The analysis of initial velocity kinetic data was used to examine the order in which fMet-tRNA and the coat cistron of genomic bacteriophage R17 or Qb RNA bind to the 30 S ribosomal subunit. These data were obtained using a quantitative assay for protein synthesis in *Escherichia coli* extracts where the rate of accumulation of protein product is dependent on the concentration of mRNA and is partially dependent on fMet-tRNA. Under the conditions of this assay, the amount of protein synthesized was proportional to the formation of ternary complexes between the mRNA, fMet-tRNA, and the 30 S ribosomal subunit. The results from the initial velocity and alternative substrate experiments are consistent with a rapid equilibrium ordered mechanism as opposed to a rapid equilibrium random mechanism. Analysis of the rate of protein synthesis at varied concentrations of mRNA and fixed concentrations of fMet-tRNA indicated that fMet-tRNA was the first substrate to bind to the 30 S subunit when either coat cistron was used as the mRNA. This scheme assumes the existence of a relatively slow step in protein synthesis that occurs after both the initiating tRNA and mRNA are bound to the ribosome and which allows substrate addition to reach thermodynamic equilibrium.

Initiation of protein synthesis in *Escherichia coli* occurs through the formation of a ternary complex containing the 30 S ribosomal subunit, messenger RNA, and fMet-tRNA (1). Three initiation factors and GTP are required to form the ternary complex (2). The mRNA in these complexes is bound at the appropriate sites so that the in-phase readout of information contained within the primary sequence of the mRNA can occur.

Shine and Dalgarno (3) proposed that sites within the mRNA are selected for initiation by the formation of complementary base pairs between the pyrimidine rich 3′ end of the 16 S ribosomal RNA and polypurine tracts found 5′ to the initiation codons of mRNAs. This proposal has been extensively confirmed using both biochemical (4, 5) and genetic (6–8) techniques. Whether additional base pairing between the initiation codon and the anticodon of fMet-tRNA is important in the selection of protein start sites is unclear. Several investigators (6, 9, 10) have suggested that the relative contributions of the codon-anticodon interaction to mRNA binding may depend upon the strength of the Shine-Dalgarno complementarity found within each mRNA. One way to judge the significance of the codon-anticodon interaction in initiation site selection is to determine the order in which fMet-tRNA and mRNA bind to the 30 S subunit. This order is as yet unresolved (11).

Analysis of initial velocity kinetic data was used by Gualerzi et al. (12) to examine the mechanism of 30 S initiation complex formation. These data were generated through the binding of synthetic RNA templates and cognate aminoacyl-tRNAs to 30 S subunits. Their results suggest that these RNA templates bind to the 30 S subunit through a random mechanism. Such a mechanism would support the conclusion of Van Duin et al. (13) that binary complexes between the coat cistrons of either MS2 RNA or Qb RNA and 30 S subunits are productive intermediates in ternary complex formation. However, a random mechanism would not be consistent with the observation of Kaempfer and Jay (14) that only binary complexes between fMet-tRNA and 30 S subunits are productive intermediates when ternary complexes are formed using R17 RNA as the mRNA.

It is unclear as to how the results of Gualerzi et al. (12) could be extrapolated to the mechanism of 30 S initiation complex formation when natural mRNAs are used as templates. The synthetic RNA templates used in their studies lacked any significant homology with the 3′ end of the 16 S ribosomal RNA. This homology is required for the selection of protein start sites on natural mRNAs by the 30 S subunit (11). Therefore, the mechanism of 30 S initiation complex formation may be different when natural as opposed to synthetic templates are used. We have analyzed initial velocity kinetic data to examine the order in which natural mRNAs and fMet-tRNA bind to the 30 S subunit during the initiation of translation. Our approach differs from that of Gualerzi et al. (12) in that we measure the synthesis of a completed protein product as an indirect assay for the formation of 30 S ternary complexes. The use of protein synthesis as an assay would measure only productive ternary complexes between the mRNA, fMet-tRNA, and the 30 S subunit. The resolution of protein products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis has provided the basis for a sensitive and accurate measurement of the rate of specific protein synthesis. In order to use this assay to examine the order of binding of these RNA ligands to the 30 S subunit, experimental conditions were adjusted so that the rate of protein synthesis was proportional to the concentration of 30 S ternary complexes.

The mRNA used in the initial velocity experiments was the coat cistron of either bacteriophage R17 or Qb RNA. The results obtained were consistent with fMet-tRNA and either coat cistron binding to the 30 S subunits through a rapid equilibrium-ordered mechanism. fMet-tRNA was the first...
substrate to bind when either coat cistron was used as the mRNA.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-[35S]Methionine was purchased from either New England Nuclear or Amersham Corp. Crude tRNA and purified tRNA

ex were obtained from Boehringer Mannheim. Leucovorin was from Lederle Laboratories, and Solune 350 was purchased from Packard Instrument Co. Q8 bacteriophage were kindly provided by Dr. T. Blumenthal, Indiana University. E. coli strain CF300 was provided by Dr. P. A. Weil, University of Iowa.

R17 and Q8 bacteriophage were prepared according to the protocol of Crooke et al. (15), with two cesium chloride gradient centrifugations added as described by Steitz (16). Special problems associated with the purification of Q8 phage were resolved as described by Weissmann et al. (17). RNA was isolated from the phage by extraction with phenol in the presence of sodium dodecyl sulfate (16).

The in vitro translation system employed is described in detail by Conditi (18). Conditi's system was derived from studies by O'Farrell and Gold (19) and a partial description has been published (20). This system was developed for coupled transcription-translation from DNA templates. We retained this feature for the range of mRNAs that limited the use of small linear DNA fragments in coupled systems.

**Methods**

**Protein Synthesis**—A typical reaction mixture (40 

µl) contained 25 mM Tris-acetate, pH 7.9, 24 µM potassium acetate, 1 mM dithiothreitol, 0.1% concentrated sodium dodecyl sulfate sample buffer (250 mM Tris-HCl, pH 6.8, 5% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 50% glycerol). 0.1% bromphenol blue was added as a marker.

**Gel Electrophoresis and Liquid Scintillation Counting**—Polyacrylamide slab gels were cast ¼ of an inch thick as described by O'Farrell (22) and Laemmli (23). Separating gels contained linear gradients of 10–15% polyacrylamide and 0–13.5% glycerol. Gels were run at 20 mA for 2.5 h at room temperature. Proteins were fixed and stained using 50% trichloroacetic acid containing 0.03% Coomassie blue R for 30 min. Gels were destained overnight in 10% acetic acid, 5% isopropanol alcohol. Destained gels were dried in vacuo onto Whatman No. 3MM paper.

Proteins of interest were cut from the gel and solubilized by adding 0.5 ml of Solune 350 and 0.15 ml of water to the protein band in a 5-ml plastic scintillation vial. The vials were capped and their contents mixed, followed by an overnight incubation at room temperature. 5 ml of toluene scintillation fluid (3.8 g of 2,5-diphenyloxazole and 95 mg of 2,2'-diphenyloxazole in 10 ml of toluene) were added to each tube. Radioactivity was monitored daily until the counts/min stabilized, which normally took 2 days. This period of time was required to obtain the maximum number of counts extractable from the gels. The efficiency of recovery of loaded radioactive controls was approximately 65%.

**Kinetic Analysis**—Kinetic results were analyzed using computer programs written by Cleland (24). Each set of data for a double reciprocal plot was fitted to the Michaelis-Menten equation using the HYPER program, and the slope (K/V) and intercept (1/V) and their standard errors were calculated. Student's t test was used to determine if these parameters were significantly different among the sets of data (p < 0.01) or if they were probably the same (p > 0.05) according to accepted criteria (25, 26). The kinetic plots show the experimentally determined points, whereas the lines are from the computer fits to the appropriate equation (competitive, noncompetitive, uncompetitive inhibition; sequential bireactant, equilibrium ordered bireactant). All kinetic constants are apparent values determined at a concentration of 0.25 mM GTP. Total amount of [35S]methionine incorporated into coat protein in a given reaction was determined by correcting the amount of [35S]methionine extracted from the coat protein region of a polyacrylamide gel for the proportion of the reaction loaded onto the gel and the efficiency of the extraction procedure. The efficiency of liquid scintillation counting was 80%. The amount of coat protein produced was determined using values of 3 and 1 for the number of methionine residues found in the R17 and Q8 coat proteins, respectively (27). These values include the initiating methionine which was retained on the proteins synthesized in vitro. Maximum velocities were calculated using values of 130 and 132 amino acids for the coat proteins of R17 and Q8 phage, respectively (27). A 106 units of ribosomes were converted into milligrams using the relationship 1 mg/ml of ribosomes equals 14.4 x 106 units; 2.7 x 106 was used as a molecular weight for low salt-washed ribosomes.

**RESULTS**

The Translation Assay—For protein synthesis to be an effective assay for 30 S ternary complex formation, the translation assay was designed so that initiation was rate-limiting. With our assay, R17 coat protein synthesis showed a linear dependence upon the amount of ribosomal fraction added (Fig. 1). Linearity was maintained at the highest and lowest concentrations of mRNA and fMet-tRNA routinely used within our assays.
The initiation factor/ribosome ratio in crude extracts of rapidly dividing E. coli cells is 0.15–0.2 (28). It is therefore probable that initiation factors were the components in the ribosomal fraction which limit translation. We made the assumption that initiation factors were present on the 30 S subunits before the binding of either of the RNA ligands. Experimental support for this assumption has been summarized by Grunberg-Manago (29); consequently, the 30 S subunit with a full complement of initiation factors was considered to be the catalyst that limits translation within the assay.

In order to use initial velocity kinetic analysis to investigate the order in which fMet-tRNA and mRNA bind to 30 S subunits, the translation assay must be dependent on the tRNA requirement. Therefore, initiating tRNA was present in proportion to the amount of tRNA\textsuperscript{Met} in the crude tRNA added to each assay. We found that R17 coat protein synthesis was limited by the amount of initiating tRNA present in the crude tRNA (Table 1). By lowering the concentration of crude tRNA, the reaction became partially dependent on added purified tRNA\textsuperscript{Met}, without significantly reducing the optimal level of R17 coat protein synthesized. A similar stimulation of R17 coat protein synthesis by tRNA\textsuperscript{Met} was observed when \[^{3}H\]tryptophan was used as a radioactive label. At a crude tRNA concentration of 3.6 \(\mu\)M, the stimulation of R17 coat protein synthesis by 0.94 \(\mu\)M tRNA\textsuperscript{Met} with \[^{35}S\]methionine or \[^{3}H\]tryptophan as the label was approximately 2.0 times the value obtained in the absence of added tRNA\textsuperscript{Met}. Thus, the stimulation of R17 coat protein synthesis in the presence of tRNA\textsuperscript{Met} did not result from the misincorporation of the methionine label into the elongating polypeptide chain. If the crude tRNA was limited in tRNA\textsuperscript{Met}, maximal amounts of protein synthesis might be attained by raising the concentration of crude tRNA to supply enough tRNA\textsuperscript{Met}. Instead, Table 1 shows that high concentrations of crude tRNA inhibit protein synthesis. This inhibition has been observed by others (31) and was correlated with an excess of tRNA over elongation factor Tu. The failure of tRNA\textsuperscript{Met} to stimulate protein synthesis at 28.5 \(\mu\)M crude tRNA may indicate that protein synthesis was already inhibited at this concentration of crude tRNA.

The dependence of protein synthesis on the concentration of either tRNA\textsuperscript{Met} or mRNA showed saturation behavior. At higher concentrations of either substrate, inhibition became apparent (data not shown). The mechanism of this inhibition was not examined, but for the initial velocity studies the concentrations of tRNA\textsuperscript{Met} or mRNA were not extended to inhibitory levels. tRNA\textsuperscript{Met} must be aminoacylated and the methionine formylated before it can function efficiently as a substrate during initiation. Aminoacylation was linear over the entire concentration range of tRNA\textsuperscript{Met} commonly used in these assays (Fig. 2), demonstrating that the aminoacyl-tRNA synthetase was not saturated. Saturation of the transformylase reaction was also not expected because the concentration of Met-tRNA\textsubscript{f} produced in the translation assays is significantly below the estimated Michaelis constant of the transformylase for Met-tRNA\textsubscript{f} (32). Thus, the ability of added tRNA\textsuperscript{Met} and R17 RNA to saturate R17 coat protein synthesis was likely the result of the saturation of binding sites on the 30 S subunit for fMet-tRNA and the R17 coat cistron.

A time course of R17 coat protein synthesis demonstrated that coat protein production reached a steady state during the translation of R17 RNA (Fig. 3). The macromolecular substrates used during translation are continually recycled. Steady state concentrations of these substrates are therefore maintained in a dynamic fashion, which would alleviate the requirement for very large concentrations of these substrates to maintain a steady state of protein synthesis. The break in the time course at 12 min correlated with the appearance of the R17 replicase protein (Fig. 4), suggesting that some of the ribosomes were channeled from the coat to the replicase cistron. Such behavior was expected from the linkage between these two cistrons (34). The second higher molecular weight band observed in Fig. 4 found in very small amounts and probably represented the accumulation of the maturation protein (\(A\) Protein) from a basal level of expression. The 2.5-min lag in coat protein production observed in Fig. 3 represented a combination of the initiation and elongation rates for this mRNA and the thermal equilibration of the system (data not shown). No significant change in the lag period was observed when a time course of R17 coat protein synthesis was performed at different concentrations of R17 RNA and

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**TABLE 1**

The dependence of R17 coat protein synthesis on the concentration of crude tRNA and purified tRNA\textsuperscript{Met}.

| Crude tRNA | Added tRNA\textsuperscript{Met} | pmol R17 coat protein |
|------------|-------------------------------|----------------------|
| \(\mu\)M   | 0 \(\mu\)M                  | 0.25 \(\mu\)M        | 0.83 \(\mu\)M        | 1.25 \(\mu\)M        |
| 3.6        | 0.42                         | 0.68                 | 0.85                 | 0.67                 |
| 7.2        | 0.53                         | 0.75                 | 0.97                 | 0.92                 |
| 28.5       | 0.66                         | 0.59                 | 0.65                 | 0.62                 |
| 71.25      | 0.48                         | 0.46                 | 0.44                 | 0.45                 |

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**Fig. 2.** The amount of methionine incorporated into tRNA as a function of input tRNA\textsuperscript{Met}. The assay conditions are described under "Experimental Procedures" except that R17 RNA was not included. The concentration of tRNA\textsuperscript{Met} was calculated using the manufacturer's analysis for the aminoacylation of the tRNA\textsuperscript{Met}, which was 1590 pmol of methionine/Am unit of tRNA. Formylation of the methionine by transformylase was 94–98%, indicating negligible contamination with tRNA\textsuperscript{Met}. Met-tRNA\textsuperscript{f} was measured as \[^{35}S\]methionine rendered insoluble in ice-cold 10% trichloroacetic acid. Acid-insoluble material was collected by filtration through 1-inch Millipore filters (type HA, pore size 0.45 \(\mu\)m). Filters were subsequently dissolved in 10 ml of Bray's (33) solution, and the amount of radioactivity was measured by liquid scintillation counting. The amount of ribosomes used was 0.43 Am unit. The cross-bar on the vertical axis represents the amount of methionine incorporated into crude tRNA. Approximately 53% of the input tRNA\textsuperscript{Met} was aminoacylated. All concentrations of fMet-tRNA\textsuperscript{f} were based on a 50% aminoacylation of input tRNA\textsuperscript{Met}, assuming 100% formylation. Added to this was an additional amount of fMet-tRNA\textsuperscript{f} synthesized from tRNA\textsuperscript{Met} which was present in the crude tRNA. The latter amount was calculated as 50% of the total methionine incorporated into the crude tRNA.
A and replicase proteins were identified that the time of incubation was varied. The amount of ribosomes conditions were as described under "Experimental Procedures" except concentrations of R17 RNA and met-tRNA. The assay elongation or initiation.

Determining step in translation, protein synthesis can be duplicate polyacrylamide gels of a single experiment. Studies obtained from purified phage particles.

As an assay for the formation of complex formation can be examined using kinetic analysis. With the ribosome as the catalyst and initiation the rate-determining step in translation, protein synthesis can be simplified to the point where the mechanism of 30 S initiation complex formation can be examined using kinetic analysis.

Initial Velocity Kinetic Analysis—The order in which substrates add to an enzyme can be analyzed using initial velocity studies (35). Our assumption that the catalyst in the translation system described, protein synthesis can be used as an assay for the formation of 30 S initiation complexes. With the ribosome as the catalyst and initiation the rate-determining step in translation, protein synthesis can be simplified to the point where the mechanism of 30 S initiation complex formation can be examined using kinetic analysis.

The results presented to this point suggest that in the translation system described, protein synthesis can be used as an assay for the formation of 30 S initiation complexes. The ribosome as the catalyst and initiation the rate-determining step in translation, protein synthesis can be simplified to the point where the mechanism of 30 S initiation complex formation can be examined using kinetic analysis.

The nomenclature of Cleland (40) was used to describe substrate concentrations and kinetic constants. A and B represent the substrates in the order that they bind. $K_a$ and $K_s$ are Michaelis constants, and $K_i$ is a dissociation or inhibition constant. $v$ and $V_i$ represent the reaction velocity and the maximum velocity, respectively. Equation 1 represents the rate equation for the rapid equilibrium ordered mechanism; Equation 2 represents the rate equation for the rapid equilibrium random mechanism or the steady state ordered mechanism.

The dependence of the reaction velocity on substrate concentrations can be used to distinguish between the mechanisms represented by Equations 1 and 2. A determination of initial velocity patterns involves variation of one substrate at several fixed concentrations of the other substrate. The rate of R17 coat protein synthesis using R17 RNA as the varied substrate at different concentrations of fMet-tRNA is shown in Fig. 5. The best fit for these data is to the equilibrium ordered mechanism, Equation 1. This judgment is based on the inability to demonstrate differences among the intercepts in the reciprocal plot, but, of course, one cannot prove that the intercepts are the same, and under other conditions differences might be demonstrated. Since the results indicate that Equation 2 is not the best description of the kinetic mechanism and that $K_s$ is much less than $K_i$, the simplest mechanism is described by Equation 1. The situation could be more complicated, but the available data do not warrant the inclusion of other steps or terms. Kinetic constants determined by the computer program are shown in Table II.

The data in Fig. 5 fit a mechanism in which fMet-tRNA binds to the 30 S subunit before the R17 coat cistron.

In this scheme, substrate addition reaches thermodynamic

\[
\frac{1}{v} = \frac{K_a}{V_i} + \frac{1}{[A]} + \frac{1}{[B]}
\]  \hspace{1cm} (1)

\[
\frac{1}{v} = \frac{K_a}{V_i} + \frac{1}{[A]} + \frac{1}{[B]} + \frac{1}{[A][B]}
\]  \hspace{1cm} (2)

The dependence of the reaction velocity on substrate concentrations was translated to a significant extent was the coat protein cistron (Fig. 4). Whereas fMet-tRNA and mRNA are present together in 30 S initiation complexes (29), the binding of these RNA ligands is sequential (35). The dependence of the reaction velocity on substrate concentrations for sequential mechanisms with two substrates can be described by the following equations (40).

**Fig. 3.** Time course of R17 coat protein synthesis at different concentrations of R17 RNA and fMet-tRNA. The assay conditions were as described under "Experimental Procedures" except that the time of incubation was varied. The amount of ribosomes used was 0.43 A_{260} unit. Each point represents the counts/min of $[^{35}S]$ methionine incorporated into the R17 coat protein extracted from duplicate polyacrylamide gels of a single experiment. 0.14 μM R17 RNA and 0.045 μM fMet-tRNA; O, 0.485 μM R17 RNA and 0.349 μM fMet-tRNA.

**Fig. 4.** Autoradiography of the polyacrylamide gel used to obtain the data in Fig. 3. The assay conditions are described in the legend to Fig. 3. Autoradiography was performed by placing the dried gel next to Kodak X AR-5 film within a tight-fitting cassette for 2 h. The film was manually developed according to the manufacturer's instructions. The R17 coat protein was identified by co-migration with marker protein obtained from purified phage particles. A and replicase proteins were identified from their published molecular weights (31).
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![Graph](Image)

**Fig. 5.** Reciprocal initial velocity as a function of reciprocal R17 RNA concentration at several fixed concentrations of fMet-tRNA. The assay conditions used are described under “Experimental Procedures.” The amount of ribosomes was 0.43 A$_{260}$ unit. Each point represents an average value for the total amount of R17 RNA concentration at several fixed concentrations of tRNA. Kinetic constants determined for this fit are shown in Table I. Velocity is expressed as picomoles of R17 coat protein produced in 8 min by 0.43 A$_{260}$ unit of ribosomes.

**TABLE I**

| mRNA               | $K_w$ | $K_b$ | $V_i$       |
|--------------------|-------|-------|-------------|
| R17 coat cistron   | 0.113 | 0.071 | 0.105 ± 0.003 |
| Qβ coat cistron    | 0.29  | 0.031 | 0.183 ± 0.003 |

The values were obtained from the data in Figs. 4 and 5 that were fitted to the equation for the equilibrium ordered mechanism. $K_w$ is the dissociation constant for fMet-tRNA, and $K_b$ is the Michaelis constant for the mRNA. $V_i$ is the maximum velocity of protein synthesis.

![Graph](Image)

**Fig. 6.** Reciprocal initial velocity as a function of reciprocal Qβ RNA concentration at several fixed concentrations of fMet-tRNA. The assay conditions used are described under “Experimental Procedures” and in the legend to Fig. 5. The amount of ribosomes was 0.32 A$_{260}$ unit. The concentrations of fMet-tRNA were 0.045 μM (O), 0.073 μM (●), 0.135 μM (▲), and 0.405 μM (●). The graph shows experimental points with lines fitted by computer to Equation 1. Velocity is expressed as picomoles of Qβ coat protein produced in 8 min by 0.32 A$_{260}$ unit of ribosomes.

Kinetic constants derived from initial velocity studies using the coat cistrons from either bacteriophage Qβ or R17 as the mRNA

The values were obtained from the data in Figs. 4 and 5 that were fitted to the equation for the equilibrium ordered mechanism. $K_w$ is the dissociation constant for fMet-tRNA, and $K_b$ is the Michaelis constant for the mRNA. $V_i$ is the maximum velocity of protein synthesis.

The order in which the coat cistrons of Qβ RNA and fMet-tRNA bind to the 30 S subunit was examined in a similar manner. The initial velocity data are shown in Fig. 6. The best fit of these data is to Equation 1, representing the equilibrium ordered mechanism. Just as for the R17 coat cistron, the Qβ coat cistron binds to the ribosome after fMet-tRNA. Kinetic constants determined for this fit are shown in Table II.

Different ribosome and supernatant preparations were used in the initial velocity experiments with Qβ and R17 mRNAs. Therefore, the small differences in the kinetic constants obtained using these two mRNAs should not be considered significant.

**Alternative Substrate Inhibition**—Further support for an equilibrium ordered mechanism of substrate addition can be obtained through inhibition studies using alternative substrates. If fMet-tRNA and the coat cistrons of R17 and Qβ RNA bind through equilibrium ordered mechanisms, the dependence of Qβ coat protein synthesis on the concentrations of fMet-tRNA and Qβ RNA in the presence of R17 RNA will be represented by the following equation:

$$\frac{1}{v} = \frac{K_w K_b}{V_i [A][B]} + \frac{1}{V_i} \left( \frac{1 + K_b}{[B]} \right) \left( \frac{1 + [B]}{K_b (1 + [B])} \right)$$

where $B'$ is the alternative substrate, R17 RNA. Equation 3 indicates that $B'$ should act as an uncompetitive inhibitor with substrate $A$, fMet-tRNA, and as a competitive inhibitor with substrate $B$, Qβ RNA. Such an alternative substrate (R17 RNA) would show the characteristic uncompetitive inhibition only if it were the second substrate to bind in a rapid equilibrium ordered system (37, 39). With the steady state ordered or rapid equilibrium random mechanisms, noncompetitive inhibition is expected. Fig. 7 shows the inhibition of Qβ coat protein synthesis by R17 RNA using fMet-tRNA as the varied substrate. These data fitted best to the equation representing uncompetitive inhibition. Although the slope of the line with the highest concentration of R17 RNA may be slightly different from the slopes of the other lines, none of the slopes were significantly different and the slope inhibition constant, although poorly estimated, is in any case much larger than the intercept inhibition constant. Kinetic constants determined by the computer program were $K_w = 0.053 ± 0.0039$ μM and $K_b = 0.044 ± 0.0016$ μM, where $K_w$ is the dissociation constant for fMet-tRNA and $K_b$ is the Michaelis constant for the alternative substrate, R17 RNA. These data support the conclusions reached from the initial velocity experiments, that the coat cistron of R17 RNA and fMet-tRNA bind to the 30 S subunit through a rapid equilibrium ordered mechanism. In addition, these results support the conclusion that fMet-tRNA binds before the R17 coat cistron.

Although Equation 3 states that an alternative mRNA substrate should show competitive inhibition with the sub-
protein region. This overlap was found to be a constant fraction of the concentration of the alternative substrate and presence of the substrate it is replacing by the following equation.

Using an alternative substrate as an inhibitor, the apparent overlap of the R17 protein band into the Qβ coat region may result from incomplete polypeptide chains of all three proteins. Control experiments indicated that the overlap was a constant fraction (9.2%) of the counts/min found in the R17 coat band. Similarly, Qβ protein synthesis overlaps into the R17 coat protein region. This overlap was found to be a constant fraction (19.2%) of the counts/min in the Qβ coat protein band and probably represents incomplete polypeptide chains of all three Qβ proteins. The correction was applied by first subtracting the overlap from Qβ protein synthesis from the counts/min incorporated into R17 coat protein. The corrected value for the R17 coat protein was used to determine the amount of Qβ coat protein synthesis from the counts/min incorporated into R17 coat protein band and probably represents incomplete polypeptide chains of all three Qβ proteins.

Using an alternative substrate as an inhibitor, $[I]$ would represent the concentration of the alternative substrate and $K_i$ would represent the Michaelis constant for the alternative substrate, corrected for the presence of the substrate it is replacing by the following equation.

$$\frac{1}{V} = \frac{1}{V_i} + \frac{K_i}{V_i[A]} + \frac{K_m}{V_i[A]}$$

Velocity is expressed as picomoles of Qβ coat protein produced in 8 min by 0.32 A$_{300}$ unit of ribosomes.

DISCUSSION

We have developed a quantitative assay for protein synthesis using *E. coli* extracts, where the rate of accumulation of the protein product is dependent on the concentration of mRNA and is partially dependent on fMet-tRNA. With this assay, protein synthesis is proportional to the ribosome concentration and shows saturation behavior with respect to the concentration of fMet-tRNA and mRNA. These results indicate that under the experimental conditions described, the synthesis of a complete protein can be used as an assay for the formation of ternary complexes between the 30 S subunits, mRNA, and fMet-tRNA.

Initial velocity kinetic analysis was used to examine the order in which fMet-tRNA and mRNA bind to the 30 S subunit during the initiation of translation. Our results using the coat cistrons of either R17 or Qβ RNA as the mRNA are consistent with an equilibrium ordered addition of substrates where fMet-tRNA is the first substrate to bind using either coat cistron as the mRNA. We have provided additional support for this mechanism through inhibition studies using the coat cistron of R17 RNA as an alternative substrate for the coat cistron of Qβ RNA. It must be emphasized, however, that the kinetic analysis is only consistent with this mecha-
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The presence of fMet-tRNA on the 30 S subunit before either coat cistron binds emphasizes the contribution that fMet-tRNA makes to the selection of initiation sites within these cistrons. The ordered mechanism implies that fMet-tRNA greatly enhances the affinity of the 30 S subunit for the two coat cistrons. The simplest explanation for the enhanced affinity of 30 S subunit-fMet-tRNA complexes over 30 S subunits alone for Qβ or R17 coat cistrons is that the additional base pairs formed between the codon and the anticodon stabilize mRNA binding. A similar conclusion has been reached by Taniguchi and Weissman (10) to explain the ability of fMet-tRNA to enhance the formation of ribonuclease-resistant 70 S initiation complexes with the coat cistron of Qβ RNA.

Whether fMet-tRNA makes a significant contribution to the binding of other mRNAs must await further study. We have attempted to expand the range of mRNAs that can be examined with this system by using fragments of DNA to synthesize discrete species of mRNA in vitro. These experiments have so far yielded equivocal results since only low concentrations of RNA can be synthesized. It is possible that the relative contribution of fMet-tRNA to mRNA binding may vary depending on the mRNA. Several investigators (6, 9, 10) have suggested that this contribution is inversely related to the degree of complementarity at the initiation site of a given mRNA for the 3' end of the 16 S ribosomal RNA. These mRNAs having fewer bases of complementarity may require the additional interaction between the codon and the fMet-tRNA anticodon to stabilize mRNA binding. In this connection, the two cistrons used in this study have only three bases 5' to the initiation codon that could form complementary base pairs with the 3' end of the 16 S ribosomal RNA (9).

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