THE SELECTIVE INHIBITION OF PROTEIN ASSEMBLY BY GOUGEROTIN

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
SHERWOOD REID CASJENS
1967

ABSTRACT

THE SELECTIVE INHIBITION OF PROTEIN

ASSEMBLY BY GOUGEROTIN

By Sherwood Reid Casjens

The mechanism by which gougerotin inhibits protein synthesis has been investigated. Gougerotin has been found to specifically inhibit the transfer of amino acids from aminoacyl-sRNA into polypeptide. Gougerotin was found to inhibit the incorporation of amino acids more strongly than the release of finished globin chains. The breakdown of polysomes, which normally occurred with protein synthesis in the cell-free system, was inhibited by the antibiotic. The action of gougerotin was not reversed by GTP or supernatant enzyme in the concentrations tested. The action of puromycin and gougerotin were compared. Gougerotin did not cause release of polypeptides from the ribosomes as did puromycin. In fact gougerotin inhibited the puromycin dependent release of peptides from ribosomes. Thus the site of action of gougerotin appeared to be primarily the inhibition of peptide synthetase. Several mechanisms for the action of gougerotin in the inhibition of protein synthesis are discussed.

THE SELECTIVE INHIBITION OF PROTEIN ASSEMBLY BY GOUGEROTIN

By

Sherwood Reid Casjens

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements

MASTER OF SCIENCE

Department of Biochemistry

1967

ACKNOWLEDGMENTS

I would like to thank Dr. Allan J. Morris for his advice and assistance during this work and also for the financial assistance received from the National Institutes of Health through him. I would also like to thank Ron Slabaugh and A. Bruce MacDonald for some stimulating discussions and the latter for use of some unpublished data.

S.R.C.

DEDICATION

To my parents

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INTRODUCTION

Aminoacyl nucleoside antibiotics (1) such as puromycin (2-5), chloramphenicol (6-8), gougerotin (9), and a number of others, as well as a number of other types of antibiotics such as cycloheximide (10,11) and streptomycin (12,13) have been found to be specific inhibitors of protein synthesis. In the past the study of the mechanism of action of these compounds has been of interest in the study of protein biosynthesis as well as in the study of antimicrobial agents, since some antibiotics have been found to inhibit specific steps in the protein biosynthetic pathway, and hence can be used to help clarify these steps. Thus the present study was undertaken with the primary goal of the determination of the mode of action of the aminoacyl nucleoside antibiotic gougerotin in the inhibition of protein synthesis and the secondary goal of making gougerotin a useful tool in the study of protein biosynthesis.

HISTORICAL

Gougerotin was first isolated from Streptomyces gougeroti and found to have antibiotic action by Kanzaki et al. (14). Its structure was first investigated by Iwasaki (15), who proposed a structure which was later shown by Fox et al. (16) to be incorrect. Fox has proposed the structure for gougerotin shown in figure 1. Gougerotin is an aminoacyl nucleoside antibiotic in that it contains peptidyl, carbohydrate and pyrimidine moieties.

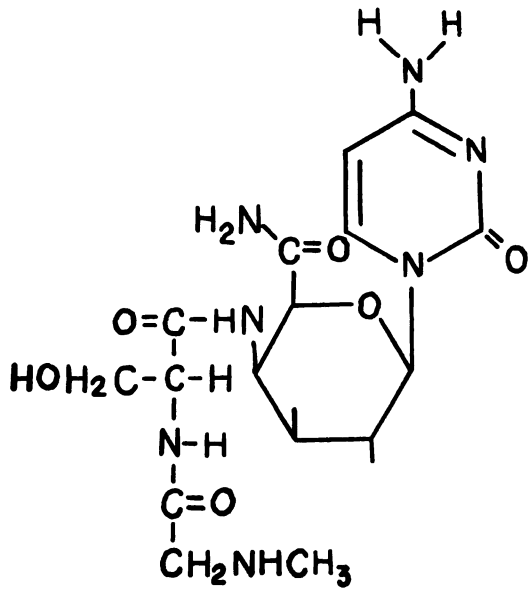
Clark and Gunther (9) showed that gougerotin was an inhibitor of protein synthesis in vitro with the use of a polyuridylic acid directed synthesizing system from Escherichia coli. they found that gougerotin inhibited protein synthesis at the stage of amino acid transfer from aminoacyl-sRNA to polypeptide. They suggested that gougerotin's action may be similar to that of puromycin since they both inhibit amino acid transfer into protein in the cell-free system.

Sinohara and Sky-Peck (17) used a cell-free amino acid incorporating system from rate liver microsomes to obtain similar results to those of Clark and Gunther, in that gougerotin had no effect on amino acid activation and appeared to inhibit protein synthesis at the transfer reaction. However they proposed that since gougerotin did not seem to fit the specificities of puromycin analogs found by Nathans et al. (18), it probably inhibited the transfer reaction in a different manner than puromycin.

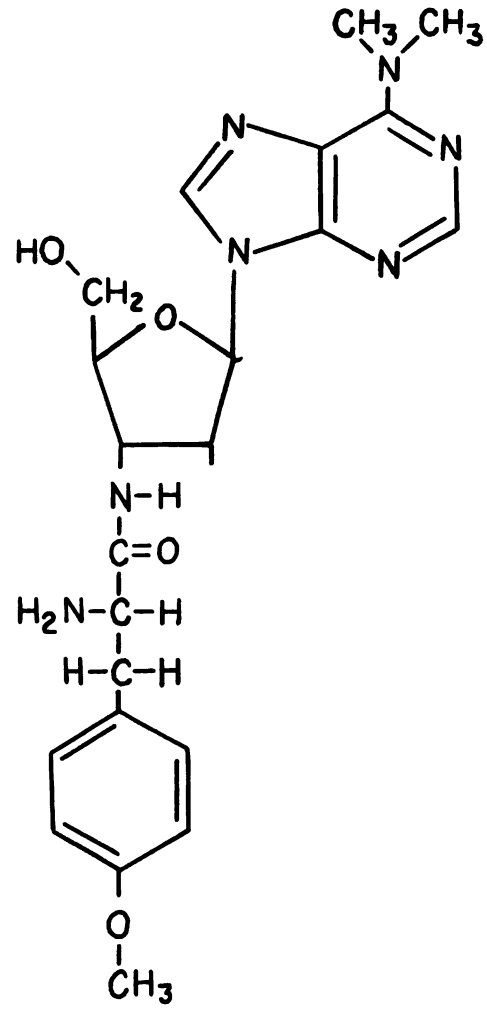
Since the time the bulk of the work described in this thesis was completed, one simultaneous study (19) and two subsequent studies have

been published (20,21) which deal with the site of action of gougerotin. These reports will be considered in the discussion.

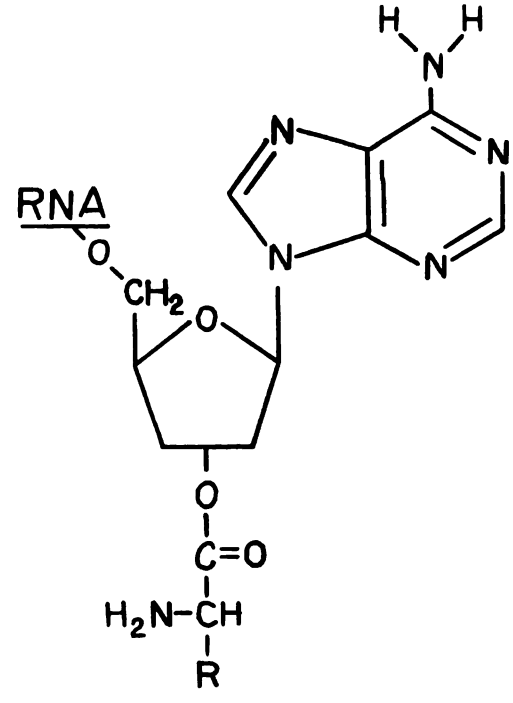
Figure 1. The structures of gougerotin (16), puromycin and and the 3' terminal nucleotide of aminoacyl-sRNA. All are similar in that they contain a nitrogen base, a carbohydrate and an amino acid type moiety. Gougerotin, however, differs from puromycin and aminoacyl-sRNA in that it contains a pyrimidine rather than a purine base, a hexose rather than a pentose carbohydrate, and a dipeptide instead of a single amino acid.



GOUGEROTIN



PUROMYCIN



AMINO ACYL-TRANSFER RNA

METHODS AND MATERIALS

COMPOUNDS

Gougerotin was a gift of Dr. J. M. Clark Jr., Biochemistry Division, University of Illinois, Urbana, Illinois and Dr. A. Miyake of Takeda Chemical Industries, Ltd., Osaka, Japan. Puromycin-methoxy (^{3}H) dihydrochloride was purchased from New England Nuclear Corp., Boston, Mass. Unlabeled puromycin dihydrochloride and unlabeled amino acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Uniformly labeled L-(^{14}C)-valine was a product of Schwartz Bio-Research Inc., Orangburg, New York. Nucleoside triphosphates were obtained from P-L Laboratories, Milwaukee, Wisconsin. Reduced glutathione was purchased from Mann Research Laboratories, Inc., New York, New York. The scintillators and thixotropic gel powder were aquired from Packard Instrument Company, Inc., Downers Grove, Illinois. Nembutal was obtained from Abbott Laboratories, North Chicago, Illinois. Dioxane, naphthalene, and phenylhydrazine hydrochloride were purchased from Distillation Products Industries, Rochester, New York. Heparin sodium and analytical grade toluene were from Fisher Scientific Company, Chicago, Illinois. All other materials were purchased from Sigma Chemical Company, St. Louis, Missouri, in the highest purity available.

BIOLOGICAL MATERIALS

I. Preparation of Rabbit Reticulocyte Ribosomes.

Male New Zealand rabbits weighing between six and eight pounds were made reticulocytic by four daily injections of 0.175 ml of 2.5% neutralized phenylhydrazine per pound of body weight. The injections were made subcutaneously. Each day's supply of phenlyhydrazine was frozen in an individual container. On the fifth day no injection was given, and on the sixth day after the initial injection the rabbit received a solution containing 2000 I. U. of heparin and 75 mg of Nembutal by intravenous injection. The blood was collected immediately by heart puncture. The blood was the cooled to 4° and centrifuged in a Sorvall cetrifuge for 20 minutes at 2000 x gravity. All further operations were done at 4°. The pelleted cells were resuspended in a solution containing 0.0075 M $MgCl_2$, 0.13 M NaCl, and 0.005 M KCl, with a volume equal to that of the supernatant plasma. The suspension was filtered through glass wool and centrifuged again for 20 minutes at 2000 x g. The supernatant liquid was removed by aspiration, and the cells were lysed by addition of 4 times the packed cell volume of a 0.025 M $MgCl_2$ solution and stirred very gently for 10 minutes. One packed cell volume of 1.5 M sucrose containing 0.15 M KCl was then added and the suspension was centrifuged at 10,000 x g for 10 minutes to remove the cell debris present. The supernatant liquid was centrifuged for 90 minutes at 78,000 x g. The high speed supernatant fluid so obtained was saved for the preparation of the supernatant enzyme fraction. The ribosomal pellets were resuspended were resuspended gently in a volume of Medium B (0.25 M sucrose, 0.017 M $KHCO_3$, and 0.002 M $MgCl_2$) which was two thirds that of the previous supernatant fluid. The solution was cetrifuged again for 90 minutes at 78,000

x g centrifugations were carried out in a Spinco Model L-2 Ultracentrifuge (22).

II. Preparation of the Supernatant Enzyme Fraction.

The supernatant enzyme fraction was prepared from the first 78,000 x g supernatant by addition of Tris-HCl buffer (pH 7.5) to a final concentration of 0.1 M. Then powdered ammonium sulfate was added to 40% saturation at 4°. The precipitate which formed was removed by centrifugation and discarded. Ammonium sulfate was added to 70% of saturation, and the resultant precipitate was removed by centrifugation and taken up in a small volume of a solution containing 0.1 M Tris-HCl buffer (pH 7.5) and 0.001 M glutathione. Ammonium sulfate was again added to 70% of saturation and the precipitate was removed as before and dissolved in a mixture containing 0.02 M Tris-HCl buffer (pH 7.5), 0.001 M EDTA, 0.001 M glutathione, and 0.001 M $MgCl_2$, and dialyzed overnight against 100 volumes of the same solution. The dialyzed preparation was then adjusted to 0.02 M glutathione and stored at -18° or -196° until it was used. The preparation, so obtained, is called the "supernatant enzyme fraction" (23).

III. Preparation of ^{14}C-Peptidyl-Ribosomes.

The preincubation of ribosomes with ^{14}C-valine to produce ^{14}C-labeled ribosomes was carried out in a medium containing 1 mM ATP, 2.5 mM phosphoenol pyruvate, 10^{-2} mg/ml pyruvate kinase, 0.05 M Tris-HCl buffer (pH 7.5), 4 mM $MgCl_2$, 0.05 M KCl, 0.02 M glutathione, 0.05 mM in each of the 20 amino acids except valine, 5 mg/ml ribosomes, 4 mg/ml supernatant enzyme fraction, and 0.05 mM in ^{14}C-valine (specific activity 10 C/M). The solution was incubated for 10 minutes at 37°, and the reaction was stopped by the addition of 10-12 volumes of

Medium B (described above) containing a 100 fold excess in unlabeled valine. The ribosomes were isolated by one centrifugation at 78,000 x g for 90 minutes. The ribosomal pellets were resuspended in a small volume of 0.25 M sucrose and centrifuged at 1000 x g to remove debris. The supernatant solution contained the ^{14}C-peptidyl-ribosomes.

CELL FREE ASSAYS

I. Assay for ^{14}C-Valine Incorporation into Protein.

The cell-free hemoglobin synthesizing system used to detect radioactive valine incorporation contained 0.25 mM GTP. 1.0 mM ATP, 5 mM phosphoenol pyruvate, 40 µg pyruvate kinase, 20 mM glutathione, 50 mM KCl, 4 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.5), 2 or 3 mg ribosomes as indicated, 4 mg of supernatant enzyme fraction, 0.05 mM of each of the 20 amino acids except valine, and 0.05 mM in L-(^{14}C)-valine (specific activity 3 C/M), in a total volume of 1.0 ml. The phosphoenol pyruvate was prepared from the barium salt, with HCl, removal of barium with K_2SO_4 treatment and neutralization with KOH. The glutathione was brought to pH 6.0 and nucleoside triphosphate solutions were brought to pH 7.0 with KOH.

II. Determination of GTP Dependent Release of Polypeptides from Ribosomes.

Studies of the effect of gougerotin on the GTP dependent release of polypeptides from ribosomes were performed by incubation of the prelabeled ribosomes in a solution containing 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM GTP, and gougerotin as indicated in a total volume of 1.0 ml. No incorporation of amino acids occurs under these conditions, although finished α and β chains are released from the ribosomes. The amount of GTP dependent release was calculated by subtracting the amount of protein non-specifically released in a similar assay to which no GTP had been added. The value of the nonspecific release was usually about 10% of the total radioactivity in the assay. Following incubation for 40 minutes at 37° the solutions were transferred to 4 ml cellulose centrifuge tubes

and centrifuged at 105,000 x g for 60 minutes. Each supernatant was then analyzed for radioactive protein (23). If the puromycin dependent release of polypeptides was to be measured, the procedure was the same except GTP was omitted and puromycin was added in the quantities indicated.

III. Assay for Incorporation of ^{3}H-Puromycin into Peptidyl-Puromycin.

The incubation mixture for the formation of peptidyl-puromycin included Tris-HCl buffer (pH 7.5), glutathione, KCl, and $MgCl_2$ in the concentrations indicated for the puromycin dependent release system (above). Ribosomes and ^{3}H-puromycin were present in the amounts indicated. The total volume of the assay was 50 microliters. The reaction was stopped by pipetting a 5 microliter aliquot into 10% (w/v) trichloroacetic acid. The resulting precipitate was analyzed for the incorporation of ^{3}H-puromycin into polypeptides as described in analytical procedures.

ANALYTICAL PROCEDURES

The concentrations of ribosomes were determined by the spectrophotometric method of Ts'O and Vinograd (24).

The analytical ultracentrifuge run was made in a Spinco Model E Analytical Ultracentrifuge at 42,040 r.p.m.

Incorporation of radioactive amino acids into protein was measured by the following procedure. Fifteen mg of bovine serum albumin was added to each of the one ml assays followed by precipitation with 5% (w/v) trichloroacetic acid. After allowing 30 minutes for complete precipitation, the precipitate was collected by centrifugation in a clinical centrifuge. This precipitate was washed by resuspension in 5% trichloroacetic acid and centrifugation. The resulting pellet was dissolved in 0.5 ml of 1 M NaOH to hydrolyze any aminoacyl-sRNA present, and reprecipitated with 5% trichloroacetic acid and the precipitate washed as before with 5% trichloroacetic acid. The pellet was then resuspended in acetone which had been made 0.1 N in HCl. The precipitate was centrifuged and suspended in a 2:3 (v/v) mixture of the acid acetone and diethyl ether. After centrifugation the pellet was suspended in diethyl ether, centrifuged and air dried. The resulting powder was transferred very carefully to a glass counting vial (the last bit was transferred with the help of 0.5 ml of 1.0 N NaOH and several drops of dioxane were added to the powder in the counting vial to aid dissolving). When all of the powder had dissolved in the NaOH solution, 15 ml of thixatropic counting fluid was added, and the vial was shaken vigorously. The

counting mixture was prepared by combining 7 g of 2,5-diphenyloxazole, 150 mg of 1,4-bis-2-(5-phenyloxazole)-benzene, 50 g of naphthalene, and 36 g of thixotropic gel powder dissolved in 200 ml of toluene, 30 ml of ethanol, and 800 ml of p-dioxane. The samples were then counted in a model 3003 Packard Liquid Scintillation Counter.

Incorporation of tritiated puromycin into peptidyl-puromycin was measured by a procedure similar to that given above for measuring amino acid incorporation except 10% trichloroacetic acid was used in place of the 5% tricloroacetic acid and the NaOH treatment and subsequent wash of the precipitate were omitted.

RESULTS

In the present studies the results of Clark and Gunther (9) and Sinohara and Sky-Peck were comfirmed and extended in that gougerotin was found to inhibit amino acid incorporation in the rabbit reticulocyte cell-free system in a manner similar to that shown by the above workers using the *E. coli* and rat liver systems. The time course of amino acid incorporation into trichloroacetic acid precipitable protein in the cell-free system is shown in figure 2. It can be seen that gougerotin causes a decrease in the total incorporation as well as a decrease in the rate of amino acid incorporation. Increasing the concentration of gougerotin resulted in further lowering of the rate of amino acid incorporation and total incorporation. At all concentrations tested the reaction was essentially complete by 40 minutes.

To characterize the effect of gougerotin in more detail, the inhibition of the amino acid incorporation system was studied as a function of antibiotic concentration. Figure 3 shows the results of the study. Gougerotin was added to the complete system in the indicated concentrations and incubated for 40 minutes at 37° (at which time the reactions were completed). The concentration required to reach maximum inhibition was approximately ten times that reported for puromycin (3).

Gougerotin also showed an effect on the breakdown of polysomal structure during protein synthesis. According to current models (25-27), protein synthesis is accompanied by an orderly breakdown process as the ribosomes reach the end of the messenger RNA molecule and become detached

Figure 2. The effect of gougerotin on amino acid incorporation into protein in a cell free system. The complete system (▲-▲) contained 3 mg of ribosomes, ^{14}C-valine (specific activity 4.0 C/M) and other components as described in METHODS (assay for incorporation of ^{14}C-valine into proteins). To the complete system gougerotin was added to a concentration of 0.1 mM (●-●) and 1.0 mM (○-○). Following incubation at 37°C for the times indicated the reaction was stopped by the addition of 4 ml of 5% trichloroacetic acid, and the total radioactive protein present was determined as described in ANALYTICAL PROCEDURES.

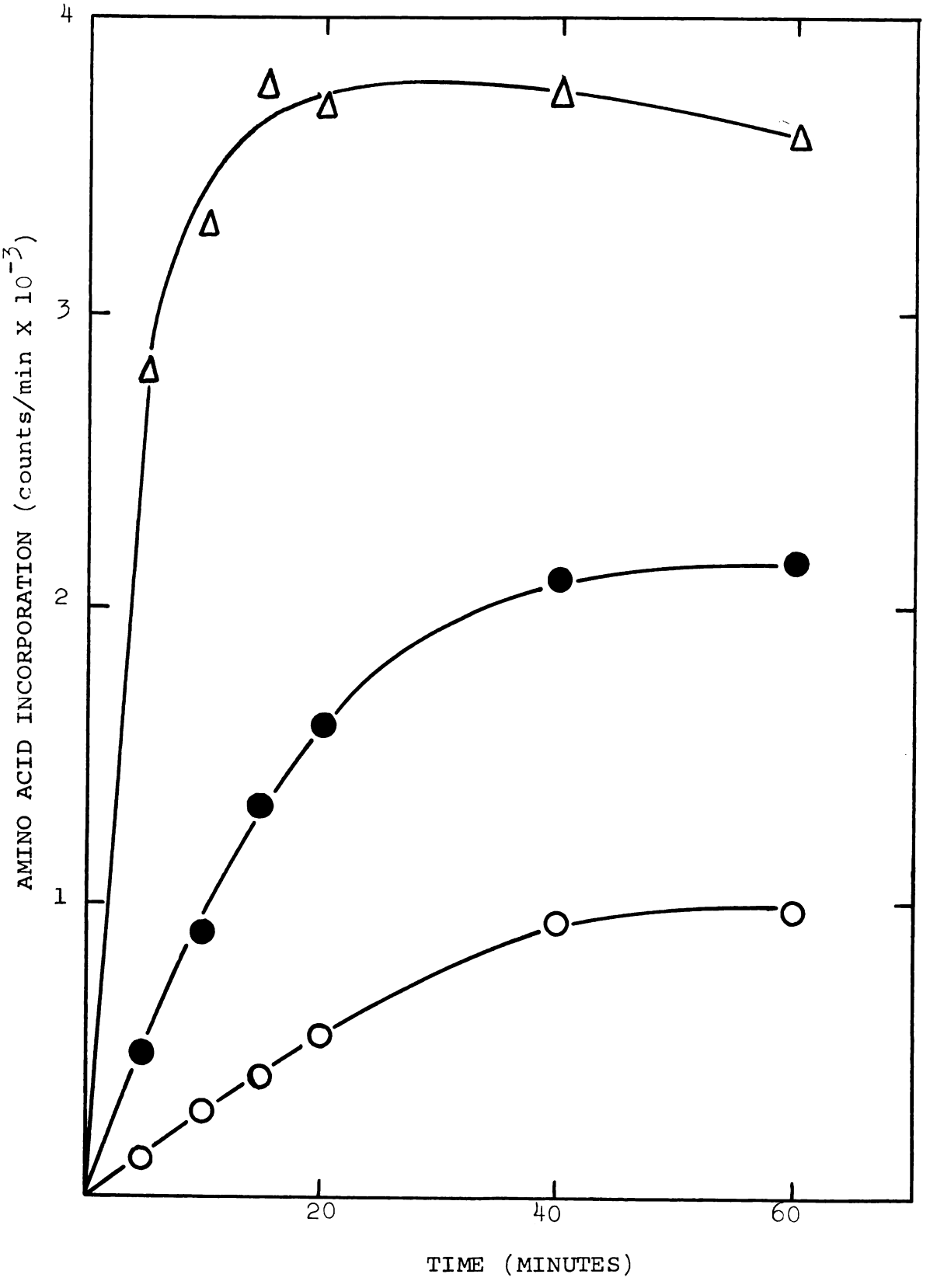
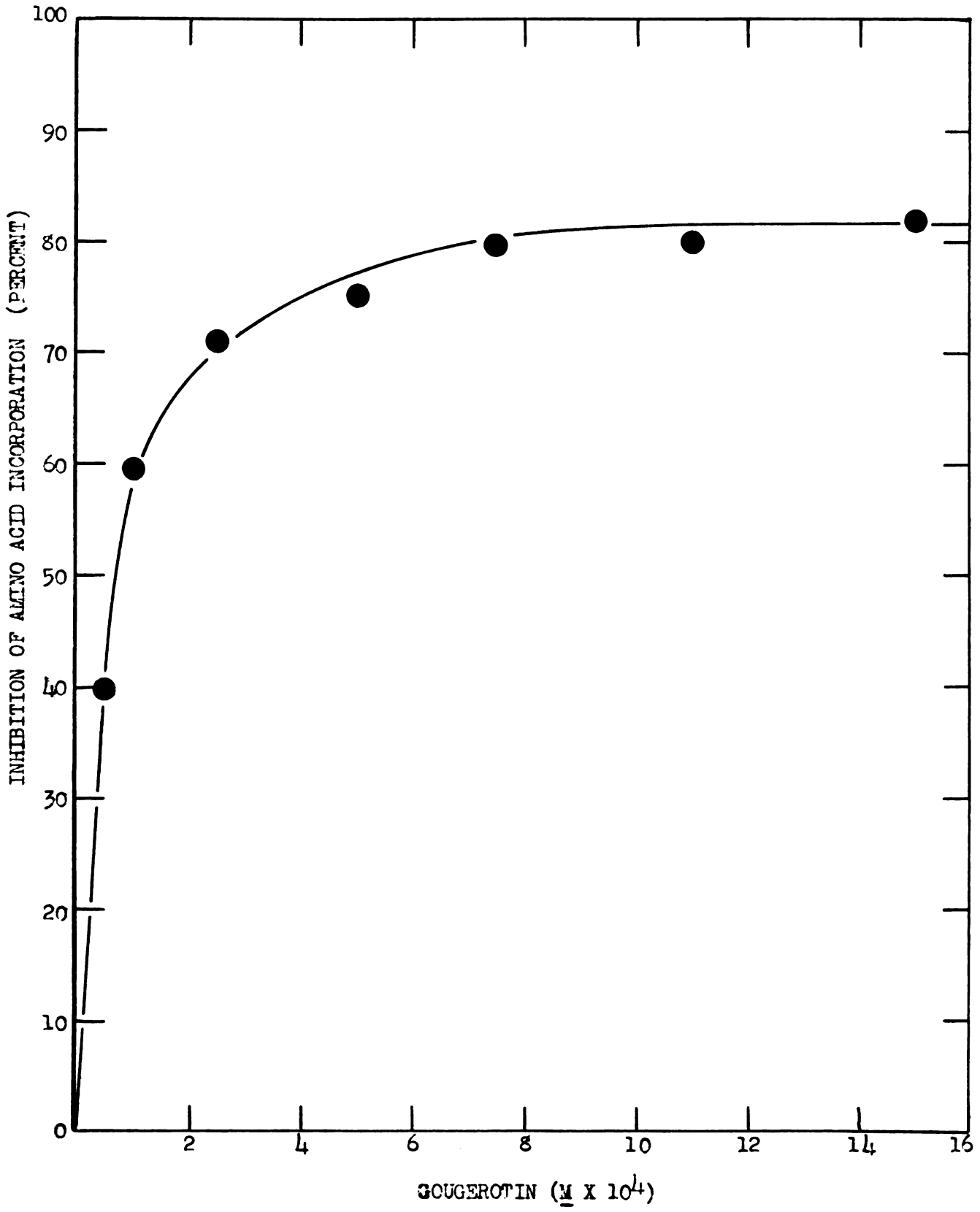

AMINO ACID INCORPORATION (counts/min x 10^{-3})
4
3
2
1
20
40
60
TIME (MINUTES)

Figure 3. The inhibition of amino acid incorporation by various concentrations of gougerotin. Antibiotic was added to the complete system (described in METHODS - assay for ^{14}C-valine incorporation into protein) in the indicated concentrations and incubated at 37°C for 40 minutes. The total radioactive protein was determined as described in ANALYTICAL PROCEDURES.


100
90
80
70
60
50
40
30
20
10
0
INHIBITION OF AMINO ACID INCORPORATION (PERCENT)
2
4
6
8
10
12
14
16
GOUGEROTIN (M X 10⁴)

from the polysome structure. Since it has been shown that only a limited amount of reattachment of single ribosomes to the messenger RNA occurs in the reticulocyte cell free system (28-31), the net effect is that the proportion of polysomes decreases with a simultaneous increase in the amount of single ribosomes as protein is synthesized.

The addition of gougerotin to a cell free system which would normally be actively synthesizing protein caused inhibition of polysome breakdown as well as inhibition of amino acid incorporation (figure 4). Concurrent studies by A.J. Morris (32) using sucrose density gradient centrifugation yielded similar results, and the use of increasing concentrations of gougerotin caused increasingly stronger inhibition of polysome breakdown by gougerotin.

The evidence available seems to implicate the utilization of GTP in ribosome movement along the messenger RNA; thus inhibition of the GTP utilization system was a possible site for the action of gougerotin. However the data shown in table I indicate that increased GTP content of the assay had no significant effect on the amount of inhibition of amino acid incorporation that was observed in the cell free system. Thus it may be stated that gougerotin is not competing with GTP for a specific ribosomal site. Similarly, the degree of inhibition was not markedly affected by variations in the levels of supernatant enzyme used in the assay. These results find some support in studies of ^{14}C-GTP binding to ribosomes and GTP hydrolysis by ribosomes by A.B. MacDonald (35). In both cases no effect by gougerotin was found.

Finished globin chains are released from the polysomes by a GTP dependent process which can be distinguished from amino acid incorpora-

Figure 4. The effect of gougerotin on polysomal breakdown during protein synthesis. Ribosomes (3 mg) were incubated in the complete system for amino acid incorporation for 20 minutes at 37°C and then chilled and transferred into a 1.2 cm prismatic cell (upper trace). An identical assay which was 1.0 mM in gougerotin was placed in the material plane cell (lower trace). The photograph was taken approximately 6 minutes after attaining 42,040 rev./minute in a Spinco Model E Analytical Ultracentrifuge (at 5°C). Sedimentation was from left to right. Total radioactivity present in protein was determined in identical assays containing 0.05 mM ^{14}C-valine (specific activity 3.0). The assay with no gougerotin contained 6607 counts/minute, and the assay with 1.0 mM gougerotin contained 805 counts/minute of radioactive protein.

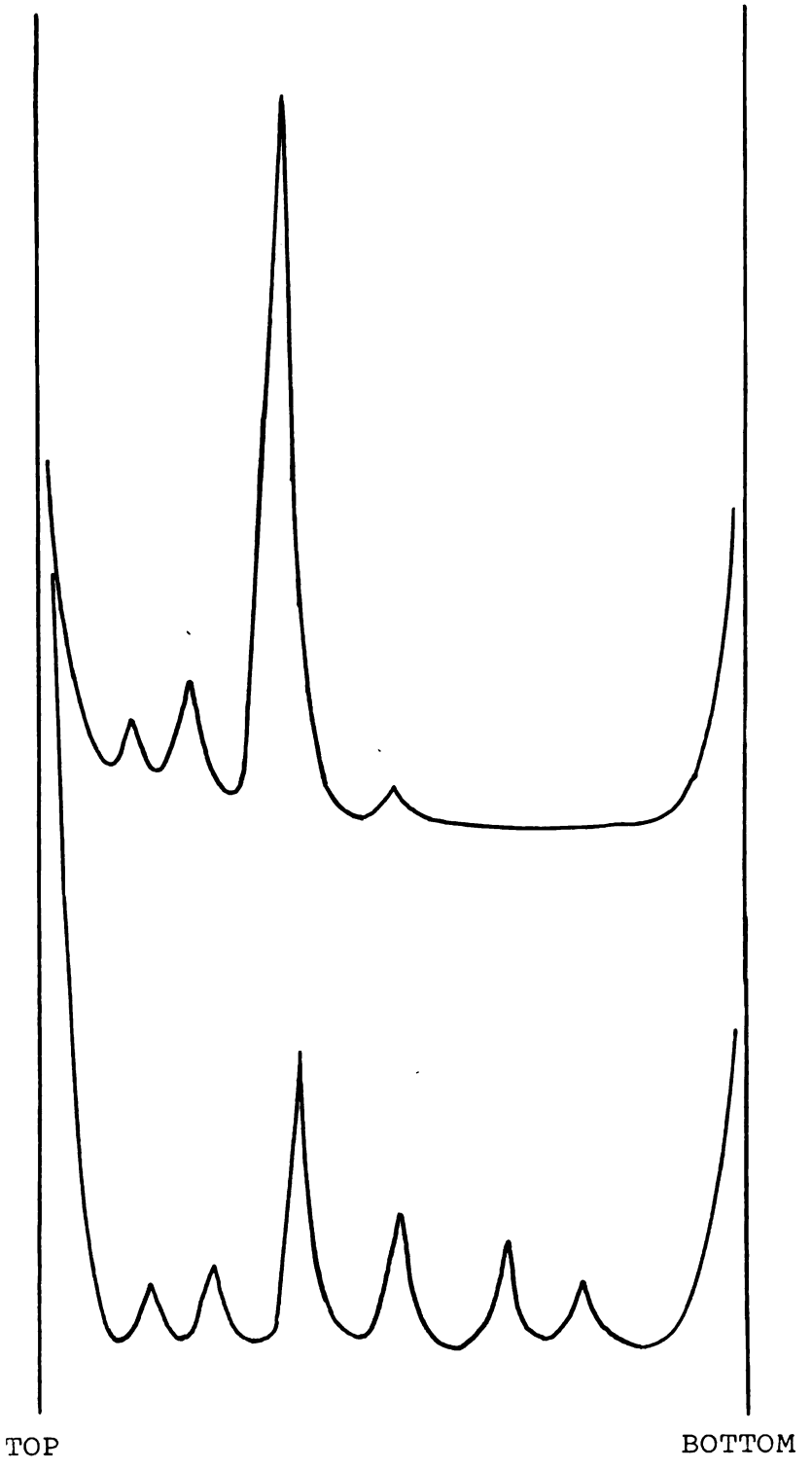

TOP
BOTTOM

Table I. Effect of supernatant enzyme preparation and GTP on the inhibition of amino acid incorporation of ^{14}C-valine (specific activity 3.0) into polypeptides was measured as described in METHODS. The assays in which GTP was varied contained 6 mg of supernatant enzyme fraction, and the assays in which the supernatant enzyme fraction was varied contained 0.25 micromoles of GTP. All assays contained gougerotin as indicated.

TABLE I

GTP ADDED (μmoles)	GOUGEROTIN ADDED (μmoles)	^{14}C-VALINE INCORPORATION counts/min	% inhibition
0.25	none	7576	0%
0.25	0.10	2206	71%
0.10	0.10	2983	61%
0.50	0.10	2832	63%
1.00	0.10	2552	66%
SUPERNATANT ENZYME ADDED (mg)			
6.0	none	7576	0%
10.0	none	7601	0%
6.0	0.10	2206	71%
10.0	0.10	2580	66%

tion into protein (23). Thus is seemed possible that gougerotin blocked polysome breakdown, and hence amino acid incorporation, at the release step. However data presented in figure 5 indicated that this step is not the primary site of action since the polypeptide release is significantly less suceptible than the amino acid incorporating system to the antibiotic (see also figure 3) both in maximum inhibition found (about 60% and 80-90% respectively) and in the concentration of gougerotin required to reach maximum inhibition (about 1 mM and 0.3 mM respectively).

The mechanism of action of puromycin in the inhibition of protein synthesis has been reasonably well characterized. Yarmolinsky and De La Haba (2) have suggested that it acts as an analog of the amino acid acceptor end of the tranfer RNA molecule, since their structures are very similar. Puromycin has since been shown to act in this manner and to release unfinished peptides (3,36) in the form of a peptide with puromycin bound to its C-terminal by a peptide bond (5,37,38). Thus puromycin acts like an aminoacyl-sRNA, however after the peptidyl-puromycin bond is formed it is released from the ribosomal particle since the remainder of the sRNA is not present with its binding sites for ribosomes. Allen and Zamecnik (5) have suggested, however, that this release of peptidyl-puromycin may not be entirely quantitative.

Clark and Gunther (9) found that gougerotin inhibited protein synthesis and suggested its effect might be similar to that of puromycin. Therefore the action of the two antibiotics was compared. Puromycin or gougerotin was added to a cell-free release system, and the appearance of ^{14}C-polypeptides was measured. Table II presents data which show that puromycin and gougerotin do not act in the same manner. Gougerotin showed no release of polypeptide from the ribosomes as did puromycin.

Figure 5. The effect of gougerotin on GTP dependent release of globin chains. Gougerotin was added to the complete releasing system described in METHODS (determination of GTP dependent release of polypeptides from ribosomes). Prelabeled ribosomes (3 mg) containing 10413 counts/minute were incubated for 40 minutes at 37°C , and the released globin chains were determined as described in METHODS. The values in the figure are average values of two identical experiments.

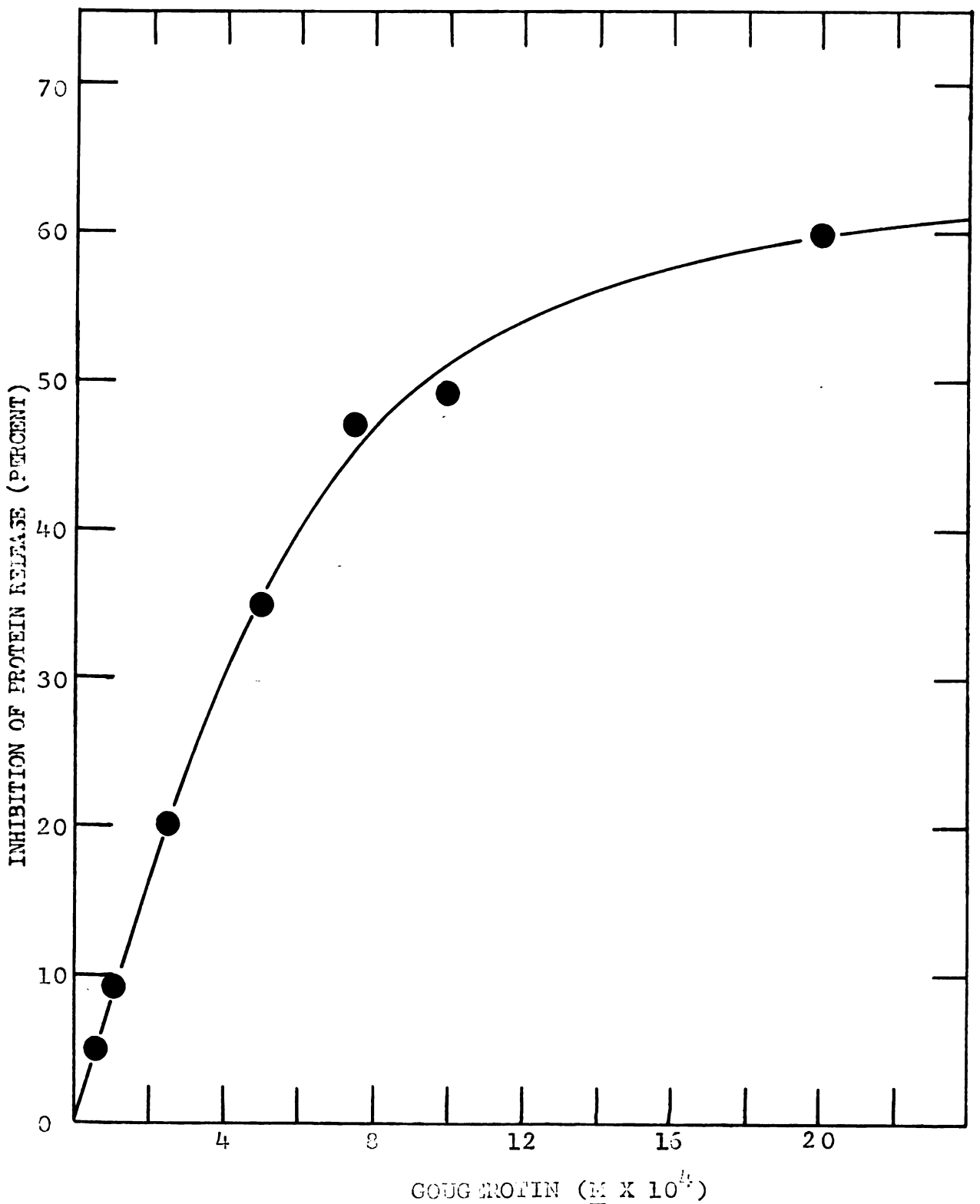

INHIBITION OF PROTEIN RELEASE (PERCENT)
0
10
20
30
40
50
60
70
4
8
12
16
20
GOUGEROTIN (M X 10⁴)

Table II. Dissociation of nascent polypeptide from ribosomes (2 mg containing 3720 counts/minute total radioactive polypeptides) by puromycin and gougerotin in a medium containing $MgCl_2$, KCl, glutathione and Tris-HCl buffer in the concentrations described in METHODS, and puromycin and gougerotin as indicated. Ribosomes were removed by centrifugation and the soluble labeled polypeptides determined as indicated in ANALYTICAL PROCEDURES.

TABLE II

Additions	Concentration ($\underline{M} \times 10^{3}$)	Soluble Polypeptide (counts/min)
none	—	481
Gougerotin	0.025	392
Gougerotin	1.00	383
Puromycin	0.025	1158
Puromycin	1.00	1773

In fact gougerotin appeared to depress the basal amount of release to a value less than that seen when no antibiotic was added to the assay. Further indication that the two antibiotics act by different mechanisms can be seen in the fact that gougerotin inhibits polysome breakdown (see figure 4), whereas puromycin has been reported to actually accelerate polysome breakdown (3,11,30).

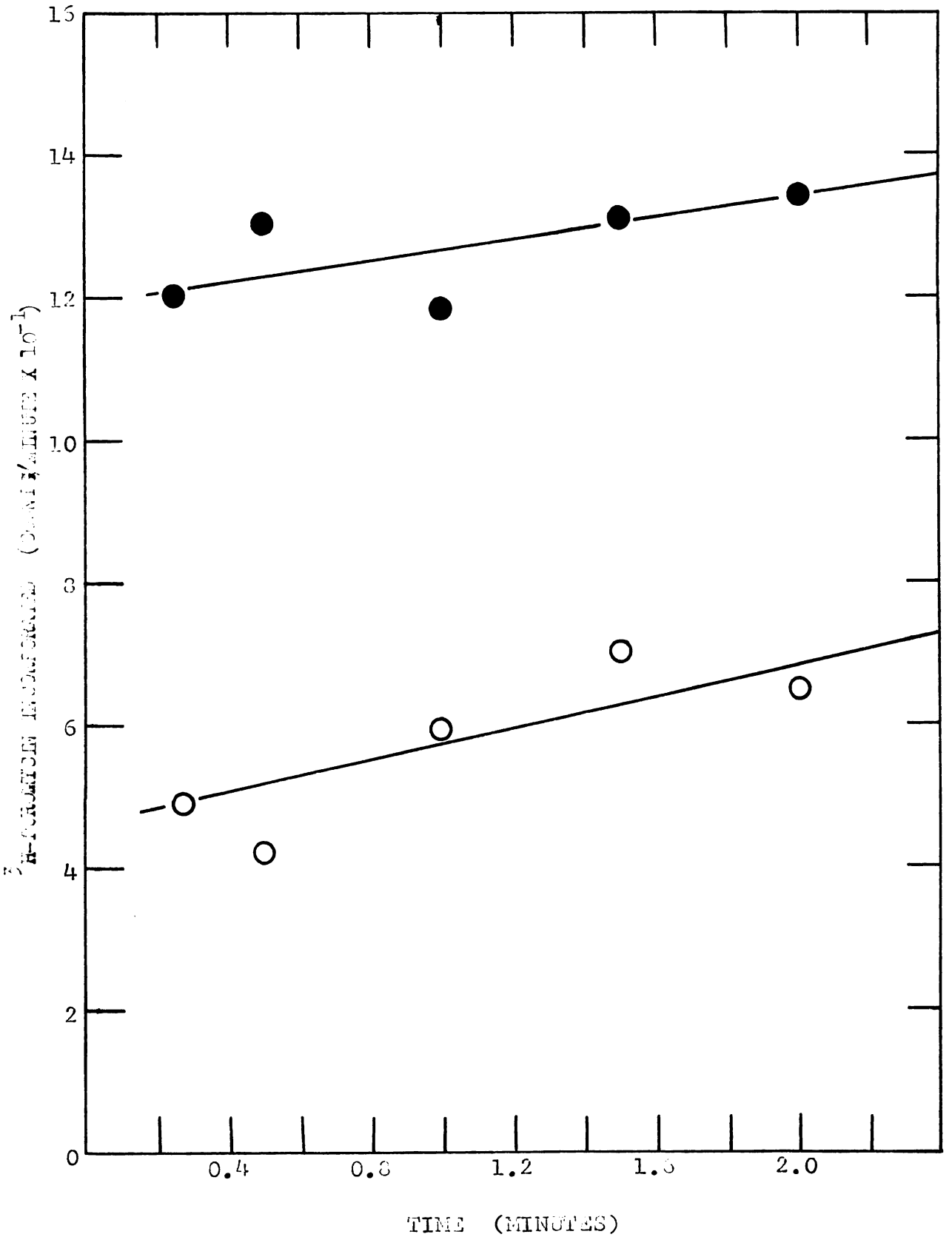
Since the formation of the puromycin-polypeptide bond probably is the only step taking place under the conditions of the release assay, it has been considered as a possible system for the study of the formation of a model peptide bond (37,39). Thus the effect of gougerotin onthe reaction between puromycin and peptidyl-sRNA may meaningful in terms of the actual peptide bond forming step in normal protein synthesis. Results of the studies of the effect of gougerotin on the puromycin dependent release are presented in table III. It is evident that gougerotin has a very marked effect on this reaction, and hence the site of action appears to be inhibition of peptide synthetase. In concurrent studies A. J. Morris defined the effects of gougerotin concentration upon the puromycin dependent release of labeled polypeptides from the ribosome in more detail using the same analytical procedure. It was found that the data best fit a Lineweaver-Burk plot (40) which showed gougerotin to be a competitive inhibitor of puromycin dependent release reaction (32). Attempts to measure the initial rate of ^{3}H-methoxy-puromycin incorporation, and hence to do more extensive kinetic studies, were not successful since the reaction rate was too fast to be measured by available techniques (see figure 6). It is evident, however, that gougerotin does

Table III. The effect of gougerotin on puromycin induced release of polypeptides from prelabeled ribosomes was determined by adding gougerotin in the indicated amounts, to the puromycin dependent release system described in METHODS. The experiments with 0.025 micromoles of puromycin were done with 2 mg of ribosomes containing 3208 counts/min. and were incubated for 20 minutes at 37°C before centrifugation. The assays with 0.50 micromoles of puromycin contained 3 mg of prelabeled ribosomes containing 4001 counts/minute and were incubated for 40 minutes at 37°C before centrifugation. Each of the counts/minute values was obtained by subtracting the amount of radioactivity in a no antibiotic blank from the amount of released radioactivity found in the assay cantaining puromycin.

TABLE III

Puromycin Added (μmoles)	Gougerotin Added (μmoles)	^{14}C-Polypeptide Released counts/min	% inhibition
0.025	none	600	0%
0.025	0.05	589	2%
0.025	0.25	414	31%
0.025	1.00	291	52%
0.50	none	1721	0%
0.50	0.05	1376	20%
0.50	0.10	1174	32%
0.50	1.00	779	55%

Figure 6. Incorporation of ^{3}H-methoxy-puromycin with time. Incorporation of puromycin into trichloroacetic acid precipitable material was measured as described in METHODS. Tritiated puromycin (specific activity 1000) was present in a concentration of 1×10^{-4} M, and there were 0.4 mg of ribosomes in the complete system (●-●). To the complete system gougerotin was added to a final concentration of 1×10^{-3} M (o-o). The total volume of the assay was 50 microliters. At the indicated times 5 microliter aliquots were pipetted into two ml of 10% trichloroacetic acid, 15 mg of bovine serum albumin was added and the incorporated ^{3}H-puromycin determined as described in ANALYTICAL PROCEDURES.


16
14
12
10
8
6
4
2
0
0.4
0.8
1.2
1.6
2.0
TIME (MINUTES)

indeed inhibit the formation of peptidyl-puromycin, as approximately one half as much tritium labeled puromycin was incorporated into trichloroacetic acid insoluble material when 1 x 10^{-3} M gougerotin was present as was incorporated in the absence of gougerotin.

DISCUSSION

The study of the mechanism of action of antibiotics which specifically inhibit protein synthesis has been very valuable in the study of the various steps in protein synthesis. This study on the mode of action of gougerotin indicates that it may also be of considerable value in studies of this type.

Since GTP dependent release did not appear to be the primary site of action of gougerotin (release was inhibited up to 50-60% at high antibiotic concentration, however at lower concentrations the incorporation reaction was inhibited 70-80% and release was only inhibited 10-20%), and the inhibition was not reversed by the concentrations of GTP and soluble enzymes tested, the step at which gougerotin acts primarily is at or before the peptide bond forming reaction. It has been shown previously that gougerotin does not effect binding of aminoacyl-sRNA to the ribosome-messenger RNA complex in an E. coli system (19) and that gougerotin does not inhibit the activation of amino acids in the rat liver system (17). These observations along with the findings that gougerotin inhibits the reaction of puromycin with peptidyl-sRNA on ribosomes (a system where it is thought that only the peptide bond forming step takes place) lead to the conclusion that gougerotin is a specific inhibitor of the peptide bond forming enzyme.

There are still several possibilities for the detailed mechanism of action of gougerotin. The first of these is pertinent if one assumes that gougerotin and puromycin do not displace each other once they have bound. Then it can be proposed that gougerotin and puromycin compete

for the same site of the peptide synthetase molecule.

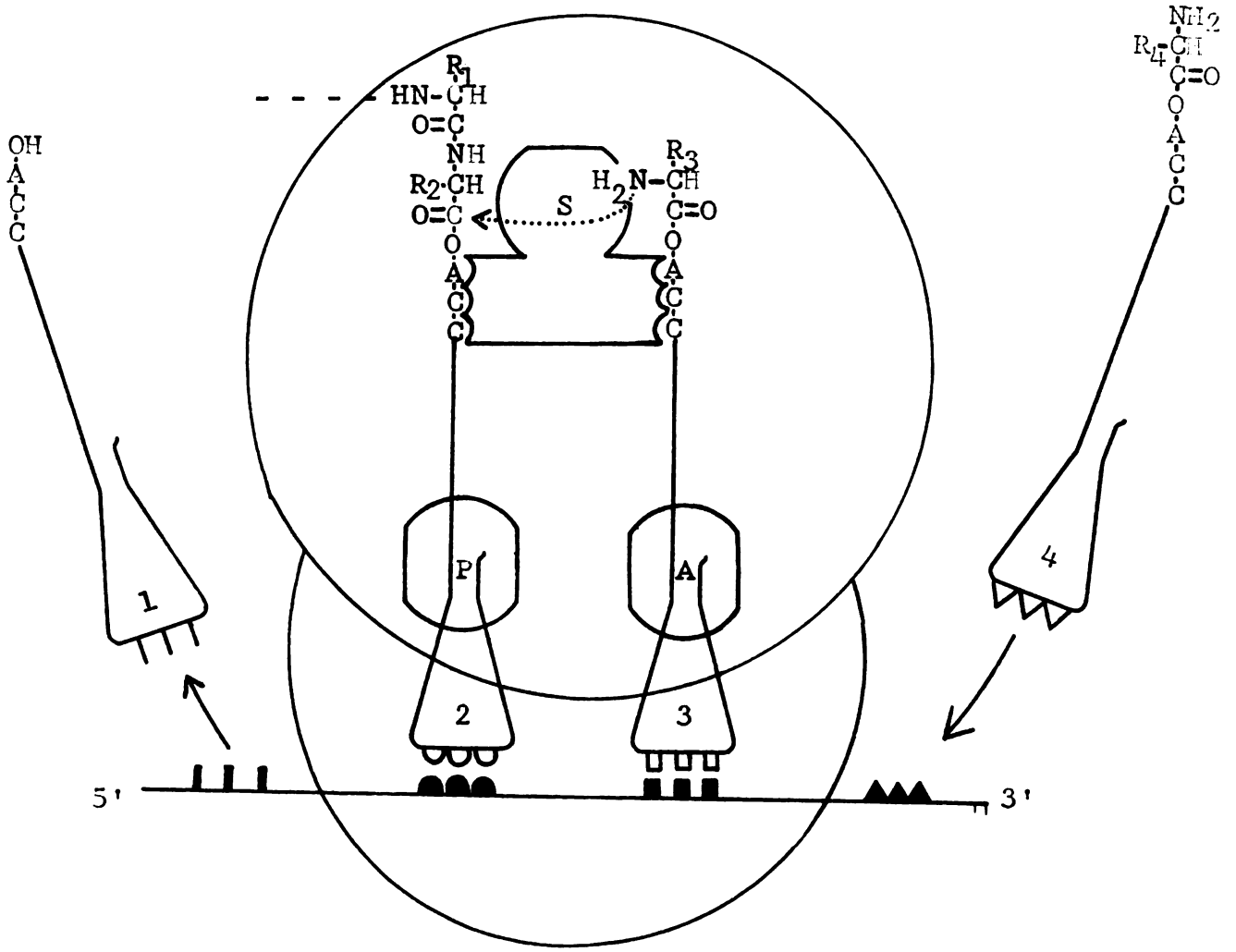
However recent reports (20,21) indicate that the above mechanism may not be entirely correct. Using E. coli ribosomes with polylysyl-sRNA Goldberg and Mitsugi (20) measured the release of polylysine by puromycin. They found that gougerotin did reduce the rate of puromycin dependent release, but Lineweaver-Burk plots of their data indicated that the inhibition was not purely competitive, but was of a mixed type (41), that is both the maximum velocity and the affinity of the enzyme for the substrate (puromycin) are altered. Coutsogeorgopoulos (21) used an E. coli system with polyuridylic acid and measured the effect of various inhibitors on the initial rate of phenylalanine incorporation. His data also show mixed type inhibition for gougerotin on the reaction. He also found that two other antibiotics which, like gougerotin have cytosine as the nitrogen base, showed kinetics very similar to that of gougerotin.

Thus at least two more hypothetical models for the action of gougerotin are possible. These are based on the fact that gougerotin shows mixed competitive and non-competitive inhibition of peptide bond formation. This is a two substrate reaction, and since the concentration of one of the reactants was held constant throughout (bound peptidyl-sRNA), the equations describing single substrate reactions are useful (42). Thus in view of the above mentioned kinetic studies it is possible that gougerotin interacts with the peptide synthetase molecule at some site unrelated spacially to the catalytic site, and after binding the conformation of the protein is changed in such a manner as to alter the maximum velocity and the binding constant of the enzyme.

The third hypothetical model for the action of gougerotin is as follows. Figure 7 shows a possible diagramatic representation of peptide bond formation. A possible mechanism for gouerotin could then be that it is an analog of one of the C's in the ACC nucleotide sequence on the 3' end of the aminoacyl-sRNA, and so it could possibly cause partial hindrance to puromycin binding as well as being close enough to the catalytic site to have some effect on the velocity of the catalysis. Coutsogeorgopolos' data (21), which do not appear to support this hypothesis, might be explained by the fact that gougerotin would be competing with bound aminoacyl-sRNA and that he was actually measuring total aminoacyl-sRNA concentration. He did, in fact, find that puromycin did not show competitive kinetics with aminoacyl-sRNA as might be expected from its accepted mode of action. These hypotheses should be viewed with extreme caution, however, since they are based on kinetic studies of very complex systems, and definitive conclusions should await further studies.

Also noted in these studies was the fact that puromycin dependent release of polypeptides from ribosomes did not require the addition of GTP. Similarly no GTP requirement was found for the inhibition of the puromycin dependent release by gougerotin. Studies on the role of GTP in peptide bond synthesis in the reticulocyte system have, to date, shown no strict stoichiometry between GTP utilization and peptide bonds formed (34), however studies in an E. coli system have shown a relationship of one peptide bond formed for each GTP hydrolyzed (43,44). The lack of a GTP requirement for the puromycin reaction reported here may indicate that GTP does not participate in the peptide bond forming step directly.

Figure 7. Apossible schematic representation of protein biosynthesis as adapted from Schweet (33) and Coutsogeorgopolos (21). The upper circle represents the 60s subunit with its binding sites for amioacyl-sRNA and peptidyl-sRNA, designated "A" and "P" respectively, and the peptide synthetase ("S"). The lower semicircle represents the 40s subunit with the messenger RNA bound to it. No attempt has been made to draw the ribosome, binding sites, and sRNA's to scale. Protein synthesis can be broken down into the following steps: activation of the amino acid by reaction with a specific sRNA and concurrent conversion of one ATP to an ATP (not shown on the diagram), binding of the aminoacyl-sRNA to the ribosome messenger RNA complex, formation of a peptide bond by peptide synthetase resulting in a peptidyl-sRNA in the "A" position, and shifting of this peptidyl-sRNA to the "P" position with the possible utilization of GTP. Special initiation and release mechanisms are needed, but are not necessary for this discussion.


OH
A
C
C
HN-CH
R1
O=C
NH
R2-CH
O=C
O
A
C
C
S
H2N-CH
R3
C=O
O
A
C
C
NH2
R4-CH
C=O
O
A
C
C
1
2
3
4
P
A
5'
3'

Thus gougerotin, as a specific inhibitor of the biosynthesis of proteins, may be of considerable value for the study of specific stages in protein synthesis such as the attachment of aminoacyl-sRNA to ribosomes and the release reaction, since it specifically inhibits the peptide bond forming reaction as it occurs on the ribosomal template.

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