SYNTHESIS OF CYTIDYLYL PUROMYCIN

Thesis for the Degree of M. S.
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EDMUND J. HENGESH
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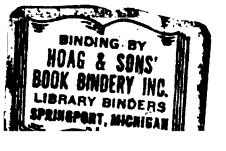
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ABSTRACT

SYNTHESIS OF CYTIDYLYL PUROMYCIN

by Edmund J. Hengesh

Methods for the synthesis of cytidylyl puromycin are compared. The structural similarity between this compound and the 3' terminus of aminoacyl tRNA and its possible implications in the inhibition of protein synthesis are discussed.

Also described are two general methods for the synthesis of ribonucleoside monophosphates from adequately blocked reactants. One of these methods involves a nucleotidyl cyclic phosphate as a necessary intermediate. With the use of alkaline phosphatase the extent of cyclization during the course of the reaction can be determined.

The dinucleoside monophosphates were purified by column chromatography. Characterization was accomplished by paper chromatography of both alkaline and pancreatic ribonuclease digestions of the reaction products. Also discussed are the applications of ultraviolet, infrared, and mass spectroscopic techniques for the identification of reaction products.

SYNTHESIS OF CYTIDYLYL PUROMYCIN

By

Edmund J. Hengesh

A THESIS

Submitted to
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TO MY WIFE GLENDA AND OUR CHILDREN

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INTRODUCTION

Transfer ribonucleic acid (tRNA) is known to be involved in at least two steps in the process of protein synthesis. It first must accept an amino acid to form the aminoacyl derivative. It then forms a ternary complex with a ribosome and messenger RNA and transfers its amino acid to a polypeptide chain (1).

All tRNA molecules are believed to have a common 3' terminal trinucleotide sequence, pCpCpA. This sequence has been substantiated through the primary sequence determinations of a number of tRNA molecules (2-7) and the terminal sequences of many others (8). Further evidence has been obtained with the characterization of enzyme fractions from Escherichia coli (9), Ehrlich ascites carcinoma cells (10), and rat liver (11-13) that are capable of the degradation and the reconstruction of this trinucleotide sequence. The terminal adenylic acid of this sequence is the acceptor for the aminoacyl synthetase catalyzed transfer of the activated amino acid (14). The ester bond which is formed occurs at either the 2' or the 3' position of the adenylate moiety (15-17). This reaction is dependent upon the integrity of the trinucleotide sequence (1). Transfer RNA preparations that have had their terminal trinucleotide sequences removed lose all ability to participate in ribosomal binding (18).

Puromycin is a compound isolated from the mold Streptomyces alboniger by J. N. Porter and co-workers in 1952 (19). The structure of puromycin was established by C. W. Waller and co-workers in 1953 (20) and confirmed by the stepwise synthesis by B. R. Baker, et. al. (21-29), during the following two year period. The chemical and physical data of the antibiotic have been reported by P. W. Fryth and co-workers (30). The original structure-activity relationships for puromycin inhibition of protein synthesis were presented by D. Nathans and A. Neidles in 1963 (31).

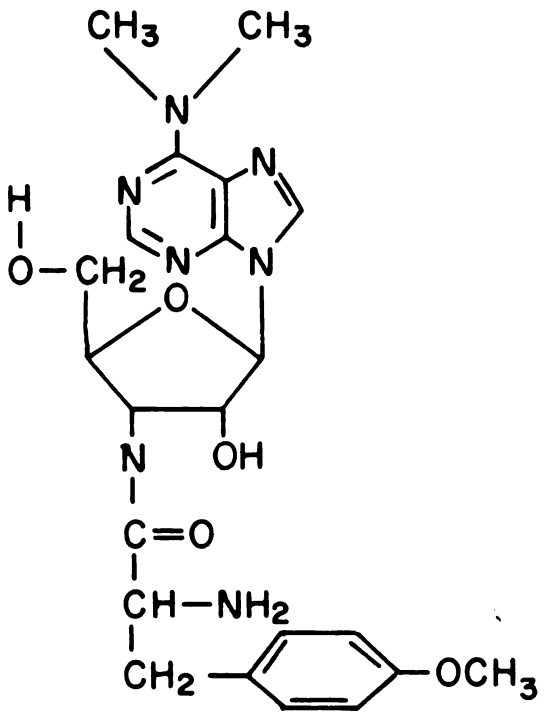
Puromycin has been shown to have a broad range of antibiotic activity being effective against both gram-negative and gram-positive bacteria (32), protozoa, parasitic worms, alga (33), and cells of mammalian origin (34-36). M. B. Yarmolinsky and De La Haba (37) were the first to report the inhibition of protein synthesis by puromycin and suggested that the similarity between puromycin and the terminus of aminoacyl tRNA may be responsible for its activity. This hypothesis has been confirmed by other workers (35, 38, 39). It is 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 receptor site on the ribosome and to bring about the premature release of the polypeptide chain as peptidyl puromycin. The formation of this bond is believed catalyzed by the enzyme "peptidyl transferase" which is an integral part of the ribosome

structure (40-43).

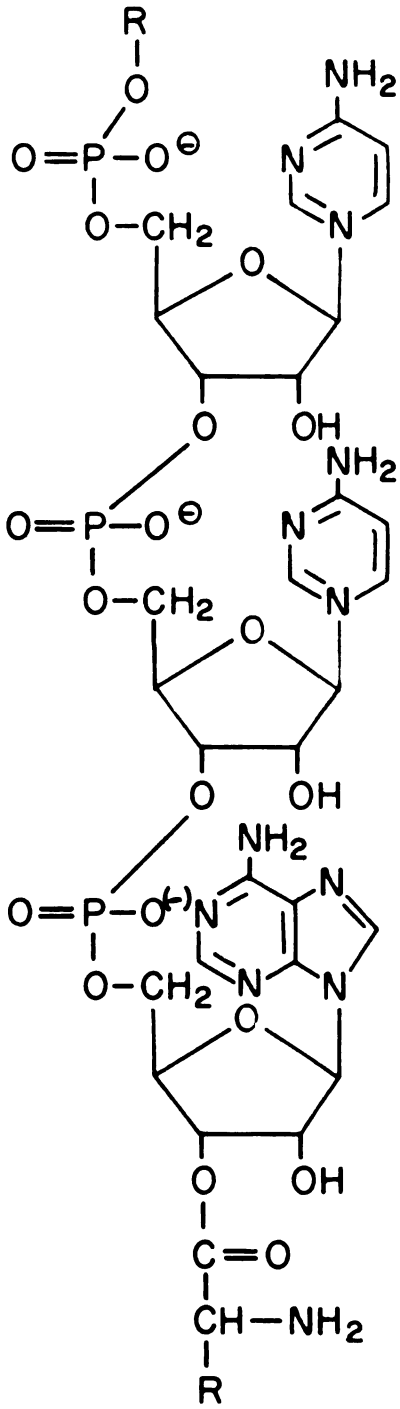
The structural similarity between puromycin and the terminus of tRNA (Figure 1) led J. P. Waller and co-workers (44) to propose that an aminoacyl adenosine should likewise inhibit protein synthesis by the same mechanism as that proposed for puromycin. To test their hypothesis they synthesized leucyl 3' adenosine and isolated phenylalanyl adenosine and tyrosyl adenosine from pancreatic ribonuclease treated aminoacyl tRNA. From their data, obtained with E. coli and poly-U cell free systems, it can be concluded that these aminoacyl adenosine compounds do inhibit protein synthesis in a manner analogous to that of puromycin but with varying efficiencies. As had been previously demonstrated with aminoacyl derivatives of puromycin (31), the most effective inhibitors were those having the aromatic amino acids, phenylalanine and tyrosine, as the aminoacyl portion.

A similar series of experiments were performed by I. Rychlik, S. Chladek, and J. Zemlicka (45). They prepared a glycine derivative of adenosine and tested its ability to inhibit protein synthesis in a poly A mediated system. It was found to be inactive. However, by adding cytidylic acid to the glycyl adenosine to obtain CpA-glycine, which more closely resembles the 3' terminus of aminoacyl tRNA, a compound was obtained that was one-half as effective as puromycin in the release of polylysyl peptides. A UpA-glycine derivative was also tested and found to be much less effective

Figure 1



Puromycin



C-C-A Terminus of amino acyl tRNA

indicating that the enhanced activity was specifically related to having cytidylic acid in the second position.

Recently R. H. Symons and co-workers (46) published a study involving nucleotidyl and amino acyl derivatives of puromycin. By measuring the release of phenylalanine peptides in a poly U mediated E. coli cell free system, they demonstrated that the proline, tryptophan, methionine, and alanine analogues of puromycin were inactive in the inhibition of protein synthesis. Leucyl puromycin was found to have some antibiotic activity but to a lesser degree than that reported by Waller for leucyl adenosine. High levels of inhibition were seen with phenylalanine, tyrosine, and S-benzyl cysteine derivatives once again demonstrating the requirement for an aromatic group. They also prepared the adenylyl, cytidylyl, guanosyl, and uridylyl analogues of 3' N-glycyl puromycin and found that only the cytidylyl derivative had antibiotic activity with an activity approaching that of puromycin itself.

Therefore, it appears as if there are at least two structural requirements for optimal antibiotic activity with puromycin analogues. The first requirement is that the amino acid portion of the molecule be aromatic as any non-aromatic modification of the O-CH_3 tyrosine of puromycin leads to a loss of activity. The reason for this specificity is not clear since the ribosomal site of protein synthesis should accommodate any amino acid with approximately equal

efficiency, and the mechanism of action of puromycin should not depend upon any particular amino acid. However, it has been suggested that the aromatic residue may be able to occupy the ribosomal binding site normally occupied by the cytosine ring thus enhancing antibiotic activity (46). The second requirement involves the nucleotidyl additions to puromycin or adenosine. The more closely the nucleotidyl analogue resembles the pCpCpA terminus of aminoacyl tRNA the more effective the compound should be in the inhibition of protein synthesis.

The work to be detailed in this thesis was undertaken to chemically synthesize a compound which would fulfill both of these structural requirements, that is, cytidylyl puromycin. This compound should provide information about the ribosomal structural requirements necessary for the peptidyl transferase reaction.

METHODS AND MATERIALS

Chemicals and reagents:

Ethylchloroformate and diphenylchlorophosphate (diphenylphosphorochloridate were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Adenosine, cytidine 2', and 3' monophosphoric acid (mixed isomers), 2', 3' diacetyladenosine, (3'-5') cytidylyladenosine, dicyclohexylcarbodiimide, and 2', 3' cyclic cytidylic acid were purchased from Sigma Chemical Company, St. Louis, Missouri. Puromycin-methoxy (^{3}H) dihydrochloride (10^{-3} M, S.A. 1000 μc/μm) was purchased from New England Nuclear Corporation, Boston, Massachusetts. Unlabeled puromycin dihydrochloride, carbobenzoxy chloride, and puromycin aminonucleoside were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Tetraethylammonium acetate was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. 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.

Paper chromatography:

Descending paper chromatography was performed with DEAE (diethylaminoethyl) cellulose paper using solvent sys-

tem I (0.1 N ammonium formate) or with Whatman No. 1 paper developed with solvent system II (ethanol -1.0 M ammonium acetate, pH 7.5, 5:2, v/v). Chromatograms were read with a UVS-12 mineralight.

Degradation of nucleotide derivatives:

Alkaline digestion: Approximately 5 O.D. units (260 mμ) of the material to be characterized were treated with 0.3 N potassium hydroxide at 37° for 18 hours.

Ribonuclease digestion: Pancreatic ribonuclease (5 μg/reaction mixture) was added to a solution of 1.5 O.D. of polynucleotide 0.020 ml of ammonium bicarbonate (0.05 M, pH 7.5). Incubation was at 37° for 24 hours. The concentration of the pancreatic ribonuclease was determined by subtracting the absorbance at 320 mμ from the absorbance measured at 280 mμ. A mg/ml solution of pancreatic ribonuclease has a corrected absorbance at 280 mμ of 0.695.

RESULTS OF THE SYNTHESIS OF OLIGONUCLEOTIDES

Preliminary studies utilizing pancreatic ribonuclease:

Due to the structural similarity between puromycin and adenosine, it was decided to use adenosine in the coupling experiments with cytidylic acid assuming that if a suitable method for the synthesis of CpA could be developed the substitution of puromycin for adenosine under the same experimental conditions should produce CpPuro. With this assumption the initial attempts to synthesize CpA were based upon the work of M. R. Bernfield (47, 48) who has extensively investigated the use of pancreatic ribonuclease and ribonuclease derivatives to enzymatically synthesize oligonucleotides of defined sequence and base composition. Under the appropriate conditions of pH, temperature, enzyme concentration, incubation time, and substrate concentration, ribonuclease can be induced to synthesize oligonucleotides. Due to the base specificity of the enzyme for pyrimidine nucleotides, the reactions usually involve the coupling of a pyrimidine 2', 3' cyclic nucleotide with a nucleoside which may be either a pyrimidine or a purine. Numerous attempts were made to synthesize CpA varying the above parameters, but no significant production of CpA was obtained. The main difficulties encountered with the enzymatic reactions were an apparent affinity of the enzyme for the pyrimidines leading

to the preferential synthesis of CpCp and the relatively poor solubility (less than 0.05 M) of adenosine in the reaction medium. This poor solubility of adenosine prohibited the attainment of sufficiently high concentrations of adenosine (0.1 M) to properly drive the reaction to the desired end product. In an attempt to increase the solubility of adenosine in the reaction medium various concentrations (20-30% v/v) of formamide were added. This technique has been successfully used by F. Rottman and M. Nirenberg (49) to solubilize poly U in a ribonuclease reaction. Increase solubility of adenosine was obtained, but no noticeable increase in CpA synthesis was detected.

Since the attempts to synthesize CpA enzymatically appeared to have been unsuccessful, the emphasis of the research was shifted to the synthesis of CpA by chemical means, primarily using the techniques of A. M. Michelson (50-52) and H. G. Khorana and co-workers (53-58).

Synthesis of cytidine 2', 3' cyclic phosphate:

To chemically synthesize CpA according to the methods of A. M. Michelson (50-52) using diphenylphosphorochloridate as the coupling agent, it is necessary that the phosphate group of the pyrimidine portion be in the cyclic ester form. Cytidine 2', 3' monophosphoric acid (mixed isomers) (1 mmole) was weighed out and placed in a flask which contained 3 ml of water. Tri-n-butylamine, freshly distilled, (0.75 ml) was then added to the flask and the reaction mixture was

stirred for 0.5 hour to insure complete solution of the cytidine 2', 3' monophosphoric acid. Ethylchloroformate (0.2 ml) was added; the flask was stoppered and shaken vigorously for 10 minutes to prepare the 2', 3' cyclic phosphate. Solvent was removed by flash evaporation and the residue dissolved in 10 ml of absolute ethanol. The solvent was again removed under vacuum and 20 ml of ether was added with vigorous shaking. A white precipitate was obtained. The yield of product was quantitative. The product was characterized by the same procedure as that described for the acetylated cyclic cytidylic acid in the following section.

Synthesis and characterization of acetylated cytidine 2', 3' cyclic phosphate:

In order to maximize the yield of the desired isomers of CpA it was necessary to form the N^4, $O^{5'}$ diacetyl derivative of cytidine 2', 3' cyclic phosphate. 2', 3' cyclic cytidylic acid was prepared by the ethylchloroformate method described above. After the solvent was removed from the cyclization mixture, the residue was made anhydrous by flash evaporation (3X) of a solution of the residue from 5 ml of anhydrous, redistilled dimethylformamide. The residue was dissolved in a solution composed of 3 ml of dimethylformamide and 3 ml of anhydrous, redistilled dioxane. Tri-n-butylamine (1.45 ml) was added and the solution was agitated. 0.5 ml of doubly distilled acetic anhydride was added to the solution,

and the reaction was stirred and allowed to react for 48 hours at room temperature in the dark. Solvent was removed by flash evaporation and 20 ml of ether was added to wash the residue. The ether was decanted and discarded. The precipitate was redissolved in 4 ml of dimethylformamide and flash evaporated. A quantitative yield of N^4, $O^{5'}$ diacetyl cytidine 2', 3' cyclic phosphate as the tri-n-butylammonium salt was obtained.

Since the integrity of the cyclic linkage is crucial to the reaction, any premature hydrolysis of this linkage would result in a reduction of the overall yield of dinucleoside monophosphate in the coupling reaction. Therefore an assay to measure the extent of cyclization was sought. It was found that alkaline phosphatase is capable of cleaving both the 2' and the 3' isomers of cytidylic acid but is unable to cleave the 2', 3' cyclic linkage. This disparity in reactivity then served as the basis for the analysis of each step of the cyclization and acetylation reactions to determine whether or not the integrity of the cyclic linkage was being maintained. The inorganic phosphate released by the alkaline phosphatase was measured by a modified method of J. B. Martin and D. M. Doty (59). The data are summarized in Table I.

It can be seen in Table I that a commercial preparation of 2', 3' cyclic cytidylic acid is resistant to the action of alkaline phosphatase releasing only 5.1×10^{-5} μm Pi/μm substrate. A preparation of the 2', 3' mixed isomers

TABLE I. RESULTS OF ALKALINE PHOSPHATASE ASSAYS

Contents	Tube Number 1	2	3	4	5
$(NH_4)_2CO_3$ (M), pH 9.0	20	20	20	20	20
Alkaline Phosphatase	10	10	10	10	10
Substrate	25 A	50 B	50 C	50 D	50 E
Water	945	920	920	920	920
µmole Pi/µmole substrate	1.1	5.1×10^{-5}	1.4×10^{-2}	9.5×10^{-3}	8.4×10^{-4}

(All volumes are in microliters)

A - Cytidylic acid, 2' and 3' mixed isomers (5.3 mg/ml).

B - Cyclic cytidylic acid (3.8 mg/ml).

C - Cyclic cytidylic acid preparation (approximately 80 mg/ml).

D - Acetylated cyclic cytidylic acid preparation (approximately 80 mg/ml).

E - Acetylated cyclic cytidylic acid preparation after DEAE cellulose chromatography (approximately 80 mg/ml).

Alkaline phosphatase (900 µg/ml) was obtained from Worthington and was chromatographically and electrophoretically pure.

of cytidylic acid was hydrolyzed completely. Alkaline phosphatase digestion of an aliquot of the cyclization mixture in assay tube number 3 released 1.4×10^{-2} μm Pi/μm substrate. Tube number 4 contained an aliquot from the acetylation reaction mixture and showed release of 9.5×10^{-3} μm Pi/μm substrate. A comparison of this value with the previous value demonstrates that although the cyclization mixture contains some uncyclized cytidylic acid no further degradation occurred in the acetylation procedure. The actual decline in value was most likely due to a difference in the blanks and was not considered significant. A quantification of the extent of contamination of the acetylated 2', 3' cyclic cytidylic acid preparation with the 2', 3' mixed isomers of cytidylic acid was achieved by comparison of the value of tube number 4 to the value of tube number 1. This comparison indicated that approximately 1% of the preparation was in the form of the monophosphate while 99% of the preparation was present in the cyclic form.

Even this minor contamination of the acetylated cyclization preparation can be removed by placing the sample over a DEAE cellulose column (94 cm x 1.5 cm) equilibrated with tri-n-butyl ammonium formate 0.1 M, pH 6.1. This buffer was prepared by titrating a 0.1 M solution of tri-n-butylamine with 1 N formic acid to the desired pH. The column was eluted with this buffer and fractions containing 4.6 ml were collected. The peak containing the acetylated cyclic cytidylic

acid eluted between fractions 40-60 which were pooled and evaporated under vacuum. A minor component eluted from the column in fractions 35-38. An aliquot of pooled peak 40-60 was subjected to alkaline phosphatase digestion and gave a value of 8.4×10^{-4} which corresponds closely to the value obtained with the commercial preparation of cyclic cytidylic acid. Therefore no non-cyclic component was present.

Identification of the products formed during the cyclization and acetylation procedures was determined by paper chromatography on DEAE cellulose paper eluted with solvent system I. Cyclic cytidylic acid moves with an R_f substantially greater than that of a standard preparation of cytidine 2', 3' monophosphate. Acetylation of the cyclic compound leads to a further increase in R_f. Typical R_f values would be: cytidine monophosphoric acid 0.17, cyclic cytidylic acid 0.53, and acetylated cyclic cytidylic acid 0.81.

Synthesis of cytidylyl adenosine (3'-5', 2'-5') mixed isomers:

Initial experiments to synthesize the dinucleoside monophosphate were conducted using acetylated cyclic cytidylic acid and adenosine. However, it was found that there is a high probability of the synthesis of the various other isomers which would involve the 2' and 3' hydroxyls of adenosine if adenosine was not blocked sufficiently. Because of this possibility, 2', 3' diacetyl adenosine was substituted for adenosine in the reaction mixtures.

A one ml solution of dimethylformamide containing 0.25 mmole of tri-n-butylammonium N^4, $O^{5'}$ diacetylcytidine 2', 3' cyclic phosphate was added to 0.25 mmole of diacetyladenosine and rendered anhydrous by three evaporations from anhydrous dimethylformamide. The residue was dissolved in a solution of one ml of dimethylformamide and one ml of dioxane to which 0.15 ml of tri-n-butylamine and 0.8 ml of diphenylphosphorochloridate were added. The reaction flask was sealed, shaken, and set aside in the dark at room temperature for 18 hours. Solvent was removed by flash evaporation and the residue was washed with ether. The residue was air-dried and dissolved in 5 ml of water yielding a yellow-colored solution pH 2.78. The pH of the solution was adjusted to pH 9.8 with dilute ammonium hydroxide and incubated at 37° C for 48 hours to remove the acetyl blocking groups. The solution was diluted and washed onto a column (1.2 cm x 12 cm) of Dowex 1x2 (formate form).

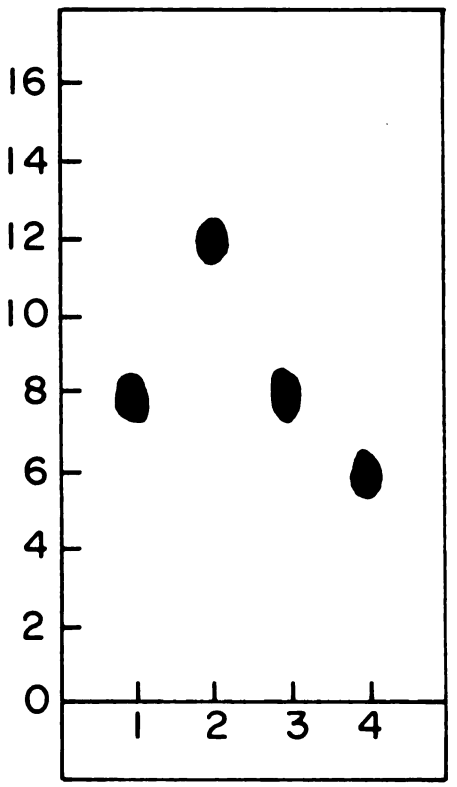
The column was first eluted with 120 ml water to remove the unreacted adenosine and a solution of 0.01 N formic acid was applied. Fractions containing 4.8 ml were collected. The dinucleoside monophosphate eluted between tubes number 83-108 (275-400 ml after addition of 0.01 N formate) with a yield of approximately 50%. These tubes were combined and evaporated to dryness under reduced pressure.

The position of the elution of CpA from the Dowex

1x2 column was confirmed by placing a sample of commercially prepared CpA through the column using a elution scheme similar to the one described above. The dinucleoside monophosphate was found to elute 60 tubes (285 ml) after the 0.01 N formic acid was applied to the column. This would correspond to tube number 85 in the reaction elution profile. Likewise, commercial preparations of adenosine and cytidylic acid were passed through the column to confirm their position in the elution profile. Adenosine eluted from the column immediately at the water front. Cytidylic acid was retained by the column and ultimately resolved into its component isomers after 720 ml of elutant which corresponds to a position far removed from the position of CpA.

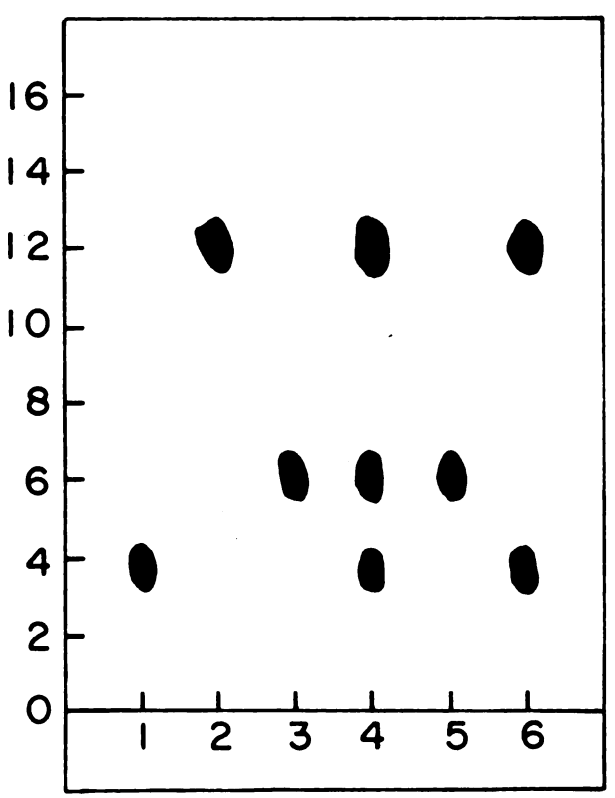
Characterization of the pooled fraction believed to contain the CpA was accomplished by paper chromatography of alkaline and pancreatic ribonuclease digestions and by spectral analysis. As can be seen in Figures II and III, the reaction product moves as a single entity in each of two chromatographic systems partitioning itself approximately one-third of the way between standards of adenosine and cytidylic acid. The chromatogram in Figure II was prepared by developing DEAE cellulose paper with solvent system I, whereas, the chromatogram in Figure III consisted of Whatman No. 1 paper developed in solvent system II. Also in Figure III alkaline digestion of the product with 0.3 M KOH gave rise to two spots which chromatograph similar to the standards

Figure II. Chromatography of Reaction Product



CODE
1. CpA Standard
2. Adenosine Standard
3. Reaction Product
4. Cytidylic Acid Standard

Figure III. Chromatography of Product Digestion



CODE
1. Cytidylic Acid Standard
2. Adenosine Standard
3. Reaction Product
4. Ribonuclease Digestion
5. CpA Standard
6. KOH Digestion

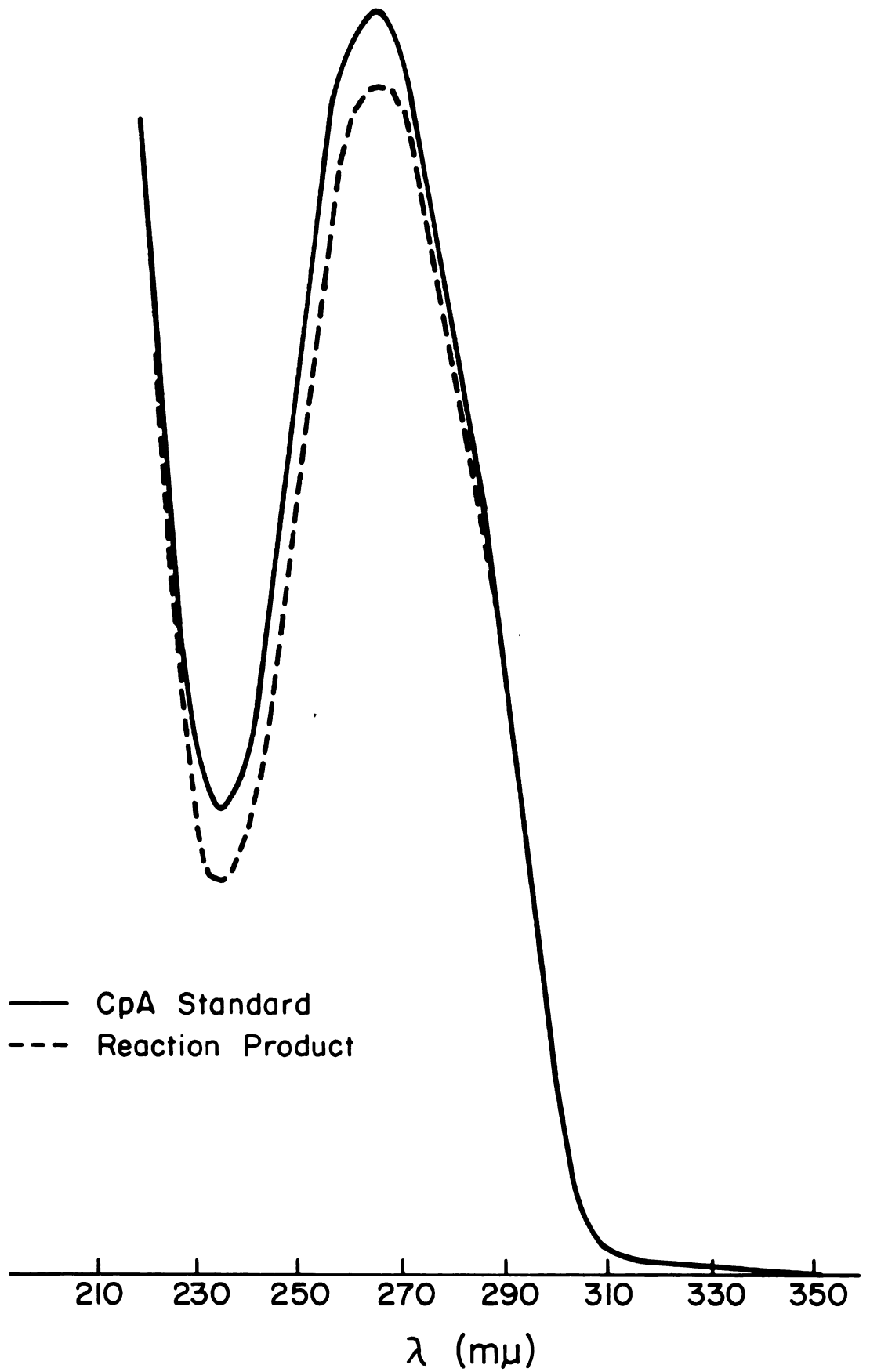
of cytidylic acid and adenosine. Pancreatic digestion of the product yielded three spots, two of which corresponded to the standards of adenosine and cytidylic acid, one of which had an R_f similar to that of the parent compound. This is to be expected because this method of chemical synthesis results in equal quantities of both the 2'-5' and 3'-5' isomers of CpA being produced. Since KOH hydrolysis is not specific for any particular isomer only two spots are seen, but pancreatic ribonuclease which is specific for the 3'-5' isomer leaves the 2'-5' isomer intact results in formation of three spots.

Additional evidence for the synthesis of CpA was obtained by comparison of the spectrum in Figure IV, of a standard preparation of CpA to the spectrum of the synthesized product. The spectra were obtained with a Carey Recording Spectrophotometer. The wavelength maximum of the standard CpA was 264.9 mμ, whereas, the wavelength maximum of the synthesized product was 264.3 mμ. This close agreement lends further confirmation as to the identity of the synthesized product being CpA.

Resolution of the 3'-5' and 2'-5' isomers of cytidylyl adenosine:

Since the diphenylphosphorochloridate method of chemical synthesis of dinucleoside monophosphates includes as a reactant cytidine 2', 3' cyclic phosphate, there is an equal probability for the formation of either the 3'-5' or the 2'-5' isomer of CpA. Resolution of these isomers was not

Figure IV. Spectrum of Reaction Product



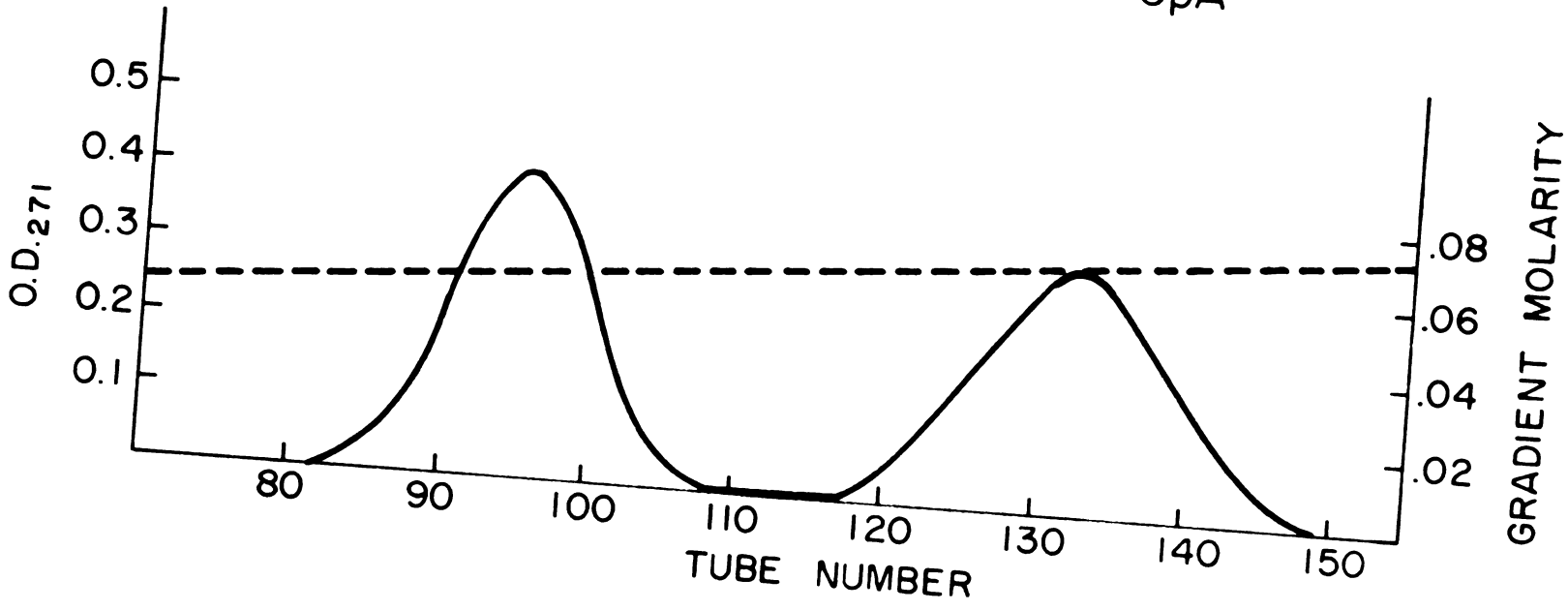
obtained with the elution of the Dowex 1x2 (formate) column with the 0.01 N formic acid. An initial attempt to resolve the isomers was performed by the use of a column of DEAE cellulose (94 cm x 1.5 cm) equilibrated with 0.1 M tri-n-butylammonium formate, pH 6.1, prepared as described previously. An aliquot of the fraction pooled from the Dowex 1x2 column was placed upon the column and eluted with the tri-n-butylammonium formate buffer. A single peak was obtained with no resolution into the component isomers.

However, resolution of the isomers was obtained by the use of a gradient procedure from P. R. Taylor and R. H. Hall (60). A Dowex 1x2 column (1.1 cm x 17 cm) was equilibrated with 0.03 M sodium formate, pH 5.0. A sample of the mixed isomers of CpA was applied to the column and eluted by means of a linear gradient containing 500 ml of 0.03 M sodium formate, pH 5.0 in the mixing chamber and 500 ml of 0.08 M sodium formate, pH 5.0 in the reservoir. As can be seen in Figure V, a clean separation of the two isomers was obtained with one eluting at a molarity of 0.05 sodium formate and the other at a molarity of 0.06. Summation of the absorbance in each of the two peaks was found to give a ratio of 1:1 confirming the equal probability for the chemical synthesis of either isomer.

Synthesis of acetylated cytidine 2', 3' monophosphoric acid (mixed isomers):

Subsequent to the studies mentioned above, another

Figure V. Resolution of the Isomers of CpA



method for the chemical synthesis of dinucleotides was developed. The second method is based upon the techniques of R. Lohrmann and H. G. Khorana (54) and utilizes a different coupling agent, dicyclohexylcarbodiimide. The N^4, $O^{5'}$, $O^{2'}$ or 3' triacetyl derivative of cytidylic acid is prepared because no cyclic phosphate bond is present during the course of the reaction. Thus, the position of the acetyl blocking groups can be directed so as to lead to the preferential synthesis of either the 2' or the 3' isomer of the dinucleotide rather than an equimolar mixture as that obtained with the use of diphenylphosphorochloridate.

Cytidine 2', 3' monophosphoric acid (0.5 mmole) and tetraethylammonium acetate 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 conclusion 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 50Wx8 ion 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, $O^{3'}$, $O^{5'}$ triacetyl cytidine 2' phosphate were obtained in quantitative yield.

Alternate synthesis of cytidylyl adenosine (3'-5', 2'-5') mixed isomers:

A pyridine solution of triacetylcytidine monophosphoric acid (0.05 mmole) mixed isomers was added to 0.1 mmole of diacetyladenosine and 35 mg of Dowex 50Wx8 (pyridinium form) and evaporated from 5 ml of anhydrous pyridine three times to render the sample anhydrous. The mixture was redissolved in one ml of pyridine, and 125 mg of dicyclohexylcarbodiimide was added. The flask was sealed and placed in the dark at room temperature with constant stirring. After five days the reaction was terminated by the addition of 0.5 ml of water. The mixture was then extracted three times with approximately 10 ml of n-pentane, which was discarded, and allowed to stand for 12 hours. The mixture was diluted with aqueous pyridine (50%) and filtered to remove the Dowex resin and the dicyclohexylurea which appears as a white precipitate upon the addition of the water to the dicyclohexylcarbodiimide. The aqueous pyridine solution was then extracted with carbon tetrachloride which was back extracted with aqueous pyridine

(50%). The aqueous pyridine layers were combined and evaporated under vacuum. The residue was dissolved in 5 ml of water and the pH of the solution was adjusted to pH 9.8 with dilute ammonium hydroxide and extracted two times with 5 ml of ether. The ether layers were discarded and the solution was incubated at 37° C for 48 hours at pH 9.8 to remove the acetyl blocking groups. The solution was then diluted and placed upon a Dowex 1x2 (formate) column (1.2 cm x 12 cm) eluted with water and then with 0.01 N formic acid. Fractions of 4.8 ml each were collected and the peak corresponding to the dinucleoside monophosphate was found to elute between tubes number 61 and 80. Tubes containing the dinucleoside monophosphate were combined and evaporated under vacuum. The yield of dinucleoside monophosphate was approximately 50%. The sample was found to be chromatographically similar to the commercial preparation of cytidylyl adenosine and was found to have a spectrum identical to the spectrum of the standard with a wavelength maximum of 264.6 mμ.

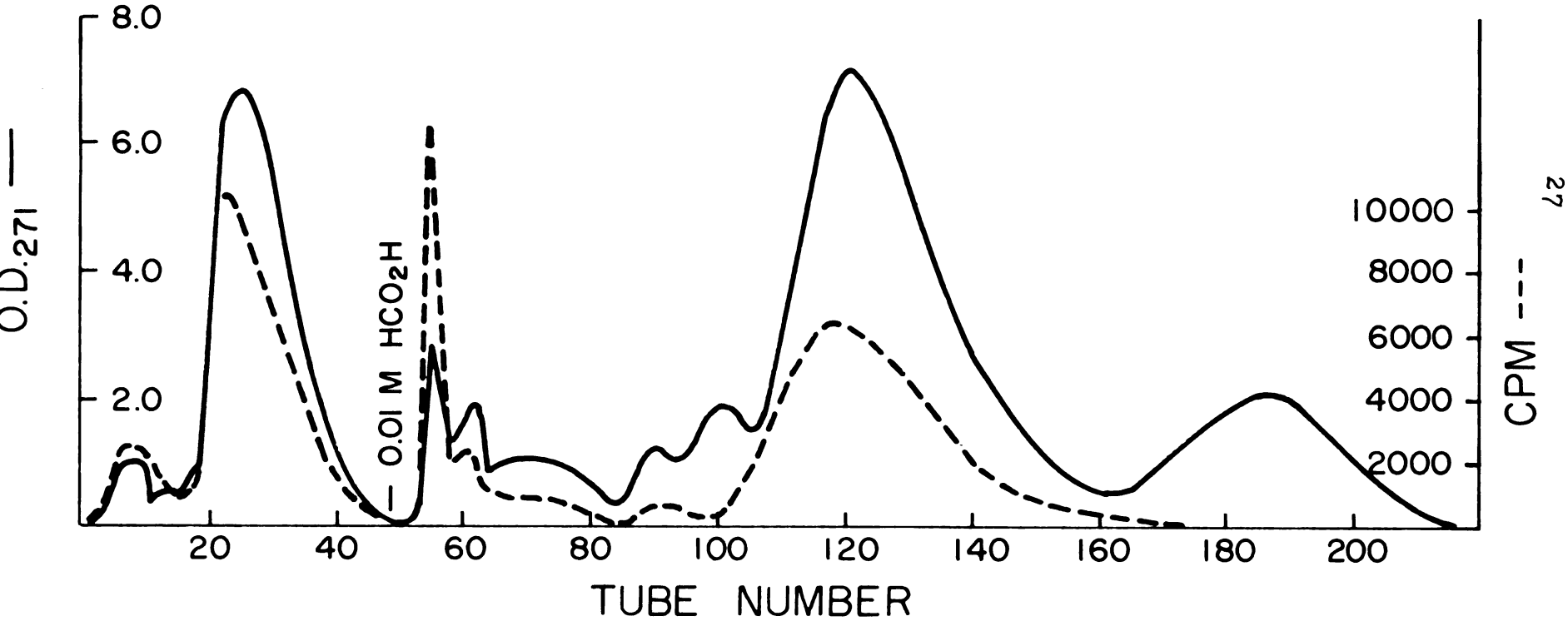
Initial synthesis of cytidylyl puromycin:

Following the successful synthesis of CpA with each of two different methods the research emphasis was directed toward the synthesis of CpPuro using the diphenylphosphorochloridate method of A. M. Michelson. One ml of a solution of dimethylformamide containing approximately 0.25 mmole of tri-n-butylammonium N^4, $O^{5'}$ diacetylcytidine 2', 3' cyclic phosphate was added to 80 mg of puromycin dihydrochloride to

which 0.2 ml of puromycin methoxy-^{3}H-dihydrochloride had been added. This mixture was then flash evaporated from 5 ml of dimethylformamide to render the sample anhydrous. The resulting residue was taken up in a solution of 0.5 ml of dimethylformamide and 0.5 ml of dioxane. To the resulting solution 0.15 ml of tri-n-butylamine and 0.08 ml of diphenylphosphorochloridate were added. The flask was sealed, shaken, and set aside in the dark for 18 hours. The solvent was then removed under vacuum, and the residue was washed with ether and the wash was discarded. The sample was air-dried and dissolved in 5 ml of water. The pH of the aqueous solution was adjusted to pH 9.8 with a dilute solution of ammonium hydroxide and incubated at 37° for 48 hours in order to remove the acetyl blocking groups. At the conclusion of the incubation period the sample was placed on a Dowex 1x2 (formate) column (1.2 cm x 12 cm) and the column was washed with water for the first 49 tubes at which time the absorbance profile had returned to base line. (See Figure VI). Fractions of 4.8 ml were collected and the optical density at 271 mμ was determined with a Gilford spectrophotometer. A solution of 0.01 N formic acid was then applied to the column and elution was continued. The absorbance and radioactivity profile can be seen in Figure VI. The radioactivity profile was obtained by counting a 25 μl aliquot of each fraction in 10 ml of Brays Solution.

The initial peaks eluted from the column with a water

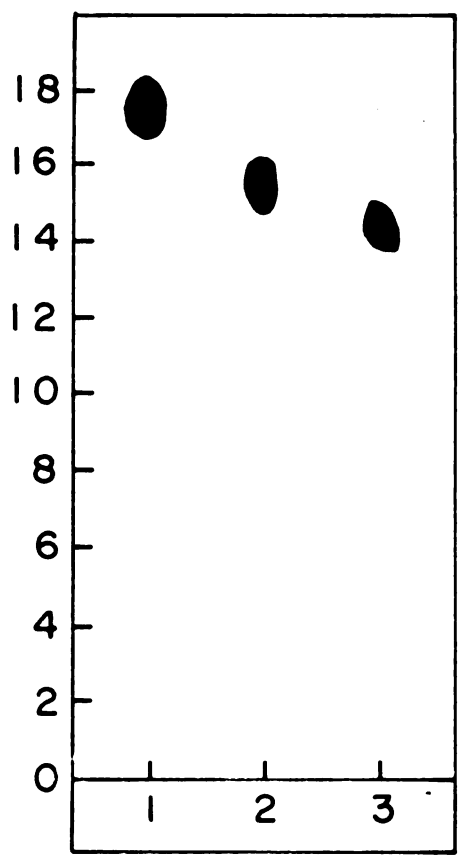
Figure VI. Elution Profile of Puromycin Reaction



rinse contained the radioactivity associated with the unreacted puromycin. The presence of free puromycin was verified by column chromatography of a sample of puromycin over the Dowex 1x2 column (formate) and by noting its elution at the solvent front. After the application of 0.01 N formic acid several unidentified side products were seen to elute which were eliminated from consideration due to the absence of radioactivity. However, at tube number 105 the beginning of a peak containing both high absorbance and radioactivity was observed. It was then assumed that this peak contained CpPuro and fractions 110-150 were pooled and evaporated. The yield based upon radioactivity was found to be 48.6%.

Upon chromatography on Whatman #1 paper eluted with solvent system II the product was found to have a mobility slightly less than that of a standard preparation of puromycin (Figure VII). This R_f was slightly greater than that of the aminonucleoside of puromycin. Treatment of the product with 0.3 M KOH resulted in the formation of two spots upon paper chromatography on Whatman #1 eluted with solvent system II as seen in Figure VIII. One of these spots corresponded to the standard cytidylic acid spot, whereas, the spot having the greater R_f moved slightly further than the standard puromycin spot. Treatment of the reaction product with pancreatic ribonuclease gave the characteristic cytidylic acid spot plus two other spots containing the radioactivity. One of these had an R_f

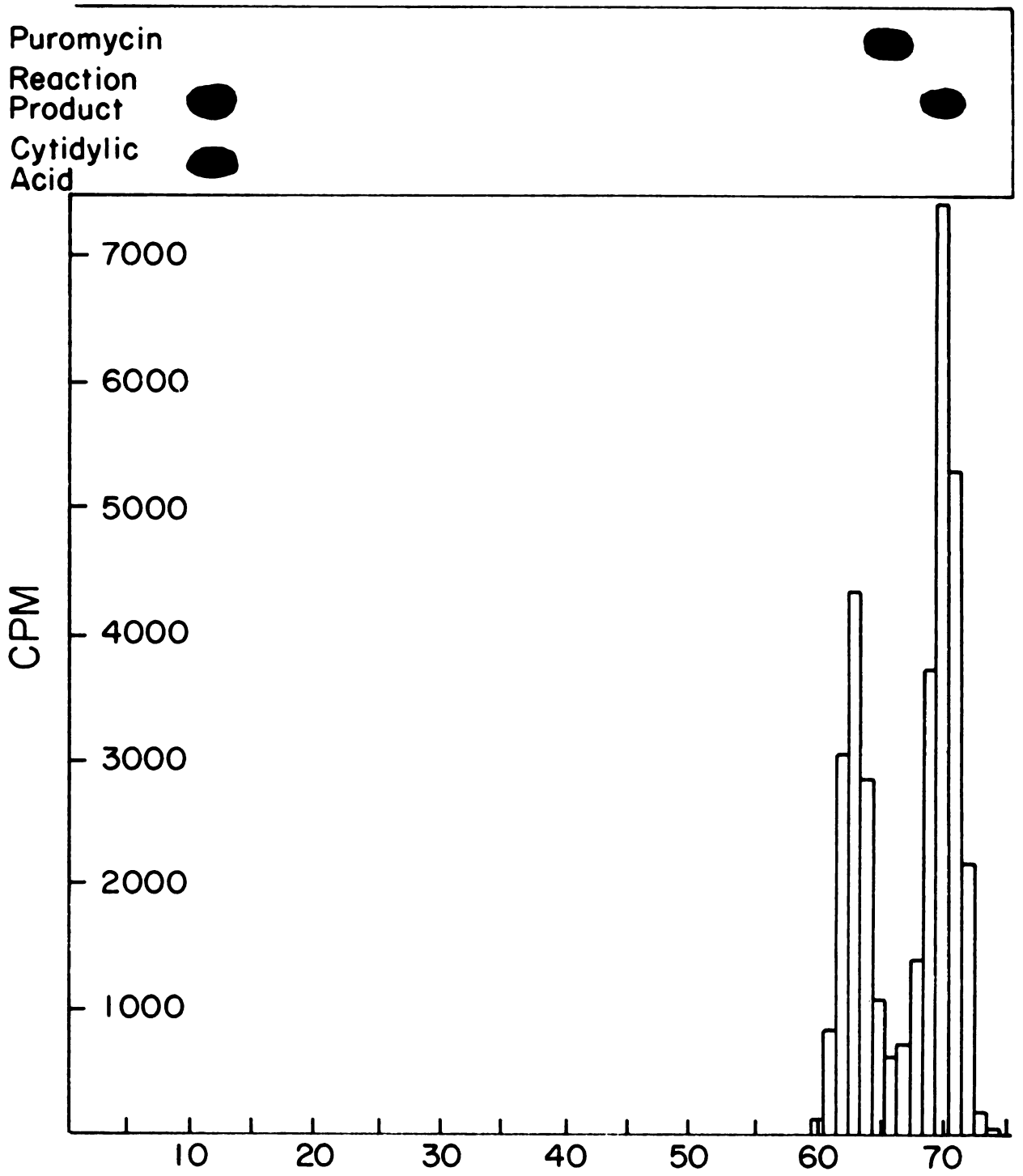
Figure VII. Chromatography of Puromycin Reaction Product



CODE

1. Puromycin Standard
2. Reaction Product
3. Aminonucleoside Standard

Figure VIII. Alkaline Digest of Reaction Product

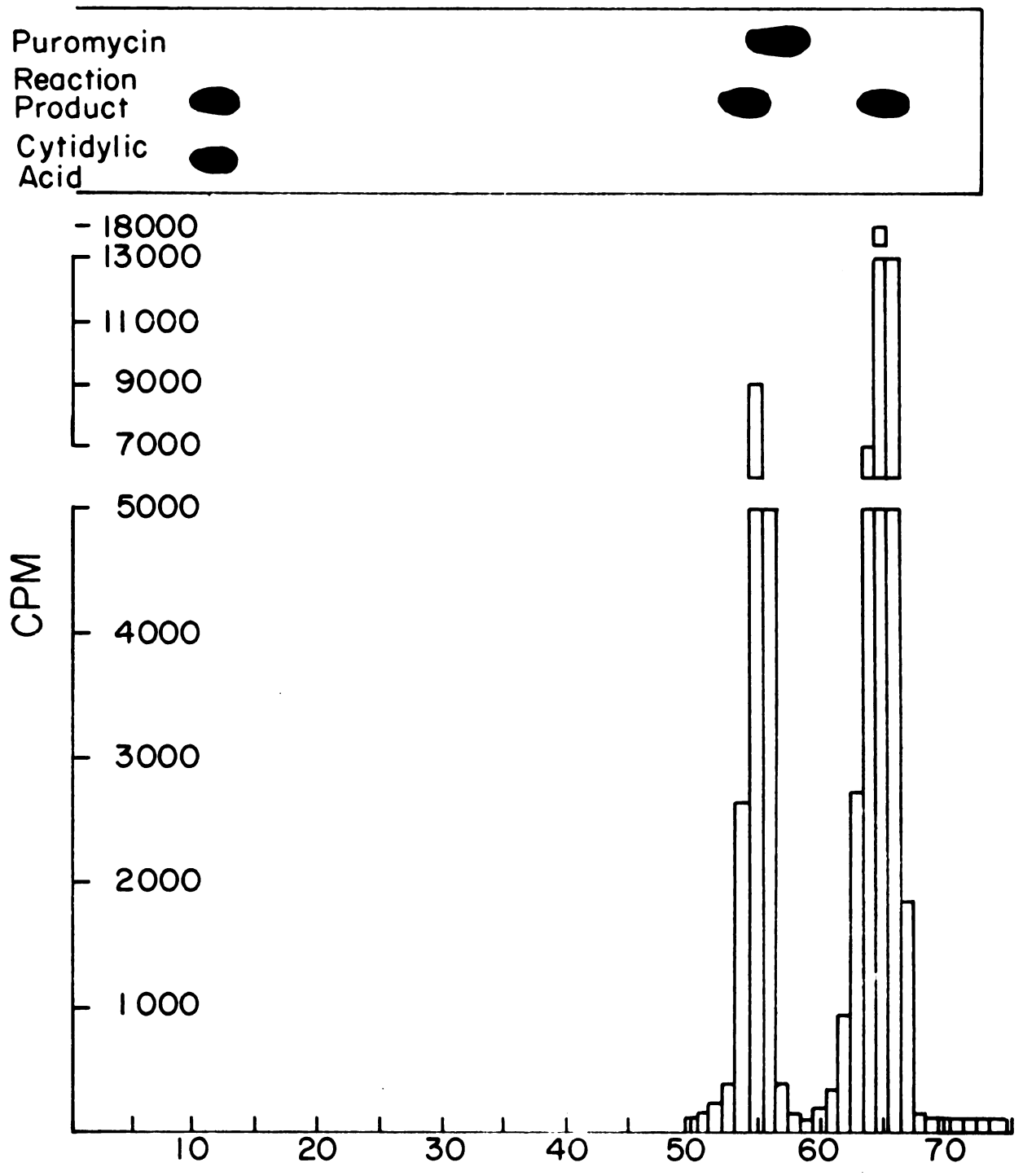


characteristic of the undigested compound, and the other had an R_f slightly greater than that of puromycin as seen in Figure IX. The dual peaks of radioactivity found on this chromatogram most probably indicated that both the 2' and 3' isomers of cytidylyl puromycin were being formed with the 3' isomer favored.

From these two chromatograms it appeared as if CpPuro was being synthesized but at the same time some modification of puromycin was indicated since in both chromatograms the puromycin spot was found to have a greater R_f than the puromycin standard. Further evidence that some modification had occurred was found in the dual peaks of radioactivity found on the alkaline digest chromatogram. One of these spots had an R_f greater than puromycin but the other was found to correspond closely to that of the puromycin standard. This would suggest that some base labile modification of puromycin had occurred.

Efforts were then directed towards the further characterization of the modified product of puromycin. It was demonstrated chromatographically and spectrally that the peak in Figure IV, previously attributed to the unreacted puromycin, also appeared to have undergone the same modification. Consequently, this material was used to characterize the modification so as to avoid the problem of having to work with the cytidylic acid portion of the molecule. Carey spectra of the modified puromycin have been compared to those

Figure IX. Ribonuclease Digestion of Reaction Product



of puromycin and have been found to be identical (Figure X). The spectral data have been interpreted to indicate that no modification of either the purine ring or the phenyl ring system had occurred because any change to that portion of the molecule will lead to a distortion of the spectra and a possible change in the wavelength maxima. Since the label in ^{3}H-puromycin was specifically in the $O-CH_3$ of the amino acid portion and since the label was still found with the compound, the methyl group was deemed to be intact. An infrared spectra of the compound showed characteristic amide I absorption, 1650 cm^{-1}, indicating that the amide bond connecting the amino acid to the ribose ring was intact. Treatment of the modified puromycin with ninhydrin gave nebulous results when compared to puromycin which developed a characteristic purple color. All of these characteristics, when taken collectively, would suggest that some modification or derivative had been formed involving the α-amino group of the amino acid. To further explore this possibility a sample of the modified puromycin was analyzed in the mass spectrometer and compared with the spectra of standard compounds of puromycin and puromycin aminonucleoside. The results, based upon the fragmentation pattern predicted by K. Biemann (61), indicated that the puromycin had undergone an addition reaction at the amino group of the amino acid which resulted in an increase of approximately 28 mass units (Table II). An increase in mass of 28 units

Figure X. Spectrum of Puromycin Reaction Product

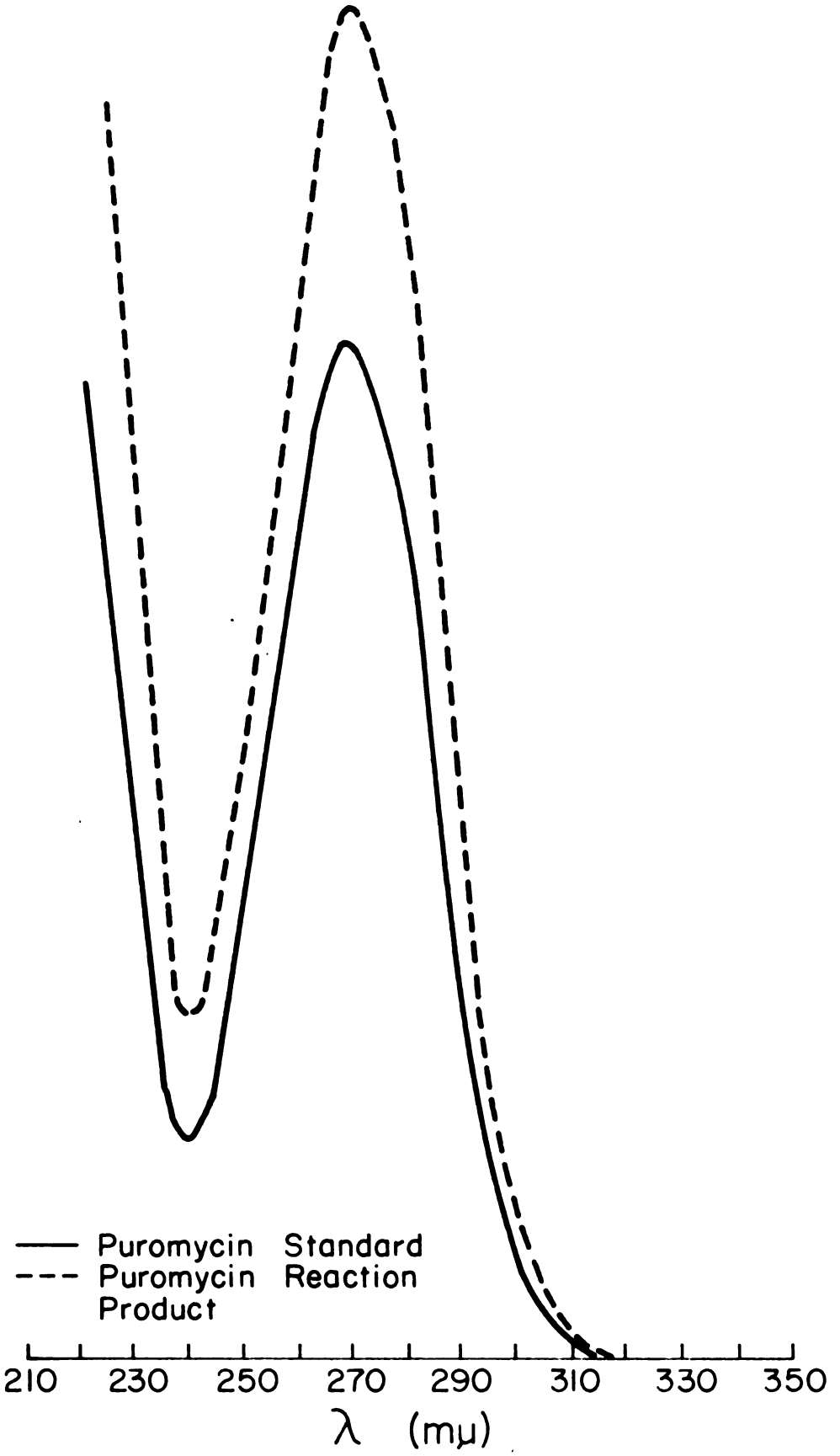


TABLE II. MASS SPECTRAL DATA

Compound	m/e							Difference
Puromycin	471	453	423	393	322	---	150	
Formyl puromycin	499	481	451	---	350	319	178	28
Acetyl puromycin	513	495	465	351	---	333	192	42

could result from the formylation of the amino group with dimethylformamide serving as the formyl donor.

To pursue the possibility of formylation having occurred at the amino group, an acetylation of puromycin was performed to obtain N-acetyl puromycin. This compound, when analyzed in the mass spectrometer should show a fragmentation pattern similar to that seen with formyl puromycin but differing by one methyl group (15 mass units). To prepare the product, 10 mg of puromycin were dissolved in a solvent system composed of 0.5 ml of dimethylformamide, 0.5 ml of dioxane, and 0.070 ml tri-n-butylamine. To this solution was added 0.003 ml of twice distilled acetic anhydride. The reaction was allowed to continue for 48 hours at room temperature after which solvent was removed under vacuum and the residue was extracted with ether. An analysis of the preparation in the mass spectometer (Table II) gave the predicted fragmentation pattern supporting the hypothesis that formylation of puromycin had occurred.

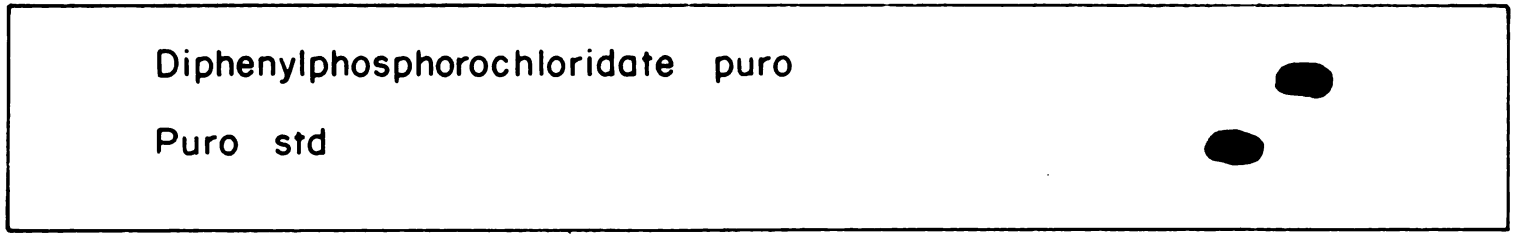
To determine when the derivatization of the puromycin occurred in the reaction sequence a reaction mixture was prepared which was complete except that the acetylated cyclic cytidylic acid was omitted. The reaction mixture contained 10 mg of puromycin, 0.010 ml of ^{3}H-puromycin, 0.02 ml of tri-n-butylamine, 0.25 ml of dimethylformamide, 0.25 ml of dioxane, and 0.010 ml of diphenylphosphorochloridate. The reaction continued for 18 hours, solvent was removed, and the

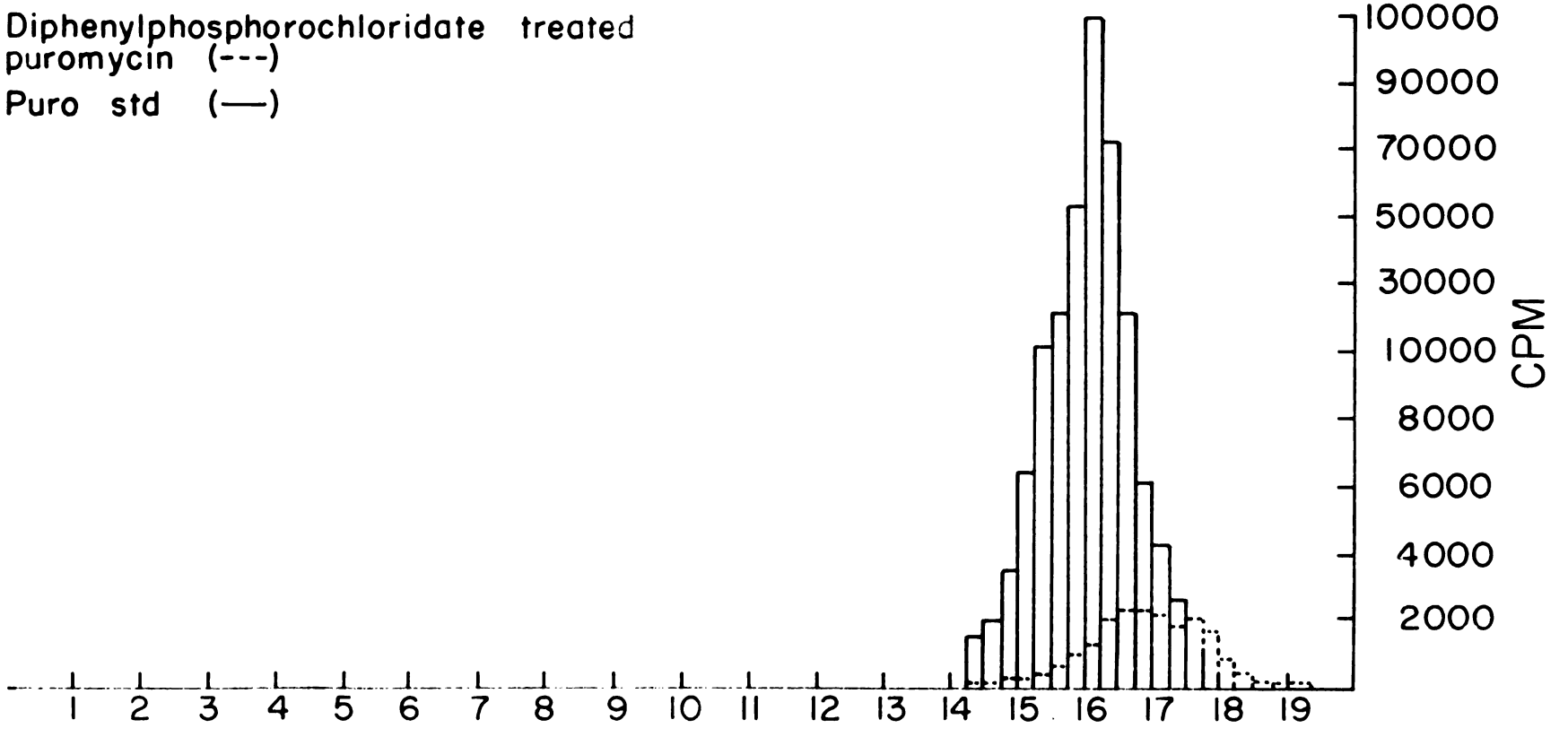
residue was extracted with ether and redissolved in water. An aliquot of this preparation was applied to a Whatman #1 paper chromatogram, and the chromatogram was developed with solvent system II. In Figure XI the diphenylphosphorochloridate-treated puromycin is found to move with a greater R_f than the puromycin standard as determined by ultraviolet absorption and radioactivity. To obtain the radioactivity profile the chromatogram was cut in 3/4 x 1/4 inch strips and counted in 10 ml of Brays Solution. These data confirmed that derivatization of puromycin, involving the α-amino group of the amino acid, occurred in the coupling reaction and did so independent of the presence of acetylated cyclic cytidylic acid.

Synthesis of the carbobenzoxy derivative of puromycin:

Since the derivatization of the puromycin molecule had been demonstrated to occur at the amino group of the amino acid, a suitable blocking group for this position was sought. The carbobenzoxy group appeared to be the method of choice because of its wide use in the field of chemical protein synthesis (62). To prepare the derivative, 95 mg of puromycin and 0.025 ml of ^{3}H-puromycin were dissolved in 20 ml of water which was adjusted to approximately pH 10 by the addition of M sodium hydroxide. To the solution was added 0.1 ml of carbobenzoxy chloride (a four-fold excess). The reaction was allowed to proceed for one and one-half hours with

Figure XI. Diphenylphosphorochloridate-treated Puromycin

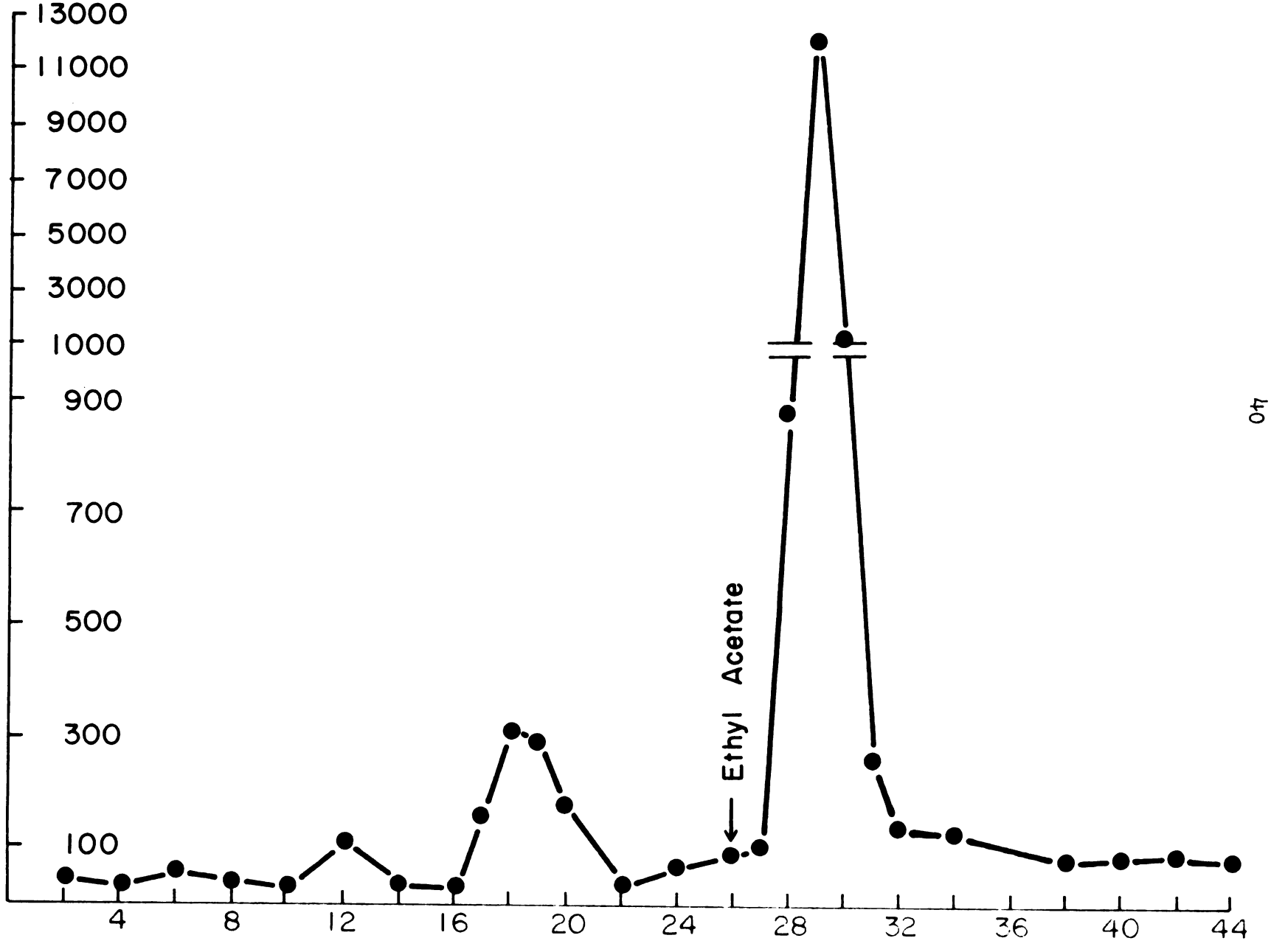




continuous adjustment of the pH with M sodium hydroxide. During the incubation a gummy white residue developed. Upon completion of the reaction, the water phase was extracted three times with 10 ml of ethyl acetate. The ethyl acetate fractions, containing 95% of the total cpm, were combined and evaporated under vacuum. The residue was then dissolved in 4 ml of chloroform and placed on a silicic acid column to remove the excess carbobenzoxy chloride. The material was eluted with chloroform and then ethyl acetate. The excess carbobenzoxy chloride was found to elute free of the column in the chloroform fractions while the majority of the radioactivity eluted immediately after the ethyl acetate was applied to the column. The elution profile which can be seen in Figure XII was obtained by counting 0.010 ml aliquots of each fraction in 10 ml of Brays Solution. Fractions (240 drops each) containing the main radioactivity peak, 79% of the total cpm, were condensed and redissolved in dimethylformamide. An aliquot of the preparation was spotted on Whatman #1 paper and developed with solvent system II. The carbobenzoxy derivative of puromycin was found to have an R_f of 0.86 as compared to an R_f of 0.75 for the puromycin standard. The Carey ultraviolet spectrum was found to be identical to that of puromycin with a wave maximum of 267.5 mμ.

To determine that the change in R_f seen with the carbobenzoxy derivative was due to the reaction with carboben-

Figure XII. Elution Profile of Carbobenzoxy Puromycin



zoxy derivative was due to the reaction with carbobenzoxy chloride and not due to the solvent system or chromatography procedure, a similar reaction mixture was prepared, but the carbobenzoxy chloride was omitted. The puromycin so treated was found to have an R_f identical to that of the puromycin standard indicating that the enhanced R_f is due exclusively to the derivatization of puromycin by carbobenzoxy chloride.

Three different methods for the removal of the carbobenzoxy group from puromycin were tested. The method involving glacial acetic acid and HBr at room temperature for 30 minutes was eliminated from consideration when chromatograms of the digestion mixture showed extensive degradation of the compound. Another method involved hydrogenation of the carbobenzoxy derivative for 30 minutes at room temperature in a solution of absolute ethanol using a palladium black catalyst. Analysis of the hydrogenation mixture by chromatographic techniques showed that the reaction was incomplete even when the incubation time was increased. The best method for the removal of the carbobenzoxy group was found to be hydrogenation of an 80% acetic acid solution of the derivative for two hours at 4° C using a platinum/charcoal catalyst. Group removal was quantitative.

Attempted synthesis of cytidylyl puromycin using the carbobenzoxy derivative:

The coupling experiments outlined earlier for the synthesis of CpPuro using diphenylphosphorochloridate as

the coupling agent were repeated using carbobenzoxy puromycin. However, problems dealing with the insolubility of the carbobenzoxy derivative soon became apparent. The derivative was soluble in the dimethylformamide: dioxane solvent system of the coupling reaction, but after the termination of the coupling reaction by the removal of the solvents with flash evaporation, it was found that the residue had to be coaxed into aqueous solution by the use of ethyl acetate as a co-solvent. The solution was then adjusted to pH 9.8 with a dilute solution of ammonium hydroxide and incubated for 48 hours at 37° C to remove the acetyl blocking groups. Upon completion of the incubation period, the solution was placed on a Dowex 1x2 column (formate form, 1.2 cm x 12 cm) at which time the incubation solution precipitated upon the top of the column. Numerous solvent systems were used but the compound would not elute from the column. Suspecting that the enhanced aromaticity of the carbobenzyoxy compound might lead to interactions with the aromatic styrene lattice of the Dowex 1x2, a QAE Sephadex column, which contains a dextrin backbone, was prepared. Once again no recovery of the carbobenzoxy compound was obtained. Hydrogenation of the reaction mixture to remove the carbobenzoxy group before placing the sample upon the column also was unsuccessful apparently due to a poisoning of the catalyst by the reaction mixture.

Further attempts to separate the components of the coupling reaction used a column procedure developed by S. Chladek (63, 64). A column of DEAE cellulose was equilibrated with 50% methanol. The reaction mixture was placed on the column and elution was with a triethylammonium bicarbonate (TEAB) gradient (0.0-0.2 M) in 50% methanol. Two distinct peaks were obtained. The first peak appeared to contain the unreacted carbobenzoxy puromycin while the second peak contained many components which could not be resolved.

Studies involving the use of a dimethylsulfoxide-dioxane solvent system:

In order to avoid the solubility problems encountered with the carbobenzoxy derivative of puromycin, a new solvent system for the coupling reaction was sought which would avoid the derivatization by the elimination of dimethylformamide from the coupling mixture. Since the dimethylformamide was thought to be the source of the formyl groups modifying the puromycin, the elimination of dimethylformamide would make the use of the carbobenzoxy blocking group unnecessary. Therefore, a coupling mixture was prepared using a dimethylsulfoxide (DMSO):dioxane solvent system after confirmation that puromycin was soluble in DMSO. After completion of the coupling reaction the acetyl blocking groups were removed by treatment with methanol saturated at 4° C with ammonia. The reaction mixture was then placed

on a Dowex 1x2 column (formate, 1.2 cm x 12 cm), and eluted with water and then with 0.01 N formic acid (Figure XIII). The fractions (240 drops/tube) that correspond to the peak which elutes immediately after the application of the 0.01 N formic acid were pooled, condensed, and characterized. The alkaline digestion and the ribonuclease digestion gave the ultraviolet spots expected from a mixture of the 2'-5' and 3'-5' isomers of CpPuro. However, a contaminant was also present that had an R_f slightly greater than that of puromycin. The contaminant was removed from the preparation by placing the sample over a Dowex 1x2 column (1.2 cm x 12 cm) which had been equilibrated with 0.03 M ammonium formate, pH 5.0. The column was eluted with a gradient of ammonium formate 0.03 M to 0.08 M, pH 5.0, and fractions of 240 drops were collected. The contaminant eluted from the column immediately at the solvent front followed by the peak containing the CpPuro. Aliquots of this peak were subjected to alkaline and ribonuclease digestion and chromatographed on Whatman #1 paper which was developed with solvent system II. In Figure XIV it can be seen that the untreated sample moves as a single spot between the standards of puromycin and cytidylic acid. The alkaline digested sample has two spots that correspond to the puromycin and cytidylic acid standards. The ribonuclease treated sample has these same spots plus another spot that has an R_f identical to that of the parent compound indicating that both

Figure XIII. Elution Profile of CpPuro DMSO Reaction Preparation

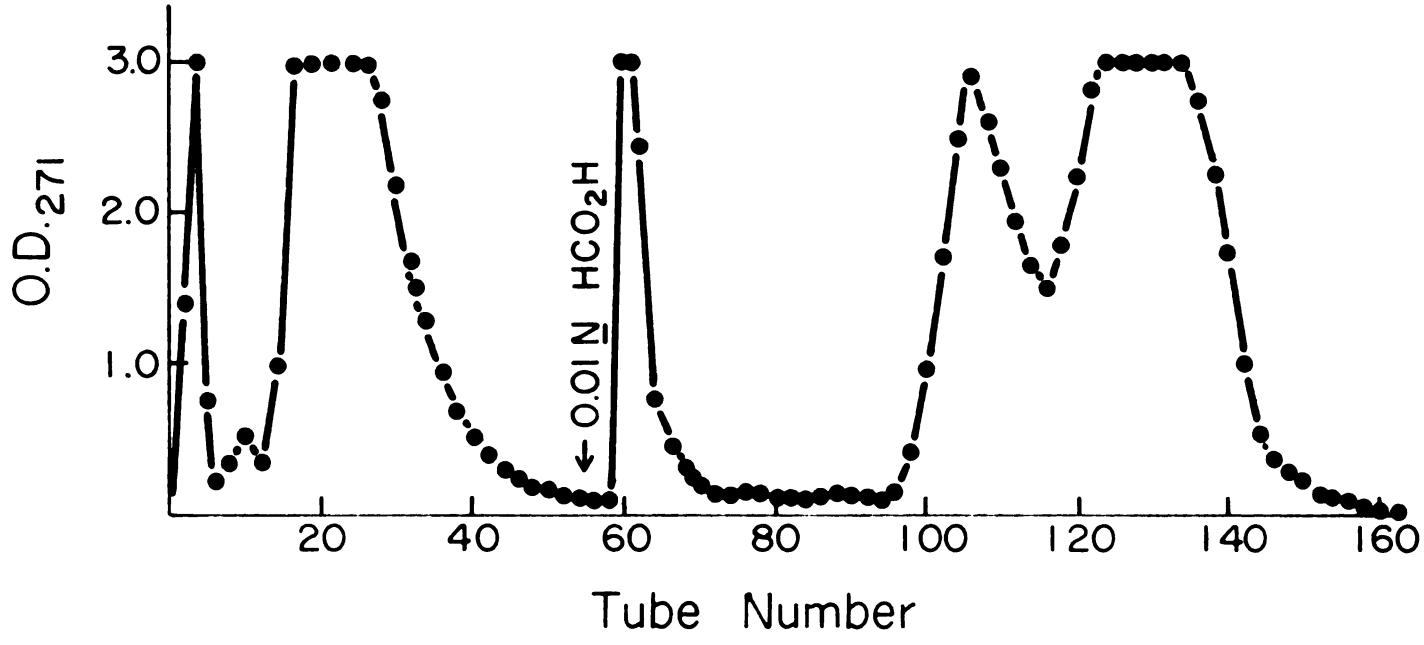
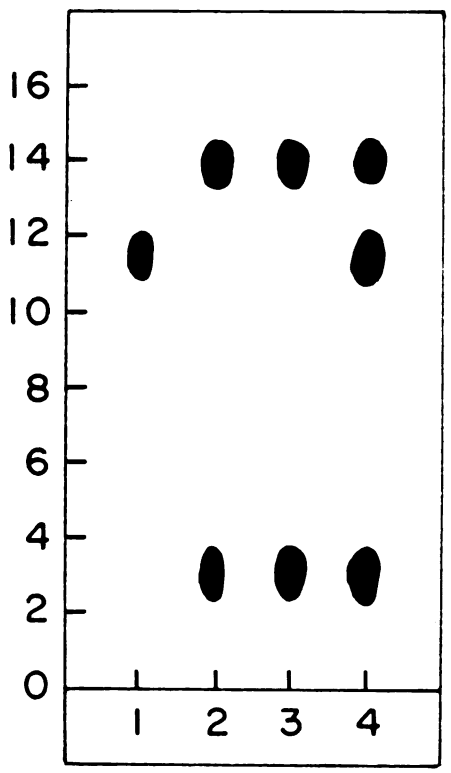


Figure XIV. Chromatography of CpPuro Digestion

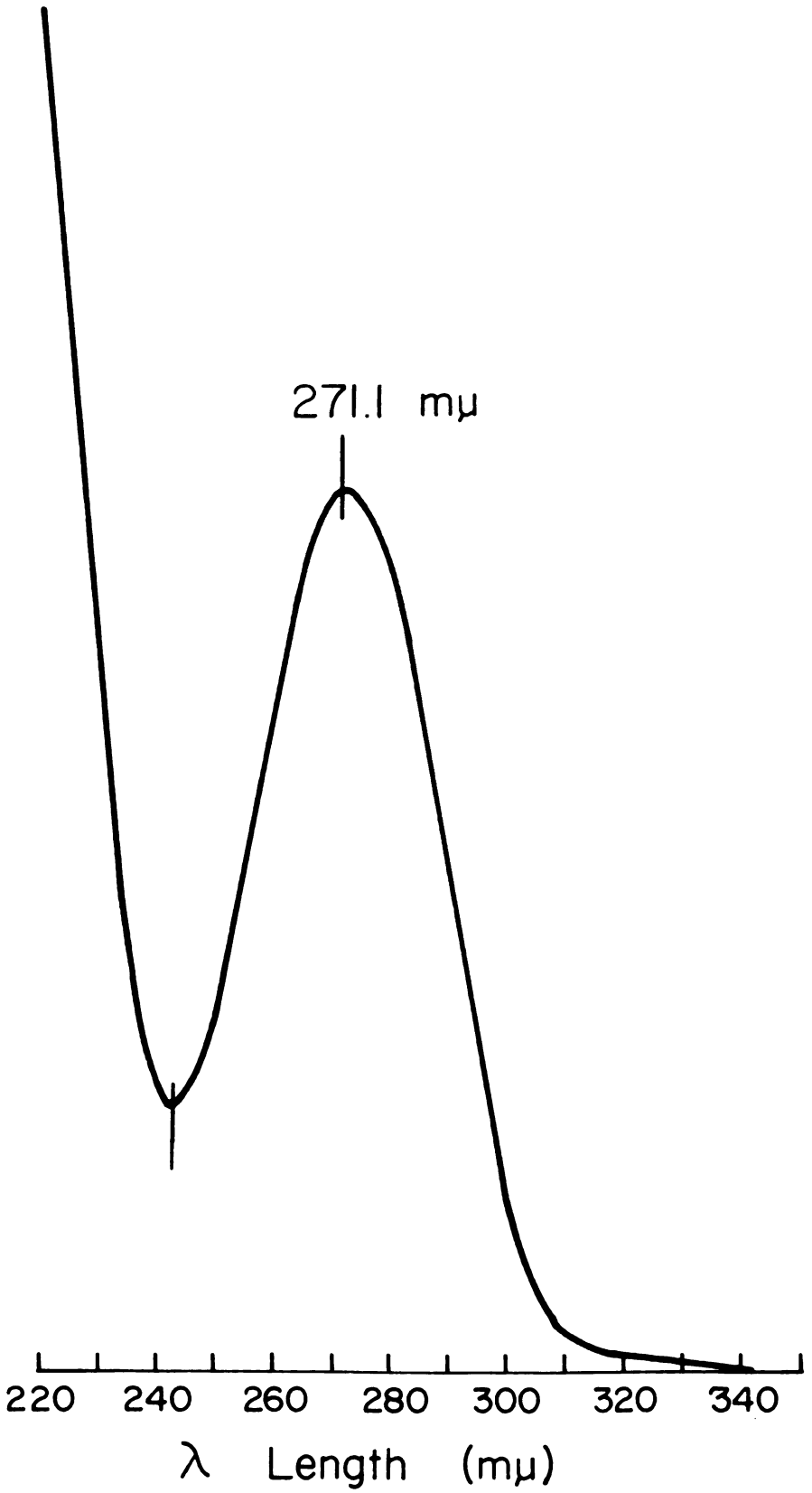


CODE

1. Pooled peak
2. Puromycin and cytidylic acid stds
3. KOH digest of peak
4. RNase digest of peak

the 2'-5' and 3'-5' isomers of CpPuro are present. The Carey spectrum of the preparation is seen in Figure XV. The wavelength maximum of the compound is 271.0 mμ which is the same wavelength maximum determined for the earlier preparations containing the modified CpPuro. Therefore, it was apparent that CpPuro had been successfully synthesized.

Figure XV. Spectrum of CpPuro



DISCUSSION

Puromycin is an antibiotic that has been well characterized and chemically synthesized. It has a mechanism of action which is related to its chemical similarity to the 3' terminus of aminoacyl tRNA. This similarity allows puromycin to substitute for aminoacyl tRNA at the receptor site of the ribosome and to cause the premature release of polypeptide chains as peptidyl puromycin. This reaction has been termed "the puromycin reaction" by R. R. Traut and R. E. Monro (65).

It has been well substantiated that the amino acid portion of the puromycin molecule is intricately involved in the puromycin reaction. Any substitution or modification of the $O\text{-}CH_3$ tyrosine moiety leads to a reduction in the antibiotic activity of the compound. It has been suggested (46) that the aromatic residue may be able to interact with the ribosomal binding site normally reserved for the cytesine ring of the 3' terminus of aminoacyl tRNA which then enhances the antibiotic activity.

The antibiotic activity of puromycin may also be influenced by the presence of 5' nucleotidyl analogues. Addition of any ribonucleotide other than cytidylic acid to glycyl puromycin was found to lead to loss of activity, whereas, the cytidylyl derivative enhanced the antibiotic

activity (46). The enhanced activity is probably due to the increased similarity between cytidylyl glycyl puromycin and the terminus of aminoacyl tRNA. In order to investigate whether or not the aromatic amino acid and the 5' nucleotidyl analogue are utilizing the same ribosomal site to enhance activity, cytidylyl puromycin was synthesized. This compound encompasses both of the structural features related to maximal activity. However, since the activity seen with both of the structural features may involve a common phenomenon, cytidylyl puromycin may be no more active than puromycin.

Before undertaking the synthesis of cytidylyl puromycin, the methods and techniques necessary for the synthesis and characterization of the compound were developed with cytidylyl adenosine, a compound which is structurally related to cytidylyl puromycin. This dinucleoside monophosphate was successfully synthesized with two different methods. The first method was based upon the works of A. M. Michelson (50-52) and involved the coupling of N^4, $O^{5'}$ diacetyl-2', 3'-cyclic cytidylic acid with 2', 3' diacetyl adenosine in the presence of diphenylphosphorochloridate. The reaction went well and in good yield as long as care was taken to insure that the cyclic linkage was preserved since it is a necessary intermediate in the reaction. The cyclic linkage is stable in the pH range 6-9 therefore reaction conditions were buffered at alkaline pH. The extent of cyclization could be

determined at any stage by an assay using alkaline phosphatase. It was determined that alkaline phosphatase is active against the 2' and 3' monophosphate esters but is inactive against the cyclic linkage. Another important factor to be considered for a successful reaction is that anhydrous conditions must be present at all times during the course of the reaction. Therefore it is necessary that all reaction solvents be redistilled and dried over calcium hydride or Linde 4-A Molecular Dieves at least several weeks prior to the reaction.

The second method for the synthesis of cytidylyl adenosine was based upon the work of R. Lohrmann and H. G. Khorana (54). It involves the reaction of the triacetyl derivative of cytidylic acid with 2', 3' diacetyl adenosine using dicyclohexylcarbodiimide as the coupling agent. Since no cyclic linkage is involved during the course of the reaction, the position of the blocking groups can be so directed as to lead to the preferential synthesis of a particular isomer. However, dicyclohexylcarbodiimide (66) is a difficult compound to handle and requires caution since it is a lachrymator. Once again anhydrous conditions were a prerequisite for a successful reaction.

Characterization of the dinucleoside monophosphates was accomplished by degradation of the compound with alkali and with pancreatic ribonuclease. Alkaline digestion is non-specific for either the 3'-5' or 2'-5' isomer and causes

a complete hydrolysis of the compound to the components. However, pancreatic ribonuclease causes hydrolysis of only the 3'-5' isomer and leaves the 2'-5' isomer intact. Paper chromatography of the digestion mixtures along with appropriate standards allows identification of the components and the determination of the presence or absence of the various isomers. Since each nucleotidyl compound has its own characteristic ultraviolet spectrum, spectral analysis is also useful in the identification of the reaction products.

Upon the completion of the preliminary work, efforts were directed towards the synthesis of cytidylyl puromycin under the same reaction conditions. Complications became apparent when it was discovered that the α-amino group of the amino acid moiety of puromycin underwent an addition reaction concomitant to the coupling reaction. The adjunct was identified as a formyl group following characterization using ultraviolet, infrared, and mass spectroscopy.

In order to avoid the side reaction, a carbobenzoxy blocking group was added to the α-amino group prior to the coupling reaction. However, the derivative and its reaction products were found to be insoluble in all but nonpolar solvents which greatly complicated the handling of the carbobenzoxy derivatives and prevented the use of the previously developed purification and characterization techniques. Problems with the removal of the carbobenzoxy group were also present. It appears as if the reaction mixtures are

capable of poisoning the catalysts used in the hydrogenation procedure.

Because of these problems, another method for the synthesis of cytidylyl puromycin was sought. Previously the adjunct on the α-amino group had been identified as a formyl group. The only source of a formyl group in the coupling reaction would be dimethylformamide. Therefore dimethylsulfoxide was substituted for the dimethylformamide under similar reaction conditions. Cytidylyl puromycin was then successfully synthesized and characterized. Yields are low (5-10%) because during the course of the coupling reaction a precipitate formed that contained approximately 50% of the puromycin (based upon radioactivity). Due to the limited solubility of the precipitate it was not characterized. The poor yield is then probably a result of a combination of the precipitation plus the steric hindrance the aromatic amino acid has been postulated to express over the 5' position. However, cytidylyl puromycin has been synthesized and can be used for further studies.

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