Protection of Ribosomes from Thiostrepton Inactivation by the Binding of G Factor and Guanosine Diphosphate*

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ABSTRACT: Prior treatment of ribosomes with the antibiotic inhibitor of translocation, thiostrepton, rapidly and irreversibly destroys their ability to bind G factor (the soluble translocation protein) and guanine nucleotides, apparently as the result of the irreversible binding of the antibiotic to the ribosome. Conversely, if G factor and guanosine diphosphate (GDP) are first bound to the ribosome, this complex decays only slowly in the presence of thiostrepton, and this decay can be accounted for by complex dissociation as measured by the rate of chase with unlabeled nucleotide. Fusidic acid, a translocation inhibitor which stabilizes the ribosome–G factor–GDP complex, provides additional protection of the complex against inactivation by thiostrepton. Following treatment with thiostrepton, the protected ribosomes not only retain the ability to bind G factor and GDP, but they retain their translocational ability as well for they are active in protein synthesis. The binding of both G factor–GDP and thiostrepton occurs on the 50S subunit. The simplest interpretation of the present results is that the mutually exclusive binding of G factor–GDP and of thiostrepton results from steric interference; *i.e.*, that these bindings occur at or near the same physical region of the 50S ribosomal subunit.

Translocation is that complicated process which results in the movement of the ribosome along mRNA and requires the participation of the soluble protein, G factor, and the hydrolysis of GTP to GDP and P_i (Nishizuka and Lipmann, 1966; Erbe *et al.*, 1969). Two antibiotics, thiostrepton (Pestka, 1970) and fusidic acid (Tanaka *et al.*, 1968; Pestka, 1968; Haenni and Lucas-Lenard, 1968), are known inhibitors of this process as well as inhibitors of the hydrolysis of GTP uncoupled from protein synthesis, *i.e.*, the hydrolysis promoted by ribosomes and G factor alone (Nishizuka and Lipmann, 1966).

During uncoupled GTP hydrolysis, a ternary complex involving the ribosome, G factor, and GDP is formed (Brot *et al.*, 1969; Parmeggiani and Gottschalk, 1969), and stabilization of this complex by fusidic acid after one round of GTP hydrolysis prevents further hydrolysis (Bodley *et al.*, 1969; Bodley *et al.*, 1970b; Brot *et al.*, 1971). We have proposed (Bodley *et al.*, 1970b) the following mechanism for the uncoupled hydrolysis of GTP and the inhibitory effect of fusidic acid.

$$\text{ribosome} + \text{G factor} + \text{GTP} \rightleftharpoons \underset{\text{(complex I)}}{\text{ribosome–G factor–GTP}}$$

$$\underset{\text{(complex I)}}{\text{ribosome–G factor–GTP}} \rightleftharpoons \underset{\text{(complex II)}}{\text{ribosome–G factor–GDP}} + P_i$$

$$\underset{\text{(complex II)}}{\text{ribosome–G factor–GDP}} \overset{\text{fusidic acid}}{\rightleftharpoons} \text{ribosome} + \text{G factor} + \text{GDP}$$

Recently, it has been shown (Bodley *et al.*, 1970a; Weisblum and Demohn, 1970b) that thiostrepton inhibits uncoupled GTP hydrolysis by quite a different mechanism; specifically, it prevents the formation of both complex I and complex II. Despite this difference, however, both thiostrepton and fusidic acid have been shown to act upon the 50S ribosomal subunit. Thiostrepton causes an irreversible inactivation which is apparently due to its binding to the 50S subunit (Pestka, 1970; Weisblum and Demohn, 1970a) while the reversible inhibition by fusidic acid (Harvey *et al.*, 1966) results from the stabilization of the ternary complex which occurs on the 50S subunit (Bodley and Lin, 1970).

In view of the facts that both G factor and the antibiotic, thiostrepton appear to bind to the ribosome and the prior binding of thiostrepton prevents the binding of G factor and guanine nucleotide, we have sought in the present report to determine if these are mutually exclusive events, perhaps as a result of their occurring upon the same physical region of the ribosome. The results presented here show that ribosomes bearing G factor and GDP are relatively refractory to irreversible inhibition by thiostrepton.

The relevance of the ribosome–G factor–GDP complex to the process of translocation is exemplified by these findings. Since ribosomes protected by the binding of G factor and GDP are competent in protein synthesis following exposure to thiostrepton, it would appear that G factor and GDP are bound to the ribosome at a site which participates in GTP hydrolysis coupled to translocation.

Materials and Methods

Materials. Both fusidic acid and thiostrepton were generous gifts of Miss Barbara Stearns of E. R. Squibb. Preparations of [^{3}H]GTP with specific activities of 1.0 and 1.4 Ci per mmole were from New England Nuclear Corp. Cellular components were prepared from either *Escherichia coli* A-19 (General Biochemicals) or *E. coli* B (Grain Processing Corp.). No significant difference relevant to the present study distinguished preparations from these two strains. S-100 was prepared and stored as previously described (Bodley, 1969). tRNA was prepared by the method of von Ehrenstein and Lipmann (1961). High salt-washed ribosomes were obtained either by

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the methods of Pestka (1968) or as described by us previously (Bodley *et al.*, 1970c). The source of materials not reported here was as previously reported (Bodley *et al.*, 1970c).

Preparation of G Factor. G factor was prepared by a combination of the methods described by Nishizuka *et al.* (1968) and by Leder *et al.* (1969). A ribosome-free extract obtained from 500 g of *E. coli* cell paste was fractionated with solid ammonium sulfate (40–65%) and dialyzed against a buffer containing 0.01 M Tris–Cl, pH 7.5–0.15 M KCl–10^{-4} M dithiothreitol. The dialysate was applied to a 4.1 × 100 cm column of DEAE-cellulose (DE-23, Whatman) and eluted with a KCl gradient (0.15–0.40 M) in the above buffer. Factors T and G elute in a single broad peak which was pooled and dialyzed against a buffer containing 0.05 M potassium cacodylate, pH 6.4–0.1 M KCl–10^{-4} M dithiothreitol. This dialysate was applied to a 2 × 100 cm column of either DEAE-cellulose (DE-52, Whatman) or DEAE-Sephadex (A-50, Pharmacia) and eluted with a KCl gradient (0.1–0.4 M) in the above cacodylate buffer. G factor emerged from this column as a single, sharp peak at approximately 0.25 M KCl nearly free of T_u activity as judged by the ribosome dependence of GDP binding to Millipore filters in the presence of fusidic acid. This material was pooled and dialyzed against 0.03 M potassium phosphate, pH 6.8, and 10^{-4} M dithiothreitol and applied to a 1.5 × 20 cm column of hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories). Elution was with a 0.03 to 0.20 M potassium phosphate gradient. In some cases, G factor was further purified by filtration on Sephadex G-100. The purified G factor was dialyzed against 0.01 M Tris-Cl, pH 7.4, and 0.001 M dithiothreitol in 50% glycerol and stored at −20°. Under these storage conditions, G factor is very stable, one preparation exhibiting negligible loss of activity in 10 months. The two G factor preparations employed here had specific activities (as described below) of 1780 and 1870 units per mg. Protein determination was based on the method of Kalckar (1947). These G factor preparations had an A_{280}/A_{260} of 1.75.

Formation and Detection of the Ribosome–G Factor–GDP Complex. The method employed for the formation and detection of ribosome-bound GDP was essentially that described before (Bodley *et al.*, 1970c) but as modified for our present purposes. The basic reaction mixture of 50 μl contained: buffer A (10 mM Tris-Cl, pH 7.4–10 mM magnesium acetate–10 mM ammonium chloride–0.1 mM dithiothreitol), 3 mM fusidic acid, 10^{-6} M [^{3}H]GTP (approximately 50,000–70,000 cpm), 12 units of G factor (see below), and 28 pmoles of ribosomes. In all cases reported here, the moles of G factor active in complex formation exceeded the moles of active ribosomes and under these conditions, complex formation is linearly dependent upon ribosomes (Bodley *et al.*, 1970c). Reactions were initiated, unless otherwise indicated, by the addition of [^{3}H]GTP and were conducted as described in the legends to the figures and tables.

Following incubation, the reactions were terminated by the addition of approximately 3 ml of buffer B (10 mM Tris-Cl, pH 7.4–10 mM magnesium acetate–10 mM NH_4Cl–10^{-5} M fusidic acid). This mixture was immediately filtered through a Millipore filter which was rapidly washed with 3 more 3-ml aliquots of buffer B. After drying, the filter was counted as described before (Bodley *et al.*, 1970c).

New Definition of G Factor Units. G factor of itself has no demonstrated enzymatic activity. In the uncoupled hydrolysis of GTP, it remains to be determined whether G factor or the ribosome acts as a cofactor or whether the enzymatic unit is created by the union of these macromolecular components. Quantitation of G factor is most commonly based upon its ability to promote the hydrolysis of GTP in the presence of ribosomes as described by Nishizuka and Lipmann (1966). These authors have defined one unit of G factor as that amount which, in the presence of an excess of ribosomes, will cause the hydrolysis of 1 μmole of GTP in 10 min at 30°. Various authors, however, employing this method report widely differing specific activities for G factor preparations which appear to be nearly homogeneous. We have also observed this type of variation and found it to result from the use of different ribosome preparations. By contrast, the G factor promoted binding of GDP to ribosomes stabilized by fusidic acid has, in our hands, yielded considerably less variation.

Recently, Weissbach *et al.* (1970) have proposed factor T_u quantitation based on its ability to cause the retention of GDP by Millipore filters. They define 1 unit of factor T_u as that amount of protein which retains 1 pmole of GDP. We wish here to propose a comparable definition for G factor; namely, 1 unit of G factor as that amount of the protein which, in the standard assay with 3 mM fusidic acid and excess ribosomes as previously described (Bodley *et al.*, 1970c), causes the retention of 1 pmole of GDP by Millipore filters. Such a definition is employed in the present report.

Separation of Ribosomes from Antibiotics. Thiostrepton was first removed from 200-μl reaction mixtures by passage through a 0.8 × 12 cm column of Bio-Gel A-1.5 m (Bio-Rad Laboratories), equilibrated, and eluted with buffer A containing 1 mM fusidic acid. The column was eluted at a flow rate of approximately 0.1 ml/min and the presence of ribosomes was detected by their absorbancy at 260 nm. The fractions containing the majority of ribosomes were pooled and dialyzed overnight against three changes of buffer A to remove fusidic acid.

Amino Acid Incorporation. The reactions were performed in a final volume of 40 μl and contained the following components: 100 mM Tris-Cl (pH 7.8), 15 mM KCl, 15 mM magnesium acetate, 10 mM β-mercaptoethanol, 5 mM phosphoenolpyruvate, 1 mM ATP, 0.05 mM GTP, 1.2 μg of pyruvate kinase, 10 μl of S-100, 6 × 10^{-6} M of [^{14}C]phenylalanine (specific activity 390 mCi/mmole, New England Nuclear Corp.), 5 μg of poly(U) (Miles Laboratories), 50 μg of uncharged tRNA, and 4 μg of ribosomes. Incubation was conducted for 30 min at 37°. The amount of polyphenylalanine product was determined by the method of Bollum (1966) as previously described (Bodley, 1969).

Results

Does Thiostrepton Cause the Destruction of the Ribosome–G Factor–GDP Complex? As shown in Figure 1 (dashed line), incubation of ribosomes (*ca.* 6 × 10^{-7} M) with thiostrepton at a concentration greater than 10^{-6} M prevents their subsequent binding of GDP. On this basis, we sought to inquire whether thiostrepton would cause the destruction of preexisting ribosome–G factor–GDP complexes. To test this, we constructed a reaction identical with that used above except that the order of addition of components was reversed. The solid line in Figure 1 shows the preexisting complexes are largely refractory to destruction by thiostrepton.

We next sought to determine which of the components of the complex are necessary to preserve the ability of the ribosomes to bind GDP in the presence of thiostrepton. The experiment illustrated in Table I show that only the combination of all three components gives full protection, G and GTP give 42%, but any component by itself or any other combination of two components is ineffective. The omission of fusidic

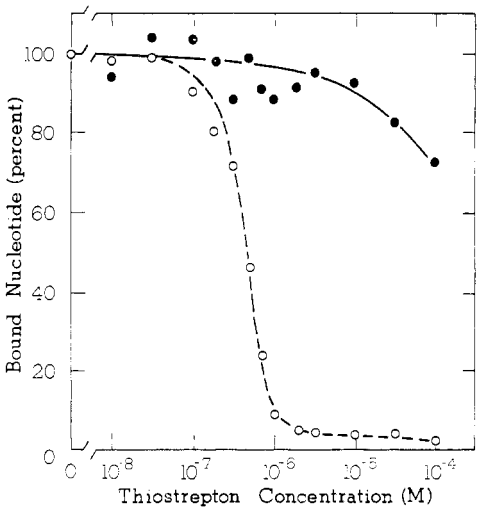

FIGURE 1: The effects of thiostrepton on the formation and stability of the ribosome–G factor–GDP complex. Two different two-step reactions were run. In the first (solid line), the complex was formed at 0° for 5 min. Thiostrepton was added at the final concentrations indicated in the figure and the reactions were terminated after 4.5 min of additional incubation. In the second reaction (dashed lined), ribosomes were incubated with thiostrepton for 4.5 min at 0°. After the addition of the components needed for complex formation and 5 min more incubation, reactions were terminated as described under Materials and Methods. One-hundred per cent is that amount of complex formed after 5 min of incubation at 0° in the absence of thiostrepton (8.2 pmoles).

TABLE I: Protection of Ribosomes from Thiostrepton Inactivation by G Factor, GTP, and Fusidic Acid.[a]

Components present prior to the exposure of ribosomes to thiostrepton	Per cent[b]
G, GTP, and fusidic acid	99
G and GTP	42
G and fusidic acid	4
G	4
GTP and fusidic acid	2
GTP	1
Fusidic acid	0

[a] In each experiment, three-step reactions were run. In the first step, ribosomes and the component(s) of the complex, as listed in the table, were incubated for 5 min at 0°. Thiostrepton (final concentration 10^{-5} M) was then added and 15 sec later, the remaining component(s) of the complex were added and incubation was continued at 0° for 45 sec. In this way, the final incubation mixtures have exactly the same composition, only the order of addition changed. The results are the averages of two separate experiments expressed as the percentage of GDP binding in the absence of thiostrepton (5.9 pmoles). The amount of nucleotide bound to ribosomes treated directly with thiostrepton (0.33 pmole) was subtracted. [b] Values listed are percentages of GDP bound in the absence of thiostrepton and after an incubation at 0° for 45 sec.

TABLE II: Protection of Ribosomes by Bound G Factor and GDP.[a]

Order of addition of GTP and thiostrepton	pmoles of bound nucleotide
1. GTP, then thiostrepton	8.58
2. GTP and thiostrepton at the same time	0.90
3. Thiostrepton, then GTP	0.10

[a] Reactions 1 and 3 were carried out in two steps. In reaction 1, complexes were formed at 0° for 4.5 min as described under Materials and Methods. Thiostrepton (10^{-5} M) was added, and the incubation was continued 5 min and then terminated. In reaction 3, ribosomes were incubated 4.5 min at 0° with 10^{-5} M thiostrepton. GTP and the rest of the components of the binding reaction were added, and the reaction was terminated 5 min later. In reaction 2, ribosomes were added to a solution which was 10^{-5} M with respect to thiostrepton and contained all the components (listed in Materials and Methods) for complex formation. Incubation was for 4.5 min at 0°.

acid in this experiment reduced binding to 29% of the control. Therefore, it would appear that the antibiotic is capable of stabilizing *preexisting* complexes

Another line of evidence supporting the conclusion that prior formation of complex prevents the binding of thiostrepton is presented in Table II. If protection from the inactivating effect of thiostrepton arises merely from the presence of G factor, GTP, and fusidic acid, then it would be expected that when all were added simultaneously with thiostrepton, the ribosome would retain its GDP binding ability. Only 11% of the normal binding activity was observed under these conditions (Table II) and it would appear, therefore, that the complex must be preformed.

Amino Acid Polymerizing Ability of Thiostrepton-Treated Ribosomes. The most straightforward interpretation of the results of the preceding experiments is that ribosomes in the form of the ribosome–G factor–GDP complex are unable to bind thiostrepton. In the simplest case, these ribosomes should retain their ability to polymerize aminoacyl-tRNA after exposure to thiostrepton. In order to test this possibility, we employed a two-step separation procedure involving gel filtration in the presence of fusidic acid to remove unbound thiostrepton, followed by dialysis to remove fusidic acid. The results of such experiments are shown in Table III, experiments B–D.

Experiment B (Table III) is a control experiment in which the ribosome–G factor–[^{3}H]GDP complex is formed and isolated by gel filtration in the presence of fusidic acid. Fusidic acid was subsequently removed by dialysis. The amount of [^{3}H]GDP excluded from this column as complex, the subsequent [^{3}H]GDP binding ability of the ribosomes as well as their phenylalanine polymerizing ability is presented in Table III (experiment B) and should be compared with experiments C and D. Treatment of ribosomes with thiostrepton prior to the formation of complex (experiment C) followed by the rapid removal of unbound thiostrepton largely, but not entirely suppressed their subsequent ability to participate in polymerization and complex formation. However, in experiment D, ribosomes in complex retained significant but not complete polymerizing and complex-forming ability following this treatment.

TABLE III: Phenylalanine Polymerizing and GDP Binding Ability of Ribosomes Separated from Antibiotics by Agarose Gel Filtration and Dialysis.[a]

Conditions and sequence of ribosome treatment	Complex recovered from column (%)	[^{14}C]Phenylalanine polymerization after treatment (%)		Complex formed after treatment (%)
		− Thiostrepton	+ Thiostrepton	
A. 1. Complex formed				
2. Thiostrepton exposure				
3. Dialysis		4	0	
B. 1. Complex formed				
2. Gel column filtration				
3. Dialysis	100 (22.9 pmoles)	100 (24.6 pmoles)		100 (4.2 pmoles)
C. 1. Thiostrepton, exposure				
2. Complex formed				
3. Gel column filtration				
4. Dialysis	2	14	0	7
D. 1. Complex formed				
2. Thiostrepton exposure				
3. Gel column filtration				
4. Dialysis	69	47		33

[a] Complex formation was conducted by incubating ribosomes, G factor, [^{3}H]GTP, and fusidic acid for 5 min at 0°. Thiostrepton exposure involved incubation for 5 min at 0° with 10^{-5} M antibiotic. The details of these procedures as well as gel column filtration and dialysis are described in Materials and Methods. The sequence of these operations for each experiment is indicated in the table.

We have previously shown that even in the presence of high concentrations of fusidic acid, the ribosome–G factor–GDP complex undergoes dissociation at a finite rate (Bodley *et al.*, 1970c). Therefore, the most reasonable interpretation of these results is that thiostrepton cannot destroy either the GDP binding or aminoacyl-tRNA polymerizing activity of ribosomes bound to G factor and GDP and that the partial inactivation that occurred under these conditions resulted from dissociation during the course of the reaction and subsequent gel filtration.

Stability of the Ribosome–G Factor–GDP Complex. If thiostrepton can bind to and hence, inactivate, only ribosomes which are *not* associated with G factor and GDP, then thiostrepton inactivation of ribosomes in complex should be a function of the stability of this complex.

In the experiment shown in Figure 2, it will be seen that both unlabeled GTP and thiostrepton effectively chase radioactive GDP from complex. Further, the rate of complex loss in the presence of thiostrepton is comparable to the loss of polymerizing and binding ability of ribosomes following treatment of complex with thiostrepton (Table III) considering the length of time required to remove the unbound antibiotic. Five different preparations of ribosomes were used for experiments of this type, and in all cases, the rate of chase of bound nucleotide by unlabeled GTP was equal to or, as in this case, slightly greater than that by thiostrepton. In no case, however, was a simple kinetic relationship observed (*e.g.*, first order) thus suggesting that these ribosomes are heterogeneous with respect to the stability of the ribosome–G factor–GDP complex.

The experiment shown in Figure 2 also examines the rate of chase of bound nucleotide by GDP and guanylyl methylenediphosphanate. GTP and GDP are equally effective in their ability to chase bound [^{3}H]GDP and more effective than either thiostrepton or guanylyl methylenediphosphanate. The latter nucleotide was the least effective of all the compounds tested in promoting the loss of bound GDP. This presumably results from the fact that while this nucleotide binds with ribosomes and G factor, the resulting complex is not stabilized by fusidic acid (Brot *et al.*, 1971; L. Lin and J. W. Bodley, unpublished data).

If the chase of bound nucleotide from complex under all of these conditions is simply the result of complex dissociation and subsequent competition for the ribosome, either for complex reformation by unlabeled nucleotide or ribosome inactivation by thiostrepton, then a combination of these agents

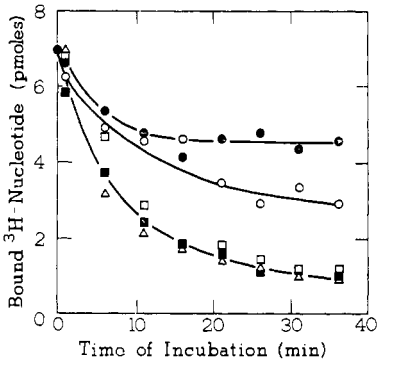

FIGURE 2: Chase of ribosome-bound [^{3}H]GDP by GTP, GDP, GMPPCP, and thiostrepton. Complex was formed for 5 min at 0° as described in the text. Following this incubation, thiostrepton (10^{-5} M) or unlabeled nucleotide (10^{-3} M) was added and the incubation continued at 0° for the indicated times. Chase by thiostrepton (○), chase by GTP (□), chase by GDP (△), chase by GMPPCP (●), and the chase by GTP and thiostrepton (■).

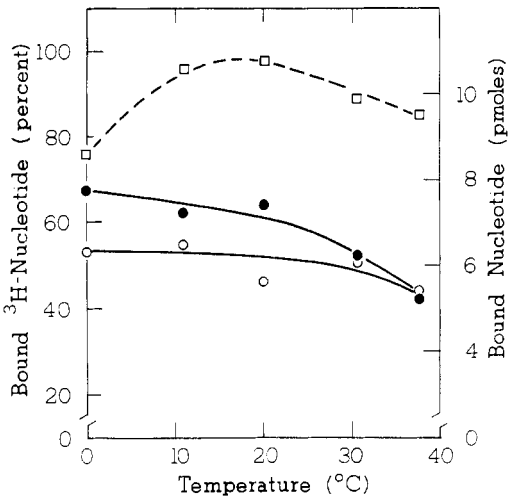

FIGURE 3: Effect of temperature on the formation and stability of complexes. To investigate complex formation as a function of temperature (dashed line), all components of the standard reaction mixture except [^{3}H]GTP were heated for 2 min at the indicated temperatures. [^{3}H]GTP was added and after an additional 5 min of incubation at temperature, reactions were terminated. To study complex stability as a function of temperature, two-step reactions were employed. The first step was the formation of complex at temperature as described above but instead of terminating the reaction, GTP or thiostrepton (10^{-3} M and 10^{-5} M, respectively) was added and after 5 min, the amount of bound [^{3}H]GDP was determined. One-hundred per cent is that amount of complex formed after 5 min of incubation at the appropriate temperature. Total complex (□), thiostrepton chase (●), GTP chase (○).

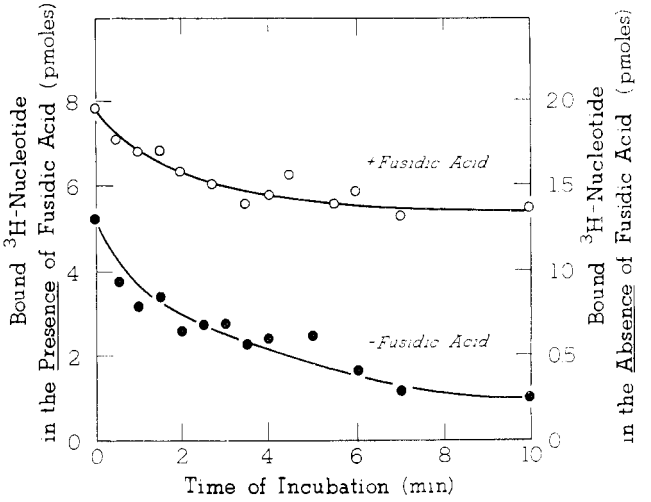

FIGURE 4: Effect of fusidic acid on thiostrepton inactivation of ribosomes. Reaction conditions were as described in the legend of Figure 2 except that in one case (closed circles) neither the reaction mixture nor wash buffer used to terminate the reaction contained fusidic acid. The open circles are the results of an experiment in which fusidic acid was present at the usual concentrations.

should chase no more effectively than the most active component. The experiments shown in Figure 2 realize this expectation; namely, the combination of thiostrepton plus unlabeled GTP chases bound, labeled GDP at exactly the same rate as does unlabeled GTP alone.

The experiments shown in Figure 3 were designed to examine the effect of temperature on the loss of bound nucleotide promoted by an excess of unlabeled GTP or by thiostrepton to determine if the relative rates of chase were due simply to the low temperature (0°) of the assay. It can be seen that the rate of loss of bound GDP by thiostrepton is surprisingly temperature independent. The total amount of complex is slightly greater at 20° than at 0° but the percentage of bound GDP remaining after treatment with either thiostrepton or an excess of unlabeled GTP is only slightly affected by temperature. In all cases, the amount of bound nucleotides surviving thiostrepton treatment equals or exceeds that surviving the chase by unlabeled GTP.

Fusidic Acid Retards Thiostrepton Inactivation. The preceding results strongly suggest that thiostrepton can bind only to ribosomes which do not bear G factor and GDP. Once this binding occurs, ribosomes are no longer capable of binding nucleotide or of participating in translocation. Since fusidic acid functions to stabilize the ribosome–G factor–GDP complex (Bodley *et al.*, 1970b; Brot *et al.*, 1971), the presence of this antibiotic should serve to retard the thiostrepton-promoted loss of bound GDP. The experiment shown in Figure 4 bears out this prediction.

Discussion

The ribosome–G factor–GDP complex serves as a significant focal point from which to examine the nature of the interaction between the ribosome and G factor, for it occurs as an intermediate in the hydrolysis of GTP by this system (Brot *et al.*, 1969; Parmeggiani and Gottschalk, 1969). The antibiotic inhibitors of translocation, fusidic acid (Tanaka *et al.*, 1968; Pestka, 1968; Haenni and Lucas-Lenard, 1968) and thiostrepton (Pestka, 1970), also inhibit uncoupled GTP hydrolysis. The inhibitory effect of fusidic acid arises from its ability to stabilize this intermediate complex and thus prevent a repetition of GTP hydrolysis (Bodley *et al.*, 1970b; Brot *et al.*, 1971). Thiostrepton, on the other hand, prevents any detectable interaction among the ribosome, G factor, and guanine nucleotides (Bodley *et al.*, 1970a; Weisblum and Demohn, 1970b).

The inhibitory effect of thiostrepton apparently results from its tight binding to the ribosome. Although this binding has not been demonstrated directly, Weisblum and Demohn (1970a) have shown that the polymerizing activity of ribosomes exposed to thiostrepton is not regained upon copious dialysis. Experiments in the present report confirm this observation (Table III, experiment A). Furthermore, only slightly more than one molar equivalence of thiostrepton is necessary to destroy the ribosome's ability to bind GDP. In addition, prolonged incubation of ribosomes with less than a molar equivalence of this antibiotic leads only to the expected, partial inactivation (Bodley *et al.*, 1970a).

The experiments presented here indicate that, while a very brief exposure of ribosomes to thiostrepton (15 sec or less) is sufficient to destroy their ability to participate in complex formation, this complex, once formed, is quite refractory to thiostrepton destruction. Moreover, the destruction of these preexisting complexes by this antibiotic proceeds at a rate which is equal to or less than the rate of dissociation of complex as measured by the disappearance of bound [^{3}H]GDP in the presence of an excess of unlabeled, unbound nucleotide. It would seem, therefore, that thiostrepton cannot bind to ribosomes in the ribosome–G factor–GDP complex and that the destruction of this complex, which occurs in the presence of thiostrepton, results from the dissociation of the more weakly bound protectors (G factor and GDP).

Clearly, fusidic acid is not required to prevent ribosomal

inactivation by thiostrepton. It behaves, however, as would be predicted on the basis of previous knowledge to delay inactivation by stabilizing the ribosome–G factor–GDP complex. In other words, ribosomes treated with thiostrepton in the presence of G factor and GTP retain a significant amount of GDP binding ability following the subsequent addition of fusidic acid. No other combination of two of these three components produces this effect. Therefore, it is still not possible to decide the order of addition to the ribosome of G factor and the guanine nucleotide. As pointed out previously (Bodley *et al.*, 1970b), other, presumably equilibrium, methods will have to be employed to determine if the ribosome interacts with G factor or whether one of these macromolecules first interacts with GTP. In any event, the hydrolytic reaction *per se* clearly requires both ribosomes and factor (Bodley *et al.*, 1970b).

The treatment of ribosomes with thiostrepton leads not only to a loss in their ability to bind GDP in response to G factor, but a loss also in their ability to polymerize aminoacyl-tRNA. The most significant result of the present experiments is that the prior binding of G factor and GDP to ribosomes protects them from thiostrepton inactivation of their polymerizing ability. While the interaction of G factor and the ribosome in the hydrolysis of GTP is widely used as a model for translocation, it has not been possible to couple hydrolysis and translocation or to directly demonstrate the relationship between these two processes. The present results provide further indirect evidence for the relationship between these two processes because the uncoupled interaction of G factor and the ribosome protects the translocational ability of the ribosome from inactivation by thiostrepton.

The 50S ribosomal subunit has several identifiable functions in protein synthesis (for a review, see Lengyel and Söll, 1969). Some, as yet unknown portion of the particle, termed peptidyl transferase, is responsible for the actual synthesis of peptide bonds. The 50S subunit is also involved in the binding of tRNAs during the course of protein synthesis, and it presumably contains at least a portion of the two tRNA binding sites (the A and P sites) believed to be involved in this process. There is also a site(s) on the 50S subunit which is involved in the interaction with and binding to the 30 subunit. Additional sites upon the 50S subunit are capable of interacting with antibiotics which interfere with protein synthesis (Weisblum and Davies, 1968).

On the basis of the present work and that reported earlier, it is clear that the 50S subunit also possesses a site recognized by G factor (Bodley and Lin, 1970). Thiostrepton also binds to this subunit and inactivates it (Weisblum and Demohn, 1970a) by destroying its ability to interact with G factor (Pestka, 1970; Bodley *et al.*, 1970a; Weisblum and Demohn, 1970b). While the present results do not rule out an allosteric relationship between the binding of G factor–GDP and thiostrepton, the simplest interpretation is that the mutually exclusive nature of these two bindings results from direct steric interference as the consequence of occurring at or near the same physical region of the 50S ribosomal subunit.

The nature of the chemical and physical results of GTP hydrolysis in this system are unknown but further investigation of the interaction of G factor and the 50S subunit and the effects of antibiotics on this process should provide significant insight into this question fundamental to an understanding of the mechanism of protein synthesis.

References

Bodley, J. W. (1969), *Biochemistry 8*, 465.

Bodley, J. W., and Lin, L. (1970), *Nature (London) 227*, 60.

Bodley, J. W., Lin, L., and Highland, J. H. (1970a), *Biochem. Biophys. Res. Commun. 41*, 1406.

Bodley, J. W., O'Dea, R. E., Gordon, J., and Goldberg, N. D. (1971), *J. Biol. Chem. 246*, 3618.

Bodley, J. W., Zieve, F. J., and Lin, L. (1970b), *J. Biol. Chem. 245*, 5662.

Bodley, J. W., Zieve, F. J., Lin, L., and Zieve, S. T. (1969), *Biochem. Biophys. Res. Commun. 37*, 437.

Bodley, J. W., Zieve, F. J., Lin, L., and Zieve, S. T. (1970c), *J. Biol. Chem. 245*, 5656.

Bollum, F. J. (1966), *Procedures Nucleic Acid Res. 1966*, 296.

Brot, N., Spears, C., and Weissbach, H. (1969), *Biochem. Biophys. Res. Commun. 34*, 843.

Brot, N., Spears, C., and Weissbach, H. (1971), *Arch. Biochem. Biophys. 43*, 286.

Erbe, R. W., Nau, M. M., and Leder, P. (1969), *J. Mol. Biol. 39*, 411.

Haenni, A. L., and Lucas-Lenard, J. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1363.

Harvey, C. L., Knight, S. G., and Sih, C. J. (1966), *Biochemistry 5*, 3320.

Kalckar, H. M. (1947), *J. Biol. Chem. 167*, 461.

Leder, P., Skogerson, K. E., and Nau, M. M. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 494.

Lengyel, P., and Söll, D. (1969), *Bacteriol. Rev. 33*, 264.

Nishizuka, Y., and Lipmann, F. (1966), *Arch. Biochem. Biophys. 116*, 344.

Nishizuka, Y., Lipmann, F., and Lucas-Lenard, J. (1968), *Methods Enzymol. 12*, 708.

Parmeggiani, A., and Gottschalk, E. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 861.

Pestka, S. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 726.

Pestka, S. (1970), *Biochem. Biophys. Res. Commun. 40*, 667.

Tanaka, N., Kinoshita, T., and Masukawa, H. (1968), *Biochem. Biophys. Res. Commun. 30*, 278.

von Ehrenstein, G., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 941.

Weisblum, B., and Davies, J. (1968), *Bacteriol. Rev. 32*, 493.

Weisblum, B., and Demohn, V. (1970a), *J. Bacteriol. 101*, 1073.

Weisblum, B., and Demohn, V. (1970b), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 149.

Weissbach, H., Miller, D. L., and Hackmann, J. (1970), *Arch. Biochem. Biophys. 137*, 262.