INHIBITION OF PEPTIDE BOND FORMATION BY NUCLEOTIDYL DERIVATIVES OF PUROMYCIN

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This is to certify that the

thesis entitled

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presented by

Edmund J. Hengesh

has been accepted towards fulfillment
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Dr. Allan J. Morris
Major professor

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ABSTRACT

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By

Edmund J. Hengesh

The ability of puromycin to inhibit protein synthesis by substituting for aminoacyl tRNA at the acceptor site of the ribosomal bound peptidyl transferase has been attributed to the structural analogy which exists between puromycin and the 3' terminus of the aminoacyl tRNA molecule. The 3' terminus of all tRNA species are thought to possess identical trinucleotide sequences (CpCpA) suggestive that this sequence is responsible for a biochemical function common to all tRNA molecules. The structural analogy between the 3' terminus of tRNA and puromycin has led us to perform structure-activity relationship studies with selected dinucleotides of puromycin.

To accomplish this study the 2' - 5' and 3' - 5' isomers of cytidylyl puromycin and cytidylyl puromycin aminonucleoside were prepared by chemical synthetic methods. The dinucleotide monophosphates were isolated

and purified by column chromatography. Characterization was accomplished by paper chromatography of alkaline and pancreatic ribonuclease digestions of the purified reaction products and by ultraviolet spectroscopy.

The 3' - 5' isomer of cytidylyl puromycin has been found to be an order of magnitude more inhibitory toward peptide bond formation in a rabbit reticulocyte cell-free system than was puromycin. The 2' - 5' isomer of cytidylyl puromycin is only 5% as inhibitory as puromycin. Cytidylyl puromycin aminonucleoside was found to have practically no effect upon protein synthesis.

These results suggest the presence of a cytosine specific binding site on peptidyl transferase and are indicative that the requirement for aromatic amino acid derivatives of puromycin is independent of that site.

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Edmund J. Hengesh

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To Glenda, Eddie, and Jeff

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
cmp	cytidine monophosphate
cpm	counts per minute
CpPAN	cytidylyl puromycin aminonucleoside
CpCpA	cytidylyl cytidylyl adenosine
CpPuro	cytidylyl puromycin
DMSO	dimethylsulfoxide
F-met – tRNA	formylmethionine transfer ribonucleic acid
GSH	reduced glutathione
GTP	guanosine triphosphate
PEP	phospho-enol-pyruvic acid
POPOP	1, 4-bis [2-(4-methyl-5-phenyl-oxazolyl)] - benzene
PPO	2,5-diphenyloxazole
TCA	trichloroacetic acid
TPS	triisopropylbenzenesulfonyl chloride
Tris	[tris] (hydroxymethyl) aminomethane

All temperatures reported in degrees centigrade

INTRODUCTION

The ribosome provides the platform upon which protein synthesis can occur. Ribosomes can be broadly classified according to their size into 70S and 80S. Mammals, higher plants, fungi, yeasts, and protozoa have 80S ribosomes; bacteria, blue-green algae, chloroplasts, and mitochondria have ribosomes of the 70S type. Besides the differences in their sedimentation coefficients, 70S and 80S ribosomes differ in their protein complement, subunit structure, and in the molecular weight of their ribonucleic acid.

Functional differences also exist between the two classes of ribosomes. There is evidence that ribosomes and supernatant fractions can be crossed between widely different systems having the same size ribosomes but not between systems using 70S and 80S ribosomes. (Vazquez and Monro, 1967; Chang, et al., 1966; Heredia and Halvorson, 1966; Parisi, et al., 1967). In spite of these seemingly unreconcilable differences between the classes of ribosomes, it is generally agreed that the basic mechanism of

protein synthesis in all systems is very similar.

All ribosomes present at least two sites for the binding of tRNA molecules (Schweet, et al., 1964; Bretscher and Marcker, 1967; Bretscher, 1966; Nomura and Lowry, 1967; Ghosh and Khorana, 1967, Mukundan, et al., 1968). Aminoacyl tRNA interacts at the "A" (or acceptor) site. Peptidyl tRNA binds at the "P" (or peptidyl) site. Polymerization of amino acids is thought to occur through nucleophilic attack by the amino group of aminoacyl tRNA upon the carbonyl group of the peptidyl tRNA in the "P" site. The formation of the peptide bond is catalyzed by peptidyl transferase, an enzyme which is believed to be an integral component of the ribosome (Monro, 1967; Maden, Traut, and Monro, 1968; and Rychlik, 1966) in both eukaryotic and prokaryotic systems. The product of the transferase reaction consists of the peptidyl moiety plus one amino acid attached to the tRNA which was originally present in the "A" site. Before another round of synthesis can occur the newly formed peptidyl tRNA species must be translocated back to the "P" site. The translocation is accomplished by supernatant factors with the concomitant hydrolysis of GTP (Nishizuka and Lipmann, 1966; Haenni and Lucas-Lenard, 1968; Skogerson and Moldave, 1968).

All of the tRNA molecules involved in the process

of protein synthesis have been found to have a common 3' terminal trinucleotide sequence, -CpCpA, as confirmed by the primary sequence determinations made of many different species of tRNA as summarized by Philipps (1969). Enzyme fractions from Escherichia coli (Preiss, et al., 1961), Ehrlich ascites carcinoma cells (Hecht, et al., 1958), and rat liver (Daniel and Littauer, 1963; Canellakis, and Herbert, 1960; Hoagland, et al., 1958) have been isolated which are capable of the degradation and the reconstruction of the trinucleotide sequence.

The importance of the -CpCpA sequence has also been demonstrated by the investigation of its involvement in tRNA charging and ribosomal binding. The terminal adenylic acid of the sequence is the acceptor for the aminoacyl synthetase catalyzed transfer of the activated amino acid. The ester bond is formed at either the 2' or 3' position of the adenylate moiety (Preiss, et al., 1959; Hecht, et al., 1959; Feldmann and Zachau, 1963) and is dependent upon the integrity of the trinucleotide sequence (Daniel and Littauer, 1965). The ribosomal binding requirements have been investigated by Pestka (1970) and others (Cannon, Krug, and Gilbert, 1963; Takanami, 1962). Transfer RNA with the terminal 3' -pA, -pCpA and -pCpCpA enzymatically or chemically removed binds to

ribosomes with progressively lower efficiency at the ribosomal "A" site.

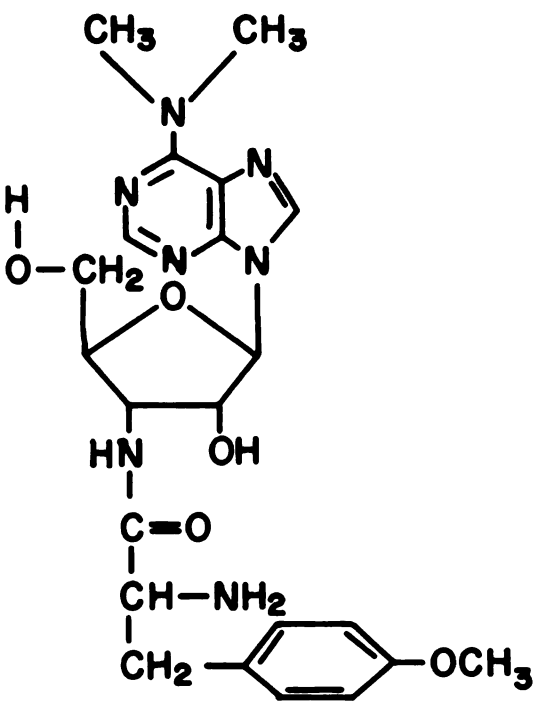
Other studies have investigated the role of the trinucleotide sequence in the interaction of peptidyl tRNA at the ribosomal "P" site. Monro, Cerna, and Marcker (1968) isolated the terminal fragments from the enzymatic digestion of F-met-tRNA, the known initiator of bacterial protein synthesis (Marcker and Sauger, 1964; Clark and Marcker, 1965). The fragments were tested in the "fragment reaction" (Monro, 1967; Monro and Marcker, 1967; Monro, 1969) where their ability to elicit the formation of F-met puromycin is measured. Terminal fragments CAACCA-, AACCA-, ACCA-, and CCA-F-met are capable of undergoing the reaction. However, CA-F-met and A-F-met are inactive (Vazquez, et al., 1969). The formyl group can be replaced by an acetyl group without any loss of activity (Monro, et al., 1969; Vazquez, et al., 1969). Oligonucleotide fragments CACCA-Ac-leu, UACCA-Ac-leu, and CCA-Ac-leu are all active. Therefore, the intact CpCpA sequence is a necessary prerequisite for interaction at the "P" site before the formation of the peptide bond.

Puromycin is an antibiotic isolated from Streptomyces alboniger by Porter, et al. (1952). It has a broad range of antibiotic activity being active in systems

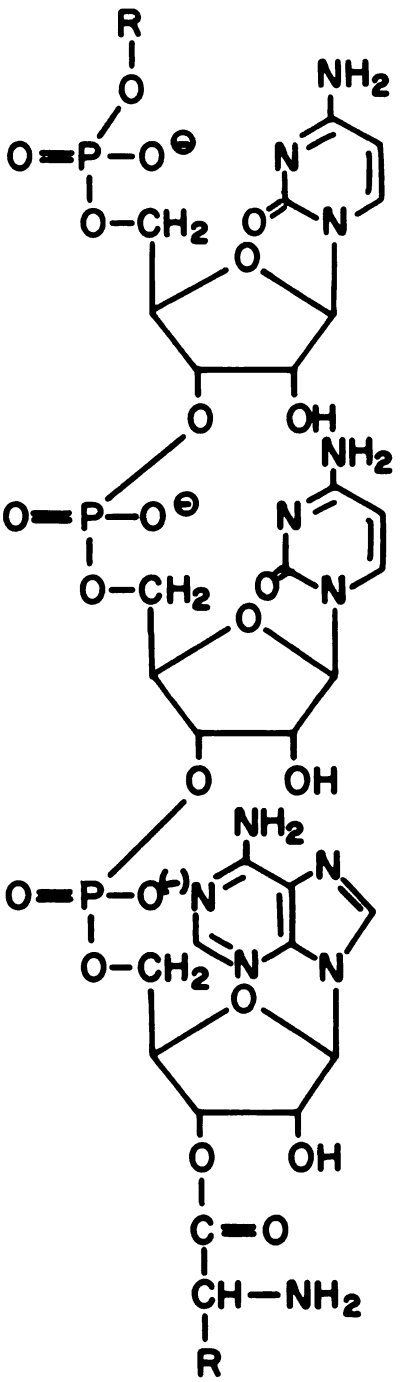
composed of either 70S or 80S ribosomes (Halliday, Bennett, and Oleson, 1955; Tomisek, et al., 1957; Rabinovitz and Fisher, 1962; Allen and Zamecnik, 1962; Morris, et al., 1963). Yarmolinsky and De La Haba (1959) were the first to suggest that the similarity between puromycin and the 3' terminus of aminoacyl tRNA may be responsible for its activity. It is now generally agreed that the ability of puromycin to inhibit protein synthesis is due to its ability to substitute for the incoming aminoacyl tRNA at the "A" site of peptidyl transferase and to bring about the premature release of the nascent polypeptide chains as peptidyl puromycin (Morris, et al., 1963; Nathans, 1964). A schematic representation of the structure of puromycin and the -CpCpA terminus of aminoacyl tRNA are presented in Figure 1.

Although many amino acid analogues of puromycin have been tested for their ability to inhibit protein synthesis (Nathans and Neidles, 1963; Hawtrey and Biedron, 1966; Symons, et al., 1969), only the analogues containing aromatic groups (tryptophan, tyrosine, benzyl-histidine, S-benzyl-cysteine, and phenylalanine) had any activity with none being as effective as puromycin itself. Therefore, a structural requirement for an aromatic amino acid to achieve optimal activity seems to exist. Based upon

Figure 1. Comparison of the structure of the CpCpA terminus of aminoacyl tRNA to the structure of puromycin.



Puromycin



C-C-A Terminus of amino acyl tRNA

this finding, Symons, et al., (1969) have suggested that there is a possibility that the aromatic amino acid requirement may be due to an ability of the aromatic group to occupy a site normally occupied by a cytosine residue of tRNA in the "A" site of peptidyl transferase.

Since puromycin resembles the 3' terminus of tRNA and inhibits protein synthesis and ribonuclease digests of aminoacyl tRNA have been found to be inhibitory (Takanami, 1964), Waller et al. (1966) proposed that the same mechanism should be applicable to aminoacyl derivatives of adenosine. Using leucyl, phenylalanyl, and tyrosyl adenosine compounds, Waller and co-workers found that the compounds did inhibit protein synthesis but with varying efficiencies. Once again the aromatic analogues were found to be the most active. Similar but more extensive studies with chemically prepared aminoacyl adenosine compounds (Zemlicka and Chladek, 1966; Zemlicka, et al., 1969; Zemlicka and Chladek, 1969; Chaldek, et al., 1970) have been done by other workers (Rychlik, et al., 1969; Rychlik, et al., 1970; Cerna, et al., 1970a; Cerna, et al., 1970b; Harbon and Chapeville, 1970) with similar results.

Rychlik, Chladek, and Zemlicka (1967) dealt not only with glycyl adenosine, but also with chemically prepared nucleotidyl derivatives of glycyl adenosine

(Chladek and Zemlicka, 1967; Chladek and Zemlicka, 1970). Glycyl adenosine by itself is not inhibitory. However, cytidylyl glycyl adenosine is almost 50% as inhibitory as puromycin, whereas, uridylyl glycyl adenosine is almost inactive. The strong preference demonstrated for cytidylyl adenosine as the dinucleotide component was investigated further by Zemlicka and Chladek (1971). They have found that replacement of cytidylyl moiety with 2' deoxycytidylyl results in a lost of activity as does replacement of adenosine with inosine (Rychlik, et al., 1970).

Nucleotidyl derivatives of glycyl puromycin aminonucleoside have also been synthesized and tested for their ability to inhibit protein synthesis (Symons, et al., 1969). They have synthesized the adenylyl, cytidylyl, guanosyl, and uridylyl analogues of 3'-N-puromycin aminonucleoside and have found that only the cytidylyl derivatives had significant inhibitory ability. The inhibition obtained with cytidylyl glycyl puromycin aminonucleoside was approximately 50% of that obtained with puromycin. Therefore, it appears as if the greater the similarity of the nucleotidyl sequence to the -CpCpA terminus of tRNA, the greater is the ability of the compound to interfere with protein synthesis, probably because of enhanced binding with peptidyl transferase.

Binding and incorporation studies with intact tRNA, nucleotidyl fragments of tRNA, and analogues of tRNA, such as puromycin, serve an important function in the localization of the site of action of antibiotics known to interact with the ribosome. Antibiotics have been classified on the basis of their binding interactions with the 70S, the 80S, or both types of ribosomes (reviewed in Vazquez and Monro, 1967). Antibiotics such as streptogramin A, carbomycin, spiramycin that compete with peptidyl tRNA (Cerna, Rychlik, and Pulkrabek, 1969) or peptidyl tRNA fragments (Monro and Vazquez, 1967; Munro, Celma, and Vazquez, 1969; Celma, Monro, and Vazquez, 1970) are implicated with the "P" site of peptidyl transferase. Similarly, inhibitors such as gougertin, chloramphenicol, sparsomycin, and amicetin that compete with aminoacyl tRNA (Vazquez and Monro, 1967; Pestka, 1970b) or puromycin (Goldberg and Mitsugi, 1967a; Goldberg and Mitsugi, 1967b; Casjens and Morris, 1965) are related either directly, or possibly allosterically, with the ribosomal "A" site.

These data combined with the evidence that the macrolide (erythromycin, spiramycin, carbomycin, oleandomycin), chloramphenical, and lincosaminide (lincomycin and celesticetin) groups of chemically distinct antibiotics bind competitively yet individually are capable of

interactions with either the "A" site or "P" site suggest the existence of a third site which appears to overlap both the "A" and "P" sites. The site or related sites, collectively called the "CM site" (Monro, et al., 1969) exists on the large (50S) subunit of the 70S ribosome (Weisblum and Davies, 1968; Vazquez and Monro, 1967; Vazquez, et al., 1969a; Vazquez, et al., 1969b).

In conclusion, much has been learned about the peptidyl transferase which exists on both 70S and 80S ribosomes. The "A" site and "P" site interactions of various analogues of the -CpCpA terminus of tRNA have helped clarify the central role played by peptidyl transferase in the process of protein synthesis. Just recently Fahnestock, et al. (1970) demonstrated that peptidyl transferase can catalyze the reaction of F-met-tRNA with a hydroxyl derivative of puromycin. The reaction also occurs with peptidyl tRNA (Fahnestock and Rich, 1971). The reaction is of interest because most of the proteolytic enzymes which catalyze the reverse reaction of peptidyl transferase are also esterases (Dixon and Webb, 1964). In both these instances hydroxyl puromycin was found to be less inhibitory than puromycin as have all other nucleotidyl and aminoacyl derivatives of puromycin which have been tested (Nathans and Neidles, 1963; Hawtrey and Biedron, 1966;

Symons, et al., 1969). It is clear that certain structural features must be present in the puromycin analogue in order to obtain maximum biological activity. The amino acid part of the molecule must be O-CH_3 tyrosine as any alteration or modification leads to a decrease in potency. It is also imperative that the nucleotidyl addition to the molecule be cytidylate. Apparently the more closely the nucleotidyl analogue resembles the 3' end of tRNA, the greater will be its biological activity. With the desired features in mind, cytidylyl puromycin was synthesized and tested for its ability to inhibit protein synthesis with hopes that even more would be learned about the interactions of peptidyl transferase with its substrates.

MATERIALS AND METHODS

Chemical Sources.

CMP (2' and 3' mixed isomers), Tris buffers, pyruvate kinase (Type II crystalline from rabbit muscle), Ribonuclease A (5 times crystallized from bovine pancreas), PEP, and crystalline BSA were obtained from Sigma Chemical Company, St. Louis, Missouri. Dowex 1 x 2 and Dowex 50W x 8 were purchased from Bio-Rad Corporation, Los Angeles, California. Puromycin dihydrochloride and puromycin aminonucleoside were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. ATP, GTP, CMP (2' isomer), and CMP (3' isomer) were obtained from P-L Laboratories, Milwaukee, Wisconsin. Phenylhydrazine hydrochloride and p-dioxane were obtained from Eastman Products, Rochester, New York. Heparin was purchased from Fisher Scientific Company, Chicago, Illinois. Nembutal was purchased from Abbott Laboratories, North Chicago, Illinois. Liquiflor and ^{3}H-puromycin dihydrochloride were obtained from New England Nuclear, Boston, Massachusetts. TPS was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. ^{14}C-CMP

(2' and 3' mixed isomers) was obtained from Schwarz Bio-research, Inc., Orangeburg, New York. Calcium hydride was purchased from K and K Laboratories, Inc., Plainview, New York. ^{14}C-leucine was obtained from International Chemical and Nuclear Corp., Irvine, California. Whatman #3MM paper and Whatman #1 paper were obtained from Reeve Angel, Clifton, New Jersey. Whatman #3MM filter paper discs (2.2 cm dia.) were purchased from Arthur H. Thomas Co., Philadelphia, Pennsylvania. Tetraethylammonium acetate, toluene, and absolute methanol were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri. Acetic anhydride, anhydrous ether, pyridine, and DMSO were obtained from J. T. Baker, Phillipsburg, New Jersey. Scintillation grade POPOP and PPO were obtained from either Packard Instruments, Downers Grove, Illinois; Beckman Instruments, Palo Alto, California; or New England Nuclear. Carbosil powder used in the thixotropic-gel counting solution was from Packard. Other chemicals and reagents were reagent grade and were obtained from commercial sources. All reaction solvents were redistilled and dried over calcium hydride or Linde 4-A molecular sieves for several weeks prior to use.

Biological Methods.

Preparation of Rabbit Reticulocyte Ribosomes.

Male New Zealand rabbits were made reticulocytic by four daily subcutaneous injections of 2.5% phenylhydrazine. The phenylhydrazine was dissolved in an isotonic saline solution (NKM) which contains 0.13 M NaCl, 5.2 mM KCl, and 7.5 mM $MgCl_2$. The pH was adjusted to approximately 7 by titration with NaOH, and glutathione was added to a concentration of 1 mM. The animals received no injections on days five and six. On day seven the animals were given 100 mg of Nembutal and 2,000 I. U. of heparin via the marginal ear vein. Blood was collected by heart puncture, and the collected blood was cooled immediately and the volume was recorded. All subsequent steps were carried out at 4^{o}.

The cells were separated from the plasma by centrifugation for 20 minutes at 2,000 x g in a Sorvall refrigerated centrifuge. The plasma was decanted and its volume recorded. The hematocrit was usually 15-20% with over 80% of the red cells as reticulocytes (Morris, 1964). The cells were washed with a volume of NKM equal to the plasma volume. The cells were first suspended in a small volume of NKM and filtered through glass wool to remove any clots or fatty material. The remainder of the NKM was added,

the suspension stirred, and the cells recovered by centrifugation. The NKM and the layer of white cells were removed by means of a water aspirator. The packed cell volume was measured in a graduated cylinder and recorded. The cells were lysed by rapidly adding four volumes of 2.5 mM $MgCl_2$ and stirring gently for 10 minutes. Isotonicity was restored to the solution by adding one volume of 1.5 M sucrose containing 0.15 M KCl. The solution was stirred and the cellular debris removed by centrifugation at 15,000 x g for 10 minutes. The supernatant solution was decanted and centrifuged for 90 minutes at 78,000 x g in the number 30 rotor of a Beckman L-2 ultracentrifuge. The high speed supernatant was decanted from the ribosomal pellet and frozen until used in the preparation of the enzyme fraction for the cell-free system. The ribosomal pellets were rinsed with 0.25 M sucrose and resuspended in a small volume of 0.25 M sucrose with the aid of a glass homogenizer and loose fitting teflon pestle. Large aggregates were removed by sedimentation at 10,000 x g for 10 minutes. The suspension, termed "1X ribosomes" (meaning once sedimented), was heavily contaminated with hemoglobin which could be removed by dilution of the 1X ribosomal suspension with Medium B (0.25 M sucrose, 0.0175 M $KHCO_3$, and 2 mM $MgCl_2$) and resedimenting at 78,000 x g. The pellet thus obtained

was resuspended in 0.25 M sucrose and was termed "2X ribosomes." The concentration of the ribosomal preparation was determined by making an appropriate dilution of an aliquot of the preparation with water and determining the absorbance at 260 mu. One mg/ml of ribosomes has an extinction coefficient of 11.3 (Ts'o and Vinograd, 1961). The preparation was divided into aliquots and stored in liquid nitrogen.

Preparation of the Cell-Free System Supernatant Enzyme Fraction.

Soluble enzymes for use in the cell-free system were prepared according to the methods of Allen and Schweet (1962). The frozen high speed supernatant obtained from the ribosome preparation was carefully thawed and maintained at 4^{o} throughout the entire procedure. The solution was brought to 0.1 M in Tris-HCl buffer by the addition of 2 M Tris-HCl, pH 7.5. The pH of the Tris buffer was adjusted at 22^{o}. When it is diluted and cooled to 4^{o} the pH is 8.1. To the supernatant fraction was added finely ground ammonium sulfate slowly, with constant stirring, to obtain a final concentration of 40% of saturation. The solution was stirred for an additional 30 minutes, and the precipitate was removed by centrifugation. The supernatant solution was removed and brought to 70% of saturation by

the addition of more finely ground ammonium sulfate. Thus the protein fraction precipitating between 40 and 70% of saturation was collected. This precipitate was dissolved in 50 ml of 0.1 M Tris-HCl, pH 7.5, containing 10^{-3} M GSH and reprecipitated by the addition of ammonium sulfate to 70% of saturation. The final protein precipitate was dissolved in a minimum volume (4 to 5 ml) of a solution containing 0.02 M Tris-HCl, pH 7.5, 10^{-3} M glutathione, 10^{-3} M EDTA, and 10^{-3} M $MgCl_2$, and dialyzed overnight at 4^{o} against 100 volumes of this same buffer solution. A precipitate which occasionally formed upon dialysis was removed by sedimentation at 10,000 x g for 10 minutes. The post dialysis enzyme preparation was brought to 2 x 10^{-2} M with glutathione, divided into small aliquots, and frozen in liquid nitrogen. The enzyme fraction appears to be stable indefinitely at this temperature.

Prelabeling of Ribosomes in the Cell-Free System.

The cell-free system used to prelabel ribosomes was a modification (Morris, 1964) of that described by Allen and Schweet (1962). The incubation medium contained ATP (1mM), GTP (0.25 mM), PEP (2.5 mM), pyruvate kinase (10 ug/ml), a mixture of 19 amino acids missing the amino acid to be added radioactive (0.05 mM each), GSH (0.02 M),

KCl (0.05 M), $MgCl_2$ (4 mM), Tris-HCl buffer, pH 7.5 (0.05 M), 2X ribosomes (1 mg/ml), purified supernatant enzymes (at a concentration determined to be saturating for each enzyme preparation), and a radioactivity labeled amino acid (0.05 mM). Incubation was carried out at 37^{o} for 7 minutes and terminated by the addition of 25 volumes of cold (4^{o}) Medium B. The ribosomes were reisolated by centrifugation at 78,000 x g for 90 minutes. The pellet of 3X ribosomes was resuspended in 0.25 M sucrose as previously described.

The Cell-Free System.

The rabbit reticulocyte cell-free system used to determine the inhibitory properties of the puromycin derivatives was based upon the methods of Morris (1964). The composition of a typical assay using the cell-free system is presented in Table 1. The assay was actually used in two forms. Some of the initial studies were done using an assay volume of one ml, whereas, more recent work used the 0.1 ml assay volume. In the larger assay the proportions are held constant but amplified 10 fold. The main difference in the two assay forms was in the method of preparation for the determination of the amount of radioactivity incorporated. The large volume assay was analyzed by the methods of Casjens and Morris (1965), the

Table 1. Composition of the typical assay for the inhibitory studies.

COMPOSITION OF TYPICAL ASSAY USED FOR THE INHIBITION STUDIES

Reagent	Concentration	µl/assay
ATP	0.05 M	2.0
GTP	0.01 M	2.5
P-enol-pyruvate	0.05 M (0.218 M K^+)	10
Pyruvate kinase	mg/ml	4.0
$MgCl_2$	0.1 M	4.0
Tris·HCl	1.0 M, pH = 7.5	5.0
GSH	0.04 M (0.375 M K^+)	5.0
KCl	1.0 M	1.0
H_2O		23
Amino acid mixture-leucine	mM each	5.0
Ribosomes	15.35 mg/ml	6.5
Supernatant enzymes	108 mg/ml	2.0
Total volume of assay mixture		70 µl
Substance to be tested		25
^{14}C-Leucine	mM, S.A. = 100 µCi/µM	5.0
Total Volume of Assay		100 µl

small volume assay was prepared for counting using the paper disc method of Manns and Novelli (1960). In both cases the incorporation of radioactive amino acid was initiated by placing the samples in a water bath at 37°. All incubations were for 40 minutes duration.

Chemical Methods.

Synthesis of Triacetylated (Cytidine 2', 3' Monophosphoric Acid Mixed Isomers)

The acetylation of cytidine 2', 3' monophosphoric acid to obtain a suitably blocked compound was done according to the methods of Lohrmann and Khorana (1964).

Cytidine 2', 3' monophosphoric acid (0.5 mmole) and tetraethylammonium acetate (5.0 mmole) were rendered anhydrous by repeated evaporations of solutions of anhydrous, redistilled pyridine. The last evaporation was continued until a viscous syrup remained in the flask. Doubly distilled acetic anhydride (1.61 ml) was added and the flask was sealed, shaken, and set aside in the dark at room temperature for 15 hours. Upon completion of the incubation period 5 ml of a methanol-pyridine (4:1) solution was added and the clear solution was maintained for 30 minutes at room temperature. The solvent was removed by flash evaporation and the residue was dissolved in 30 ml of cold 10% aqueous pyridine. The aqueous pyridine solution was

passed through a column (2.5 cm x 30 cm) of Dowex 50 W x 8 ion exchange resin (pyridinium form) in the cold room (4° C). The column was eluted with 10% aqueous pyridine, and three column volumes of effluent were collected (approximately 500 ml). The solution was concentrated by flash evaporation to a small volume and rendered anhydrous by repeated evaporation of pyridine. The pyridinium salts of N^4, $O^{2'}$, $O^{5'}$ triacetylcytidine 3' phosphate and N^4, $O^{3'}$, $O^{5'}$ triacetylcytidine 2' phosphate were obtained in quantitative yield. The product could be isolated in crystalline form by dissolving the residual gum in anhydrous pyridine (5-10 ml) and then slowly adding the solution under vigorous stirring to an excess of anhydrous ether. After centrifugation and washing with ether the compound could be dried over phosphorous pentoxide.

Synthesis of N^4, $O^{2'}$, $O^{5'}$ Triacetylcytidine 3' Phosphate.

The synthesis of triacetylcytidine 3' phosphate is accomplished in the same manner as described above for the mixed isomers. However, the nucleotide component of the reactants is exclusively the 3' phosphate isomer.

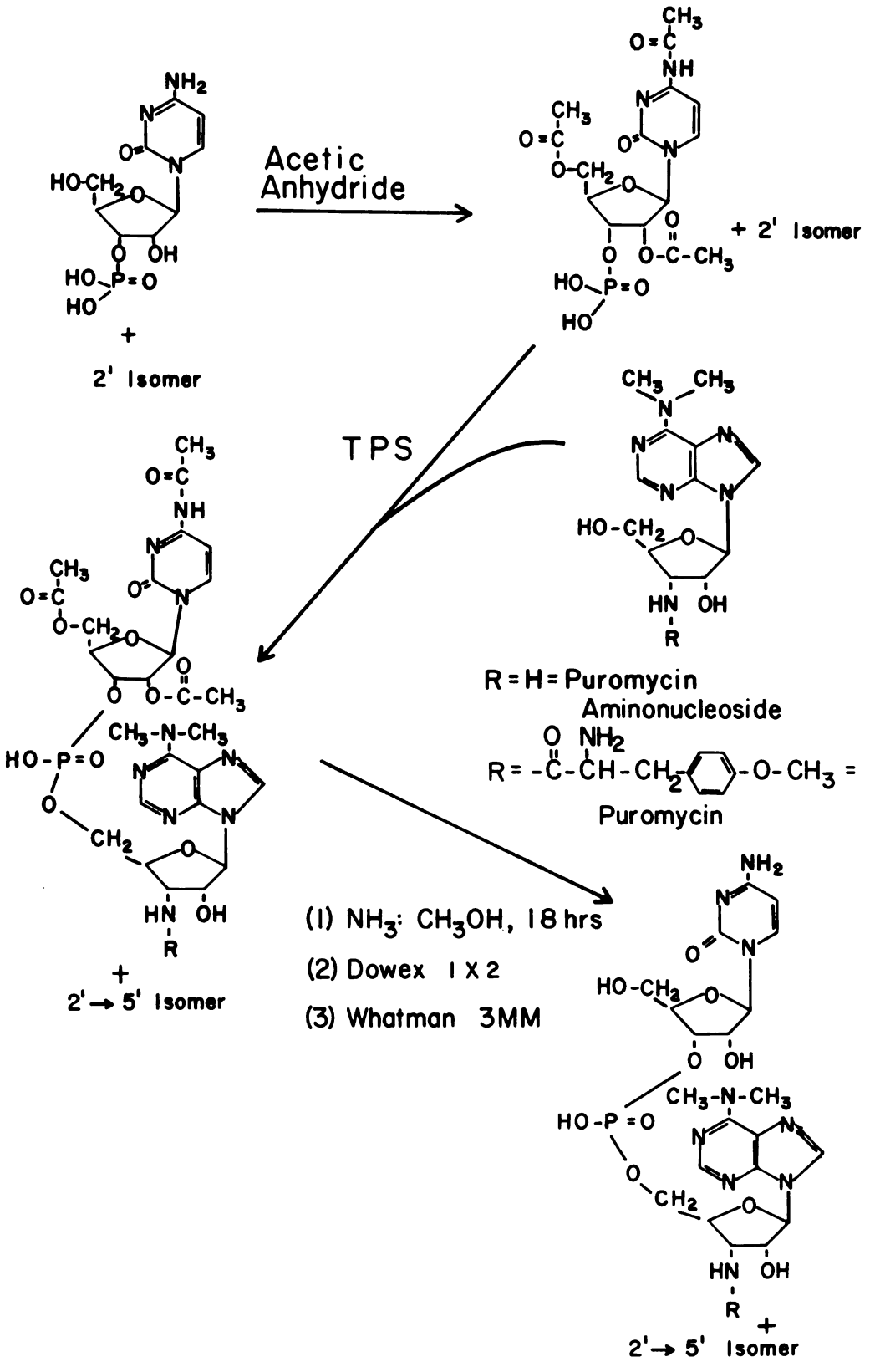
Synthesis of 2'-5', 3'-5' Cytidylyl Puromycin, Mixed Isomers.

The synthesis of cytidylyl puromycin was performed using the general methods of Lohrmann and Khorana (1966a)

and Lohrmann, et al. (1966b). A schematic flow chart of this reaction and other closely related reactions is presented in Figure 2.

A mixture of N^4, $O^{2'}$, $O^{5'}$ and N^4, $O^{3'}$, $O^{5'}$ triacetylcytidylic acid (0.05 mmole) and puromycin (0.1 mmole) were rendered anhydrous by 4 or 5 evaporations of anhydrous pyridine. The last evaporation was continued until about 0.5 ml of solution remained in the flask. Then, triisopropylbenzenesulfonyl chloride (0.1-0.2 mmole) was added under exclusion of moisture, and the reaction flask was sealed for 8 hours and stored in the dark. The reaction was terminated by the addition of 0.5 ml water and allowed to stand at room temperature for approximately 12 hours. After the addition of 50% aqueous pyridine (5 ml), the solution was subjected to flash evaporation. The residual gum was again rendered anhydrous by the repeated evaporations of anhydrous pyridine and 7 ml of cold (4^o) ammonia saturated methanol was added to remove the acetyl blocking groups. The deacylation solution was prepared by adding liquid ammonia (approximately 130 ml) to a solution of absolute methanol (200 ml) previously cooled in an ice bath. The mixture was allowed to equilibrate at ice bath temperature for 48 hours being careful not to seal the flask completely. The mixture was sealed and shaken for 18 hours at room

Figure 2. Flow diagram showing the general reaction sequence used for the synthesis of cytidylyl puromycin and cytidylyl puromycin aminonucleoside.


Acetic
Anhydride
+ 2' Isomer
2' Isomer
TPS
R = H = Puromycin
Aminonucleoside
R = Puromycin =
(1) NH3: CH3OH, 18 hrs
(2) Dowex 1 X 2
(3) Whatman 3MM
2'→ 5' Isomer
2'→ 5' Isomer

temperature to insure complete deacetylation. The solution was evaporated and dissolved in water. The gum dissolved slowly and could be coaxed by the addition of some heat. The solution was applied to a Dowex 1 x 2 (formate) column (1.2 cm x 12 cm), eluted with water to remove nucleosides and then eluted with a gradient of formic acid (0.005 N-0.015 N). The peak corresponding to the dinucleoside monophosphate which elutes 10-20 tubes after application of the gradient, was pooled, evaporated, redissolved in water, streaked on citrate washed Whatman #3MM paper, and subjected to descending paper chromatography using ammonium acetate, pH 7.5, (1 M) and ethanol in the ratio 2:5, as the developing system. The area of the paper containing the cytidylyl puromycin was cut into small strips (1/4" x 3/4") which were placed in test tubes and washed with a number of 10 ml fractions of water. The total aqueous fraction was filtered to remove the paper fibers and the volume was reduced by flash evaporation. The overall yield of 2'-5', 3'-5' cytidylyl puromycin was about 10%.

Synthesis of 3'-5' Cytidylyl Puromycin.

The synthesis of 3'-5' cytidylyl puromycin was accomplished according to the same methods presented for the synthesis of 2'-5', 3'-5' cytidylyl puromycin, mixed

isomers. The one difference was that the nucleotidyl component of the reaction was N^4, $O^{2'}$, $O^{5'}$ triacetylcytidine 3' phosphoric acid. The isolation procedures used were also identical. Overall yields of 3'-5' cytidylyl puromycin ranged from 5-10%.

Preparation of 2'-5' Cytidylyl Puromycin.

Cytidylyl puromycin (2'-5') was obtained from a preparation of cytidylyl puromycin (mixed isomers). The mixed isomers were digested with pancreatic ribonuclease, and the products of the digestion were separated by paper chromatography on Whatman # 3MM paper developed with solvent system II. Since ribonuclease is specific for only the 3'-5' isomer, the 2'-5' isomer of cytidylyl puromycin was isolated intact by elution from the chromatogram using the methods described above.

Synthesis of Cytidylyl Puromycin Aminonucleoside:

The synthesis of cytidylyl puromycin aminonucleoside was also accomplished by the methods of Lohrmann and Khorana (1966) as previously presented for the synthesis of cytidylyl puromycin. Triacetylcytidylic acid was coupled to puromycin aminonucleoside under the influence of triisopropylbenzenesulfonyl chloride. The reaction solution was passed through a Dowex 1 x 2 (formate)

eluted with water and then a gradient of formic acid (0.005-0.015). The peak corresponding to the dinucleoside monophosphate which elutes after the application of the gradient was pooled, evaporated, redissolved in water, and streaked on Whatman #3MM paper and developed in solvent system II. The area of the paper containing the compound was eluted with water as described above, and the volume was reduced by flash evaporation. The overall yield of cytidylyl puromycin aminonucleoside was approximately 10%.

Analytical Techniques.

Degradation of Dinucleoside Monophosphates.

Dinucleoside monophosphate compounds were degraded by the use of two different methods: (1) Alkaline digestion, approximately 5 A_{260} units of the material to be digested were treated with 0.3 N potassium hydroxide at 37° for 18 hours. (2) Pancreatic ribonuclease, ribonuclease (5 μg/ reaction mixture) was added to a solution of 1.5 OD of polynucleotide, 1 mM in ammonium bicarbonate, pH 7.5. The concentration of the pancreatic ribonuclease was determined by subtracting the absorbance at 320 mu from the absorbance measured at 280 mr. A mg/ml solution of pancreatic ribonuclease has a corrected absorbance at 280 of 0.695.

Preparation of Dowex-50W x 8 Ion Exchange Columns in the Pyridinium Form.

Dowex 50W x 8 columns (pyridinium salt) used in the acetylation procedures were generated according to the procedures of Khorana and Conners (1966). Dowex 50 was treated with 1 N NaOH overnight, washed thoroughly with water, and converted to the acid form by slow passage of 2N HCl (2 liters of the acid solution/50 ml of wet resin) through the resin packed in a column. The resin was stored as the free acid. Just before use a Dowex 50W x 8 column (8 cm x 1 cm) was prepared in the pyridinium form by passing an excess of 10% aqueous pyridine through the resin and washing at a flow rate of 1 ml/minute for at least two hours.

Preparation of Dowex 1 x 2 Columns in the Formate Form.

A column of Dowex 1 x 2 resin (12 cm x 1.2 cm) in the chloride form was washed with 9 column volumes of 1 N NaOH to convert the resin to the hydroxide salt. The column was then washed with at least two column volumes of 1 N formic acid to convert the column to the formate form. Excess formic acid was removed by rinsing the column with water until a pH of 4.8 was achieved.

Preparation of Ultaviolet Spectra.

Ultaviolet spectra were obtained on a Gilford Recording Spectrophotometer. Three different solvent systems were employed: (1) 0.1 N hydrochloric acid, (2) 0.1 N soidum hydroxide, and (3) distilled, deionized water. Standards were analyzed under the same conditions for comparative purposes. Whenever possible, spectra were compared to those published in the pamphlet entitled "Pabst Research Biochemicals - Specifications," circular OR-17.

Molar extinction coefficients for the common nucleotides and nucleosides were also obtained from Pabst circular OR-17. The extinction values for puromycin and puromycin aminonucleoside were obtained from Baker, et al. (1954) or The Merck Index, seventh edition. The extinction coefficient for cytidylyl puromycin and cytidylyl puromycin aminonucleoside were calculated from spectra of their component parts assuming no hyperchromicity. Verification of the theoretical coefficient was achieved by comparison with the extinction obtained with equimolar quantities of the component nucleotides and nucleosides. The calculated extinction coefficient for cytidylyl puromycin in water and base was 28,300, in acid the extinction was 30,200. Cytidylyl puromycin aminonucleoside had a calculated extinction of 27,030 in water, 28,930 in acid, and 24,500 in base.

Paper Chromatography.

Descending paper chromatography was performed with DEAE (diethylaminoethyl) cellulose paper using solvent system I (0.1 N ammonium formate) or with Whatman #1 paper developed with system II (ethanol - 1.0 ammonium acetate, pH 7.5, 5:2, v/v). When paper chromatography was used as a preparative method, Whatman #3MM filter paper was substituted for Whatman #1 using solvent system II for the development. The nucleotidyl components were visualized with a UVS-12 Mineralight.

Paper Electrophoresis.

Paper electrophoresis was used as a means to resolve both pancreatic ribonuclease and alkaline digestions of dinucleoside monophosphates and as a check of the purity of compounds. The instrument used was a Gilson Model D High Voltage Electrophorator. Electrophoretic mobilities were investigated at three different pH values on Whatman #3MM paper. The pH and the composition of the buffer to produce that pH are as follows: (1) pH 8.9 - 1% ammonium carbonate, (2) pH 4.75 - pyridine-acetic acid-water, 1.25:1.25:97.5, (3) pH 2.0 - acetic acid - formic acid-water, 20:5:225. The electrophoretic mobilities of the unknown compounds were compared to the

mobilities of authentic standards analyzed under the same conditions. Usual conditions of electrophoresis were 2000 volts for a period of one hour.

Counting Procedures.

Aqueous samples were counted by using 15 ml of Bray's solution (Bray, 1960). Bray's solution contains per liter: 60 gm naphthalene, 4 gm PPO, 200 mg POPOP, 100 ml absolute methanol, 20 ml ethylene glycol, and p-dioxane to volume.

Radioactivity incorporated into protein in the cell-free system was measured by two different methods. The first method consists of preparing the sample according to the methods of Casjens and Morris (1965). After completion of the incubation procedure bovine serum albumin (15 mg) was added to each sample followed by precipitation with 5% trichloroacetic acid (TCA). The precipitate was washed by resuspension and resedimentation in 5% TCA. The resultant pellet was dissolved in 0.5 ml of 1 N NaOH, washed once more, and then suspended in acetone containing 0.1 N HCl. The precipitate from the acetone step was washed with a mixture of acid-acetone and ether (2:3), and finally with ether alone. The resulting powder was transferred to a glass counting vial with the aid of 0.5 ml

of 1 N NaOH. When the protein powder had completely dissolved (heat increased the rate of solution), 15 ml of thixotropic counting mixture was added, and the contents of the vial were vigorously shaken. The thixotropic counting mixture contained 7 gm of PPO, 150 gm of POPOP, 50 gm naphthalene and 35 gm of thixotropic powder dissolved in 200 ml of toluene, 30 ml of ethanol, and 800 ml of p-dioxane.

The second method for determining the radioactivity incorporated into protein by the cell-free system is based upon the procedure of Manns and Novelli (1960). This method has the advantage that many more samples can be processed than by using the previous method. A comparison of the relative efficiencies of the two methods is presented in the results section. According to this method 0.050 ml aliquots of the samples from the cell-free system were spotted on Whatman #3MM filter paper discs (2.2 cm diameter). The paper discs were mounted on straight pins to facilitate handling of the samples and to prevent the discs from sticking to each other. The discs were dried and placed into a solution of 10% TCA which is 2% in ^{12}C-leucine. The volume of the TCA wash was usually 100 ml when 30 or fewer discs were being assayed. With occasional stirring the discs were allowed

to stand for approximately one hour. The TCA was then poured off and the discs were washed a second time with the same volume of TCA but without the 2% leucine. After 15 minutes the discs were removed and plunged into 5% TCA at 90^{o} and held at this temperature for 30 minutes. Next, the discs were collected, suspended in ether-ethanol (1:1), and incubated for 30 minutes at 37^{o}. Finally, the discs were placed in ether for 15 minutes and then air-dried before placing them into glass counting vials. Scintillation fluid (5 ml) was added and the samples were counted in a Nuclear of Chicago Scintillation counter. The scintillation fluid contained 0.4% PPO and 0.01% POPOP in 100% toluene.

RESULTS

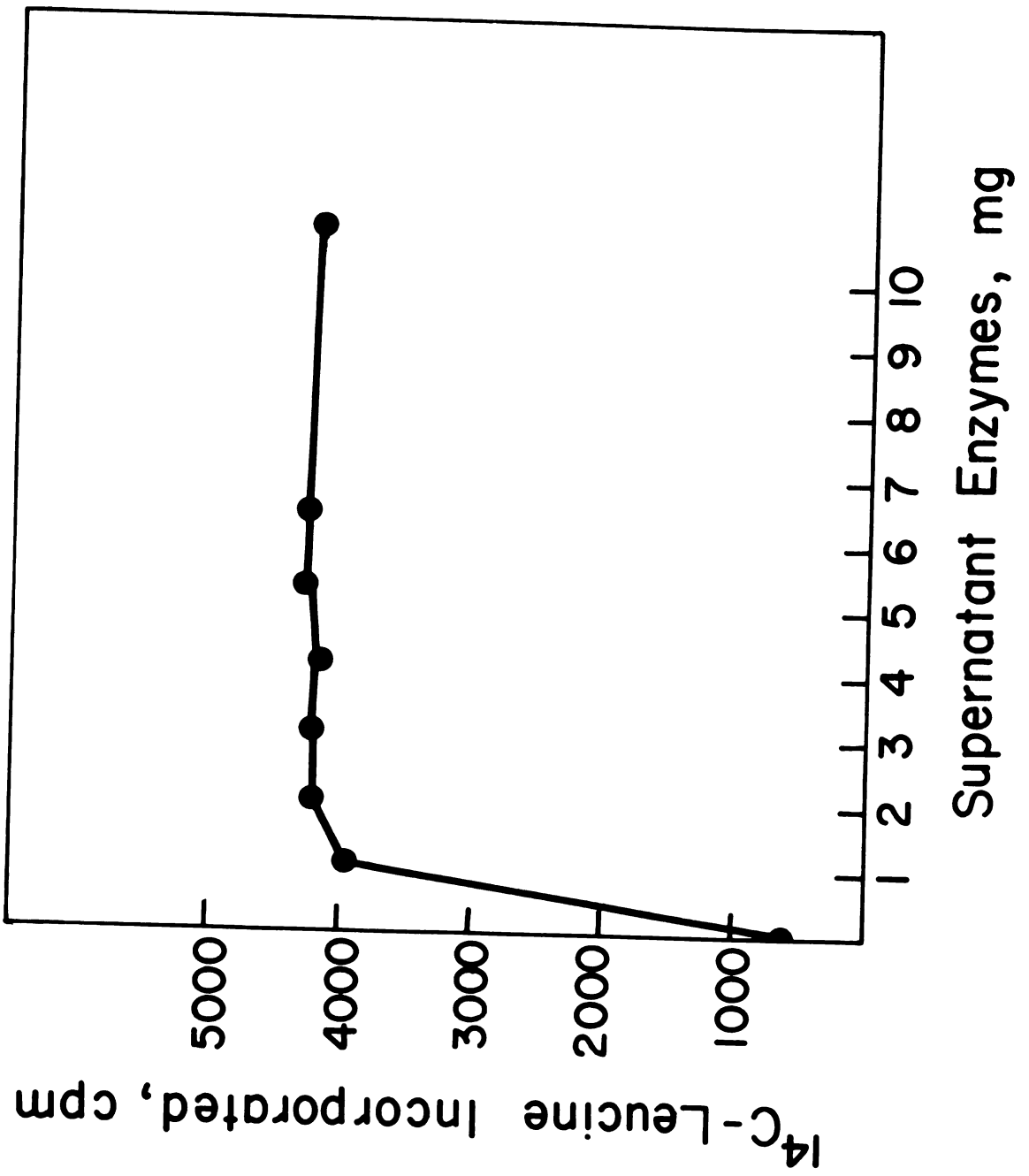
Determination of the Concentration of Supernatant Enzyme Necessary for Saturation.

For the optimum functioning of protein synthesis in the cell-free system prepared from rabbit reticulocytes it is necessary that the supernatant enzymes be present in sufficient concentration so as to not constitute a limiting factor. Determination of the optimal supernatant enzyme concentration is achieved by using a modification of the standard cell-free system. All components are present in the usual concentrations except that the reaction is initiated by the addition of various concentrations of supernatant enzymes. The data obtained are plotted as ^{14}C-leucine incorporated versus enzyme concentration, and a typical plot is seen in Figure 3. With this particular enzyme preparation the saturation of amino acid incorporation occurred at an enzyme concentration of 1 mg/ml.

Comparison of the Counting Procedure of Manns and Novelli with that of Casjens and Morris.

The counting procedure of Casjens and Morris (1965) is very efficient and highly reproducible. However, it

Figure 3. Determination of the concentration of supernatant enzymes necessary to obtain maximum incorporation of ^{14}C-leucine into protein.

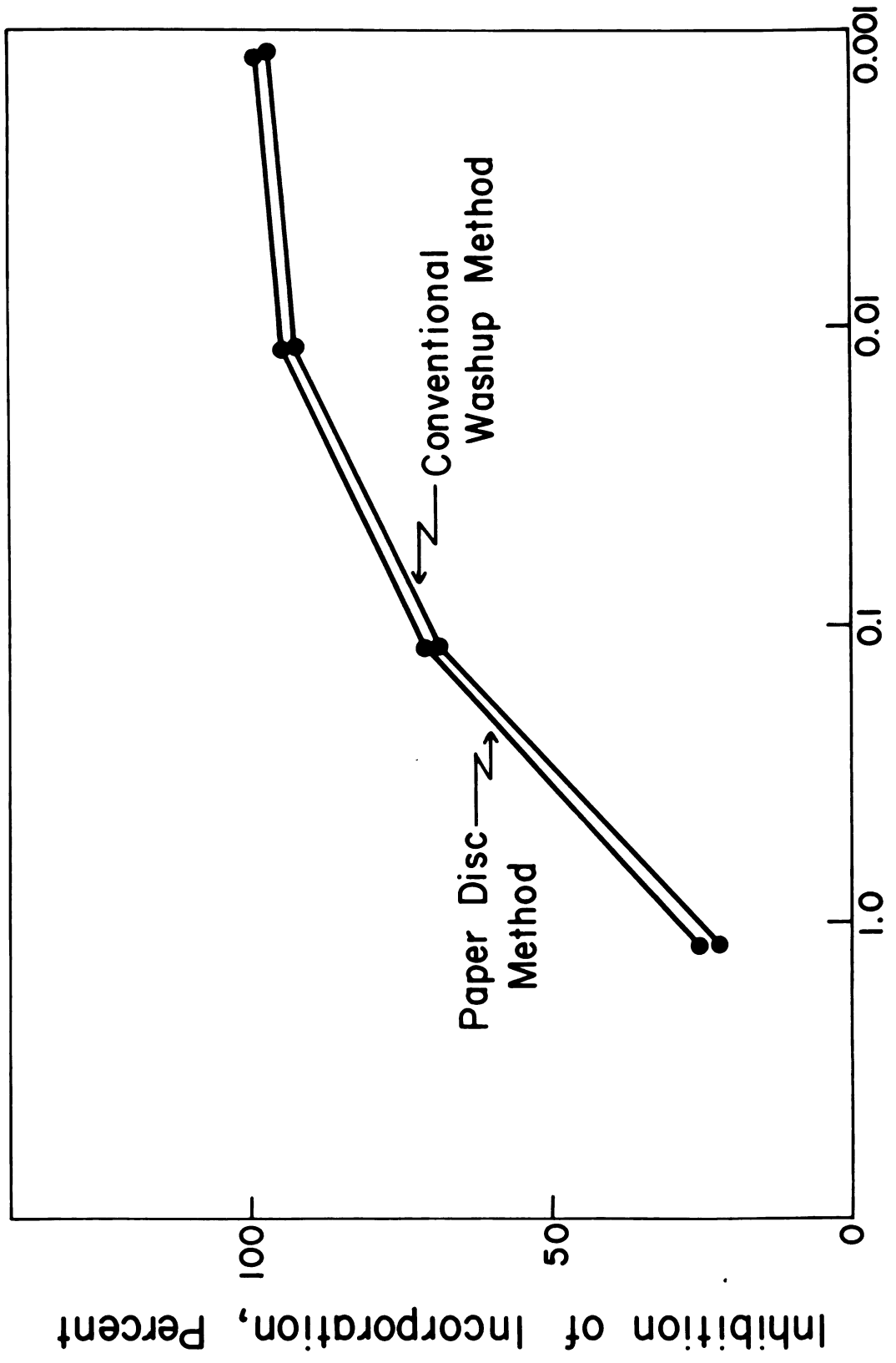

^{14}C-Leucine Incorporated, cpm
5000
4000
3000
2000
1000
1 2 3 4 5 6 7 8 9 10
Supernatant Enzymes, mg

suffers from a limitation of the number of samples which can be processed at any one time. To overcome this limitation upon the number of samples, the paper disc method of Manns and Novelli (1960) was investigated. The method of Manns and Novelli (1960) has the additional advantage of being a semi-micro method which allows a reduction in the sample volume by 90% provided that the specific activity of the radioactive amino acid in the cell-free system is increased 10 fold.

A four point standard curve demonstrating the inhibitory properties of puromycin upon both the 1.0 ml and the 0.1 ml versions of the cell-free system, was obtained using each of the counting procedures. In Figure 4 are seen the curves corresponding to both the disc method (0.1 ml assay) and the conventional method (1.0 ml assay). The relationship of the percent of inhibition to the concentration of puromycin are seen to be almost identical with both methods suggesting that the counting efficiency is nearly identical with both counting procedures.

The relationship of aliquot size to radioactivity incorporated as determined with the method of Manns and Novelli (1960) was also investigated. Increasing volumes of a bulk sample obtained from a cell-free assay were

Figure 4. Comparison of the radioactivity counting procedure of Manns and Novelli to the procedure of Casjens and Morris.


Inhibition of Incorporation, Percent
100
50
0
1.0
0.1
0.01
0.001
Concentration, µM
Conventional Washup Method
Paper Disc Method

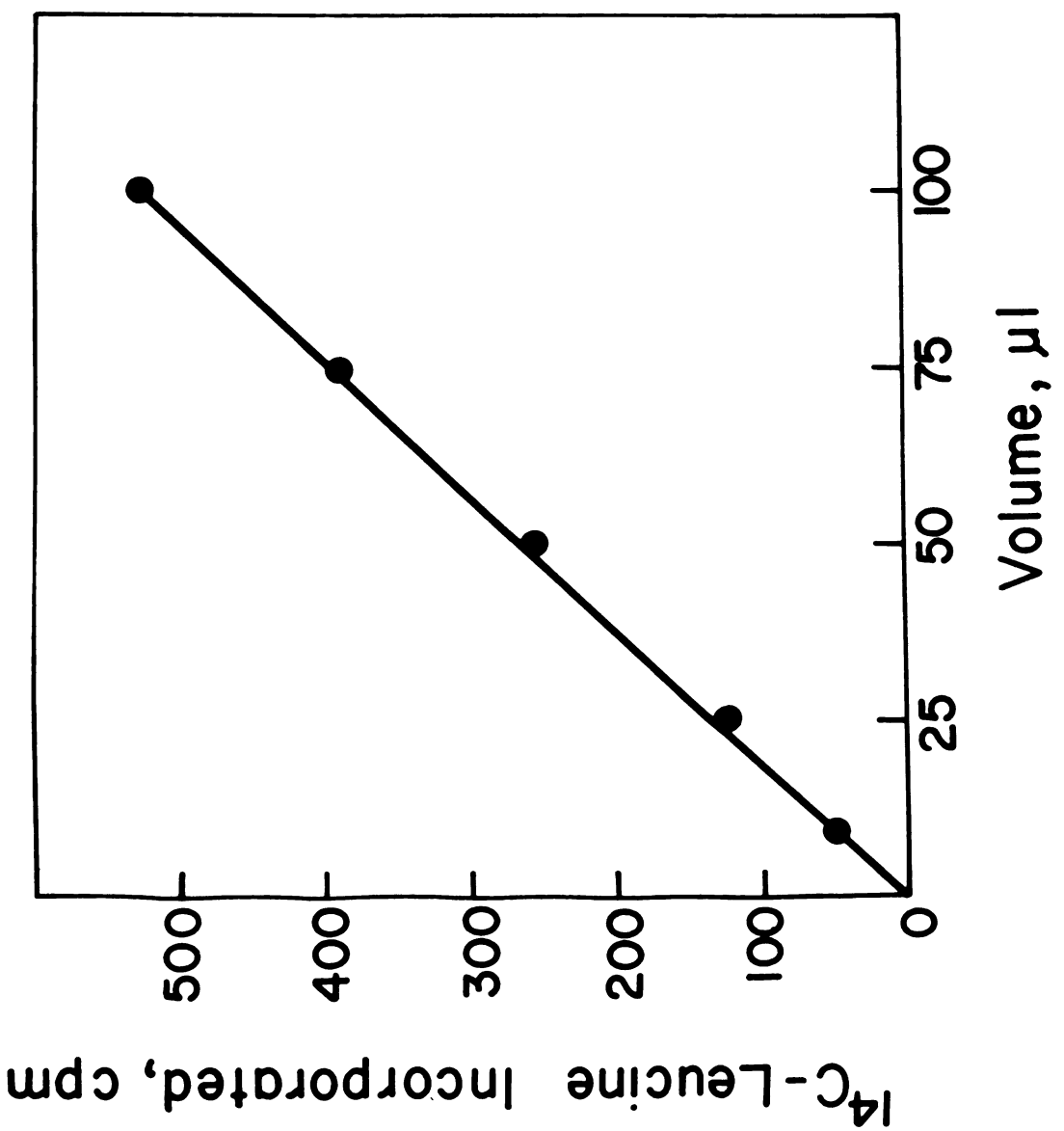
spotted on filter paper discs. The discs were processed in the usual fashion and the radioactivity incorporated on each disc was measured. The amount of radioactivity incorporated into protein compared to the aliquot size is shown in Figure 5. The line is linear and passes through the origin indicating that the results of the cell-free assay are independent of the aliquot processed in the paper disc counting procedure.

Therefore the paper disc method of Manns and Novelli was found to be as efficient as the conventional method of Casjens and Morris (1965). It appears to be independent of the aliquot size processed and is more convenient for the present studies by allowing the processing of more samples and by allowing a reduction of the volume of the cell-free assay to 0.1 ml.

Characterization of Cytidylic Acid and Its Acetylated Products.

Cytidylic acid was acetylated both as the 2' and 3' isomeric mixture and exclusively as the 3' isomer. Efforts were made to obtain the specific acetylated products by resolution of the isomeric mixture after acetylation. However, the lability of the acetyl group with continuous handling rendered this approach unfeasible.

Figure 5. Determination of the relationship of aliquot volume spotted on the filter paper disc to the radioactive leucine incorporated into protein.


^{14}C-Leucine Incorporated, cpm
0
100
200
300
400
500
25
50
75
100
Volume, µl

The isomeric purity of the 3' preparation obtained commercially was analyzed by adding a small amount of ^{14}C-2', 3' cytidylic acid to the preparation and by passage of the mixture through a Dowex 1 x 2 (formate) column (2.2 cm x 18 cm). The Dowex column was washed with 0.01 N formic acid and 240 drop aliquots were collected. One ml of every other sample was counted in Bray's solution, and the absorbance at 278 mu was determined with a Gilford spectrophotometer. Both the absorbance and radioactivity profiles are shown in Figure 6. The position of the elution of the 2' and the 3' isomers of cytidylic acid are indicated by the two peaks of radioactivity. The second peak of radioactivity also corresponds to the absorbance peak of the 2' isomer indicating that there is little, if any, contamination of the 3' isomeric preparation with 2' cytidylic acid.

The extent of acetylation of preparations of cytidylic acid was analyzed by paper chromatographic and spectrophotometric techniques. The degree of acetylation can be readily demonstrated by chromatography on Whatman #1 paper and development with solvent system II. The greater the degree of acetylation, the greater the increase in the Rf value. Typical Rf values would be: cytidylic acid 0.36 and triacetyl cytidylic acid 0.62. The mono

Figure 6. Dowex 1 x 2 (formate) column chromatography of a commercial preparation of cytidylic acid (3' isomer) to which has been added ^{14}C-cytidylic acid (2' and 3' isomers). The solid line corresponds to the absorbance of the commercial preparation and the dashed line corresponds to the radioactive profile of the labelled isomers.

Absorbance, 278 mμ
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
10
20
30
40
50
60
70
80
90
100
110
Fraction Number
100
200
300
14C - Cytidylic Acid, cpm

and diacetyl derivatives have Rf values greater than 0.36 but less than 0.62. The ultraviolet spectrum of triacetyl cytidylic acid showed a λ_{max} at 297 and 247 mu. These λ_{max} are identical to the λ_{max} demonstrated by Lohrmann and Khorana (1964) with triacetyl cytidylic acid. Only preparations of triacetylcytidylic acid demonstrated to be completely acetylated were used in the coupling reactions.

Demonstration of the Efficiency of the Deacetylation Reaction.

Not only is it necessary that the cytidylic acid be completely blocked prior to the coupling reaction, but it is extremely important that all of the blocking groups can be quantitatively removed after the coupling reaction. The effectiveness of the deacylation reaction was followed using the methods outlined above. A sample of triacetylated cytidylic acid (8.7 mg) was rendered anhydrous and treated with 5 ml of NH_3: CH_3OH solution for 18 hours. The solvent was removed, the residue dissolved in water, radioactive cytidylic acid added, and the solution placed over a Dowex 1 x 2 (formate) column. The radioactivity and the absorbance were monitored and are presented in Figure 7. Aliquots from each peak were subjected to paper chromatography along with standards of cytidylic acid and were found to have

Figure 7. Dowex 1 x 2 (formate) column chromatography of a sample of deacetylated cytidylic acid (2' and 3' isomers). N^4, O^5, $O^{2'}$ or 3'triacetylcytidylic was treated with 5 ml of ammonia saturated methanol solution for 18 hours. The solution was evaporated, dissolved in water, and an aliquot of ^{14}C- cytidylic acid was added. The sample was placed on the column and eluted with 0.01 N formic acid. The solid line corresponds to the absorbance of the deacetylated cytidylic acid and the dashed line corresponds to the labelled isomers.

Absorbance, 278 mμ
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
10
20
30
40
50
60
70
80
90
100
110
120
Fraction Number
200
400
600
800
1000
^{14}C-Cytidylic Acid, cpm

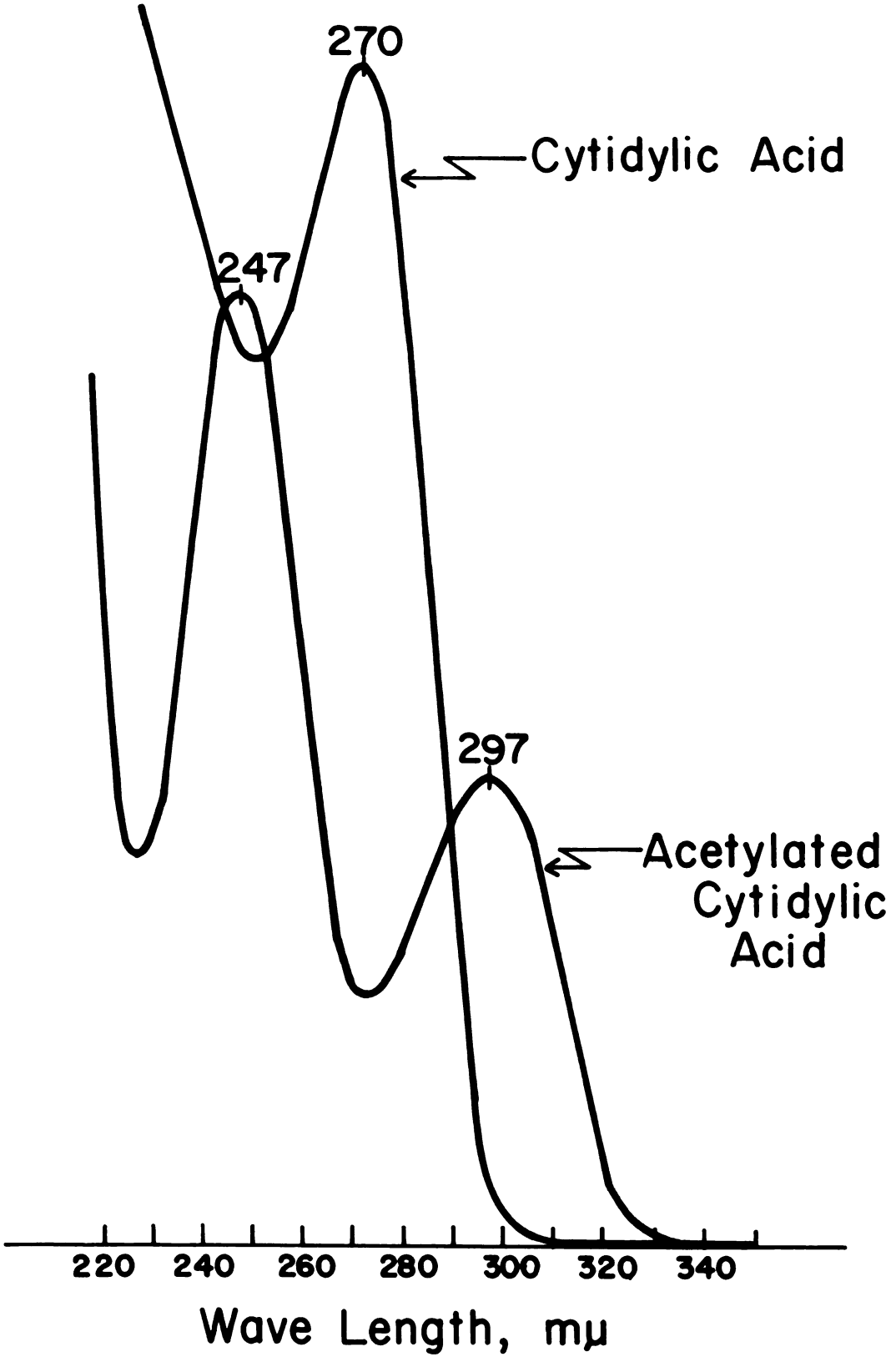
identical Rf values. This result and the close correlation of the absorbance and radioactivity peaks demonstrate that the acetyl groups were completely removed by the ammonia: methanol treatment.

The deacetylation reaction with ammonia saturated methanol can also be evaluated spectrophotometrically as presented in Figure 8. The acetylated cytidylic acid has a λ_{max} of 247 mμ and at 296 mμ, whereas, the deacetylated product which has been previously treated with 5 ml ammonia saturated methanol for 18 hours has a λ_{max} of 270 mμ similar to that of cytidylic acid in water.

Determination of the Extent of Sulfonylation Using Triisoprophylbenezenesulfonyl Chloride.

Arylsulfonyl chlorides are considered to be very satisfactory agents for the synthesis of interribonucleotide bonds (Narang, S.A., and H. G. Khorana, 1965). One drawback to their extensive use in the synthesis of nucleotidyl bonds is the possibility of sulfonylation occurring at the primary hydroxyl group of the nucleoside and to a lesser extent at the secondary hydroxyls (Jacob and Khorana, 1964). This problem has been minimized by the preparation of more sterically hindered arylsulfonyls chlorides such as triisopropylbenzenesulfonyl chloride (Lohrmann and Khorana, 1966).

Figure 8. Comparison of the spectra of N^4, $O^{5'}$, $O^{2'}$ or 3' triacetylcytidylic acid before and after treatment with ammonia saturated methanol to remove the acetyl groups.


270
Cytidylic Acid
247
297
Acetylated
Cytidylic
Acid
220
240
260
280
300
320
340
Wave Length, mμ

The extent of sulfonylation occurring with puromycin and puromycin aminonucleoside using triisopropylbenzenesulfonyl chloride (TPS) was determined by the methods of Lohrmann and Khorana (1966). Two separate anhydrous reaction mixtures were prepared. One contained puromycin (0.05 mmole) and TPS (0.1 mmole) and the other contained puromycin aminonucleoside (0.05 mmole) and TPS (0.1 mmole) in pyridine. The reactions were incubated for 8 hours then terminated by the addition of 0.5 ml water and allowed to stand at room temperature for 12 hours. Aliquots of the reaction mixture were treated with equal quantities of water and applied to Whatman #1 paper which had been twice washed with a mixture of dimethylsulfoxide and benzene (1:4 v/v). The chromatogram was eluted with carbon tetrachloride, and the two spots corresponding to the sulfonylated products and the parent compounds (identified by standard compounds) were eluted and the absorbances were compared. The extent of sulfonylation of puromycin was 25.5%, whereas, puromycin aminonucleoside was sulfonylated to the extent of 21.6%. However, it was later demonstrated that if the reaction mixtures were subjected to alkaline treatment prior to paper chromatography, which would be a routine procedure during a normal synthesis, the yield of sulfonylated

product was minimal. Therefore, sulfonylation of puromycin by TPS is most likely not of major concern as far as alteration of the final reaction products are concerned, but the sulfonylation reaction may present a competing reaction during the coupling reaction which would, in effect, reduce the efficiency of the coupling reaction.

Characterization of Cytidylyl Puromycin:

The preparations of cytidylyl puromycin obtained from Dowex column chromatography and subsequent paper chromatography of Whatman #3MM were characterized by digestion with pancreatic ribonuclease and with base hydrolysis. Base hydrolysis of a dinucleoside monophosphate is not specific for either the 2' - 5' or the 3' - 5' isomer as both are hydrolyzed equally well. Pancreatic ribonuclease is specific for the 3' - 5' isomer (Brown and Todd, 1955) leaving the 2' - 5' isomer intact. A comparison of the cleavage products with standards via paper chromatography can then identify the composition, as well as, the isomeric makeup of the dinucleoside monophosphate.

Paper chromatography on Whatman #1 with development by solvent system II of the products resulting from the hydrolysis of aliquots of cytidylyl puromycin prepared

with the isomeric mixture of acetylated cytidylic acid is illustrated in Figure 9. Undigested cytidylyl puromycin moved as a single spot between the standards of puromycin and cytidylic acid. The alkali digested sample had two spots that corresponded to the puromycin and cytidylic acid standards. The ribonuclease digested sample had these same spots plus another spot that had an R_f identical to that of the untreated cytidylyl puromycin. The data suggests that this particular preparation of dinucleoside monophosphate was composed of a mixture of both the 2' - 5' and 3' - 5' isomers of cytidylyl puromycin. Chromatography of the ribonuclease and alkali treated digests on DEAE paper with development by solvent system I produces a chromatogram which is almost identical to the one described above. When cytidylyl puromycin was prepared using exclusively 3' acetylated cytidylic acid, the cleavage results were similar except that the ribonuclease treated sample lacked the spot having the R_f identical to the parent compound. This indicates that the cytidylyl puromycin preparation was composed exclusively of the 3' - 5' isomer.

Additional data related to the composition of cytidylyl puromycin was obtained by spectral and electrophoretic methods. In Figure 10 are presented the spectra

Figure 9. Whatman #1 paper chromatography of the hydrolysis products obtained by alkaline and pancreatic ribonuclease digestions of cytidylyl puromycin. Chromatogram was developed with solvent system II.

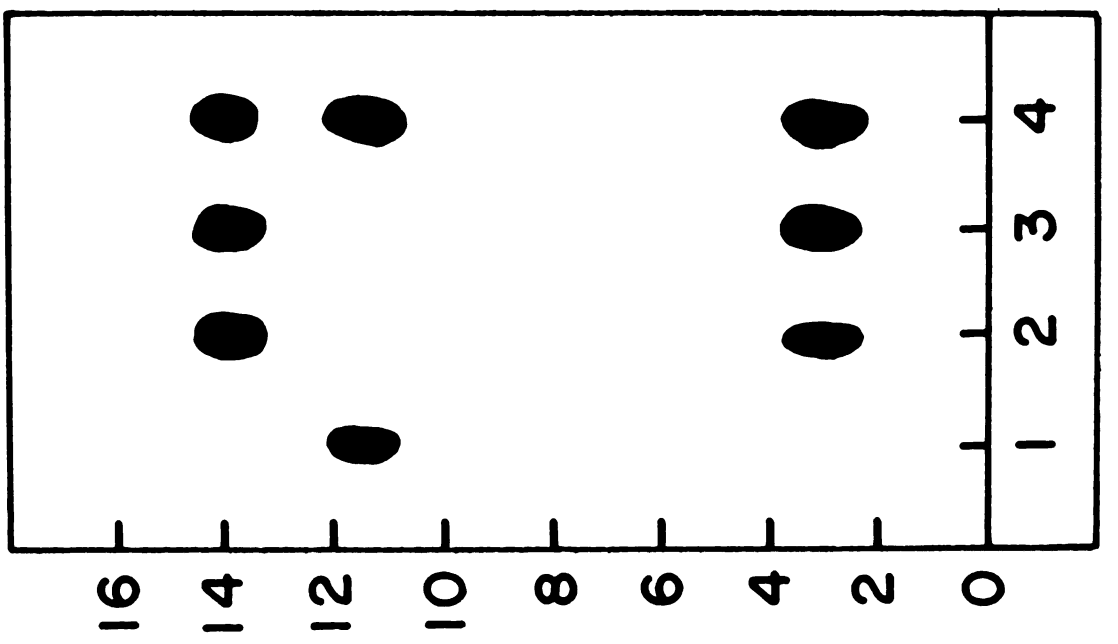
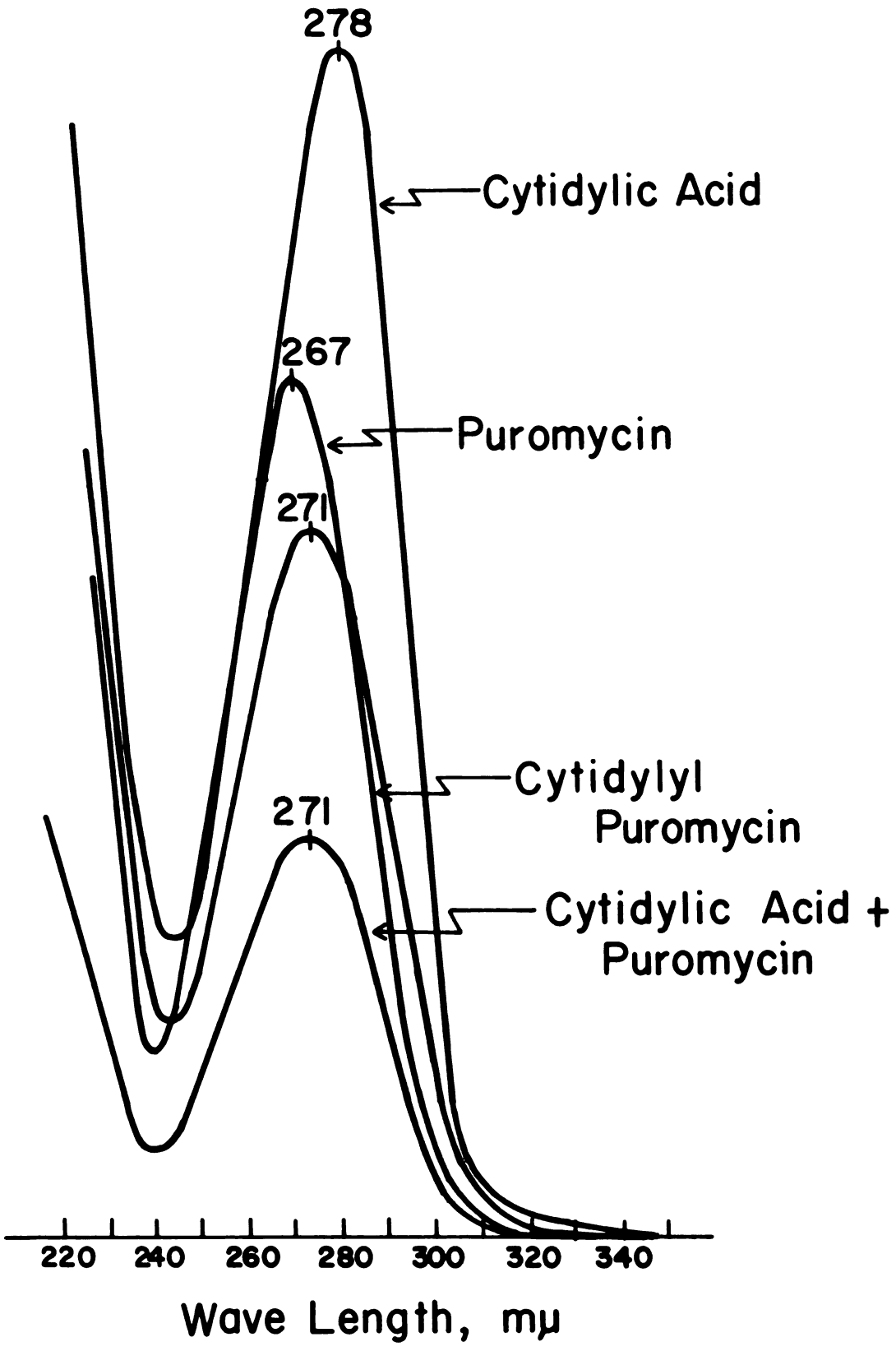

CODE
1. Pooled peak
2. Puromycin and cytidylic acid stds
3. KOH digest of peak
4. RNase digest of peak
16
14
12
10
8
6
4
2
0
1
2
3
4

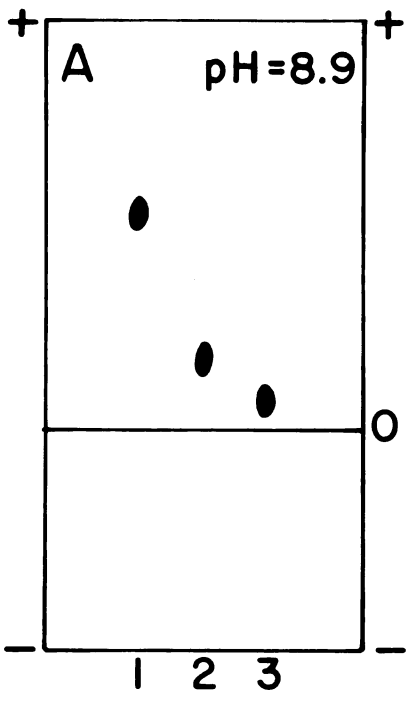
Figure 10. Comparison of the spectra of standard solutions of cytidylic acid and puromycin to cytidylyl puromycin and to a equimolar mixture of cytidylic acid and puromycin. All spectra were determined in 0.1 N HCl.

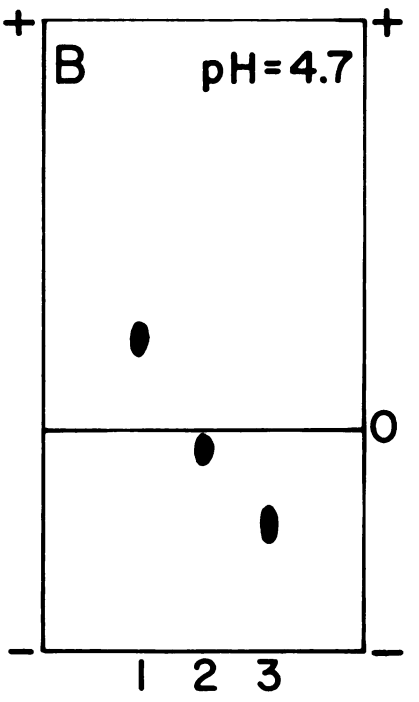

278
Cytidylic Acid
267
Puromycin
271
Cytidylyl
Puromycin
271
Cytidylic Acid +
Puromycin
220
240
260
280
300
320
340
Wave Length, mμ

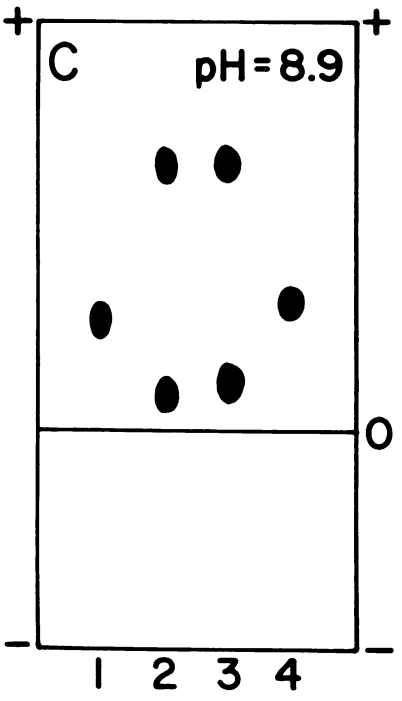
of standards of puromycin and cytidylic acid, an equimolar mixture of the two compounds, and cytidylyl puromycin. Puromycin has a λ_{max} of 267 mμ, cytidylic acid has a λ_{max} of 278 mμ, and cytidylyl puromycin has a λ_{max} equal to 271 mμ. The agreement of the λ_{max} of cytidylyl puromycin with that of the equimolar mixture of the two compounds strongly supports the composition data obtained with the digestions as any modification of the chromophore will most likely cause a shift in the λ_{max}. Paper electrophoresis of cytidylyl puromycin, mixed isomers, at pH 4.8 and pH 8.9 as shown in Figure 11 (A and B) caused the compound to migrate as a single entity. The 3' - 5' preparation of cytidylyl puromycin was also found to migrate as a single spot (see C and D of Figure 11). These data demonstrate the homogenicity of the various preparations of cytidylyl puromycin. No difference in electrophoretic mobilities could be demonstrated for the 2' - 5' and 3' - 5' isomers of cytidylyl puromycin.

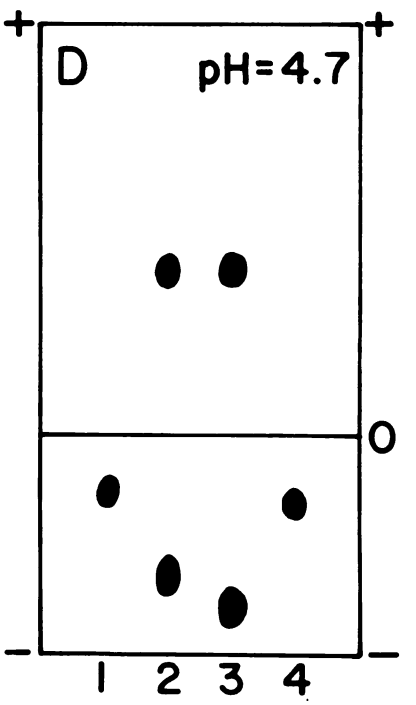
The intactness of the α amino group of cytidylyl puromycin was determined by reacting the compound with ninhydrin. Puromycin is ninhydrin positive. However, addition reactions involving the α amino group (Hengesh, 1969) render the compound ninhydrin negative. Both the

Figure 11. Comparison of the electrophoretic mobilities at pH 8.9 and pH 4.8. The code for part A and B is: (1) cytidylic acid standard, (2) cytidylyl puromycin, and (3) puromycin standard. The code for parts C and D is: (1) cytidylyl puromycin, (2) cytidylic acid plus puromycin standards, (3) cytidylic acid plus puromycin aminonucleoside standards, and (4) cytidylyl puromycin aminonucleoside.


A
pH=8.9
+
+
0
−
−
1 2 3


B
pH=4.7
+
+
0
−
−
1 2 3


C
pH=8.9
+
+
0
−
−
1 2 3 4


D
pH=4.7
+
+
0
−
−
1 2 3 4

preparation of cytidylyl puromycin, mixed isomers, and the preparation of cytidylyl puromycin, 3' - 5' isomer, were found to be ninhydrin positive suggesting that the α amino group was not modified.

Determination of the Isomeric Ratio of Cytidylyl Puromycin (Mixed isomers).

An aliquot of the mixed isomers of cytidylyl puromycin was subjected to exhaustive digestion with pancreatic ribonuclease for 24 hours at 37°. The digested preparation was then applied to pre-washed Whatman #3MM paper and developed with solvent system II. Areas of the chromatogram containing the absorbance due to the presence of puromycin, cytidylic acid, and 2' - 5' cytidylyl puromycin (not hydrolyzed by ribonuclease) were cut out and eluted with water. The absorbance recovered with the elution of each of the three areas of the paper (cytidylic acid 0.575 O.D./ml, puromycin 0.830 O.D./ml, and 2' - 5' cytidylyl puromycin 1.63 O.D./ml) was compared to the total absorbance recovered. The results indicate that 65% of the isomeric mixture was not susceptible to pancreatic ribonuclease, and, therefore, it was concluded that the isomeric mixture consisted of 65% 2' - 5' cytidylyl puromycin. The recovery yields in moles of puromycin and cytidylic acid were calculated and found to be 4.3×10^{-8} moles of puromycin and 6.4×10^{-8} moles

of cytidylic acid. Therefore, puromycin and cytidylic acid are present in approximately a 1:1 ratio which indicates that cytidylyl puromycin is present as the dinucleoside monophosphate.

The spectrum of each of the three products, cytidylic acid, puromycin, and 2' - 5' cytidylyl puromycin, obtained by the ribonuclease digestion were compared with those of the standard compounds as seen in Figures 12 and 13. The similarities in the spectra lend credence to the structural characterization of cytidylyl puromycin.

Characterization of Cytidylyl Puromycin Aminonucleoside.

The characterization of cytidylyl puromycin aminonucleoside was performed in a similar manner to that presented above for cytidylyl puromycin. Pancreatic ribonuclease and alkaline digestions were chromatographed on Whatman #1 paper and developed in solvent system II. The paper chromatographic data is presented in Figure 14. Cytidylyl puromycin aminonucleoside moved intermediate between standards of cytidylic acid and puromycin aminonucleoside. Cytidylyl puromycin aminonucleoside was found to move as a single entity when electrophoresed at pH 4.8 and pH 8.9. See Figure 11 (C and D). The treatment with

Figure 12. Comparison of the spectra of standard solutions of cytidylic acid and puromycin to the spectra of puromycin and cytidylic acid recovered from pancreatic ribonuclease digestion of cytidylyl puromycin. Digestion was done as described in Methods. The digestion products were subjected to paper chromatography on Whatman #1, eluted with water, evaporated, and then dissolved in 0.1 N HCl. All spectra were determined in 0.1 N HCl.

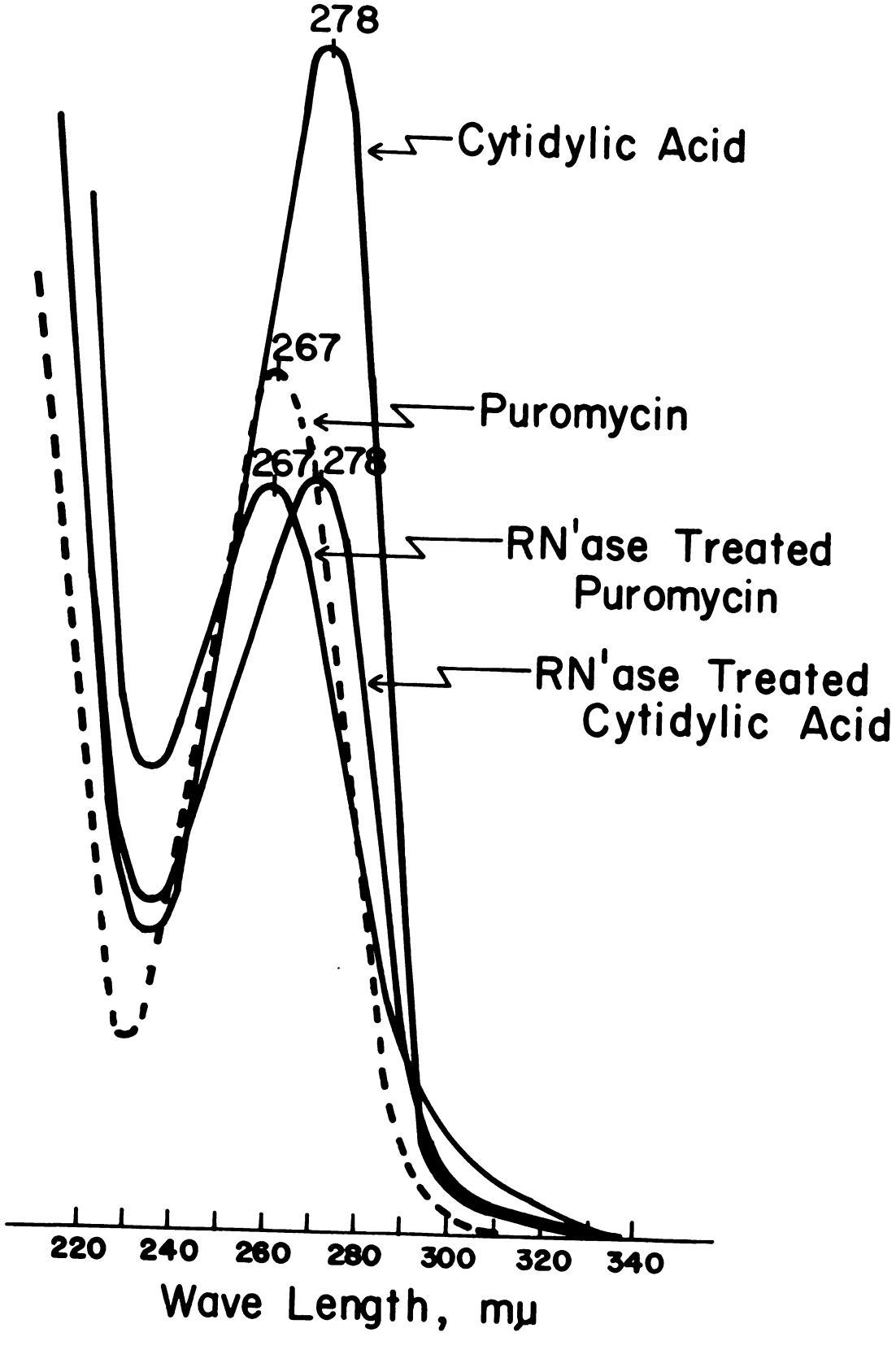

278
Cytidylic Acid
267
Puromycin
267
278
RN'ase Treated
Puromycin
RN'ase Treated
Cytidylic Acid
220
240
260
280
300
320
340
Wave Length, mμ

Figure 13. Comparison of the spectrum of 2' - 5' cytidylyl puromycin obtained by pancreatic ribonuclease digestion of cytidylyl puromycin as described in Figure 11 to the spectrum of cytidylyl puromycin (mixed isomers).

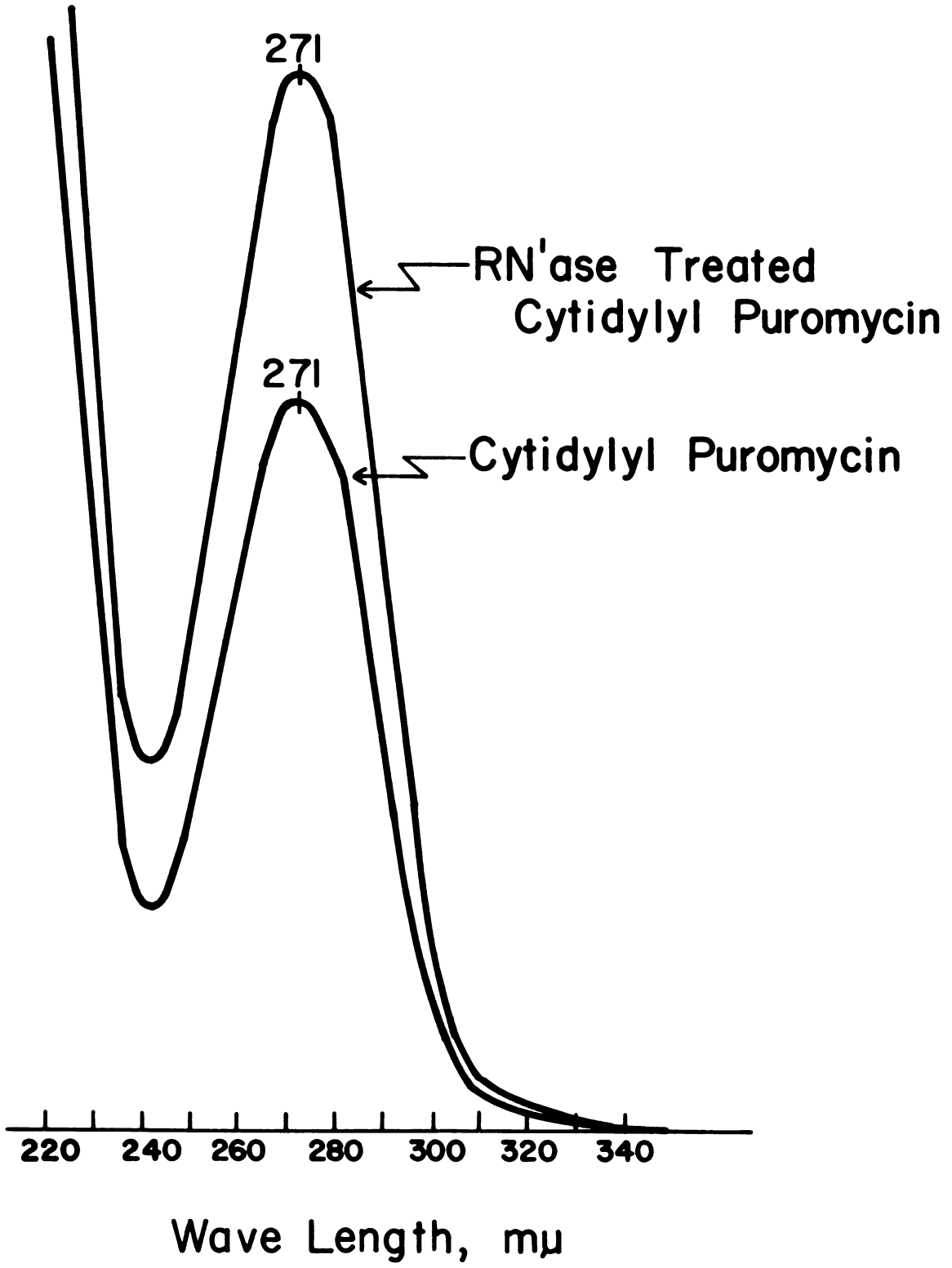
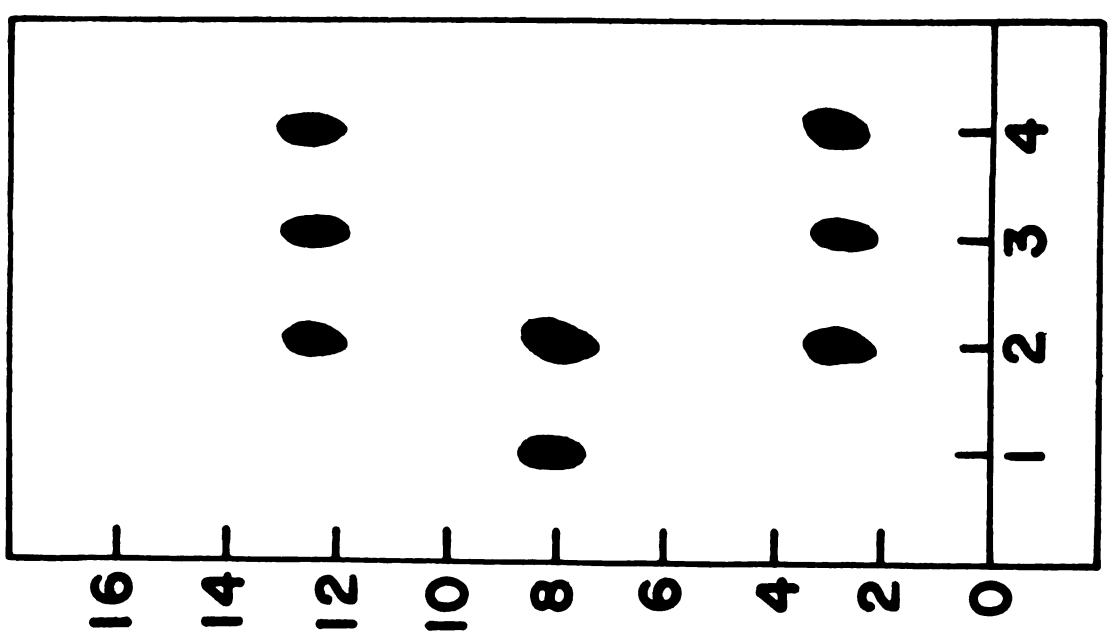

271
RN'ase Treated
Cytidylyl Puromycin
271
Cytidylyl Puromycin
220
240
260
280
300
320
340
Wave Length, mμ

Figure 14. Whatman #1 paper chromatography of the hydrolysis products obtained by alkaline digestion and pancreatic digestion of cytidylyl puromycin aminonucleoside. The chromatogram was developed with solvent system II.


CODE
1. Pooled peak
2. RN'ase digest of peak
3. KOH digest of peak
4. CP+ PAN stds
16
14
12
10
8
6
4
2
0
1
2
3
4

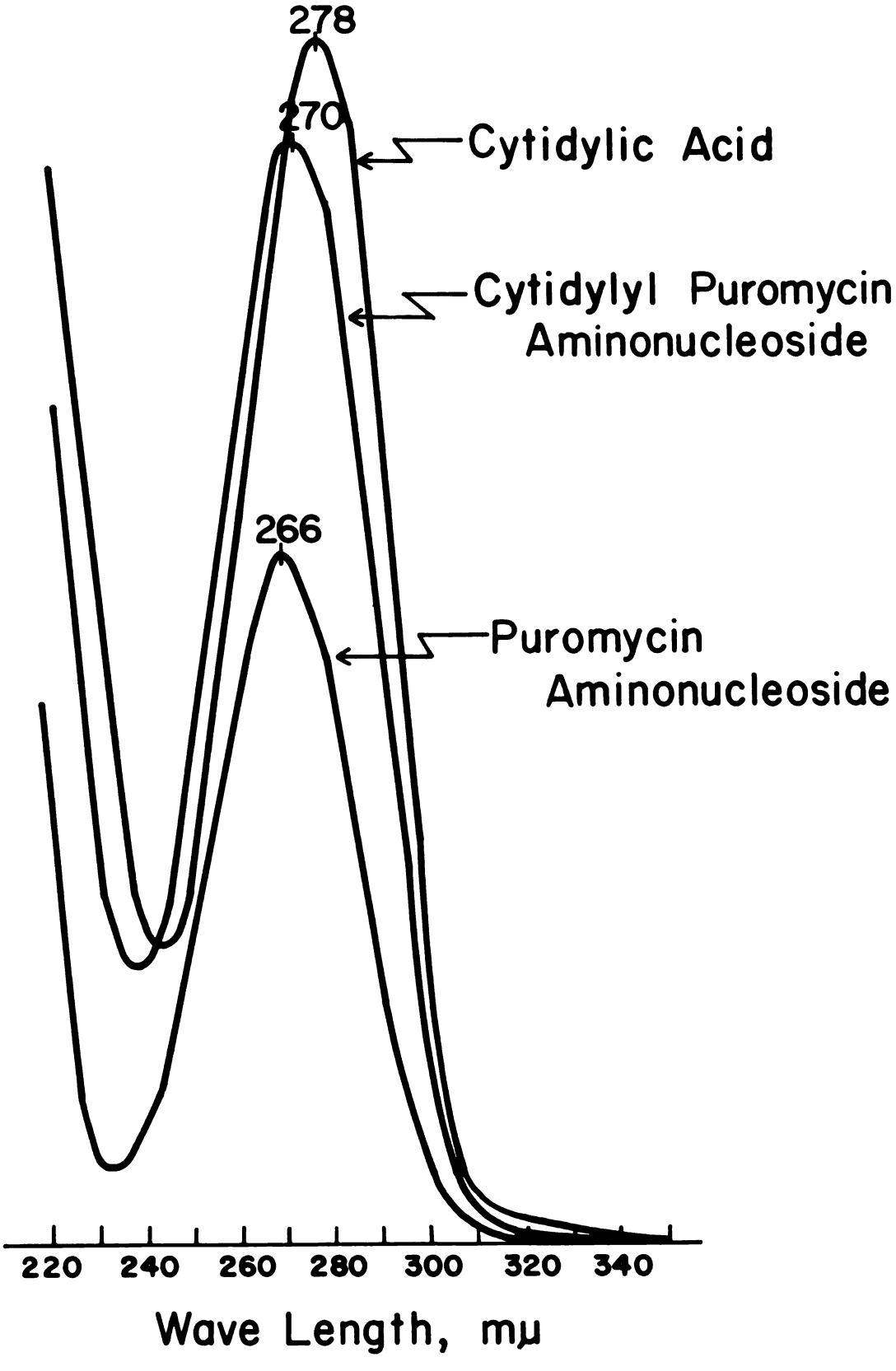
pancreatic ribonuclease resulted in the formation of three spots two of which correspond to the standards of cytidylic acid and puromycin aminonucleoside, whereas, the third corresponds to cytidylyl puromycin aminonucleoside. Since alkaline digestion produced two spots having R_f values corresponding to the standards, the preparation was deemed to be an isomeric mixture of 2' - 5' and 3' - 5' cytidylyl puromycin aminonucleoside.

The spectra of cytidylyl puromycin aminonucleoside and its component parts were obtained and are presented in Figure 15. Puromycin aminonucleoside had a λ_{max} of 266 mμ, cytidylic acid had a λ_{max} of 278, and cytidylyl puromycin aminonucleoside had a λ_{max} of 270 mμ.

Inhibitory Properties of Puromycin Analogues in the Rabbit Reticulocyte Cell-Free System.

Various concentration of puromycin analogues were tested for their ability to inhibit protein synthesis in a rabbit reticulocyte cell-free system. An aliquot from each reaction mixture was analyzed for TCA precipitable radioactivity using the paper disc assay of Manns and Novelli (1960). All assays were done at least in duplicate, and the results presented on each graph were obtained on the same day. The data is presented as percent inhibition where 100% inhibition would be the lack of

Figure 15. Comparison of the spectra of solutions of standards of cytidylic acid and puromycin to the spectrum of cytidylyl puromycin aminonucleoside. All spectra were determined in 0.1 N HCl.


278
270
Cytidylic Acid
Cytidylyl Puromycin
Aminonucleoside
266
Puromycin
Aminonucleoside
220
240
260
280
300
320
340
Wave Length, mμ

incorporation. Zero percent inhibition corresponded to between 2000 and 3000 cpm. Inhibitor concentrations are plotted in terms of μmolar with the range encompassing from 10^{-7} to 10^{-3} molar.

A summary of the results follows with a more detailed discussion of the results found in the discussion section. Figures 16 and 17 compare the inhibitory properties of 2' - 5', 3' - 5' cytidylyl puromycin (mixed isomers) to those of puromycin. In both cases cytidylyl puromycin is seen to be twice as inhibitory as puromycin at a concentration of 1.0 μmolar. The second graph has an additional line which represents the inhibition demonstrated by a equimolar mixture of cytidylic acid and puromycin. The similarity between the inhibition demonstrated by the mixture and that seen with puromycin indicates that the presence of free cytidylic acid has no synergistic effect. Figure 18 extends the comparison to include an investigation of the inhibitory properties of cytidylyl puromycin aminonucleoside, a compound which appears to have very little inhibitory activity.

Figures 19, 20, and 21 show data obtained in a study of the relationship of the inhibitory properties of the individual isomers of cytidylyl puromycin to the inhibition produced by a mixture composed of both isomers

Figure 16. Comparison of the inhibition produced by cytidylyl puromycin (2' - 5' and 3' - 5' mixed isomers) to the inhibition produced by puromycin.

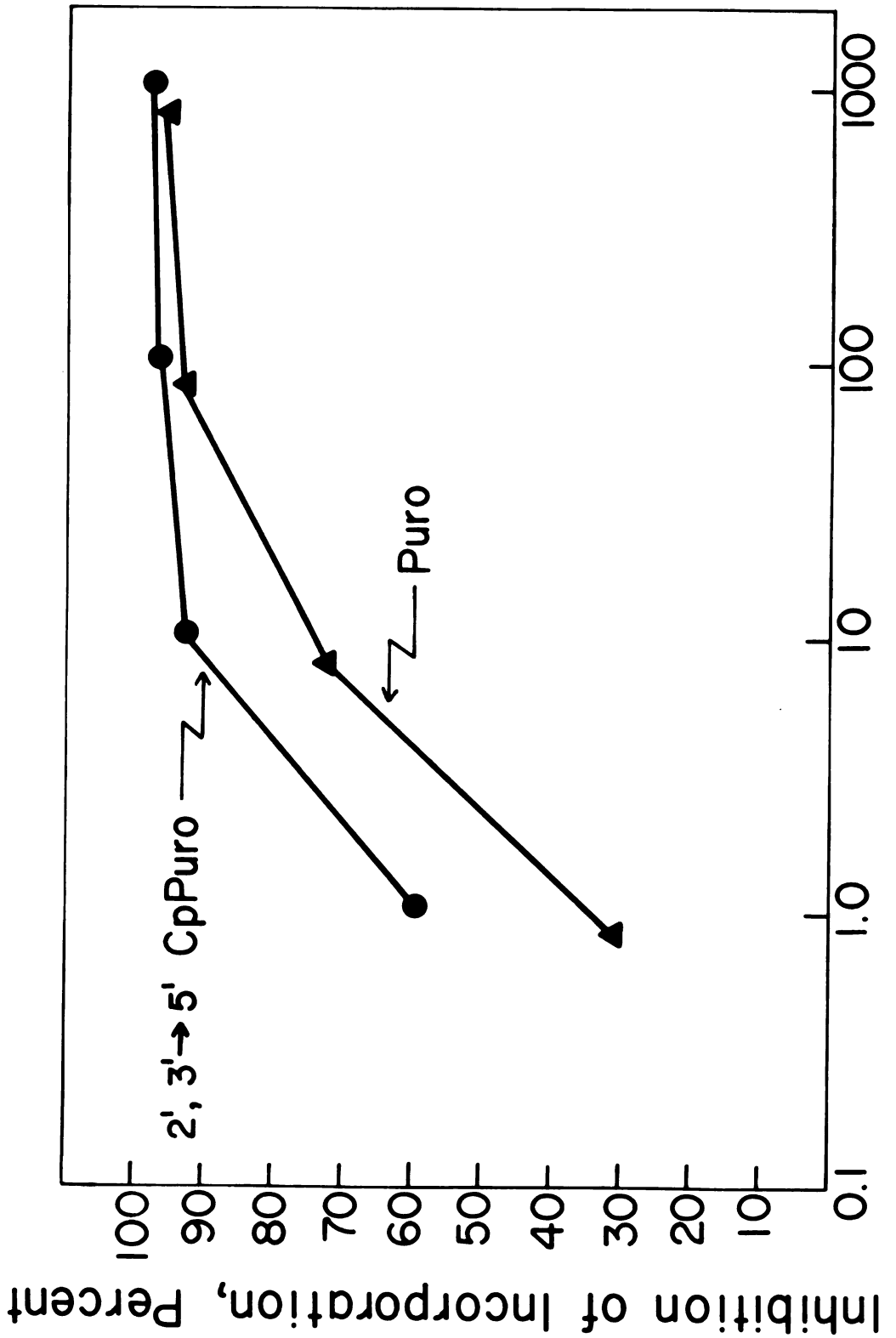

Inhibition of Incorporation, Percent
100
90
80
70
60
50
40
30
20
10
0.1
1.0
10
100
1000
Concentration, μM
2', 3'→5' CpPuro
Puro

Figure 17. Comparison of the inhibition produced by cytidylyl puromycin (2' - 5' and 3' - 5' mixed isomers) and a equimolar mixture of cytidylic acid and puromycin to the inhibition produced by puromycin.

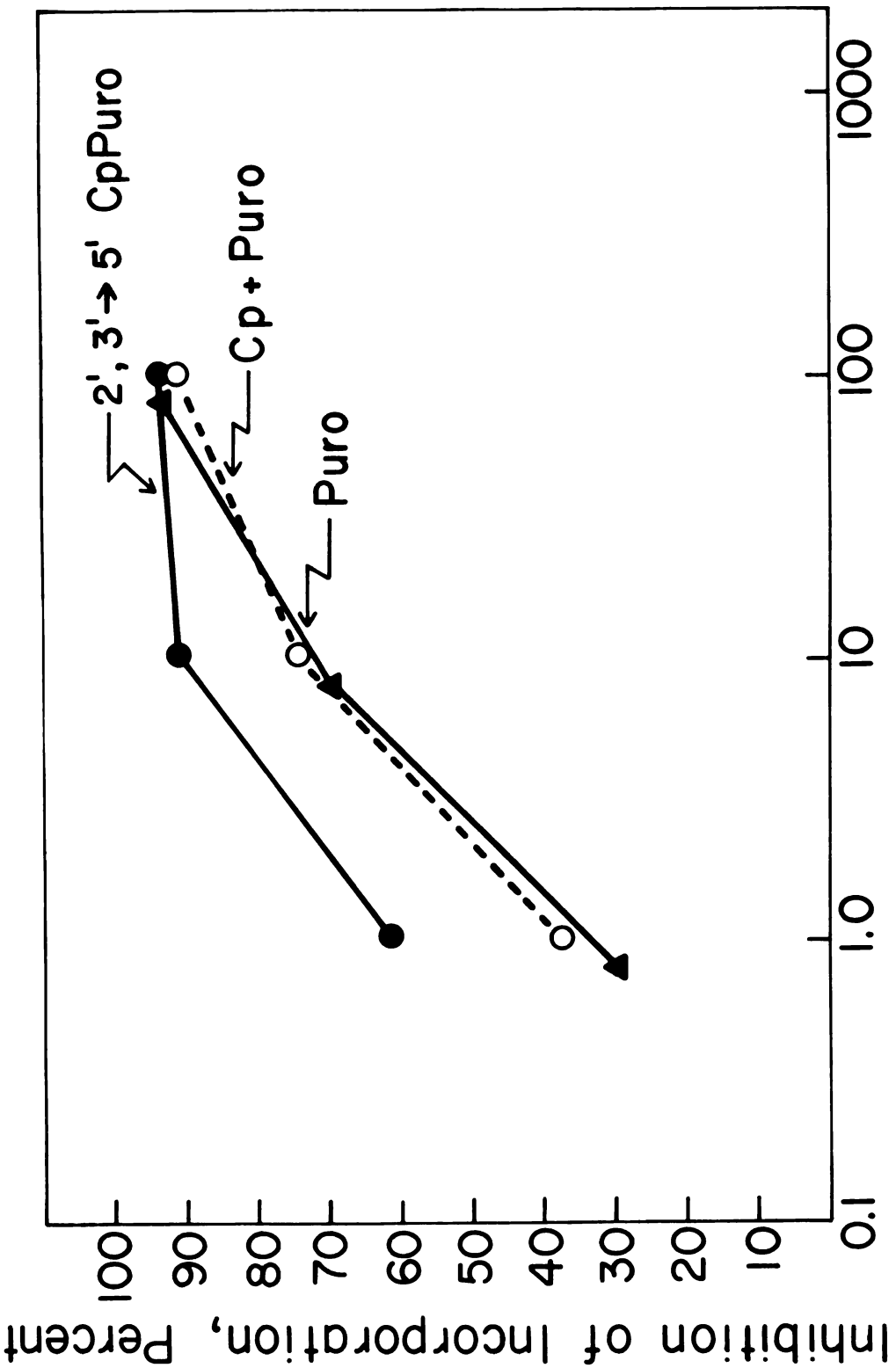

Inhibition of Incorporation, Percent
100
90
80
70
60
50
40
30
20
10
0.1
1.0
10
100
1000
Concentration, μM
2', 3'→ 5' CpPuro
Cp + Puro
Puro

Figure 18. Comparison of the inhibition produced by cytidylyl puromycin aminonucleoside to the inhibition produced by puromycin.

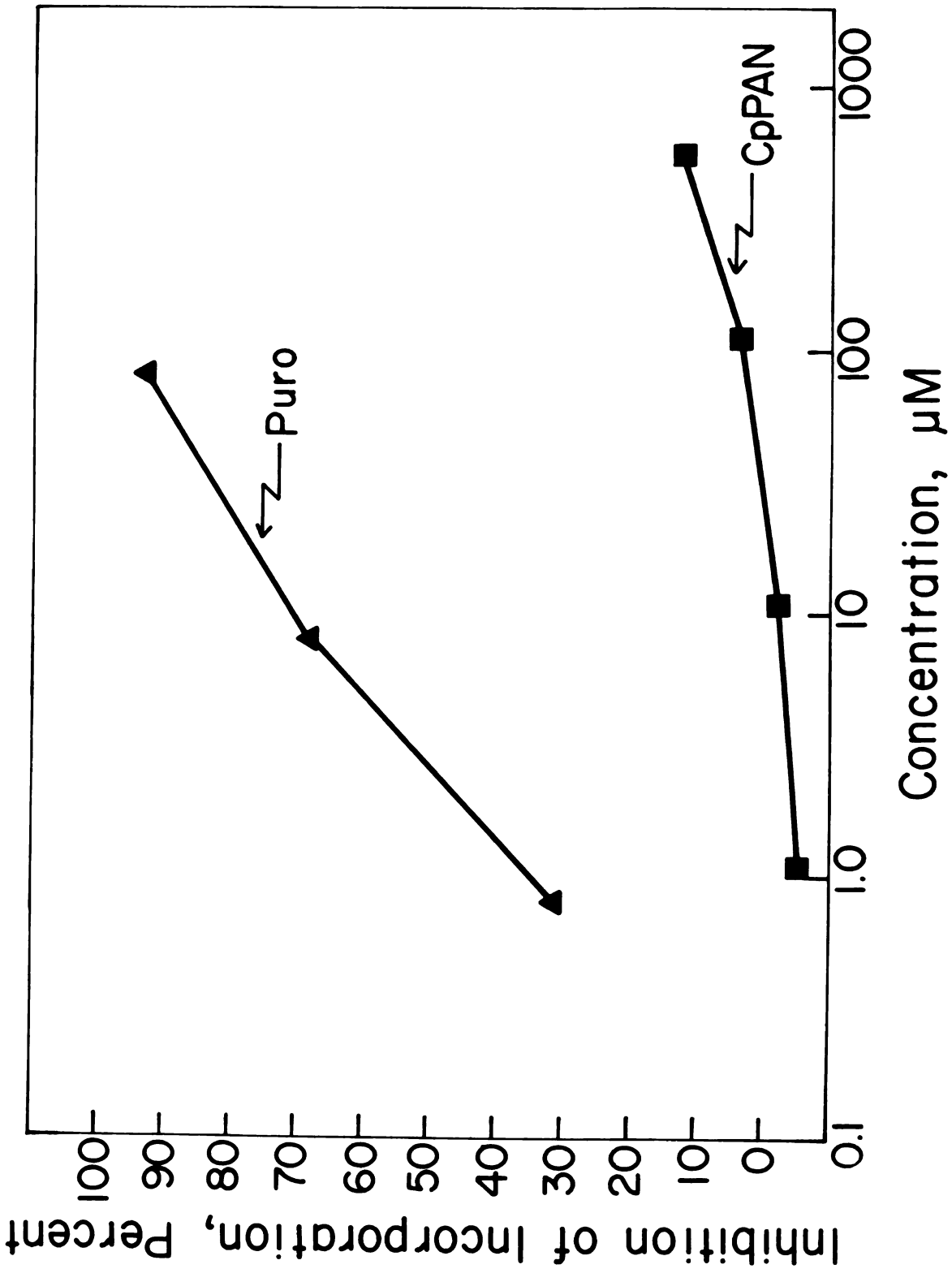

Inhibition of Incorporation, Percent
100
90
80
70
60
50
40
30
20
10
0
0.1
1.0
10
100
1000
Concentration, μM
Puro
CpPAN

Figure 19. Comparison of the inhibition produced by cytidylyl puromycin (2' - 5' and 3' - 5' mixed isomers) and cytidylyl puromycin (3' - 5' isomer) to the inhibition produced by puromycin.

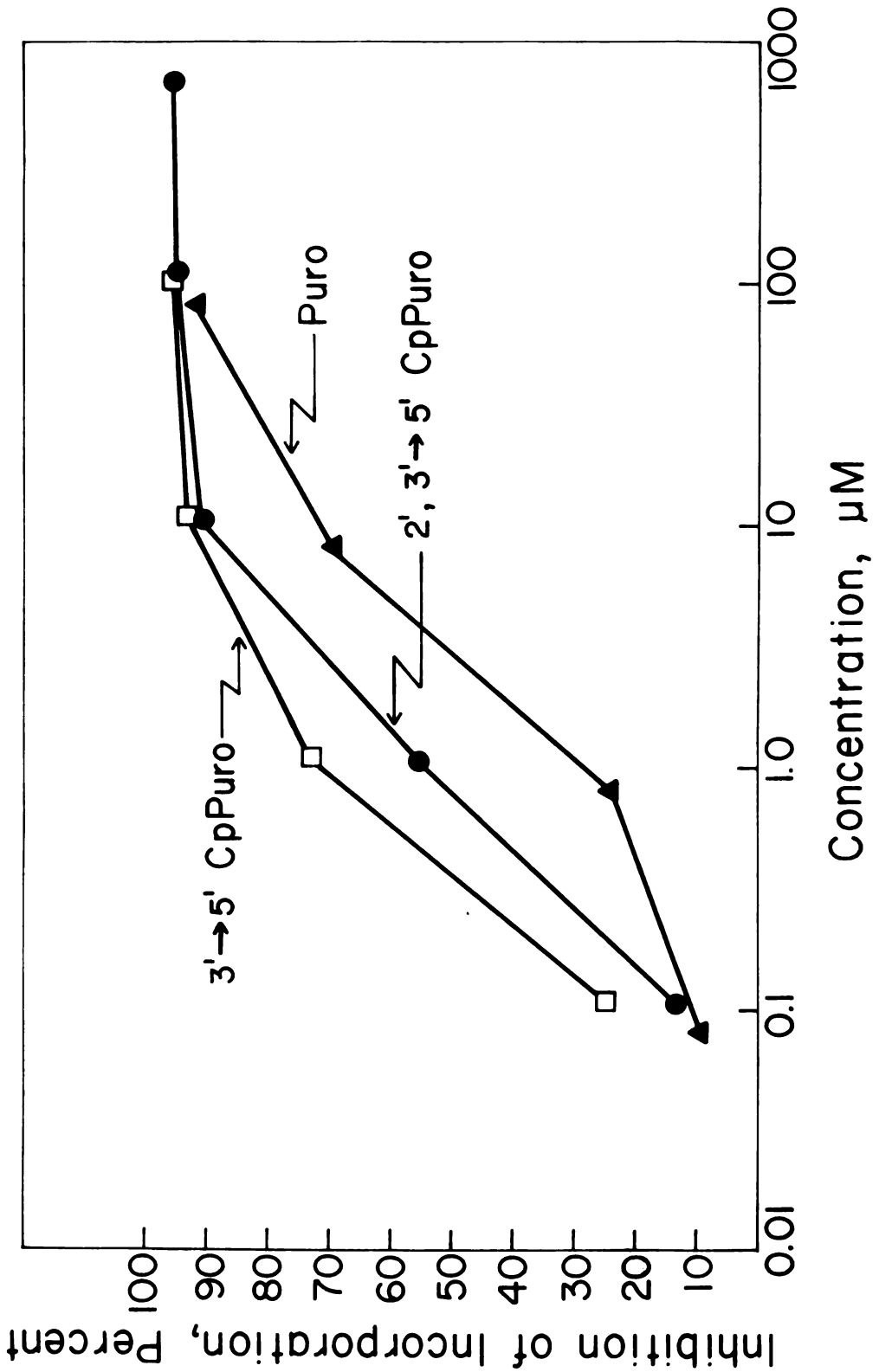

Inhibition of Incorporation, Percent
100
90
80
70
60
50
40
30
20
10
0.01
0.1
1.0
10
100
1000
Concentration, μM
3'→5' CpPuro
Puro
2', 3'→5' CpPuro

Figure 20. Comparison of the inhibition produced by cytidylyl puromycin (2' - 5' and 3' - 5' mixed isomers) to the inhibition produced by cytidylyl puromycin (3' - 5' isomer).

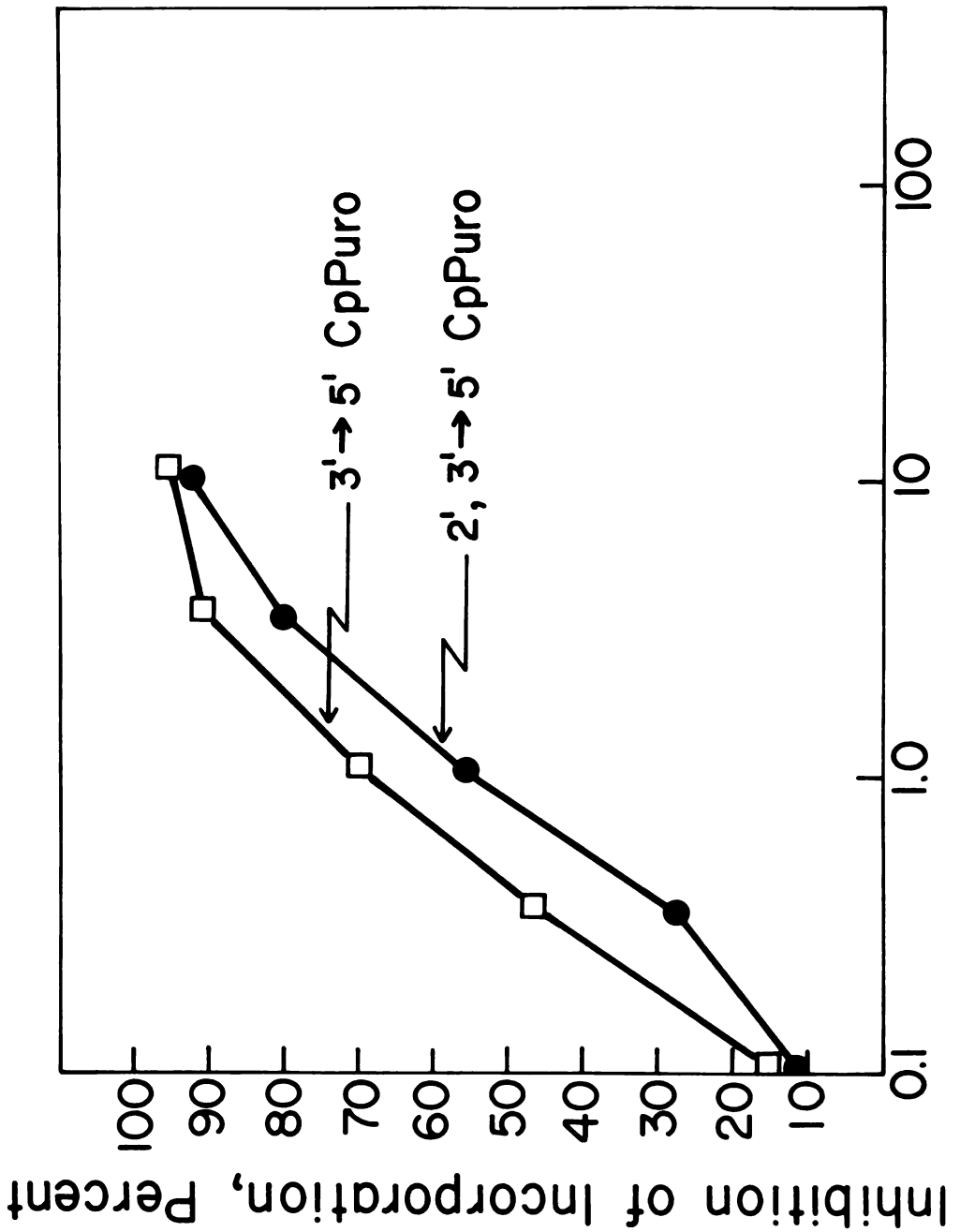
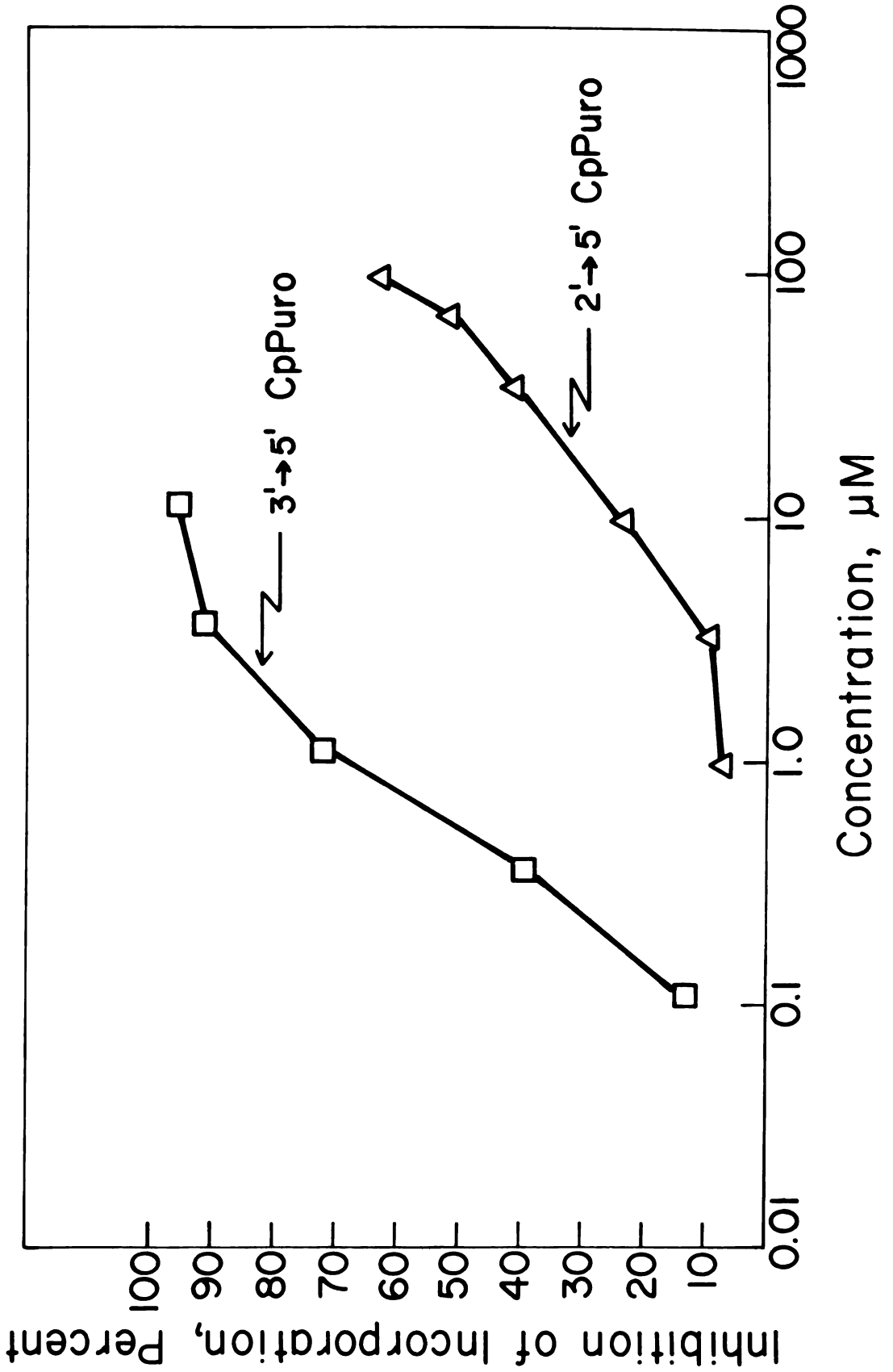

Inhibition of Incorporation, Percent
100
90
80
70
60
50
40
30
20
10
0.1
1.0
10
100
Concentration, μM
3'→5' CpPuro
2', 3'→5' CpPuro

Figure 21. Comparison of the inhibition produced by cytidylyl puromycin (2' - 5' isomer) to the inhibition produced by cytidylyl puromycin (3' - 5' isomer).


Inhibition of Incorporation, Percent
100
90
80
70
60
50
40
30
20
10
0.01
0.1
1.0
10
100
1000
Concentration, μM
3'→5' CpPuro
2'→5' CpPuro

These data are discussed more thoroughly in the Results section. However, it may be readily seen that the 2' - 5' isomer of cytidylyl puromycin has very low activity. Most of the ability to inhibit the incorporation of amino acids into protein apparently resides in the 3' - 5' isomer of cytidylyl puromycin.

Characterization of the Reaction Products of Puromycin and Cytidylyl Puromycin in the Cell-Free System.

The product of the reaction of puromycin in the cell-free system has been identified as peptidyl puromycin (Morris, et al., 1963; Nathans, 1964) which is released from the ribosome in the supernatant. By analogy, the product of the reaction of cytidylyl puromycin in the cell-free system would be expected to be peptidyl cytidylyl puromycin. To test for the release of nascent peptides into the supernatant, ribosomes were prelabeled in the cell-free system (see methods section). The prelabeled ribosomes were assayed in the standard cell-free system using cold amino acids. Duplicate assays containing puromycin, cytidylyl puromycin, or neither were incubated for 40 minutes at 37^{o}. Half of the duplicate assays were centrifuged at 78,000 x g. for 90 minutes to sediment the ribosomes. The supernatant was analyzed for the presence

of TCA precipitable counts. The other half of the assays were analyzed directly.

The results are presented in Table II where the TCA precipitable cpm present in the supernatant fraction or ribosomes plus supernatant fraction are recorded. In the absence of puromycin 52% of the TCA insoluble radioactivity are released into the supernatant probably through normal termination. When either puromycin or cytidylyl puromycin is present, the percent of release was less amounting to an average of 34% and 32% respectively. The data is consistent with that of Morris, et al. (1963) where 75% of the cpm were released in the absence of puromycin while 48% were released in the presence of the antibiotic. The ratio $\frac{\text{cpm released in presence of antibiotic}}{\text{cpm released in the absence of antibiotic}}$ was 0.64. This compares favorably with the ratios obtained in like manner from the data in Table I: 0.65 for cytidylyl puromycin and 0.68 for puromycin.

Table II. Release of ^{14}C-Labeled Peptides From Ribosomes by Puromycin and Cytidylyl Puromycin.

Release of ^{14}C-Labeled Peptides from Ribosomes by Puromycin and Cytidylyl Puromycin

Incubation Conditions	Acid Insoluble (cpm)		$\left(\frac{\text{Supernatant + Ribosomes}}{\text{Supernatant}}\right)$
Whole System Plus	Supernatant	Supernatant + Ribosomes	
Nothing	92	178	.52
Cytidylyl Puromycin (3.7×10^{-6} M)	63	173	.36
Cytidylyl Puromycin (1.1×10^{-7} M)	56	174	.32
Puromycin (8.2×10^{-5} M)	74	180	.41
Puromycin (8.2×10^{-6} M)	74	228	.32

DISCUSSION

The goal of the research presented in this thesis was to study the substrate specificity of the "A" site of peptidyl transferase by synthesizing structural analogues of the CpCpA terminus of aminoacyl tRNA which is believed to interact with peptidyl transferase. Monro, et al. (1969) and Vazquez, et al. (1969) have done similar studies demonstrating the involvement of -CpCpA in the ribosomal "P" site. The inhibitory properties of puromycin are related to its ability to substitute for aminoacyl tRNA at the "A" site of the ribosome. Testing of a large number of amino acid analogues of puromycin for their ability to inhibit protein synthesis has demonstrated that a structural requirement for an aromatic amino acid, preferably O-CH_3 tyrosine, exists (Nathans and Neidles, 1964; Symons, et al., 1969). Other studies have shown that antibiotic activity can be enhanced with cytidylyl nucleotidyl analogues (Rychlik, et al., 1967). Therefore cytidylyl puromycin, a compound that possesses both of

these structural features, and other related analogues were synthesized.

The synthesis of cytidylyl puromycin and the related analogues required that new techniques and methods be learned or developed in order to properly handle the reactions under anhydrous conditions. Efforts to block the amino group of puromycin with carbobenzoxy chloride were abandoned when it was discovered that the blocked compound was insoluble in all but the most non-polar solvents.

Three different coupling agents were investigated for their ability to form the phosphodiester bond between puromycin and cytidylic acid. The use of diphenylphosphorochloridate and dicyclohexylcarbodiimide as coupling agents has been discussed previously (Hengesh, 1969). Triisopropylbenzenesulfonyl chloride (TPS) was the coupling agent used in these series of experiments. It was found to produce higher yields with fewer side products in a shorter reaction time.

The most prevalent side reaction that occurs with TPS involves a sulfonylation at the primary hydroxyl, and perhaps the secondary hydroxyls of the nucleosidyl reactant (Jacob and Khorana, 1964). It was estimated that approximately 25% of the nucleoside was sulfonylated when treated

directly with TPS. However, the sulfonylation products appear to be unstable and disappear during the subsequent workup of the reaction mixture. Since these sulfonylation reactions were done in the absence of the nucleotidyl reactant which would also compete directly for the primary hydroxyl, it was felt that sulfonylation of the reaction products was not of major concern. Besides, the sulfonylated product had a characteristic R_f, having a mobility greater than either of the parent nucleosides, which made the presence of the product readily identifiable.

Characterization of the dinucleotide monophosphates was performed by hydrolysis back to the original starting materials using base hydrolysis or pancreatic ribonuclease digestion. Treatment with ribonuclease allowed the isomeric mix to be calculated since only the 3' - 5' isomer is susceptible to hydrolysis by that enzyme. Spectral analysis of the products of ribonuclease digestion demonstrated that the characteristic λ_{max} of the respective components is unchanged by the coupling procedure. If sulfonylation of puromycin had occurred a shift in the λ_{max} of puromycin would be expected since the triisopropylbenzene group is a strong chromophore.

The compounds were assayed for their ability to inhibit protein synthesis by using a miniturized version

of the methods of Allen and Schweet (1962). TCA precipitable radioactivity was determined by the paper disc method of Manns and Novelli (1960). This method is faster, easier, and allows a large number of assays to be processed at the same time, thereby eliminating the problem of day to day assay variation. The assay has approximately the same counting efficiency as the assay of Casjens and Morris (1965). Each assay contained slightly more than 465,000 cpm of which only 1.0% were incorporated into TCA precipitable cpm.

The product of the reaction of puromycin in the cell-free system is known to be peptidyl puromycin (Morris, et al., 1963; Nathans, 1964). Aminoacyl adenosine compounds which inhibit protein synthesis similar to puromycin have also been shown to form a peptidyl compound (Rychlik, et al., 1969; Rychlik, et al., 1970). Therefore, it is probable that cytidylyl puromycin elicits the formation of an analogous compound, peptidyl cytidylyl puromycin. The data presented is consistent with that hypothesis but is not conclusive. No effort was made to identify the presence of TCA soluble cpm which are formed under the continuous presence of puromycin in the cell-free system (Morris, et al., 1963). Nor was the release of TCA insoluble material in the absence of energy demonstrated. However, the

circumstantial evidence is consistent with the formation of peptidylyl puromycin.

The first preparations of cytidylyl puromycin were prepared using a mixture of 2' and 3' acetylated cytidylic acid and puromycin. Consequently, the product of the reaction was an isomeric mixture. Figure 16 presents a comparison of the inhibitory properties of 2' - 5', 3' - 5' mixed isomers of cytidylyl puromycin with puromycin. At a concentration of 1.0 μmolar cytidylyl puromycin is twice as inhibitory as puromycin. The same relationship is found to exist in Figure 17. Cytidylyl puromycin is again twice as inhibitory at 1.0 μmolar, and the presence of free cytidylic acid in the assay had no synergistic effect.

Another preparation of cytidylyl puromycin was prepared using exclusively 3' acetylated cytidylic acid. The compound was characterized as the 3' - 5' isomer of cytidylyl puromycin on the basis of its complete susceptibility to pancreatic ribonuclease. A comparison of the 3' - 5' cytidylyl puromycin with the isomeric mixture is presented in Figure 19. The 3' - 5' isomer is found to be even more inhibitory than the mixture. Fifty percent inhibition of amino acid incorporation is produced by: 0.35 μmolar 3' - 5' cytidylyl puromycin, 0.8 μmolar cytidylyl

puromycin mixed isomers, and 3.0 μmolar puromycin. The inhibitory effects of 3' - 5' cytidylyl puromycin and the mixed isomers are examined in greater detail in Figure 20 where additional concentrations of inhibitors have been analyzed. The same relationship is still found to exist. The greater inhibitory ability of 3' - 5' cytidylyl puromycin when compared to the mixture, coupled with the evidence indicating that the isomeric mixture was 35% 3' - 5' cytidylyl puromycin, suggested that most, if not all, of the inhibition produced by the mixture was due to the presence of the 3' - 5' species.

In order to examine this hypothesis 2' - 5' cytidylyl puromycin was isolated from a ribonuclease digestion of the isomers and tested for its ability to inhibit protein synthesis. The inhibitory properties of the 2' - 5' and 3' - 5' isomers of cytidylyl puromycin are compared in Figure 21. The data indicates the 2' - 5' cytidylyl puromycin is relatively inactive with 50% inhibition occurring at 65 μmolar.

A comparison of the concentrations of puromycin, 2' - 5' cytidylyl puromycin 3' - 5' cytidylyl puromycin, and the isomeric mixture of cytidylyl puromycin, required to inhibit the incorporation of labeled amino acid in the reticulocyte cell-free system by 50%, is summarized in

Table III. 3' - 5' cytidylyl puromycin is an order of magnitude more inhibitory than puromycin. It is worthy of note that 3' - 5' cytidylyl puromycin is the first derivative of either puromycin or aminoacyl adenosine to be more inhibitory than puromycin. Due to the low inhibitory properties of 2' - 5' cytidylyl puromycin and its large contribution to the molarity values, practically all of the inhibition produced with the isomeric mixture can be attributed to the presence of the 3' - 5' isomer.

The importance of the aminoacyl part of cytidylyl puromycin was investigated by determining the ability of cytidylyl puromycin aminonucleoside to inhibit protein synthesis. In Figure 18 cytidylyl puromycin aminonucleoside is shown to have practically no inhibitory effect even at concentrations approaching 1 mmolar.

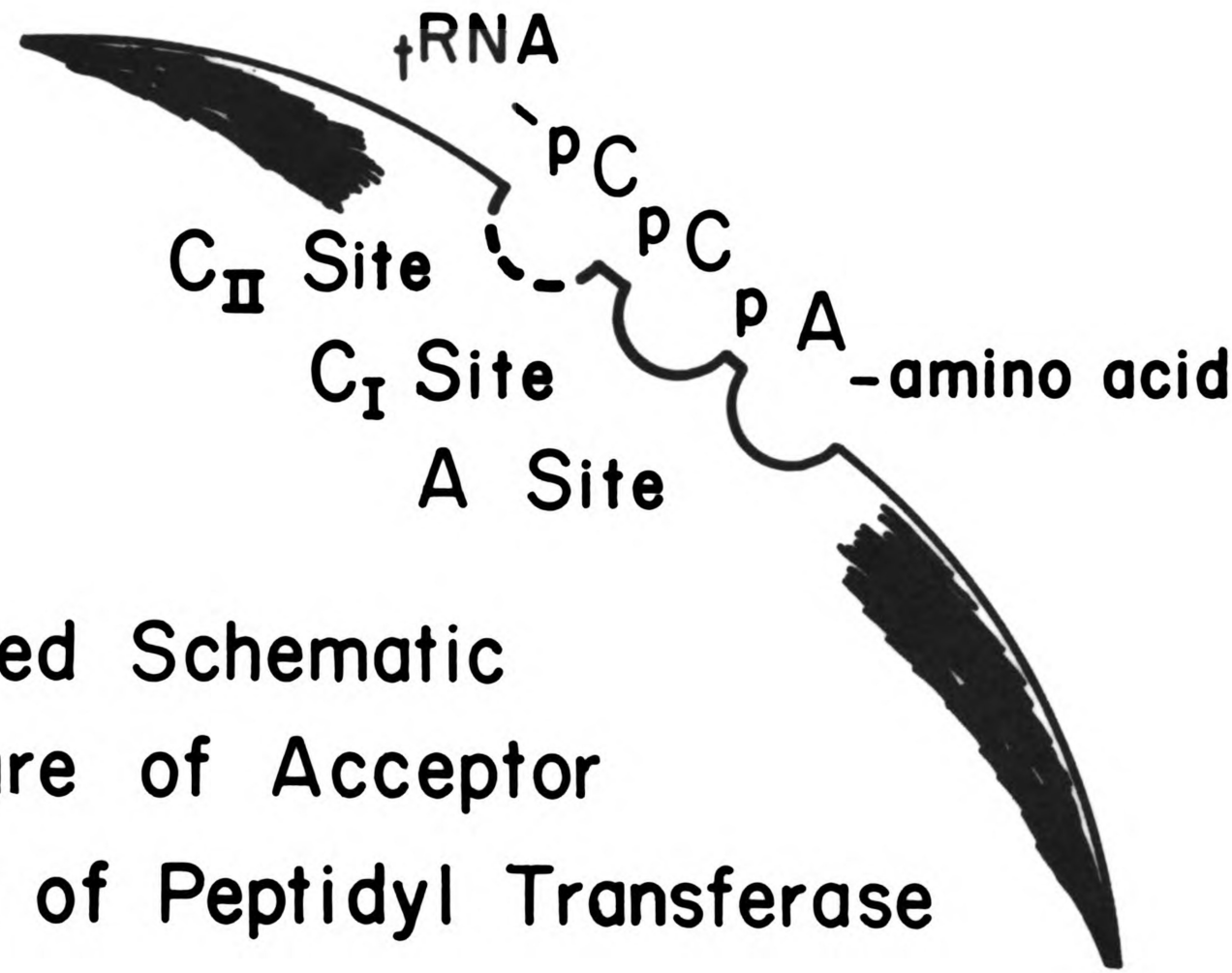
These data with the data of Rychlik et al. (1967) and of Symons, et al. (1969) support the concept of the existence of a cytosine specific site or sites in the acceptor region of peptidyl transferase which is capable of interacting with the 3' terminus of aminoacyl tRNA as demonstrated in Figure 22. In addition the aromatic amino acid specificity of puromycin has been investigated. Symons, et al. (1969) suggested that there is a possibility that the aromatic amino acid may be able to occupy a site

Table III. Summary of the inhibition produced by puromycin, cytidylyl puromycin (2' - 5' and 3' - 5' mixed isomers), cytidylyl puromycin (2' - 5' isomer), and cytidylyl puromycin (3' - 5' isomer).

INHIBITION OF INCORPORATION

Antibiotic	Concentration at 50% Inhibition
Puromycin	30×10^{-7} M
2'→5', 3'→5' Cytidylyl Puromycin	8.0×10^{-7} M
3'→5' Cytidylyl Puromycin	3.5×10^{-7} M
2'→5' Cytidylyl Puromycin	650×10^{-7} M

Figure 22. Proposed schematic structure of acceptor region of peptidyl transferase.



Proposed Schematic
Structure of Acceptor
Region of Peptidyl Transferase

normally occupied by a cytosine residue of tRNA. If indeed this is the case, the aromatic amino acid and the cytidylyl moiety would be competing for the same ribosomal binding site, and cytidylyl puromycin which contains both the aromatic amino acid and cytidylate moiety, would be predicted to be no more inhibitory than puromycin. However, cytidylyl puromycin is an order of magnitude more inhibitory than puromycin suggesting that both the amino acid and the nucleotidyl derivative are important factors in the inhibition of protein synthesis. Cytidylyl puromycin aminonucleoside contains cytidylate but no amino acid and is inactive. Cytidylyl glycyl puromycin aminonucleoside also contains cytidylate, but it also contains an amino acid that is not aromatic. This compound was found by Symons, et al. (1969) to be only 25% as inhibitory as puromycin. Since substitution of a non-aromatic amino acid into cytidylyl puromycin results in a lower potency, and complete omission of the amino acid totally inactivates the molecule one is lead to conclude that the structural requirement for the aromatic amino acid must still exist but has an affinity for an independent binding site.

In conclusion, data presented in this thesis support the postulate that the "A" site of peptidyl transferase

interacts with the 3' terminal adenosine of tRNA and at least one, if not both of the two cytosine residues all tRNA molecules have in common. The nucleotidyl specificity of the "A" site appears to be identical to the specificity demonstrated by Monro, et al. (1969) and Vazquez, et al. (1969) for the "P" site. Peptidyl transferase can distinguish between 2' - 5' and 3' - 5' interribonucleotidyl bonds and appears to have an independent binding site for aromatic amino acids. This conclusion will be supported by Harris, et al. (1971).

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