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Peptidyl-Puromycin Synthesis on Polyribosomes from *Escherichia coli**

(peptide bond synthesis/transpeptidation/peptidyl-tRNA/Hill equation)

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ABSTRACT **Peptide bond synthesis was studied with native polyribosomes of *E. coli*. With the use of this system for transpeptidation, it was possible to show that a single K^+ activates the ribosome monomers of polyribosomes; that protonation of a single group (probably imidazole or an N-terminal amino group) with a pK_a equal to about 7.2 inactivates the transpeptidase complex; that Mn^{++} can substitute for Mg^{++}, but that Ca^{++}, spermidine, and putrescine do so only very poorly; and that the K_m for puromycin in this system is about 2.4×10^{-6} M.**

Since several antibiotics that inhibit peptide bond synthesis with washed ribosomes do not appear to inhibit peptide bond formation in the intact cell (2–4), we decided to again examine peptide bond synthesis in a system as nearly physiologic as possible. The reaction of peptidyl-tRNA with puromycin on polyribosomes can be considered a model of peptide bond synthesis. Thus, the reaction of puromycin with peptidyl-tRNA of native polyribosomes, which were obtained from *Escherichia coli*, was measured. Peptidyl-puromycin synthesis on native polyribosomes has distinctly different characteristics and requirements from those of peptidyl-puromycin synthesis with synthetic donors and washed ribosomes when such assays as acetylphenylalanyl-puromycin or formylmethionyl-puromycin synthesis are used. This communication reports the characteristics and requirements of peptidyl-puromycin synthesis on native bacterial polyribosomes in a cell-free system.

EXPERIMENTAL PROCEDURE

Chemicals, Materials, and Cell Extracts. The sources of chemicals, enzymes, and cell extracts have been given in a previous publication (5). [^{3}H]Puromycin (740 mCi/mmole) was obtained from New England Nuclear Corp. *E. coli* B polyribosome extracts containing nascent polypeptides were prepared by modification of the method of Godson and Sinsheimer (6). The specific details of the procedure have been reported (5).

[^{3}H]Puromycin Incorporation into Nascent Peptides. Each 0.050-ml reaction mixture contained the following components unless otherwise indicated: 0.10 M KCl; 0.01 M NH_4Cl; 0.005 M $MgCl_2$; 0.05 M Tris·acetate (pH 7.2); 0.1% (w/v) Brij 58 contributed by the polyribosome extract; 2.8 A_{260} units of polyribosome extract; and 4×10^{-6} M [^{3}H]puromycin (or as specified). Unless otherwise noted, the polyribosome extract was added last to start reactions. Incorporation of [^{3}H]puromycin into nascent polypeptides was measured as follows: reactions were stopped by addition of 2 ml of cold 10% (w/v) trichloroacetic acid and placement in an ice bath for at least 5 min; the contents of each tube were filtered through a polyvinyl chloride Millipore filter (type BDWP, 0.6 μm pore size, 25 mm in diameter); each tube and filter were washed three times with 3-ml portions of 5% (w/v) trichloroacetic acid; the filter was washed eight times with 3-ml portions of absolute ethanol at room temperature to remove unreacted puromycin; radioactivity was determined as described (5). In the absence of puromycin, under the conditions of the assay for peptidyl-puromycin synthesis, leucine incorporation into polypeptides was negligible.

Analysis and Computation of Data. The Hill equation was used for evaluation of the molecular order of participation of substrates, activators, and inhibitors on peptidyl-puromycin synthesis. For substrates, $\log[v/(V_{max} - v)] = n \log [S] - \log K$; and for inhibitors, $\log[v/(V_0 - v)] = \log K' - n \log [I]$, where v = reaction rate, V_0 = uninhibited reaction rate, [S] = substrate concentration, [I] = inhibitor concentration, n = interaction coefficient, and K = apparent K_m or $S_{0.5}$. When $n = 1$, $K' = [(K_m + [S])K_t]/K_m$ for competitive inhibition and $K' = K_t$ for noncompetitive inhibition, where K_m represents the Michaelis constant or $S_{0.5}$ and K_t, the apparent K_t for a given substrate concentration or $I_{0.5}$ (7–10). For some plots, a straight line was fit to the data by the least squares method (11) by computer program. Thus, the slope of the line and the intercept were calculated by computer.

RESULTS AND DISCUSSION

Kinetics of Peptidyl-Puromycin Formation. The kinetics of peptidyl-[^{3}H]puromycin formation at various temperatures is shown in Fig. 1. The initial rate of peptidyl-puromycin formation is nearly a linear function of temperature throughout the temperature range studied. The extent of peptidyl-puromycin synthesis at 15 min also increases with temperature. An Arrhenius plot provides an estimate of 5.6 kcal/mol for the activation energy of peptidyl-puromycin synthesis; this is about the same as that for acetylphenylalanyl-puromycin formation above 30°C (12). The value for 0°C (*solid square*) does not fall on the straight line as do the other points representing temperatures higher than 0°C.

Peptidyl-Puromycin Synthesis and Monovalent Cation Concentration. Since the polysome extract contained 0.05 M NH_4Cl, all reaction mixtures for evaluation of the effect of monovalent cations contained 0.01 M NH_4^+. Addition of K^+ beyond 0.01 M NH_4^+ concentration increased the rate of

* This paper is no. XVIII in the series, Studies on Transfer Ribonucleic Acid-Ribosome Complexes. The preceding publication in this series is listed as ref. 1.

peptidyl–puromycin synthesis slightly; nevertheless, peptidyl–puromycin synthesis occurred in the absence of additional K^+ or NH_4^+ ions (Fig. 2). The optimum concentration of NH_4^+ was found to be 0.05–0.1 M. At higher NH_4^+ concentrations, the rate of peptidyl–puromycin synthesis was reduced; and at 1.0 M NH_4^+, the rate of synthesis was less than half that in the absence of any additional monovalent cations. Na^+ and Li^+ were markedly inhibitory.

When polyribosomes are centrifuged through a sucrose gradient containing no K^+ or NH_4^+, the resulting polyribosomes are inactive in synthesizing peptidyl–puromycin (Fig. 3). In contrast to the results with polyribosomes prepared and stored in the presence of 0.05 M NH_4^+ (Fig. 2) where additional K^+ or NH_4^+ is only slightly stimulatory, these polyribosomes washed free of NH_4^+ are relatively inactive in forming peptidyl–puromycin unless they are activated by prior incubation with relatively high concentrations of K^+ or NH_4^+. That activation rather than a direct effect on the reaction rate is involved is suggested by the data of Figs. 2 and 3. If polyribosomes are incubated at 37°C for 1 min in the presence of K^+ or NH_4^+, substantial activity is seen (*left panel*, Fig. 3); in contrast, however, incubation at 24°C for 1 min produced a relatively small amount of peptidyl–puromycin synthesis (*center panel*, Fig. 3). If the polyribosomes are incubated at 37°C for 5 min and then assayed for activity at 24°C for 1 min (*right panel*, Fig. 3), it can be seen that substantial activity was restored during the 5-min incubation at 37°C. Under none of these conditions was Li^+ or Na^+ active. From a double reciprocal plot of the data in Fig. 3, the V_{max} for the appropriate experiment can be obtained (*right panel*, Fig. 4). In the three cases shown, the molecular interaction coefficients for K^+ were found to be

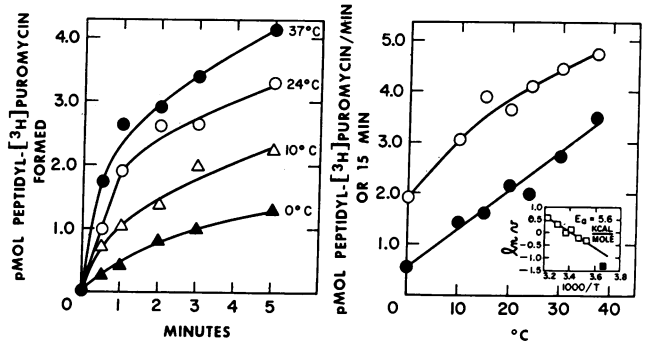

FIG. 1. Kinetics and extent of peptidyl–[^{3}H]puromycin formation at various temperatures. Each 0.050-ml reaction mixture contained the components indicated under *Experimental Procedure*. Reaction mixtures were incubated at various temperatures and assayed at the times indicated on the *abscissa* of the *left panel*. Reactions were started by addition of [^{3}H]puromycin to reaction mixtures last, and tubes were not prewarmed. For the determination of the rate of peptidyl–[^{3}H]puromycin formation as a function of temperature (*right panel*, ●), the pmol of peptidyl–puromycin synthesized/min (v) was determined from the initial slope of the time curves (not all of which are shown in the *left panel*); this *rate* (pmol/min) is plotted as a function of temperature in the *right panel*. In addition, the *extent* of peptidyl–puromycin synthesis (pmol at 15 min) is also given at various temperatures (○). An Arrhenius plot (*insert of right panel*) yields an activation energy (E_a) of 5.6 kcal/mol for peptidyl–[^{3}H]puromycin synthesis. The data for the Arrhenius plot are given as (□) for all temperatures except 0° C, which is plotted as (■).

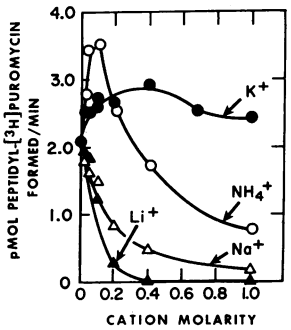

FIG. 2. Effect of monovalent cations on the rate of peptidyl–puromycin formation. Each 0.050-ml reaction mixture contained the components indicated under *Experimental Procedure* except that the concentration of monovalent cation is specified on the *abscissa*; 0.01 M NH_4Cl was present in each reaction mixture as the contribution from the polysome suspension. Reactions were incubated at 37°C for 1 min. To start reactions, [^{3}H]puromycin was added last, and tubes were not prewarmed.

0.88, 0.93, and 0.96 from the Hill plot (*left panel*, Fig. 4): thus, approximately one K^+ is necessary for converting inactive into active polyribosomes. Furthermore, inhibition of peptidyl–puromycin synthesis by Na^+ appears to involve only one Na^+ (data not shown). Since the interaction coefficient for K^+ activation and Na^+ inhibition are both about 1, these ions may compete for a similar ribosomal site.

The lack of a requirement for the presence of high concentrations of K^+ or NH_4^+ in the reaction mixtures distinguishes the reaction from acetylphenylalanyl–puromycin (12), phenylalanyl–phenylalanine (13), polyphenylalanyl–puromycin (14),

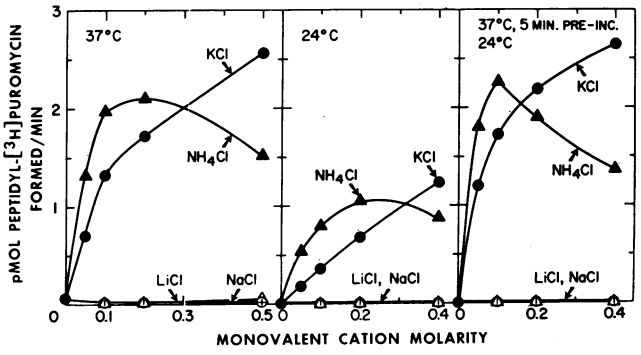

FIG. 3. Effect of monovalent cations on rate of peptidyl–[^{3}H]puromycin formation with washed polyribosomes. For these experiments, washed polyribosomes were used. These were prepared by layering 0.25 ml of the polyribosome preparation onto a 10–30% (w/v) sucrose gradient containing 0.005 M $MgCl_2$ and 0.005 M Tris·HCl (pH 7.2). The gradient was centrifuged in a Spinco SW65 rotor at 60,000 rpm for 80 min at 0°C. The polyribosome pellet was resuspended in 0.005 M $MgCl_2$ and 0.005 M Tris·HCl, (pH 7.2). Each 0.050-ml reaction mixture contained the components indicated under *Experimental Procedure* in addition to the following: 1.0 A_{260} unit of washed polyribosomes; 0.005 M Tris·acetate (pH 7.2); no Brij 58 was present in reaction mixtures; and monovalent cation concentration was as indicated on the *abscissa*. Reaction mixtures were incubated for 1 min at 37 or 24°C (*left* and *center panels*, respectively). In the *right panel*, reaction mixtures were incubated at 37°C for 5 min before addition of [^{3}H]puromycin; after addition of [^{3}H]puromycin, they were incubated at 24°C for 1 min.

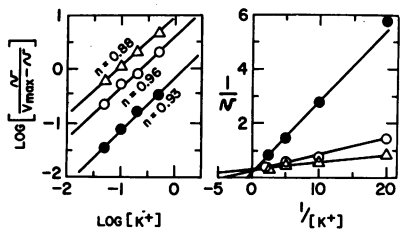

FIG. 4. Molecular order of activation of polyribosomes by K^+. The data of Fig. 3 in which K^+ was used is plotted. In the *right panel*, a double-reciprocal plot of the data is presented. With the V_{max} values obtained from the double-reciprocal plot, $\log[v/(V_{max} - v)]$ was calculated and plotted as a function of log [K^+] (*left panel*). Referring to the data of Fig. 3: Δ = the experiments where reaction vessels were incubated at 37°C for 5 min before assay at 24°C for 1 min; ● = the data where reactions were performed at 24°C for 1 min; and ○ = the data for reactions performed at 37°C for 1 min without prior incubation.

and formylmethionyl-puromycin (14) synthesis. For native polyribosomes, high K^+ or NH_4^+ concentrations are necessary for activating polyribosomes, but are not necessary in concentrations above 0.01 M NH_4^+ for maintaining the active state (Figs. 2 and 3). Activation and inactivation of ribosomes and subunits with respect to transpeptidation have been described (15–17).

The requirement for a single K^+ per ribosome monomer suggests a possible mode of controlling protein synthesis. A cellular protein that could avidly bind or replace this single K^+ could shut off protein synthesis instantaneously at a ratio of one molecule of protein per ribosome. That changes in intracellular K^+ can alter the rate of cellular protein synthesis has been shown by Lubin and Ennis (18). In addition, Scheps *et al.* (19) have shown that ribosomes from stationary phase *E. coli* require activation with 0.56 M NH_4^+ to function efficiently in protein synthesis. Perhaps, K^+ plays a regulatory role in controlling transpeptidation and protein synthesis in the intact cell. Also, the effectiveness of some antibiotics and other agents that inhibit protein synthesis may be related to the function of this K^+ binding site.

Effect of Mg^{++} and Other Cations. Optimum magnesium concentration for peptidyl-puromycin synthesis was 0.004 M (Fig. 5), which is lower than that necessary when acetylphenylalanyl-tRNA is a donor (20). It is about the same as the optimum Mg^{++} concentration for release by puromycin of polyphenylalanine from ribosomes containing polyphenylalanyl-tRNA (14). Polyribosomes washed through a sucrose gradient also showed similar Mg^{++} dependencies. The maximal rate of peptidyl-puromycin formation occurred at the following cation concentrations: 0.004 M Mg^{++}, 0.0013 M Mn^{++}, 0.0012 M Ca^{++}, 0.01 M putrescine, and 4×10^{-4} M spermidine. All showed a stimulatory phase at low concentrations and an inhibitory phase at cation concentrations above the optimum. Excess Mg^{++} inhibited the optimal rate (Fig. 5) and extent (data not shown) of peptidyl-puromycin synthesis about 50%. Although excess Mg^{++} could only partially inhibit peptidyl-puromycin synthesis, excess Mn^{++} or spermidine produced essentially total inhibition. Why excess of these cations inhibits both the rate and extent of peptidyl-puromycin synthesis is not clear: perhaps Mg^{++} stabilizes peptidyl-tRNA on the ribosome so that the molecule cannot properly interact with the components at the site for transpeptidation. Transpeptidation involves nucleophilic attack by the α-amino group of aminoacyl-tRNA or another appropriate acceptor on the carboxyl ester bond of peptidyl-tRNA with subsequent transfer of the carboxyl group of the peptidyl moiety to the α-amino group to form a peptide bond (21). We have previously suggested that the aminoacyl-end of aminoacyl-tRNA is the fixed entity, and that the peptidyl-donor is mobile at the C-C-A(peptidyl) terminus (22). A reduction in mobility of the C-C-A(peptidyl) terminus of peptidyl-tRNA by magnesium-phosphate bridges or otherwise by Mg^{++} may, therefore, inhibit transpeptidation. The polyribosome extracts contained factor G and GTP. Even when washed polyribosomes were used, washing was not performed in high salt concentrations. It is, therefore, impossible at this time to exclude the possibility of the occurrence of some minimal translocation as well as transpeptidation.

The Hill plot with respect to Mg^{++} (*insert*, Fig. 5) suggests there may be several classes of Mg^{++} binding to polyribosomes necessary to maintain functional peptidyl-puromycin synthesis. Below 0.001 M Mg^{++}, multiple Mg^{++} binding sites ($n > 4$) appear necessary for maintaining the integrity of transpeptidation; at these low concentrations the cations sup-

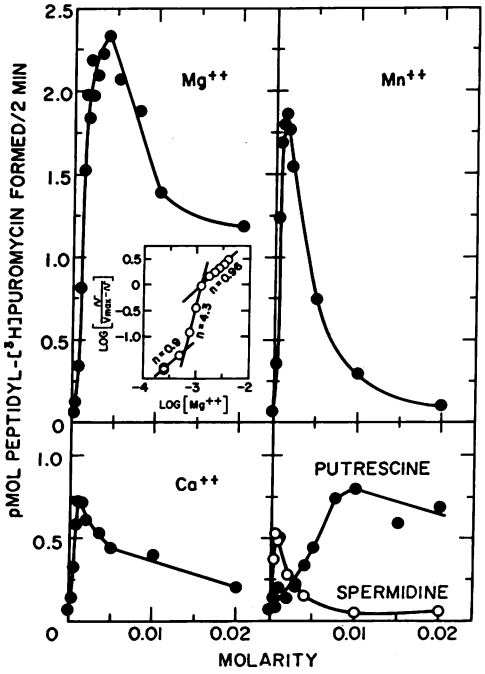

FIG. 5. Effect of divalent cations and oligoamines on rate of peptidyl-puromycin synthesis. Each reaction mixture contained the following in a total of 0.20 ml: divalent cation or oligoamine concentration as indicated on the *abscissa*; 0.10 M KCl; 0.05 M Tris·acetate (pH 7.2); 2.5 A_{260} units of polyribosome preparation; and 1×10^{-6} M [^{3}H]puromycin. Polysomes were added last, and reaction mixtures were incubated at 24°C for 2 min. Reaction volumes were 0.20 ml instead of the usual 0.05 ml so that the Mg^{++} in the polyribosome preparation could be sufficiently diluted; the final concentration of Mg^{++} because of Mg^{++} in the polyribosome preparation was 2.5×10^{-4} M. The cation concentrations on the *abscissa* refer to the concentrations of the cations in addition to this 2.5×10^{-4} M Mg^{++}; however, for Mg^{++}, the actual total Mg^{++} concentration is plotted. A Hill plot of the data for the Mg^{++} curve is also presented as an *insert* to the Mg^{++} portion of the figure.

port transpeptidation in the following order: $Mn^{++} > Mg^{++} > Ca^{++} >$ spermidine > putrescine. At concentrations greater than 0.001 M Mg^{++}, it seems that a single specific Mg^{++} is essential for activity ($n = 0.96$). This specific Mg^{++} site can be partially replaced by Mn^{++}, but only poorly by Ca^{++}, spermidine, or putrescine. A similar hierarchy of cations was found with respect to binding of C-A-C-C-A-(Phe) to ribosomes (22). It is likely that the multiple site interactions represent relatively nonspecific binding sites for maintaining structural integrity of the ribosome monomers. The other cations may simply be sparing nonspecifically the small quantity of Mg^{++} (2.5×10^{-4} M) present in the reaction mixture for specific interaction with the site necessary for transpeptidation; although Mn^{++} can substitute for this specific site fairly well, the other cations cannot. Similarly, Kimes *et al.* (24) and Weiss and Morris (23) have also suggested two classes of Mg^{++} binding sites on *E. coli* ribosomes. Nevertheless, it should be noted that most of the Mg^{++} bound to ribosomes is bound to the primary phosphoric acid groups of ribonucleic acid (25–27).

Effect of pH on the Rate of Peptidyl-Puromycin Synthesis. The rate of peptidyl-puromycin formation as a function of pH is shown by the data of Fig. 6. The rate of the reaction increases as the H^+ concentration is decreased. The optimum pH for the rate of peptidyl-puromycin formation was found to be 9.1. Above pH 9.1, the velocity of the reaction decreases.

Since addition of H^+ at the optimum pH decreases the reaction velocity, H^+ may be considered an inhibitor of the reaction, and the kinetics of the reaction can be analyzed in several ways. By plotting the data according to the Hill equation, one can obtain the molecular order of participation of H^+ as an inhibitor (Fig. 7*A*). Since $n = 1.01$, a single protonated group appears involved in the inhibitory process. From the intercept of the *abscissa* where $\log[v/(V_0 - v)]$ is zero, $I_{0.5}$ is equal to pH 7.3; from the intercept of the *ordinate*, pK_a is equal to 7.36. In addition, a Dixon plot (28) of the data indicates that the pK_i is equal to pH 7.2 (Fig. 7*B*); furthermore, the intersection of the lines at the baseline indicates that inhibition of peptidyl-puromycin synthesis by H^+ is noncompetitive. A similar value of pH 7.2 for the pK_i is obtained by plotting log v as a function of pH according to

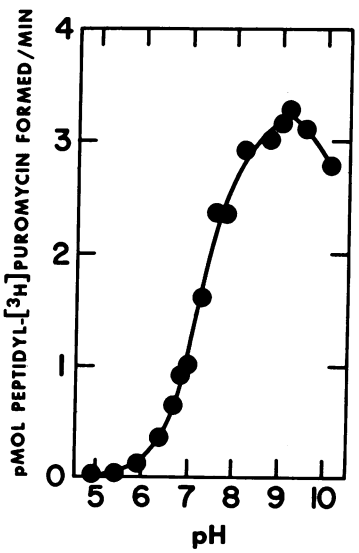

FIG. 6. Effect of pH on rate of peptidyl-[^{3}H]puromycin synthesis. Each 0.20-ml reaction mixture contained the following components: 0.10 M potassium acetate; 0.0025 M NH_4Cl; 0.00425 M $MgCl_2$; 0.025% Brij 58 (w/v); 2.8 A_{260} units of polyribosome extract; and 1×10^{-6} M [^{3}H]puromycin; Tris concentration was 0.05 M; the appropriate pH was obtained by prior adjustment with acetic acid at the temperature of the reaction mixture. Reaction mixtures were incubated at 24°C for 15, 30, and 60 sec at each pH. From this curve, the initial rate of peptidyl-puromycin synthesis was determined and is presented.

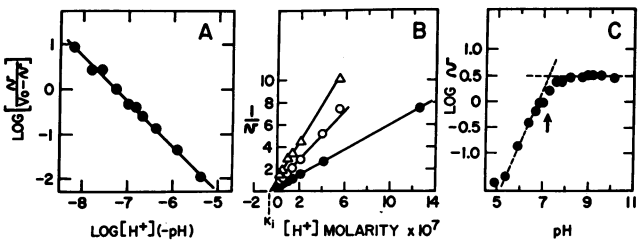

FIG. 7. Kinetic analyses of effect of H^+. (*A*) The data of Fig. 6 are plotted according to the Hill equation: $n = 1.01$, the number of interacting sites for H^+; log K', the intercept, equals −7.36. The uninhibited reaction rate (V_0) is assumed to be the reaction velocity (v) at the optimum pH. $I_{0.5}$, the concentration where $v = V_0/2$ and where $\log[v/(V_0 - v)] = 0$, is 5.0×10^{-8} M (pK = 7.3).

(*B*) The data of Fig. 6 are plotted as (●) according to the method of Dixon (28) for 1×10^{-6} M puromycin. In addition, similar experiments as described in Fig. 6 were performed at 2.5×10^{-7} and 5×10^{-7} M [^{3}H]puromycin and plotted as (△) and (○), respectively. The intersection of the three lines is on the baseline where $K_i = 5.82 \times 10^{-8}$ M ($pK_i = 7.24$).

(*C*) The data of Fig. 6 are plotted according to Dixon and Webb (10): log v as a function of pH. *Arrow* = pH 7.2.

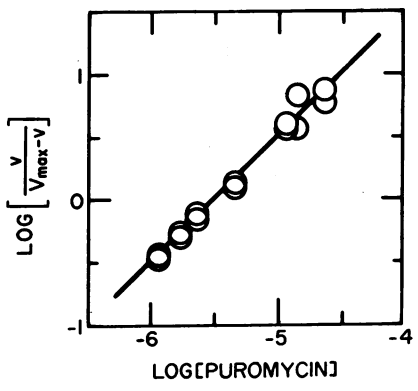

FIG. 8. Hill plot for puromycin participation in peptidyl-puromycin synthesis. Each 0.050-ml reaction mixture contained the components described under *Experimental Procedure* except that the puromycin concentration was varied and reactions were performed at 24°C for 1 min. The data were plotted according to the Hill equation. The interaction coefficient, n, was found to be 0.996, and K_m was determined as 3.3×10^{-6} M for puromycin (log $K_m = -5.48$, the intercept of the ordinate). The curve from which these data are taken corresponds to Figs. 2 and 4 of †. When $v = V_{max}/2$, $\log[v/(V_{max} - v)]$ equals 0, and $K_m = 3.0 \times 10^{-6}$ M ($pK_m = 5.52$), as determined from the intercept of the *abscissa*.

Dixon and Webb (10) as shown in Fig. 7C. With the use of polyribosomes washed through a sucrose gradient containing 0.05 M KCl, 0.01 M Tris·HCl (pH 7.2), and 0.005 M $MgCl_2$, the pH profile was virtually identical to that of Fig. 6, and a Hill plot yielded a value of 1.0 for n. In cases where H^+ simply alters the ionization state of the enzyme without causing irreversible inactivation, this method under appropriate conditions may afford a simple approach for determination of the pK of the ionizable group involved. With other assays for transpeptidation, it has been reported that the pH optimum for transpeptidation is 8.5 or greater (21, 29). A similar pH dependence was observed when α-hydroxy-puromycin was used as an acceptor substrate (29); this eliminated the α-amino group of puromycin as responsible for the pH profile. For H^+, the pK_i should be equivalent to pK_a of the protonated group. The pK_a of 7.2 (Fig. 7B) is consistent with the possibility of a single imidazole residue (or possibly an N-terminal amino group) being involved at the active center of the transpeptidase complex and in the rate-determining step for transpeptidation (30). If the pH optimum for peptide bond formation is about 9, this reaction in the intact cell may be occurring at a substantially reduced rate compared to its potential capacity. On the other hand, the pH optimum for peptide bond synthesis with puromycin as an acceptor may differ from the pH optimum for normal peptide bond formation between peptidyl- and aminoacyl-tRNA.

Molecular Order of Participation of Puromycin in the Reaction. Since it has been already reported that one puromycin molecule attaches to a single polypeptide chain of peptidyl-tRNA, the analysis of n and K_m by the Hill plot for puromycin was used as a control for the methodology of these experiments. As the data of Fig. 8 show, $n = 0.996$ and $K_m = 3.3 \times 10^{-6}$ M. The K_m determined independently by the Lineweaver-Burk (31) double reciprocal plot yields a value of 2.4×10^{-6} M for the K_m for puromycin†. It thus appears that the methodology used may be useful in understanding the mechanisms involved in peptidyl-puromycin synthesis on native polyribosomes. The value for the K_m is about two orders of magnitude lower than the K_m determined for acetylphenylalanyl-puromycin or formylmethionyl-puromycin synthesis (12, 29).

Other Comments. The observations that some antibiotics inhibit acetylphenylalanyl-puromycin synthesis, polylysyl-puromycin synthesis, and formylmethionyl-puromycin synthesis, but not protein synthesis on polyribosomes in the cell seemed contradictory (4). In a previous publication (4) we suggested that the apparent discrepancy may be due to the inability of certain antibiotics to interact properly with ribosomes on polyribosomes in the intact cell. The fact that these polyribosome preparations seem to behave similarly to intact cells in this respect (5)† suggests that the study of transpeptidation on native polyribosomes as described in this communication may be a useful tool for understanding transpeptidation as well as protein synthesis in the intact organism.

† Pestka, S. & Hintikka, H., manuscript in preparation.

1. Hishizawa, T. & Pestka, S. (1971) *Arch. Biochem. Biophys.* **147**, 624–631.
2. Cundliffe, E. (1969) *Biochemistry* **8**, 2063–2066.
3. Ennis, H. (1970) *Proceedings of the Sixth International Congress of Chemotherapy, Tokyo, 1970* (University of Tokyo Press, Tokyo), pp. 489–498.
4. Pestka, S. (1971) *Annu. Rev. Microbiol.* **25**, 487–562.
5. Pestka, S. & Hintikka, H. (1971) *J. Biol. Chem.* **246**, 7723–7730.
6. Godson, G. N. & Sinsheimer, R. L. (1967) *Biochim. Biophys. Acta* **149**, 476–488; 489–495.
7. Monod, J., Changeux, J.-P. & Jacob, F. (1963), *J. Mol. Biol.* **6**, 306–329.
8. Loftfield, R. B. & Eigner, E. A. (1969) *Science* **164**, 305–308.
9. Atkinson, D. E. (1966) *Annu. Rev. Biochem.* **35**, 85–124.
10. Dixon, M. & Webb, E. C. (1964) in *Enzymes* (Academic Press, New York), 2nd Ed.
11. Mills, F. C. (1955) in *Statistical Methods* (Henry Holt and Co., New York), 3rd Ed., p. 584.
12. Pestka, S. (1970) *Arch. Biochem. Biophys.* **136**, 80–88.
13. Pestka, S. (1970) *Arch. Biochem. Biophys.* **136**, 89–96.
14. Maden, B. E. H. & Monro, R. E. (1968) *Eur. J. Biochem.* **6**, 309-316.
15. Miskin, R., Zamir, A. & Elson, D. (1968) *Biochem. Biophys. Res. Commun.* **33**, 551–557.
16. Zamir, A., Miskin, R. & Elson, D. (1969) *FEBS Lett.* **3**, 85–88.
17. Miskin, R., Zamir, A. & Elson, D. (1970) *J. Mol. Biol.* **54**, 355–378.
18. Lubin, M. & Ennis, H. (1964) *Biochim. Biophys. Acta* **80**, 614–631.
19. Scheps, R., Wax, R. & Revel, M. (1971) *Biochim. Biophys. Acta* **232**, 140–150.
20. Weissbach, H., Redfield, B. & Brot, N. (1968) *Arch. Biochem. Biophys.* **127**, 705–710.
21. Monro, R. E., Maden, B. E. H. & Traut, R. R. (1967) in *Fed. Eur. Biochem. Soc. Symp.* (April, 1966), ed. Shugar, D., (Academic Press, London), pp. 179–202.
22. Pestka, S., Hishizawa, T. & Lessard, J. L. (1970) *J. Biol. Chem.* **245**, 6208–6219.
23. Weiss, R. L. & Morris, D. R. (1970) *Biochim. Biophys. Acta* **204**, 502–511.
24. Kimes, B. W., Weiss, R. L. & Morris, D. R. (1971) *Fed. Proc.* **30**, 1204.
25. Goldberg, A. (1966) *J. Mol. Biol.* **15**, 663–673.
26. Choi, Y. S. & Carr, C. W. (1967) *J. Mol. Biol.* **25**, 331–345.
27. Sheard, B., Miall, S. H., Peacocke, A. R., Walker, I. O. & Richards, R. E. (1967) *J. Mol. Biol.* **28**, 389–402.
28. Dixon, M. (1953) *Biochem. J.* **55**, 170–171.
29. Fahnestock, S., Neumann, H., Shashoua, V. & Rich, A. (1970) *Biochemistry* **9**, 2477–2483.
30. Cohn, E. J. & Edsall, J. T. (1943) in *Proteins, Amino Acids, and Peptides* (Reinhold Publishing Co., New York), p. 445.
31. Lineweaver, H. & Burk, D. (1934) *J. Amer. Chem. Soc.* **56**, 658–666.