 (in

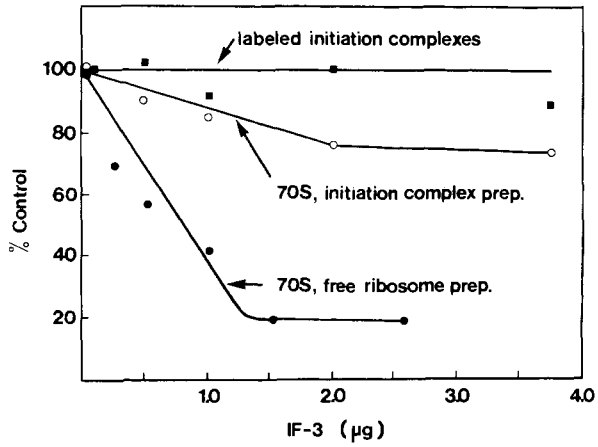

FIGURE 2: Effect of varying IF-3 on centrifuged GTP initiation complexes and on free ribosomes. Initiation complexes were made with R17 RNA, centrifuged (after eightfold dilution with filtration buffer), and resuspended as described under Materials and Methods. 0.5 A_{260} unit of this preparation (○), or of NH_4Cl-washed ribosomes (●) was incubated for 3 min at 37° in TAM_3D_1 with varying amounts of IF-3. Samples were analyzed as in Figure 1. Surviving initiation complexes (■) were determined as counts in the 70S region. Control without IF-3 = 100%.

amounts that completely dissociated comparable quantities of free ribosomes). However, this result turned out to be due to another artefact. Benne and Voorma (1972) and Dubnoff et al. (1972) have observed that during gel filtration GMPPCP complexes readily lose IF-2 and GMPPCP, and this loss, like that following GTP hydrolysis and release, allows the fMet-tRNA to become puromycin reactive. It turns out that centrifugation has a similar effect: uncentrifuged GMPPCP complexes (made with phage R17 RNA) were mostly unresponsive to puromycin (20% release), but after centrifugation they became as responsive as control complexes made with GTP (80% release). Maturation was also shown by tests in gradients containing 175 m*M* KCl instead of the usual 50 m*M*, which dissociate free ribosomes but not mature initiation complexes (Beller and Lubsen, 1972). The purified preparations thus could not be used to characterize the complexes formed with GMPPCP.

The GMPPCP complexes rendered puromycin reactive by centrifugation could be made nonreactive again by addition of IF-2 and GMPPCP (Table I), just as has been observed with GMPPCP complexes rendered reactive by gel filtration (Benne and Voorma, 1972). This reversibility suggests that the shift from an intermediate to a mature complex involves only a small energy change.

Apparent Dissociability of Uncentrifuged GMPPCP Complexes. To avoid alteration of the GMPPCP complexes by centrifugation the original reaction mixtures were tested with IF-3, after dilution of the Mg^{2+} to the usual 3 m*M*. Though mature complexes in similar preparations had suffered some artificial enzymatic breakdown during incubation enough survived to raise the specific activity of the 70S peak (see Figure 1 above); hence unpurified complexes could be used to test for dissociability by IF-3.

This test, under the standard conditions (3 m*M* Mg^{2+}), proved unsatisfactory with GMPPCP complexes, for even without IF-3 the fMet-tRNA was rapidly lost (Figure 3). (GTP complexes, in a parallel incubation, are seen to have exhibited only a slight loss, which was probably due to enzymes carried over from the first incubation.) Moreover, when incubated for 3 min at various Mg^{2+} concentrations

Table I: Restoration of Puromycin Unreactivity by Addition of IF-2 and GMPPCP to Centrifuged Initiation Complexes.

Addition during Preincubation	Release of fMet-tRNA by Puromycin (%)
None	63
IF-2 (10 μg)	42
GMPPCP (1 m*M*)	55
IF-2 + GMPPCP	1

[a] A preparation of "intermediate" initiation complexes made with f[^{3}H]Met-tRNA, poly(U,G), and GMPPCP, and including unreacted free ribosomes, was centrifuged, and the particles were resuspended and diluted in TAM_5D_2. Samples of 0.5 A_{260} in 50 μl were preincubated for 5 min at 22° with additions as indicated. Release by puromycin was then determined (see Materials and Methods).

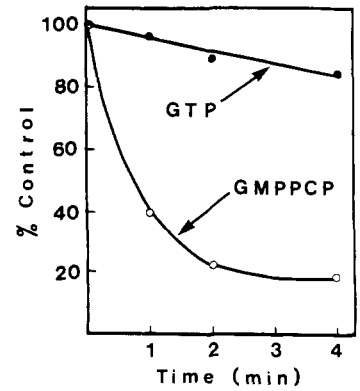

FIGURE 3: Time course of decay of GTP and GMPPCP initiation complexes at 3 m*M* Mg^{2+}. Initiation complexes, directed by poly(U,G), were made in parallel 100-μl mixtures with either GMPPCP or GTP (incubation for 15 min at 22°). Samples (20 μl) were diluted fivefold in $TAM_{2.4}D$, incubated for indicated times at 37°, and chilled by adding 2 ml of cold filtration buffer (10 m*M* Mg^{2+}). Bound f[^{3}H]Met-tRNA was determined by filtration (see Materials and Methods). Zero time control = 100% initiation complexes.

the GMPPCP complexes lost half their fMet-tRNA at 3 m*M* Mg^{2+}, while the GTP complexes required lowering to 2 m*M* (Figure 4), a concentration that strains the ribosomes enough to cause visible dissociation of free ribosomes (Subramanian and Davis, 1970). It is therefore clear that the GMPPCP complexes are less stable to low Mg^{2+} than the GTP complexes, and their apparent responsiveness to IF-3 under the standard conditions (at 3 m*M* Mg^{2+}) could be explained by their rapid conversion to free ribosomes.

Stability of GMPPCP Complexes at an Adequate Mg^{2+} Concentration. The GMPPCP complexes were therefore studied at a slightly higher Mg^{2+} concentration, 4 m*M*. Under these conditions they were found to be stable: even large amounts of IF-3 did not cause any loss of fMet-tRNA, as measured by filtration (Figure 5b). Moreover, after incubation with IF-3 a gradient analysis showed negligible (13%) loss of counts in the 70S region, while the free ribosomes (36% of the 70S particles) were dissociated (Figure 6). It thus appears that at a Mg^{2+} concentration adequate to hold together GMPPCP complexes they are no longer reversible by IF-3, in contrast to free ribosomes; hence they evidently are not in equilibrium with free ribosomes or with their subunits.

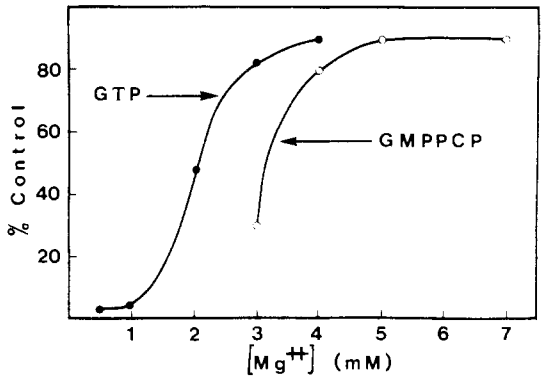

FIGURE 4: Decay of GTP and GMPPCP initiation complexes as a function of [Mg^{2+}]. Conditions as in Figure 3, except that the preparations were diluted into buffers yielding final Mg^{2+} as indicated. Samples were then incubated at 37° for 3 min, chilled, and analyzed as in Figure 3. Amount of complexes at zero time was taken as 100% control.

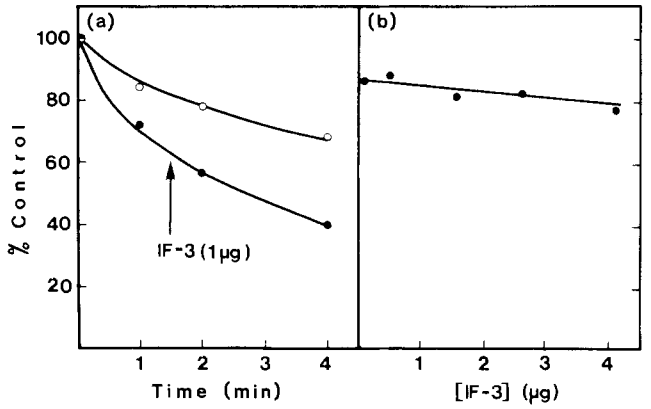

FIGURE 5: Decay of GMPPCP complexes at 3 and 4 mM Mg^{2+}. (a) GMPPCP complexes were formed and diluted as in Figure 3, with final Mg^{2+} = 3 mM. Tubes with (●) or without (○) addition of 1 μg of IF-3 were incubated for indicated times at 30°, chilled, and assayed by filtration. Zero time control = 100% initiation complexes. (b) Same as (a) except that [Mg^{2+}] was 4 mM, IF-3 was added as indicated, and incubation was for 3 min at 37°. Zero time control (100%) was without IF-3.

At 3 mM Mg^{2+}, at which GMPPCP complexes undergo rapid spontaneous breakdown, this breakdown could be accelerated by a moderate concentration of IF-3 (Figure 5a). (The spontaneous breakdown is slower than in Figure 3 because of the lower temperature.) The significance of this finding will be discussed below.

Discussion

At some stage during or following initiation the coupling between ribosomal subunits becomes irreversible, i.e., IF-3 no longer causes their net dissociation. We have sought to determine whether this irreversible state is attained at an intermediate stage of initiation (i.e., with the fMet-tRNA stabilized in a puromycin-unreactive position by GMPPCP and IF-2) or only at a later stage (e.g., with the fMet-tRNA in a puromycin-reactive position).

The incubation mixtures for complex formation were found to contain enzymes that caused partial breakdown of the GTP complexes, and so it seemed desirable to work with complexes that had been separated from their incubation mixtures. However, it turned out that such separation by centrifugation (at 10 mM Mg^{2+}), like gel filtration (Benne and Voorma, 1972; Dubnoff et al., 1972), radically altered

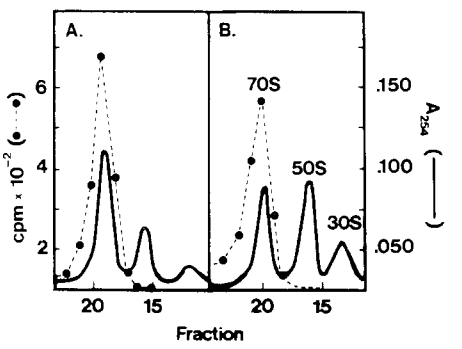

FIGURE 6: Effect of IF-3 on GMPPCP complexes and free ribosomes at 4 mM Mg^{2+}, assayed by gradient centrifugation. Conditions as in Figure 5b, with 3 μg of IF-3. Samples taken before incubation (A) and after incubation (B) were chilled, layered on gradients, and analyzed.

the GMPPCP complexes: the fMet-tRNA became puromycin reactive, presumably by loss of the GMPPCP and IF-2. Hence it was necessary to revert to the use of unpurified complexes.

Another artefact, opposite in effect, was then encountered with GMPPCP complexes. In the standard test for dissociation by IF-3, at 3 mM Mg^{2+}, these complexes not only were dissociated but were dissociated as extensively as free ribosomes. This unexpectedly high reactivity was explained by the finding that at this Mg^{2+} concentration GMPPCP complexes (unlike mature complexes) rapidly lose their fMet-tRNA, even without IF-3 (Figures 3 and 4). At 4 mM Mg^{2+}, however, GMPPCP complexes no longer exhibited spontaneous breakdown. Moreover, under these conditions they did not lose any fMet-tRNA when treated with an excess of IF-3 (Figure 5b), which dissociated the free ribosomes present (Figure 6). If there were a spontaneous equilibrium between the 70S GMPPCP complexes and their precursor 30S complexes IF-3 would be expected to shift that equilibrium, since it can displace fMet-tRNA from 30S initiation complexes (Dubnoff et al., 1972). Hence the failure of IF-3 to cause net dissociation of the intermediate 70S complexes, at the minimal Mg^{2+} concentration required to hold them together, suggests that under physiological conditions their formation is effectively irreversible.

At 3 mM Mg^{2+} GMPPCP complexes rapidly lose their fMet-tRNA, and IF-3 accelerates that loss (Figure 5a). Since the net dissociation of free ribosomes by IF-3 appears to depend on its shifting the reversible equilibrium between these ribosomes and free 30S subunits (Noll and Noll, 1972; Kaempfer, 1973; Gottlieb, Davis, and Thompson, unpublished), its acceleration of the release of fMet-tRNA from GMPPCP complexes at low Mg^{2+} suggests that this release is reversible.

Though GMPPCP complexes lose their fMet-tRNA at a low Mg^{2+} concentration, we have seen that at a higher concentration they not only retain that ligand but can move it into a puromycin-reactive position, if subjected to centrifugation (Table I) or to gel filtration (Benne and Voorma, 1972; Dubnoff et al., 1972). These procedures evidently wash off the GMPPCP and the IF-2. It is of interest that the recognition step in protein synthesis is similarly capable of artificial maturation, without the hydrolysis of GTP. Thus when GMPPCP is used instead of GTP in the "enzymatic" binding (with EFTu) of aminoacyl-tRNA to initiation complexes, centrifugation through sucrose solution can wash off the GMPPCP and EFTu. The aminoacyl-tRNA

then reaches a reactive position and forms a dipeptide with the fMet (Yokosawa et al., 1973), just as after GTP hydrolysis.

Mature complexes can be reversed to an intermediate, puromycin-unreactive form by the addition of GMPPCP and IF-2 (Benne and Voorma, 1972; Table I above). This finding suggests that the free energy difference between these two forms of the ribosome is small, despite the fact that under physiological conditions the transition is associated with the effectively irreversible hydrolysis of GTP. Accordingly, the energy drop in formation of the intermediate initiation complex may well contribute a large fraction of the stability of the final complex.

In contrast to the stability of GMPPCP complexes to dissociation by IF-3, they did not appear to be more stable than free ribosomes to dissociation by altered ionic conditions (Beller and Lubsen, 1972). The present findings may provide a trivial explanation: at the ion concentrations required for visible dissociation of free ribosomes GMPPCP complexes would already have lost their ligands. The same studies showed that the mature initiation complex is more stable to ionic dissociation, and early growth of the polypeptide chain adds further stability.

References

Beller, R. J., and Davis, B. D. (1971), *J. Mol. Biol. 55,* 477.

Beller, R. J., Davis, B. D., and Gottlieb, M. (1974), *Biochemistry 13,* 939.

Beller, R. J., and Lubsen, N. H. (1972), *Biochemistry 11,* 3271.

Benne, R., Ebes, F., and Voorma, H. O. (1973a), *Eur. J. Biochem. 38,* 265.

Benne, R., Naaktgeboren, N., Gubbens, J., and Voorma, H. O. (1973b), *Eur. J. Biochem. 32,* 372.

Benne, R., and Voorma, H. O. (1972), *FEBS Lett. 20,* 347.

Dubnoff, J. S., Lockwood, A. H., and Maitra, U. (1972), *J. Biol. Chem. 247,* 2884.

Dubnoff, J. S., and Maitra, U. (1971), *Methods Enzymol. 20,* 248.

Gesteland, R. F., and Boedtker, H. (1964), *J. Mol. Biol. 8,* 496.

Gottlieb, M., Lubsen, N. H., and Davis, B. D. (1974), *Methods Enzymol. 30,* 87.

Hershey, J. W. B., and Thach, R. E. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57,* 759.

Kaempfer, R. (1973), *J. Mol. Biol. 71,* 583.

Kohler, R. E., Ron, E. Z., and Davis, B. D. (1968), *J. Mol. Biol. 36,* 71.

Mazumder, R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2770.

Modolell, J., and Davis, B. D. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 1279.

Nirenberg, M. W., and Leder, P. (1964), *Science 145,* 1399.

Noll, M., and Noll, H. (1972), *Nature (London) New Biol. 238,* 225.

Sabol, S., Meier, D., and Ochoa, S. (1973), *Eur. J. Biochem. 33,* 332.

Subramanian, A., and Davis, B. D. (1970), *Nature (London) 228,* 1273.

Subramanian, A., and Davis, B. D. (1973), *J. Mol. Biol. 74,* 45.

Subramanian, A., Davis, B. D., and Beller, R. J. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 223.

Thach, S. S., and Thach, R. E. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1791.

Vermeer, C., deKievit, R. J., van Alphen, W. J., and Bosch, L. (1973), *FEBS Lett. 31,* 273.

Yokosawa, H., Inoue-Yokosawa, N., Arai, K.-I., Kawakita, M., and Kaziro, Y. (1973), *J. Biol. Chem. 248,* 375.