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INTERACTIONS OF MITOMYCIN C WITH RNA

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INTERACTIONS OF MITOMYCIN C WITH RNA

by

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ABSTRACT

INTERACTIONS OF MITOMYCIN C WITH RNA

by

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Binding of reductively activated mitomycin C (MC) to RNA was studied at various MC:RNA reaction ratios, and binding curves were similar to those for DNA-MC complexes, with binding ratios reaching almost 1 mole MC/1 mole ribonucleotide in RNA when reactions were run in .01 M salt with high MC:RNA ratios. Results also paralleled those for DNA by exhibiting higher binding to single-stranded than to double-stranded RNA under identical reaction conditions. The RNA-MC bond was quite stable to heat, repeated gel exclusion chromatography, low pH, and 7 M urea, and it protected the complex from degradation by T_1 and pancreatic ribonucleases.

Experiments with synthetic homopolymers of AMP, CMP, GMP and UMP, and with poly (U,G), indicated that the binding of MC was guanine-specific. A small amount of binding to polymers which did not contain guanine was observed in reactions run in low salt (0.01 M), but this was almost entirely suppressed when the salt concentration in the reaction mixture was raised to 0.20 M.

Reactions were carried out between MC and poly O^6-methyl GMP, poly IMP, and poly GMP in which 40% of the

guanine residues had been methylated at the N-7 position. Binding of MC was significantly inhibited only in the case of poly IMP, pointing to the 2-amino group of the guanine residues in nucleic acids as the probable major binding site of MC.

Formation of a covalent compound between mononucleotides, or GpC, and MC, to be used as a model for investigating the complex formed between MC and nucleic acids, was attempted by reductive activation of MC in the presence of the nucleotide under the conditions used for complex formation, but this was unsuccessful. RNA-MC complexes were hydrolyzed by 0.3 M KOH, and the digestion products separated by 2-dimensional thin layer chromatography or by paper electrophoresis, in another attempt to isolate a nucleotide-MC adduct, but no such adduct was isolated, and base ratios after hydrolysis and release of MC were identical to the base ratios of hydrolyzed control RNA.

Finally, poly G-MC and poly (U,G)-MC complexes were degraded by T_1 and pancreatic ribonucleases, and the products analyzed. The MC-containing products isolated were a very small amount of a labile compound with the UV spectrum of a GMP-MC adduct, and a high molecular weight, water-insoluble complex containing UMP and GMP. The isolation of large, stable, enzyme-resistant segments of polynucleotide-MC complex with high binding ratios from the original complex, which had an overall binding ratio of 0.1, suggests that the binding may be cooperative. Covalent

binding of MC to guanine may be stabilized by stacking interactions between the MC molecules. This type of noncovalent interaction may also account for the nonspecific binding observed when reactions are run in low salt.

ACKNOWLEDGEMENTS

I shall remember with deep respect and gratitude the expert guidance and continual encouragement of my research advisor, Dr. Maria Tomasz. Discussions with Dr. Carmen Mercado, Richard Pelc, and Dr. David Kaplan, were extremely helpful. Finally, many thanks to Roslyn Lipman, who provided each day in the lab both highly capable professional advice and assistance, and warm emotional support.

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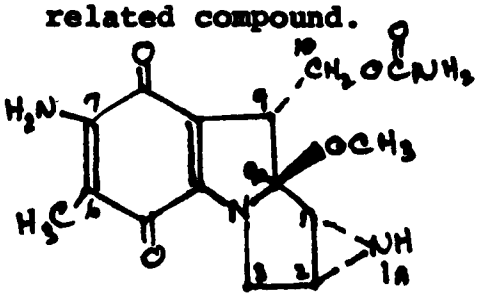
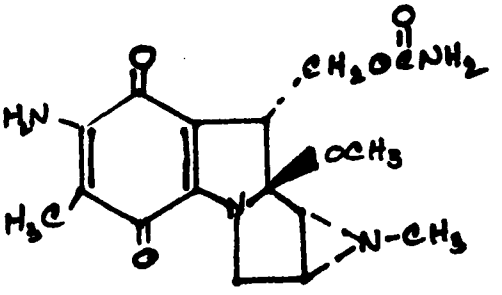
INTRODUCTION

Mitomycin C (MC) has been the most intensively studied of the mitomycin compounds, a group of potent antibiotics (Iyer and Szybalski, 1963) and antitumor agents (Wakaki, et al., 1958) produced by certain species of Streptomyces.

Its structure, determined by chemical means (Webb, et al., 1962) and by X-ray crystallography (Tulinsky, 1962), is shown below, with the structure of porfiromycin, a closely related compound.



Mitomycin C

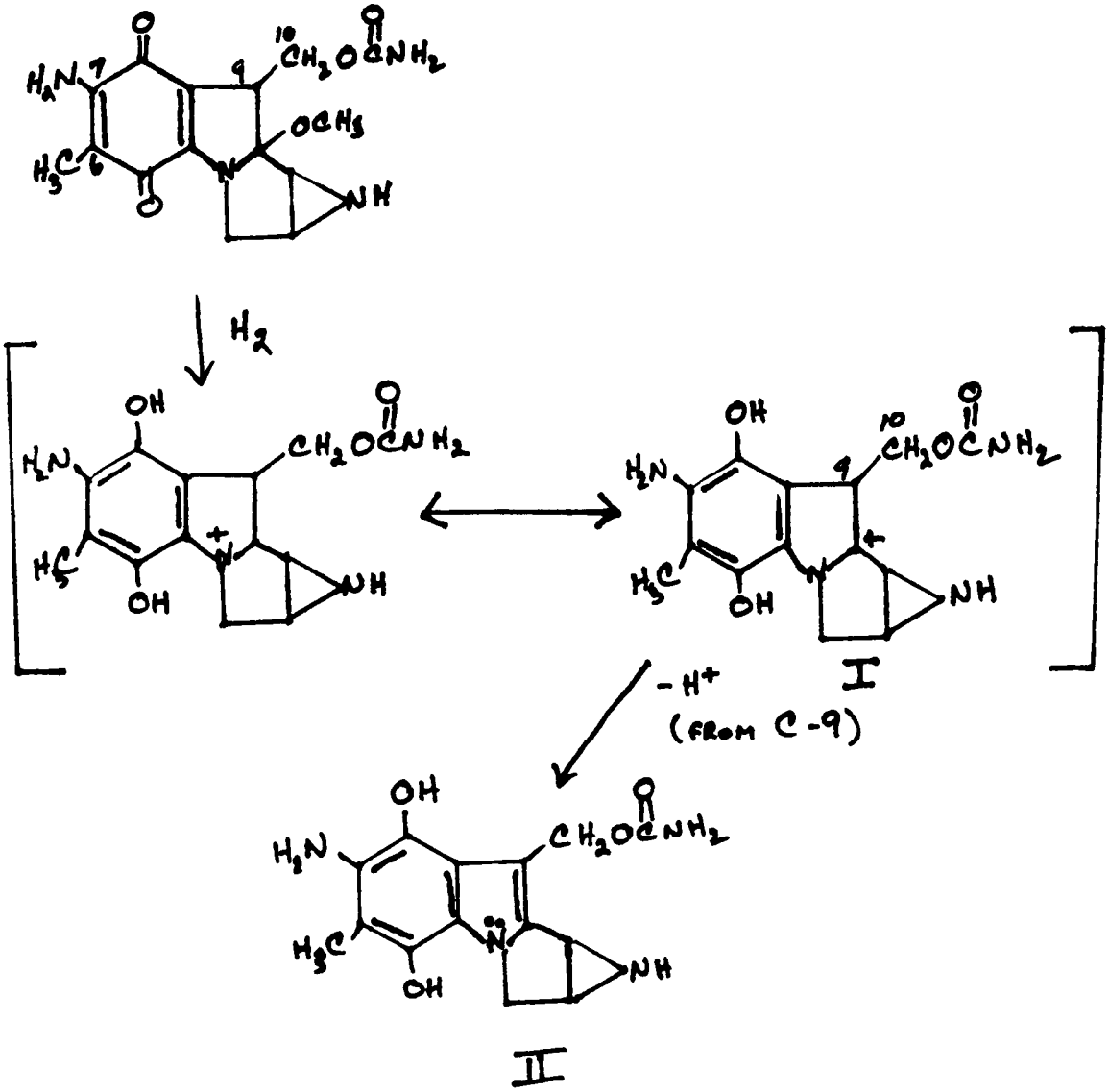


Porfiromycin

The antibiotic acts by selectively inhibiting DNA synthesis (Shiba, et al., 1959). DNA from bacteria which had been exposed to MC was found to be covalently cross-linked, on the evidence of spontaneous renaturation after heating (Iyer and Szybalski, 1963). This cross-linking of native DNA by MC has also been demonstrated in vitro, but only in the presence of either a cell extract containing NADPH or a chemical reducing agent, such as $Na_2S_2O_4$ (Iyer and Szybalski, 1964; Weissbach and Lisio, 1965). Of the total MC bound, only 10% or less is involved in bifunctional binding to create crosslinks, and the remainder appears to be monofunctionally bound (Weissbach and Lisio,

1965). Both the covalent nature of the bond, more rigorously tested by Tomasz (Tomasz, et al., 1974), and the requirement of reduction for activation make MC unusual among antibiotics which bind to DNA (Goldberg and Friedman, 1971).

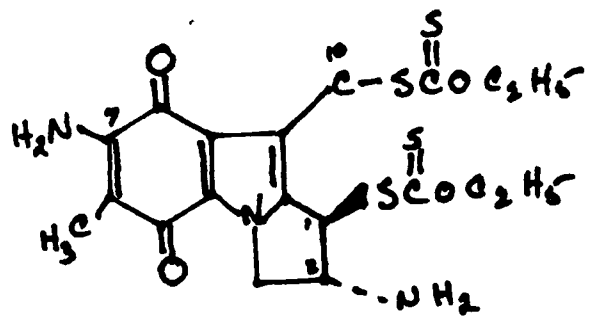
Iyer and Szybalski (1964) proposed the following mode of activation of MC:



According to their theory, the lack of reactivity, without reduction, of the usually easily cleaved (March, 1968) aziridine ring might be due to the electron-withdrawing effect of the quinone ring. Reduction of the quinone leads to the observed release of methanol and formation of the aromatic indole system (II) which can stabilize the positive charge of the transition state of aziridine ring opening. Thus, the aziridine-bearing C-1 may become a center for nucleophilic attack. They proposed the carbamate-bearing C-10 as the second center for nucleophilic attack; however, they did not rule out the 7-amino group as an alternative. Thus, in the crosslinking reaction the reduced MC intermediate could alkylate a nucleophile on each strand of the DNA.

In support of this theory, it has been demonstrated that MC analogs lacking the aziridine ring (Mercado and Tomasz, 1972) or the carbamoyl group (Otsuji and Muroyama, 1972) were somewhat less effective antibacterial agents than MC, and that several analogs lacking the aziridine ring did not crosslink DNA in vitro (Tomasz, et al., 1974; Lipman, et al., 1978).

Furthermore, Hornemann et al. (1979) have carried out reduction and reoxidation of mitomycin C in the presence of the nucleophile potassium ethylxanthate, and have isolated a product substituted at both the C-1 and C-10 positions:



1,10-Diethylxanthyl-2,7-diaminodecarbamoylmitosene

Most evidence now points to the guanine residues in nucleic acids as the site of attachment of MC. Iyer and Szybalski (1964) noted that the degree of crosslinking increased with DNA's of high G+C content. Comparisons of binding to synthetic polymers of ribonucleotides (Lipsett and Weissbach, 1965) and of deoxyribonucleotides (Tomasz, et al., 1974) showed a decided preference for poly G, or poly (U,G), and for poly dG. Later, under reaction conditions which generally increase the amount of MC binding, a positive linear relationship between increased G+C content of DNA and increased MC bound was demonstrated (Lipman, et al., 1978).

Some agents which alkylate nucleic acids are mutagenic or carcinogenic, or both; therefore, this field has been extensively studied (for example, see reviews by Lawley, 1966; Singer, 1975; Lijinsky, 1976), and a great deal of information is now available about the possible sites of alkylation of guanine in DNA and RNA.

The site in both DNA and RNA most reactive toward alkylating agents is N-7 of guanine (Lawley and Brookes, 1963), and crosslinking of DNA by bifunctional nitrogen mustards, e.g. mechlorethamine (HN2): $CH_3-N(CH_2-CH_2-Cl)(CH_2-CH_2-Cl)$ is known to involve the N-7 of guanines on opposite strands (Lawley, 1963). For these reasons, Lipsett and Weissbach (1965) suggested that N-7 is also the site of attack by MC. However, this now seems unlikely for a number of reasons. Iyer and Szybalski (1964), on the basis of experiments with models, reported that the distance between guanine N-7 sites could not be spanned by MC if the sites of attachment on MC were actually C-1 and C-10, and suggested interstrand linking between the O-6 of guanine and the amino groups of adenine or cytosine. They also noted that the thermal stability of MC crosslinking argues against N-7 alkylation, which causes thermal instability through labilization of the glycosidic bond and leads to spontaneous loss of 7-alkylated purines from DNA. The latter observation was confirmed by Tomasz, et al. (1974), who made a direct comparison of the stability of crosslinks induced by MC and by nitrogen mustard (HN2). After heating in DSC[1] for 2 hours at 60°, at pH 7.2, MC-DNA showed no loss of crosslinking, while HN2-DNA showed 76% loss.

Further evidence against N-7 alkylation was also provided by Tomasz (1970a, 1970b), who designed an assay for 7-alkylation of guanine residues in DNA based on the

fact that methylation at N-7 labilizes the C-8 hydrogen. Using DNA specifically labelled with tritium at the C-8 position of guanine, she found after alkylation with HN2 that the yield of alkylated guanine residues was almost identical with the yield of tritium released to the solvent. However, when alkylation was carried out with MC, there was no indication of release of tritium from the DNA. This would, of course, also rule out C-8 as a binding site.

Finally, Hsuing et al. (1976) found that the effect of alkylation of DNA by MC, as measured by decrease in fluorescence of intercalated ethidium, was distinctly different from that of alkylation by dimethyl sulfate or nitrogen mustard at N-7, since it was neither pH-dependent nor time-dependent.

Lawley and Shah (1972) have reported that the principal minor product of N-methyl-N-nitrosourea alkylation of RNA is O^6-methyl guanine. O^6-methyl deoxyguanosine has also been isolated from DNA, after treatment with N-methyl-N'-nitro-N-nitrosoguanidine (Lawley and Thatcher, 1970). They suggest that the weakly nucleophilic O-6 position can be alkylated by nitroso compounds and possibly by other alkylating reagents which are believed to react in part through an S_N1 mechanism, but probably not by S_N2 reagents such as dimethyl sulfate. O^6-methyl guanine is unstable under the conditions for acid hydrolysis of nucleic acids and rapidly hydrolyzed at pH 1, but stable at pH 2 at 23^o. It is

relatively stable to KOH digestion of RNA and to enzymatic digestion (Lawley and Thatcher, 1970).

The yield of guanine residues alkylated at N-1, N-3, or the 2-amino group by the above reagents is relatively low (Lawley, et al., 1972; Lawley and Shah, 1972; Singer, 1975). However, a number of bulky polycyclic aromatic carcinogens bind covalently to the 2-amino group of guanine, as well as to other sites in both DNA and RNA. These include derivatives of benzo[a]pyrene, benz[a]-anthracene, and 2-acetamidofluorene.

Benz[a]pyrene, which is now believed to bind to DNA as its 7,8-dihydroxy 9,10-epoxide derivative (Osborne, et al., 1976; Jennette, et al., 1977) binds to guanine residues primarily at the N-2 position (Weinstein, et al., 1976; Koreeda, et al., 1976; Jeffrey, et al., 1977; King, et al., 1979), and possibly also forms phosphotriesters with nucleic acids (Koreeda, et al., 1976). The adducts formed are stable to lengthy enzymatic hydrolysis and chromatographic analysis (Feldman, et al., 1980).

Derivatives of benz[a]anthracene also form a number of adducts with guanine residues, all bound to the 2-amino group (Dipple, et al., 1971; Lieberman and Dipple, 1972; Jeffrey, et al., 1976). These adducts are stable to conditions which convert nucleosides to bases (0.1 N HCl at 100°, 15 minutes for deoxyribosides; 1.0 N HCl at 100°, 1 hour for ribosides).

Dipple, et al. (1971) noted that 7-bromomethylbenz [a]-anthracene in dimethylacetamide reacts with N-7 of guanosine, while in aqueous solution the reaction apparently involves an intermediate of greater ionic character which carries out an electrophilic attack on the 2-amino group in preference to the N-7. They likened this to the reaction of acylating agents with guanine (Shapiro, et al., 1969), in which the amino group is attacked in preference to the ring nitrogen, leading to an electrically neutral species through loss of a proton.

Some binding of 2-acetamidofluorene to N-2 of guanine has also been observed, although its primary site of attachment is C-8 (Kriek, 1972; Grunberger and Weinstein, 1979).

Jeffrey, et al. (1976) and Pulkrabek, et al. (1977) have suggested that a large molecule can attack and bind to the 2-amino group of deoxyguanosine in native DNA with little distortion of the helix conformation, since it is relatively exposed in the minor groove of the helix.

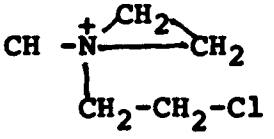
Another site in nucleic acids which may be attacked is the phosphodiester bond. As is the case with alkylation of the guanine O-6, it appears that alkylating reagents which react by a mechanism having at least some S_N1 character, such as nitrosoureas or ethyl methanesulfonate, are able to form phosphotriesters (Lawley and Thatcher, 1970). Because mutagenicity and carcinogenicity of alkylating reagents appears to increase as the S_N1 character of their reactions increases, the nature of these phosphotriesters

and the ability of various reagents to form them has been a subject of great interest.

Phosphotriesters in DNA are relatively stable at neutral pH (Rhaese and Freese, 1969; Bannon and Verly, 1972). These phosphotriesters are resistant to the activity of DNase I, DNase II, micrococcal nuclease, S1 nuclease, venom exonuclease, and spleen exonuclease (Bannon and Verly, 1972; Jensen and Reed, 1978). However, alkaline treatment of DNA containing phosphotriesters rapidly leads to strand breakage (Shooter and Merrifield, 1976; Shooter, 1976).

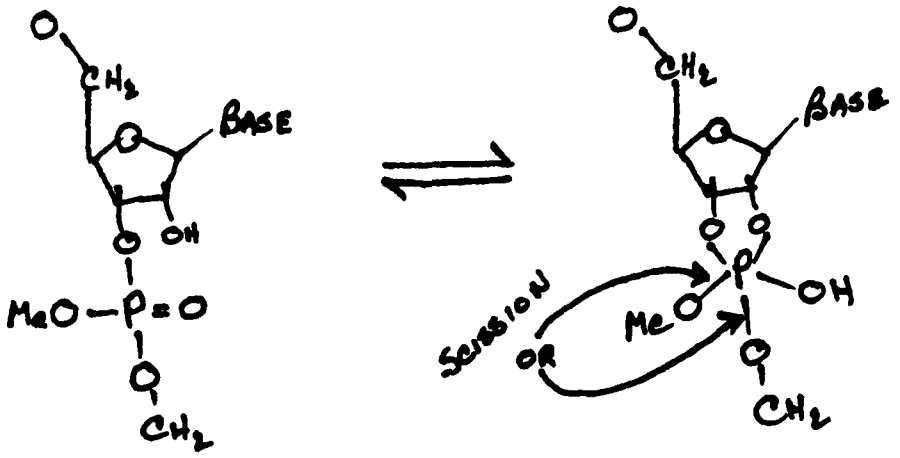
In RNA, on the other hand, the formation of phosphotriesters may cause strand breakage (Ludlum, 1969; Brimacombe, et al., 1965; Lawley and Shah, 1972; Shooter, et al., 1974a and 1974b; Shooter, 1975).

Shooter (1975) devised an assay for mutagenicity and carcinogenicity of alkylating agents based on their ability to cause breakage of a single-stranded RNA bacteriophage, R17. He found that, although there was no degradation of the RNA by sulfur mustard, the nitrogen mustard HN2 did cause breakage, possibly by reacting in the form of an immonium ion (protonated aziridine ring) after loss of chloride ion:



The hydrolysis is thought to proceed through a cyclic intermediate followed by a scission which, depending on the

cleavage site, may either release the alkyl group or cause a strand break (Shooter, et al., 1974a).



Hydrolysis of methyl triesters formed was complete within the time scale for the alkylation reaction, 30 minutes at 37°.

However, the stability of the phosphotriester may be influenced by the size and complexity of the alkyl group. Shooter, et al. (1974b) found that phosphotriesters involving an ethyl or isopropyl group hydrolyzed more slowly than methyl triesters, but attributed this to a decrease in pH during the reactions with the larger groups. The isopropyl triester has a 1/2 time for hydrolysis of 10-13 hours at pH 7, which was decreased to 1 hour at pH 9.

Sun and Singer (1975) reported that when the alkylating agent was ethyl nitrosourea, the major site of alkylation in the DNA of HeLa cells was the O-6 of guanine, and about 70% of the total alkylation was in the form of phosphotriesters, as opposed to 20% or less for dimethyl sulfate or

ethylmethane sulfate. Similarly, about 60% of alkylation of RNA by this reagent was in phosphotriesters (Singer and Fraenkel-Conrat, 1975) which appeared to be quite stable. The ethyl ester bond of the triester was more labile than the ribose-phosphate bonds.

A convenient model for studying the position of the MC-guanine bond in DNA would have been guanine or guanosine bound to MC, which could be studied by NMR, UV, and mass spectroscopy. However, despite the covalent nature of the bond in DNA, MC did not bind to guanine, guanosine or GMP under conditions leading to complex formation with polynucleotides. Attempts to isolate a crosslink or a guanine-MC adduct by acid hydrolysis or depurination of DNA-MC complexes were likewise unsuccessful, leading to loss of MC and recovery of adenine and guanosine in the same ratio as control, with no evidence of modification of these bases by MC binding. This suggested that the bond to MC was unstable to acid. However, the MC-DNA complexes had proven quite stable to base. Enzymatic degradation of DNA-MC complexes by pancreatic DNase had shown that the MC-bound areas of DNA were resistant to enzymatic hydrolysis, but has produced a mixture of MC-containing DNA oligomers of heterogeneous size (Sarker, 1967; Tomasz, et al., 1974).

At the time this study was begun, Tomasz had recently discovered that binding could be increased from 50 or more nucleotides per molecule MC to only a few nucleotides per molecule MC in the in vitro reaction by changing the method

of reductive activation of the antibiotic. In the new method, the reducing agent was added in five aliquots at 5-minute intervals, instead of all at one time, so that most of the binding took place in the presence of excess unreduced MC (Tomasz, et al., 1974). With this new method available, it seemed worthwhile to try once more to isolate a model compound from a reaction between MC and a mononucleotide.

If this was again unsuccessful, an investigation of RNA-MC complexes seemed promising. No particular biological significance had been claimed for MC binding to RNA, and the antibiotic does not crosslink double-stranded RNA. However, it had been shown to bind to poly G (Lipsett and Weissbach, 1965) and to ribosomal RNA (Tomasz, et al., 1974) in vitro, and if the MC-RNA bond proved to have the same stability as the MC-DNA bond, there might be several advantages in this approach. Since the bond to MC appeared to be stable to base, it might be possible to isolate a nucleotide-MC adduct from an alkaline hydrolysate of RNA-MC complex. Furthermore, it seemed that enzymatic degradation of RNA-MC complexes might produce better-defined MC-containing oligomers, since the RNA can be cleaved at specific bases by readily available endonucleases.

It was with these objectives that this study was undertaken.

MATERIALS

Mitomycin C was obtained from Kyowa Hakko Kogyo Co., Tokyo, Japan.

Ribosomal RNA, 30% 16S and 70% 23S, and transfer RNA, both from E. coli K-12 MO, were purchased from Miles Laboratories, Elkhart, Indiana. Double-stranded RNA, f2 phage replicative form, was the gift of Dr. N. Zinder, Rockefeller University, New York. Its double-stranded conformation was confirmed by its resistance to digestion by bovine pancreas ribonuclease. Additional studies of binding to transfer RNA were carried out with yeast transfer RNA, purchased from Schwartz Bioresearch, Orangeburg, N.Y. [^{32}P]-labelled ribosomal RNA from B. subtilis was prepared in Dr. Rivka Rudner's laboratory according to the procedure published by Pace, et al. (1973).

The synthetic polyribonucleotides poly A, poly C, poly U, poly I, poly G and poly (U,G) were purchased from Biogenics Research Corp., Chagrin Falls, Ohio.

Poly O^6-methyl GMP was the gift of Dr. David B. Ludlum, Albany Medical College, prepared by his published procedures (Gerchman et al., 1972; Mehta and Ludlum, 1976). Alkaline hydrolysis of a small sample of this polymer and chromatography of the hydrolysate on cellulose thin-layer plates (Eastman) in isopropanol:NH_4OH:H_2O (7:1:2, v/v/v) gave a single spot with the blue fluorescence character-

istic of O^6-methyl guanine and its derivatives, and migrating well ahead of a GMP standard, which was not fluorescent.

Poly G in which about 40% of guanine was methylated at the N-7 position was prepared by the method of Michelson and Pochon (1966). Extent of methylation was determined by acid hydrolysis of the polymer, chromatography of the digest on Whatman 40 paper (descending method) in methanol: conc. HCl:H_2O (7:2:1, v/v/v), elution of guanine and 7-methyl guanine from the paper with 0.1 N HCl, and quantitation of the material eluted by its UV absorbance at 250 nm (7-methyl guanine) or 248 nm (guanine). (Brookes and Lawley, 1963)

[1a-^{3}H]Porfiromycin (p. 1) was prepared by methylation of mitomycin C with [^{3}H]methyl iodide (Amersham), by a procedure published by Stevens, et al. (1965). Excess methyl iodide was added to MC dissolved in acetone, and refluxed at 61°, in the presence of anhydrous K_2CO_3, for 4.5 hours. The product was purified by descending chromatography in 1-propanol:1% NH_4OH (2:1, v/v) on Whatman 40 paper, and eluted from the paper in the same solvent.

Ribonuclease T_1, bacterial alkaline phosphatase, and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp., Freehold, N.J. Bovine pancreas ribonuclease was from Schwartz Bioresearch, Inc.

METHODS

(1) Preparation of Nucleic Acid-Mitomycin C Complexes

A. In the standard method of complex preparation, mitomycin C, from a stock solution in 30% aqueous methanol (Spectrograde) kept at -15° in the dark, was added to a 0.67 mM solution of RNA in 0.017 M phosphate buffer, pH 7.4, in a small reaction flask. The flask was then stoppered, wrapped in foil to protect it from light, and the reaction mixture deaerated by bubbling helium (Matheson "research grade," 99.9999% purity) through it for 20 minutes. The reducing agent, $Na_2S_2O_4$, was dissolved in similarly deaerated sterile distilled water in a second flask. The required amount of reducing agent, 1.5 micromoles/micromole MC, was added by syringe to the reaction mixture in five aliquots at 5-minute intervals, while helium was continuously bubbled through. Reduction of MC was evidenced by a change in color from purple to pale yellow, which was reversed when the mixture was again exposed to air. To determine the effect of varying ratios of MC to RNA in the reaction mixture, the concentration of RNA was held constant in each preparation, and the concentration of MC changed.

B. Reactions between synthetic polynucleotides and [1a-^{3}H]porfiromycin, reaction ratio 1 micromole MC:1 micromole nucleotide, generally followed the above procedure, except that reactions were run at 37° and the buffer used was Tris-HCl, pH 7.4, with parallel reactions for each done

in two buffer concentrations: 0.01 M and 0.20 M. In the case of poly O^6-methyl GMP there was enough polynucleotide only for one reaction with [1a-^{3}H]porfiromycin, which was done in 0.01 M Tris. The reaction with poly (7-methyl GMP, GMP) was somewhat different: reaction ratio was 2 micromoles MC: 1 micromole nucleotide, and it was done with non-radioactive MC in 0.20 M Tris only. Extinction coefficients for the homopolynucleotides were provided by the manufacturer. For the other polymers they were determined by an assay for nucleotide phosphate (Ames and Dubin, 1960) and were: poly (U,I) (U:I ratio 1.0:1.14), 7,600 at 260 nm; poly O^6-methyl GMP, 9,500 at 249 nm; poly (7-methyl GMP, GMP) (40% methylated), 10,200 at 260 nm; and poly (U,G) (U:G ratio 1.0:1.02), 7,600 at 260 nm.

C. Chromatography on Sephadex G-100 in SSC[1] to separate the complex from unbound drug was a standard step in the procedure. It was also useful in determining the stability of the bond to MC. Complexes of known binding ratio were subjected to conditions which might be expected to release MC if it were not covalently bound, including repeated chromatography alone, or exposure to heat, acid, or urea, followed by rechromatography on Sephadex. The binding ratio of the material recovered in the complex fraction was then redetermined.

D. To determine the binding ratio of each complex, expressed as moles MC per mole nucleotide, the nucleotide

concentration was found by the phosphate assay of Ames and Dubin (1960) and the MC concentration by its UV absorbance at 310 nm (extinction coefficient 11,500) or, when [1a-^{3}H] porfiromycin was used, by radioactivity counted in Aquasol (New England Nuclear) in a liquid scintillation counter. In a few instances when there was insufficient material for the phosphate determination, binding ratios were calculated from UV absorbance at 260 nm for nucleic acid and at 310 for MC. An adjustment was first made by subtracting 260 nm absorbance due to MC, determined from its UV spectrum to be 110% of its absorbance at 310 nm.

E. In attempts to bring about complex formation between 5'-mononucleotides (or GpC) and MC, the standard procedure for complex formation was followed, except that the solvent was water rather than buffer. The reaction ratio was usually 5 micromoles MC: 1 micromole nucleotide. After reduction of MC in the presence of nucleotide, the reaction mixture was concentrated and chromatographed on Whatman 40 sheets (descending method) or on cellulose or silica gel thin layer plates. Solvent systems used were: (A) isobutyric acid: 0.5 M NH_4OH (10:6, v/v); (B) 1-propanol:conc. NH_4OH:H_2O (6:3:1, v/v/v); (C) ammonium acetate:ethanol (3:7, v/v); and (D) 1-propanol: 1% NH_3 (2:1, v/v).

(2) Determination of Base Ratios after Alkaline Hydrolysis of RNA-MC Complexes

A. Base ratios of nucleotides recovered after alkaline hydrolysis of RNA:MC complexes were compared to those of RNA controls. Because initial results with nonradioactive RNA were somewhat ambiguous, further experiments were carried out with [^{32}P]-labelled ribosomal RNA. In both cases, the method of hydrolysis was incubation in 0.3 M KOH at 37^{o} for 18 hours. The digests were neutralized either with perchloric acid or by adding a few beads of Dowex 50-X8 resin, 20 to 50 mesh, H^{+} form (Bio-Rad), stirring until neutralized, and filtering out the resin.

B. A number of chromatographic methods were investigated for separating the components of the nonradioactive alkaline digests. They included separation of nucleotides on Sephadex G-10 and G-25 (fine) columns in SSC; on Bio-Rad AGI-X8, 200 to 400 mesh, formate form, in 0.25 M ammonium formate, pH 3.3 (Singhal, 1974); and on polyethyleneimine thin layer plates (Brinkmann Instruments) in 1.0 M acetic acid followed by 0.3 M LiCl (Randerath and Randerath, 1967) or on Whatman 40 sheets in Solvent A (Wyatt, 1955); as well as degradation of the nucleotides to nucleosides by alkaline phosphatase, and subsequent chromatography on Sephadex G-10 in 0.13 M ammonium formate, pH 6 (Uziel, et al., 1968). However, a more effective method than any of these was two-dimensional thin layer chromatography on cellulose thin layer plates (Eastman; without fluorescent indicator, on

plastic backing), first in solvent A; then in 2-propanol: conc. HCl: H_2O (68:17:15, v/v/v) (Nishimura, et al., 1967). Nucleotide spots were located under UV, cut from the plates, with appropriate blanks, eluted overnight in 1.2 ml 0.01 N HCl, and the eluants centrifuged to remove gel particles. UV absorbance was determined for each nucleotide at the absorbance maximum for that nucleotide at pH 2, and the molar ratios were calculated from extinction coefficients.

C. Paper electrophoresis was used to determine base ratios of hydrolyzed $[^{32}P]$-labelled rRNA in a control and in two RNA-MC complexes of binding ratios .127 and .180. Aliquots of hydrolysate, mixed with cold carrier 2' and 3' mononucleotides, were spotted on Whatman 3 paper and subjected to electrophoresis, with cold nucleotide standards, in 0.02 M citrate buffer, pH 3.5, for four hours at 77 V/cm. Spots were then located under UV, and the areas of the electropherogram containing the radioactive hydrolysate were cut into small strips, added to scintillation vials containing toluene-PPO-POPOP, and the cpm counted in a liquid scintillation counter.

The radioactivity in the CMP area, close to the origin, was extraordinarily high in both control and complex hydrolysates. Electrophoresis of hydrolyzed $[^{32}P]$rRNA in 0.05 M Na_2HPO_4 buffer, pH 7.2, for 2.5 hours, which moved all mononucleotide standards well away from the origin, left a residue of radioactive material (about 20% of total radioactivity) at the origin, presumably a phosphorylated non-

nucleic acid compounds. Base ratio calculations were therefore adjusted to exclude radioactivity due to this material.

(3) Enzymatic Digestion of Polynucleotide-MC Complexes

A. The preliminary study of enzymatic degradation of an rRNA:MC complex was analagous to the procedure of Horwitz (1971). 2.0 ml of rRNA (5.2 A_{260} units) or rRNA-MC complex (binding ratio 0.5) (8.2 A_{260} units, 3.8 A_{310} units) in DSC, pH 7.4, was placed in a dialysis bag with either 30 units of T_1 ribonuclease or 300 units of bovine pancreatic ribonuclease, or both, and dialyzed against 10 ml of DSC at room temperature for 24 hours. UV absorbance of material remaining in the bags was determined, a second aliquot of enzyme was added, and dialysis continued vs. fresh DSC for an additional 24 hours, when the absorbance of the material remaining in the bag was again determined.

B. Enzymatic digestion of poly G and poly G-MC complex followed the procedure of Uchida and Egami (1967), with the adaptation that 7 M urea was added because, according to Warrington (1974), this tends to enhance the action of ribonuclease T_1. (Preliminary tests showed slightly increased efficiency of digestion by T_1 in urea over digestion in aqueous buffer.) A typical reaction mixture contained 30 A_{260} units of poly G in 2.5 ml 7 M urea, to which had been added 2.5 ml 0.2 M Tris-HCl, pH 7.5, 1.0 ml 20 mM EDTA, 3.0 ml 7 M urea, and 1.0 ml aqueous

solution containing 3,200 units of T_1. A few drops of $CHCl_3$ were added to inhibit bacterial growth, and the mixture incubated 24 hours at 37°.

As a control for separation of guanylate oligomers on DEAE-cellulose, poly G was partially hydrolyzed by incubation in 0.1 M KOH at 70° for 10 minutes (Bock, 1967), and neutralized with HCl before being applied to the column.

C. For enzymatic digestion of small amounts of poly (U,G) and poly (U,G)-MC complex, a typical reaction mixture contained 10 A_{260} units poly (U,G) in .45 ml H_2O, to which was added 0.25 ml 0.2 M Tris-HCl, pH 7.4; 0.10 ml 0.2 mM EDTA, 0.10 ml T_1 solution containing 300 units, and 0.10 ml pancreatic RNase solution containing 240 units, and a few drops $CHCl_3$. The digest was incubated at 37° for 24 hours (procedure for digestion by pancreatic RNase from Billeter and Weissman, 1966).

D. For removal of terminal phosphate from oligonucleotides, bacterial alkaline phosphatase was added in a ratio of 0.1 mg enzyme/1.0 mg nucleotide to the oligonucleotide in 5 mM $MgCl_2$, 0.2 M Tris-HCl, pH 8.6, and the mixture was incubated at 37° overnight (Parish, 1972).

E. Some fragments from endonuclease digests of poly (U,G)-MC complex were subjected to further digestion by snake venom phosphodiesterase. Samples in 0.01 M Tris, pH 9, were heated in a 90° water bath 3 minutes to minimize aggregation, transferred to a 37° bath for two minutes, and bacterial alkaline phosphatase was added to an enzyme-

substrate ratio of 1:10. The mixture was incubated at 37° for 30 minutes. $MgCl_2$ was added to 0.002 M and snake venom phosphodiesterase to an enzyme-substrate ratio of 1:10, and the mixture was incubated at 37° for 60 minutes. (Min Jou and Fiers, 1969)

F. DEAE-cellulose (Cellex D, Bio-Rad) columns in 7 M urea, pH 7.8, were prepared and samples applied as described by Tener (1967). Urea was removed from fractions on a column of DEAE-cellulose, bicarbonate form, by the method of Rushizky and Sober (1962).

DEAE-Sephadex A25 (Pharmacia) was washed with 2 M NaCl, then with distilled water until Cl^- free. It was then equilibrated with the 7 M urea buffer for at least 2 days. Columns in 7 M urea, 0.01 M Tris-HCl, pH 7.4, .0002 M EDTA, were prepared and digests eluted with linear gradients of guanidinium chloride according to the method of Olson and Volkin (1972), but at ambient temperature.

Nucleotides and nucleosides released from enzymatic digests were identified by their elution volume on a Sephadex G-25 (Fine) column which had been calibrated for this purpose, and by their ultraviolet spectra obtained by using a Cary 219 spectrophotometer.

[1] Abbreviations used: MC, mitomycin C; PM, porfiromycin; SSC, standard saline citrate buffer (.15 M NaCl and .02 M citric acid, pH, 7.4); DSC, SSC diluted tenfold with distilled water.

(1) Attempts to Bring about Binding between MC and Nucleotides, or the Dinucleoside Monophosphate GpC

Paper and thin layer chromatography of the reaction mixture of MC reduced in the presence of mononucleotides or of GpC showed no mixture components other than those in the controls: nucleotide alone, and MC reduced in the absence of nucleotide. Variations in the reaction procedure, such as reducing only a portion of the MC so that the reaction was run in the presence of unreduced MC, reversing the reaction ratio to 1 micromole MC/5 micromoles nucleotide, using sodium borohydride instead of sodium dithionite as the reducing agent, or chromatographing a larger quantity of the reaction mixture on Sephadex G-25 rather than on paper or thin layer plates, also failed to show any evidence of a mononucleotide-MC adduct. As a further check, a mixture of MC reduced in the presence of GpC was cochromatographed on paper with a mixture, in the same concentrations, of GpC plus unreduced MC, in solvent systems B and C, and the GpC then quantitively eluted from the paper with .017 M phosphate buffer, pH 7.4. Recovery of GpC from reaction mixture and control was approximately equal. (Table 1)

Table 1. Recovery of GpC from Reaction Mixture after Reduction in the Presence of MC

Volume Applied to Paper	A_{260} Units			
	Solvent B*		Solvent C*	
	Reaction	Control	Reaction	Control
50 microliters	.393	.387	.361	.362
100 microliters	.612	.639	.595	.633
100 microliters	.620	.641	.615	.625

* See Methods, p. 17.

(2) Binding Curves: RNA-MC Complexes

Complexes between MC and three forms of RNA were produced, isolated, and binding ratios determined as described in Methods. Various ratios of MC to RNA were tested for each form of RNA. Results are tabulated in Table 2. Figure 1 compares these binding curves to each other and to binding of MC to native and denatured DNA under the same reaction conditions. Single-stranded nucleic acids are more effective in binding the drug, with native DNA and dsRNA binding only about half as much MC as denatured DNA and rRNA, respectively. Transfer RNA binds somewhat less than rRNA.

Binding to tRNA is not strictly comparable to binding to rRNA at reaction ratios above 5 micromoles MC/1 micromole nucleotide. During isolation of complex from unbound drug on Sephadex G-100, rRNA, and consequently all rRNA-MC complexes, were of sufficiently high molecular weight to be totally excluded. The tRNA, and some tRNA-MC complexes, were retained by the column (V_e/V_o = 1.4). However, when the reaction ratio was higher than 5 micromoles MC/1 micromole nucleotide, two complex peaks were eluted from the column, one in the same volume as the rRNA complex and the second in the usual volume for tRNA (Figure 2). The higher molecular weight complex had a binding ratio close to 1.0.

Table 2. Binding Ratios of RNA-MC Complexes

micromole MC/ micromole nucleotide in reaction		Binding Ratio (Mole MC/Mole RNA-P) E. coli tRNA	E. coli rRNA	f2 dsRNA
1.0		.065	.068	.043
2.0		.109	.149	.071
3.0				.123
5.0		.255	.427	
10.0	Peak I	.909	.720	
	Peak II	.357		

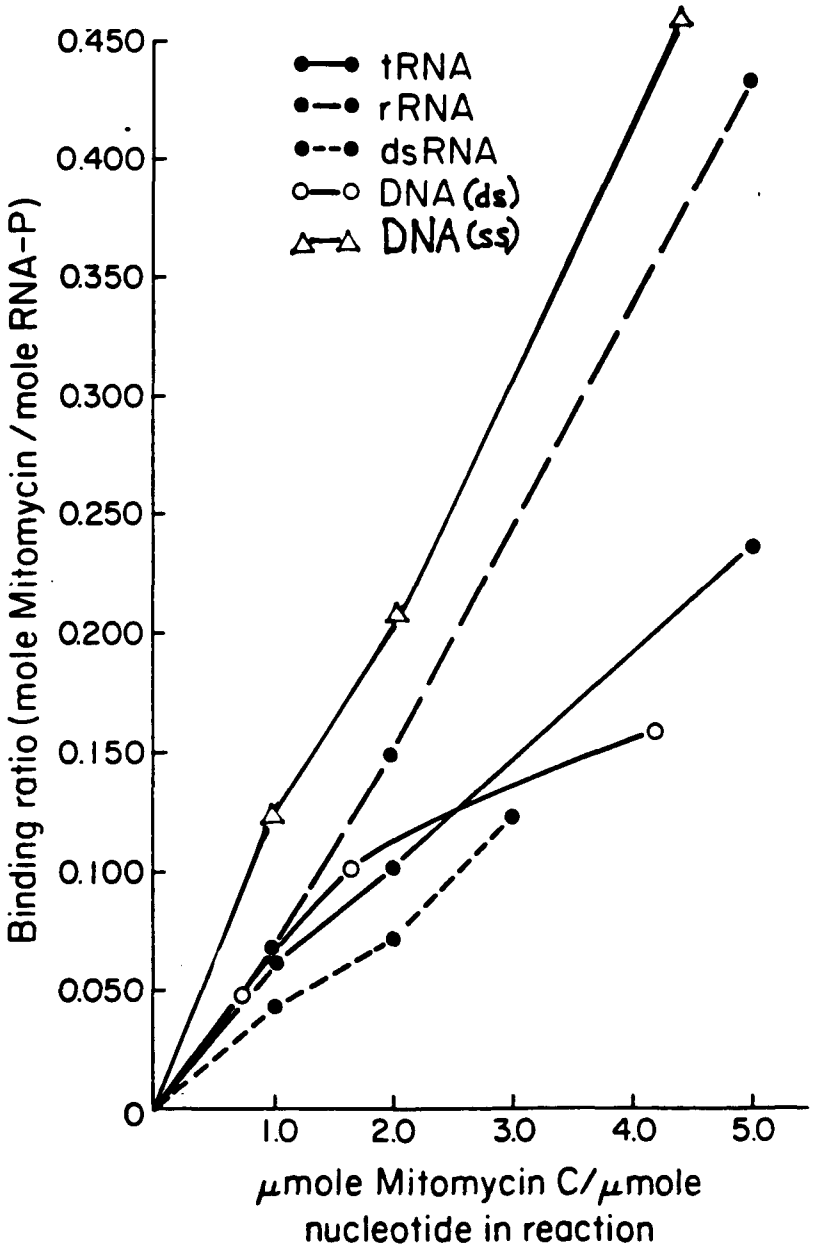


Figure 1 **Nucleic Acid-MC Binding Curves**

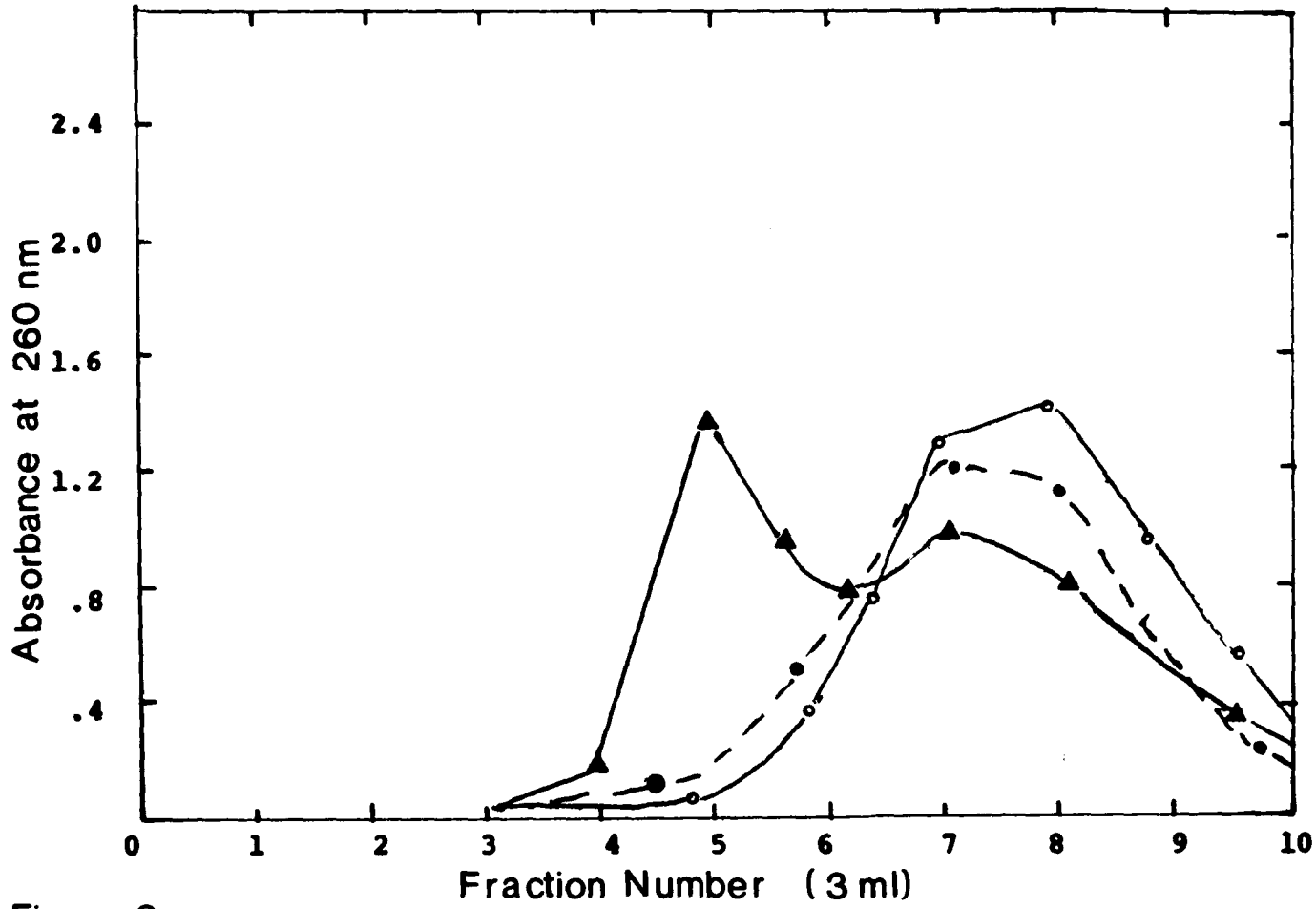


Figure 2. **Elution Pattern of tRNA-MC Complexes on Sephadex G-100.** A tRNA control (●—●) and two tRNA-MC complexes, reaction ratios in micromole nucleotide:micromole MC, 1:1 (○—○) and 1:10 (▲—▲), were eluted from a 1.5 x 19 cm column of Sephadex G-100 in SSC, pH 7.4.

(3) Stability of RNA-MC Complexes

Tables 3 and 4 show the effect of repeated chromatography on Sephadex G-100. Loss of absorbance from the complex-containing fraction appeared to be attributable as much to degradation of the tRNA as to loss of MC from the complex.

To determine the stability of the complex to mild acid treatment, complexes were incubated at pH 1 at room temperature for 30 minutes, neutralized with 1% NH_4OH, and chromatographed on Sephadex G-25 (Fine). Under these conditions, recovery of a poly G control was 92%. This procedure was used to test stability to acid of complexes MC formed with poly (U,G), poly G, and tRNA. Table 5 shows the results of mild acid treatment. Under conditions which caused little degradation of the polynucleotides, the bond to MC was quite stable.

In testing for stability of the complex to heating at 100°, it was found that tRNA controls were somewhat degraded after more than 10 minutes at this temperature. Therefore, tRNA-[^{3}H]-PM complexes were treated for only 10 minutes at 100° and rechromatographed on Sephadex G-100. The thermal stability of the MC-polynucleotide bond was also investigated with a poly G-MC complex after it was determined that poly G itself was stable to heating at 100° for one hour. The complexes, heated for one hour at 100°, were rechromatographed on Sephadex G-25 (Fine).

Stability of RNA-MC Complexes to Repeated Chromatography on Sephadex G-100

Chromatography:	Binding Ratios (Moles MC/Moles RNA-P) and % Recovery								
Time after Complex Formation	Complex A			Complex B			Complex C		
	B.R.	Recovery $\%A_{260}$	Recovery $\%A_{310}$	B.R.	Recovery $\%A_{260}$	Recovery $\%A_{310}$	B.R.	Recovery $\%A_{260}$	Recovery $\%A_{310}$
Table 3. Yeast tRNA-MC Complexes									
Immediate	.068	100	100	.357	100	100	.478	100	100
Same day	.058	88	86	.313	87	72	.377	70	61
24 hours	.057	83	78	.345	76	72	.338	60	49
7 days	.078	67	77	.294	64	50	.338	48	39
Table 4. dsRNA-MC Complexes									
Immediate	.032	100	100	.086	100	100	.184	100	100
Same day	.040	85	100	.077	87	73	.162	82	71
48 hours	.041	71	87	.077	75	69	.142	74	55

Table 5. Stability of Polynucleotide-MC Complexes at pH 1

Binding Ratios (Moles MC/Moles RNA-P) and % Recovery

	Poly G-MC Complex			Poly (U,G)-MC Complex			tRNA-MC Complex		
		Recovery			Recovery			Recovery	
	B.R.	$\%A_{260}$	$\%A_{310}$	B.R.	$\%A_{260}$	$\%A_{310}$	B.R.	$\%A_{260}$	$\%A_{310}$
Before incubation at pH 1	.049	100	100	.455	100	100	.476	100	100
After incubation at pH 1	.068	83	95	.435	77	71	.422	84	80

Figure 3 shows the elution pattern on Sephadex G-100 of a tRNA-[1a-^{3}H]porfiromycin (^{3}H-PM) complex before and after heating at 100° for 10 minutes. For a complex made with poly G, after heating at 100° for one hour, the binding ratio had been changed from .085 to .084, with 98% recovery of A_{260} units and 90% recovery of A_{310}.

Portions of a poly (U,G)-MC complex which had been resistant to degradation by endonucleases T_1 and pancreatic ribonuclease were tested for stability to 7 M urea at pH 7.4 and room temperature for 5 days. This study was undertaken because a portion of the complex was to be chromatographed on DEAE-Sephadex in 7 M urea. The polymer was considerably degraded after 5 days at room temperature, with or without urea, but in the material recovered in the complex fraction, the binding ratio was somewhat higher:

	Binding Ratio	% A_{260}	% A_{310}
Original poly (U,G)-MC complex	.070	100	100
Control: 5 days, room temperature, pH 7.4	.132	49	85
In 7M urea: 5 days, room temperature, pH 7.4	.095	42	58

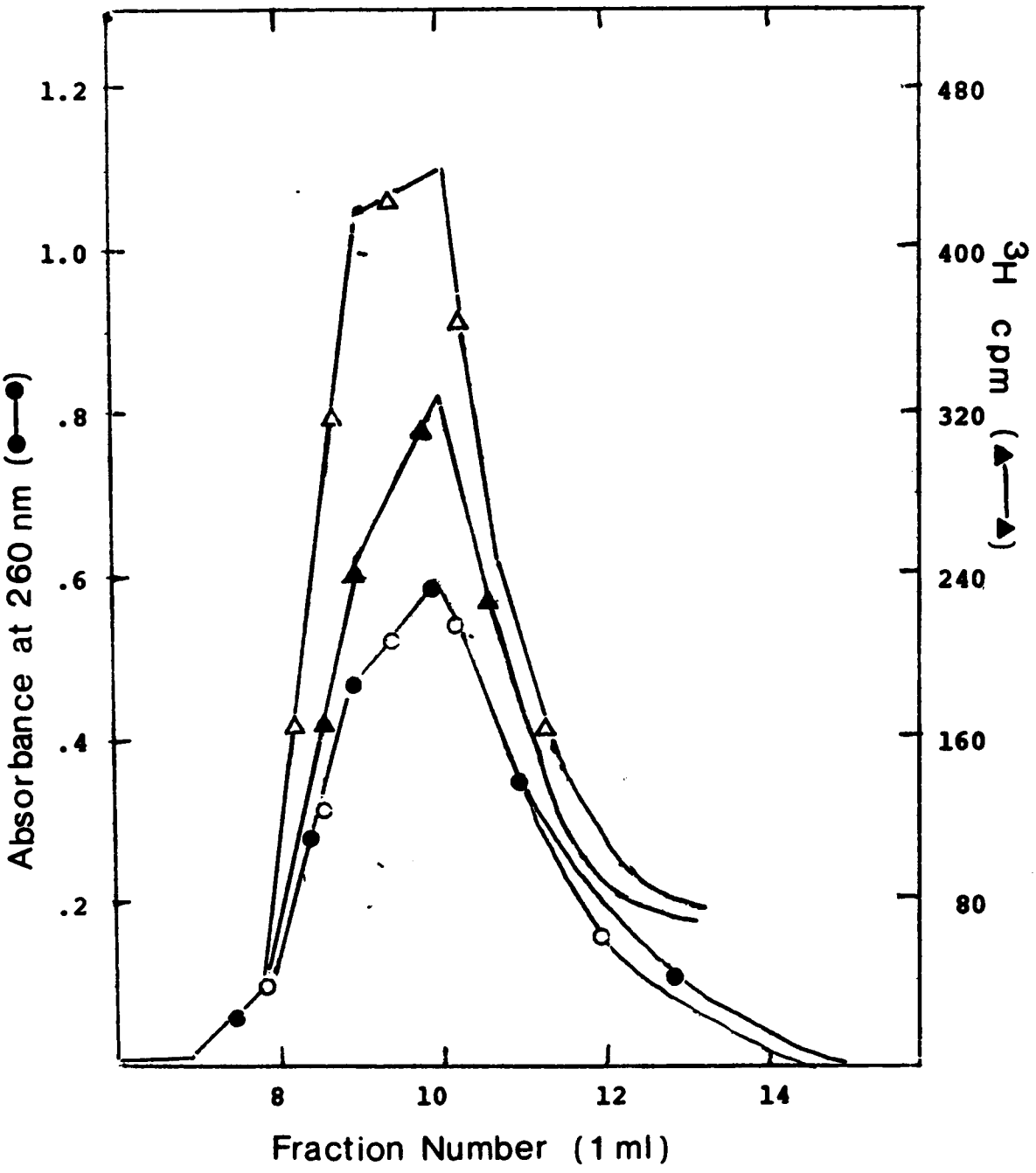


Figure 3. Stability of tRNA-[^{3}H]-Porfiromycin Complex at 100°. Complex, binding ratio .125, was chromatographed on Sephadex G-100 in 5 mM TEAB, pH 7.4, column size 1.1 x 26 cm., before (O—O) and after (●—●) being heated for 10 min. at 100° C.

(4) Base Ratios after Alkaline Hydrolysis of RNA-MC Complex

A. Nonradioactive Methods. tRNA-MC complexes used for alkaline hydrolysis had rather high binding ratios, on the order of .2, and had a definite purple color before hydrolysis, which turned to a yellow-brown after. This same color change was observed in unbound MC when it was reduced, reoxidized, and incubated in KOH.

When a denatured DNA-MC complex with a similar binding ratio was given comparable alkaline treatment, the same color change was observed. 63% of the bound MC was retained in the complex, indicating that the bond to MC was quite resistant to alkaline hydrolysis. However, when the RNA was degraded to nucleotides by KOH, it appeared that all the MC was released.

Paper chromatography of an rRNA control hydrolysate in solvent A showed the expected 3 spots: AMP, CMP and the slowest moving spot containing both UMPand GMP. The rRNA-MC complex hydrolysate showed an additional brown spot which remained at the origin. Control MC, reduced and then incubated in KOH, remained as a brown spot at the origin.

Figure 4 shows the results of two-dimensional chromatography on cellulose thin layers. The second solvent resolves the GMP/UMP mixture into three separate spots: GMP, UMP, and another very faint spot. Since this was a complex containing tRNA, the third spot is probably ΨMP (Nishimura, et al., 1967). The complex chromatogram also showed a brown spot which remained at the origin and which,

Figure 4.

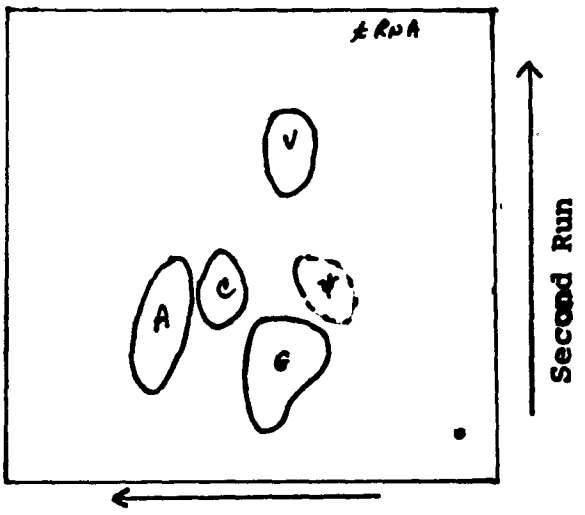
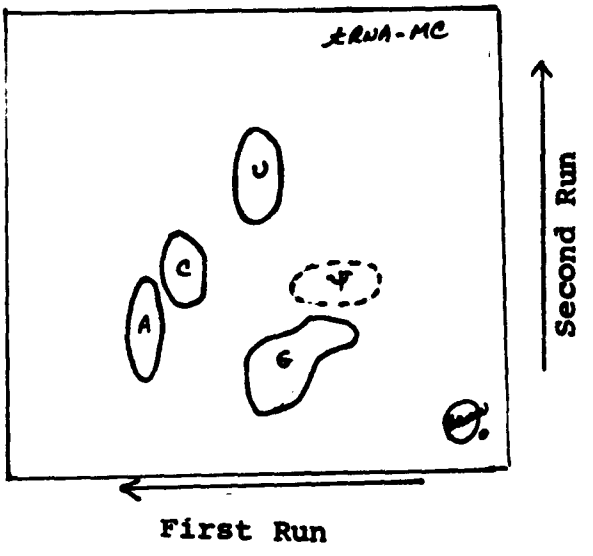


Figure 5.



Two-Dimensional Thin Layer Chromatography of Alkaline Digests of tRNA and tRNA-MC Complex

when eluted from the plate, had the UV spectrum characteristic of reduced MC. Since there was no significant absorbance at 310 nm in any of the other spots eluted, it appeared that no nucleotide-MC adduct had been present in the digest.

Representative nucleotide ratios are given below and show some variation between complex and control. However, these variations were not strictly reproducible. Therefore, another approach, using radioactive rRNA, was used to continue this investigation.

	Base Ratios			
Hydrolysate	GMP	AMP	UMP	CMP
Yeast tRNA	.26	.26	.27	.21
Yeast tRNA-MC, Binding ratio .2	.34	.23	.21	.22

B. Radioactive method. Paper electrophoresis of hydrolyzed [^{32}P] rRNA complexes and controls, as described in Methods, gave excellent resolution of nucleotides and showed no significant change in nucleotide ratios after binding and subsequent release of MC:

	Base Ratios			
Hydrolysate	GMP	AMP	UMP	CMP
rRNA control	.30	.29	.22	.19
rRNA:MC, Binding ratio .18	.29	.31	.22	.18
rRNA:MC, Binding ratio, .33	.28	.30	.22	.20

(5) Base Specificity - Binding of MC to Synthetic Polynucleotides

The results of these reactions are shown in Figure 6 and Table 6. For complexes made at the lower salt concentration, a preference for guanine was evident, but there was also a significant amount of binding to polymers containing no guanine. At the higher salt concentration, this was almost entirely suppressed, and binding was observed only to the two polymers containing guanine.

Because the stable secondary structure of poly G may involve the MC-binding site in G-G interactions and make it inaccessible to the drug (Lipsett and Weissbach, 1965), poly (U,G) (U:G ratio 1.0:1.02) in which no such secondary structure is evident, was also used. Based on a binding ratio of .222 for poly (U,G) and .048 for poly U in .01 M Tris, the estimated binding to G alone in poly (U,G) was calculated as follows:

.444 micromoles MC/2 micromoles poly (U,G) (which contains one micromole U and one micromole G) - .048 micromoles MC/ one micromole U = .392 micromoles MC/one micromole G.

This, of course, is considerably higher than the binding ratio of .14 for poly G.

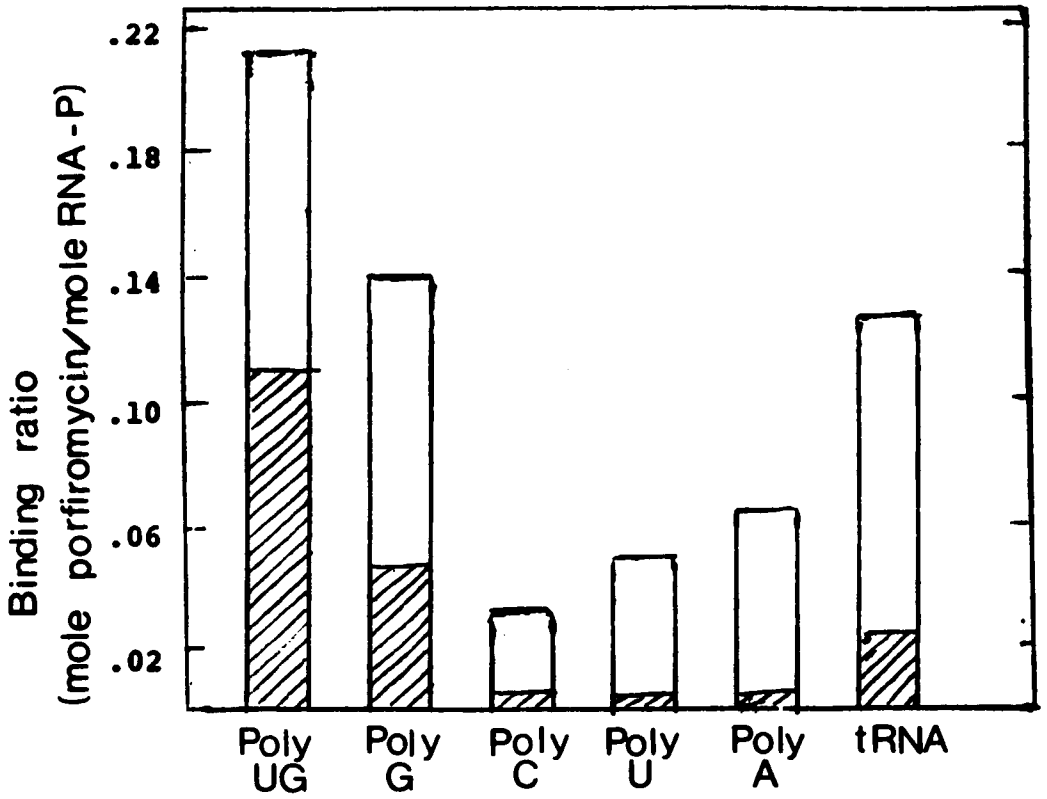


Figure 6. Binding of [^{3}H]-Porfiromycin to Synthetic Polynucleotides. Shaded portion of bar represents binding in reaction run in 0.2 M Tris; total height of bar, binding in 0.01 M Tris.

Table 6. Binding of [^{3}H] Porfiromycin to Synthetic Polyribonucleotides

Polyribonucleotide	Binding Ratio in 0.01 M Tris	Binding Ratio in 0.20 M Tris
Poly (U,G) (1:1)	0.22	0.109
Poly G	0.14	0.045
Poly C	0.03	$\leq$ 0.005
Poly U	0.05	$\leq$ 0.004
Poly A	0.07	$\leq$ 0.004
Yeast tRNA	0.12	0.023

(6) Binding of MC to Polymers of Modified GMP

Figure 7 compares binding ratios of MC complexes with polymers containing GMP, O^6-methyl GMP, and IMP. In low salt, at 37°, the melting temperature of poly O^6-methyl GMP (Gerchman, et al., 1972), binding to this polymer is .30 micromoles MC/1.0 micromole nucleotide, greater than the binding to poly G, but somewhat less than the calculated binding to G alone in poly (U,G).

Binding to poly G and to poly I was almost identical in .01 M Tris. However, poly I is believed to have a secondary structure similar to that of poly G (Zimmerman, et al., 1975; Cech and Tinoco, 1975), therefore poly (U,I) was also used in this series. For poly (U,I) and poly (U,G) in 0.20 M Tris, binding ratios of .011 and .109, respectively, show considerably less binding to IMP.

The experiments with poly 7-methyl GMP were done somewhat differently, using a reaction ratio of 2 MC:1 G; therefore the binding ratio cannot be directly compared with the data above. However, a control complex of poly G-MC made with the same reaction ratio had a binding ratio of .086, while the complex with 40% 7-methyl GMP had a much higher binding ratio, .44. In a converse reaction, methylation of a poly G-MC complex (binding ratio .12) produced about 43% methylation, essentially the same as methylation of poly G not complexed with MC.

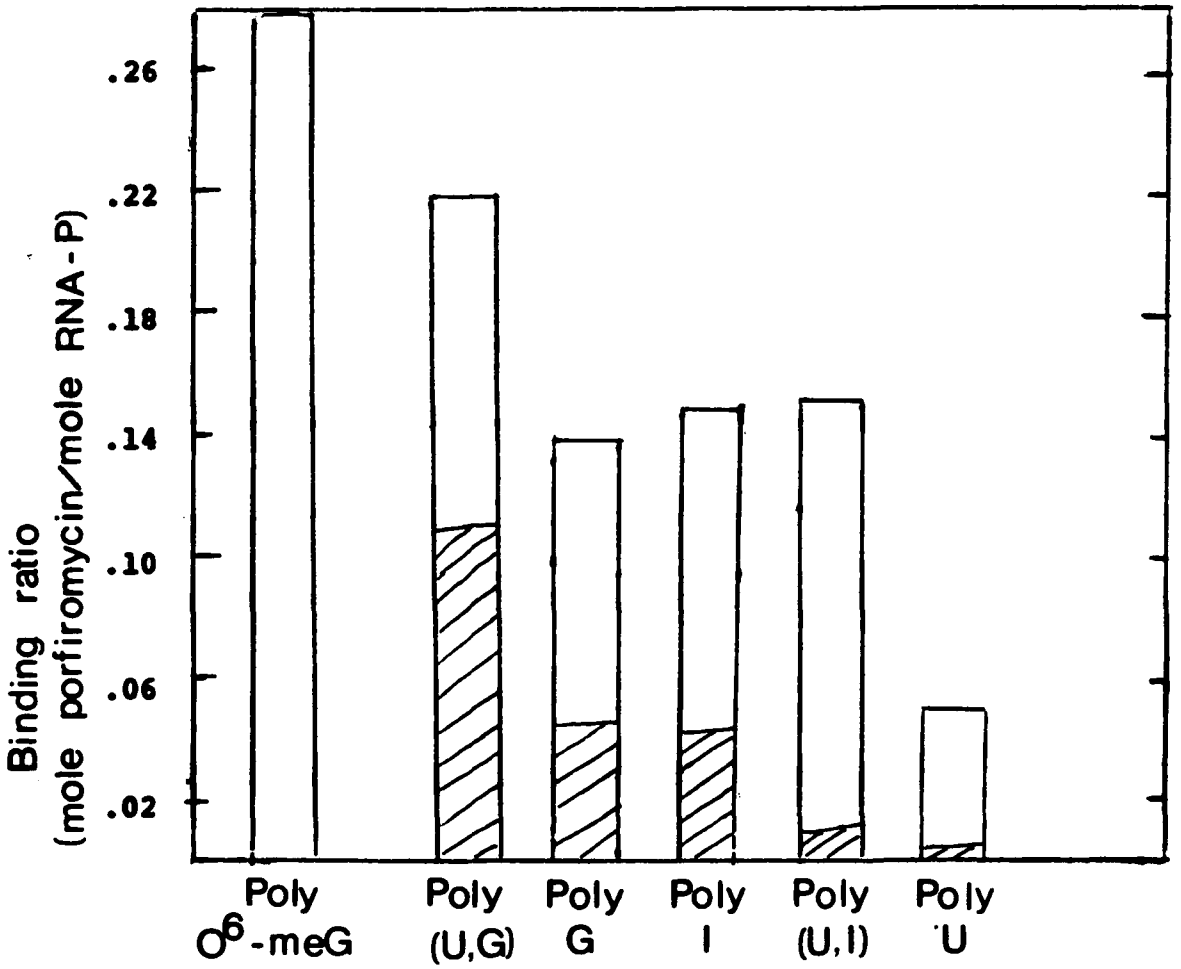


Figure 7. **Binding of [^{3}H]-Porfiromycin to Polymers of Modified GMP.** Shaded portion of bar represents binding in reaction run in 0.2 M Tris; total height of bar, binding in 0.01 M Tris. For poly O^6-meG, reaction was run only in 0.01 M Tris.

(7) Enzymatic Digests of Polynucleotide-MC Complexes

A. RNA-MC Complexes. For a preliminary study of the effect of enzymatic degradation on an RNA-MC complex, digestion was carried out in dialysis bags and its effectiveness measured by the amount of material rendered dializable. Table 7 shows the percent of initial absorbance of the material remaining in the dialysis bags after 24 and 48 hours, and the calculated binding ratio of the enzyme-resistant portion of the complex.

It is apparent that MC conferred considerable protection against degradation by these endonucleases, and particularly against T_1, supporting the theory that the drug was bound primarily to guanine.

To determine the extent of degradation of the undialyzed portion (hexanucleotides or larger, according to Vandenbussche and Fiers, 1966), it was chromatographed on Sephadex G-100 in SSC. Figure 8 compares the elution patterns of undialyzable RNA control and RNA-MC complex after digestion by T_1. Of the total sample complex chromatographed, about 97% of both 260 nm and 310 nm UV absorbance was excluded by the column, indicating that this material was still of high molecular weight. In contrast, of the small amount of RNA which had not been degraded to dialyzable size, about half was small enough to be retained by the gel.

B. Poly G-MC Complexes. Since MC bound to RNA protected it against degradation by ribonuclease T_1, it

Table 7. Stability of rRNA-MC Complexes to Enzymatic Digestion in Dialysis Bags

	% UV Absorbance Remaining in Bag After						
	24 hours			48 hours			
	Control rRNA	Complex		Control rRNA	Complex		Binding
	A_{260}	A_{260}	A_{310}	A_{260}	A_{260}	A_{310}	Ratio
No enzyme	92	100	89	96	88	89	.60
T_1	65	78	94	36	88	94	.55
Panc. RNase	53	65	94	29	70	99	1.00
T_1+ panc. RNase	26	63	99	13	55	78	.61

2.0 ml of RNA or RNA-MC complex (binding ratio approximately 0.5) in DSC buffer, pH 7.4, was placed in a dialysis bag. Either 30 units of T_1 or 300 units of pancreatic RNase or a combination of both, was added and dialysis against DSC continued at room temperature.

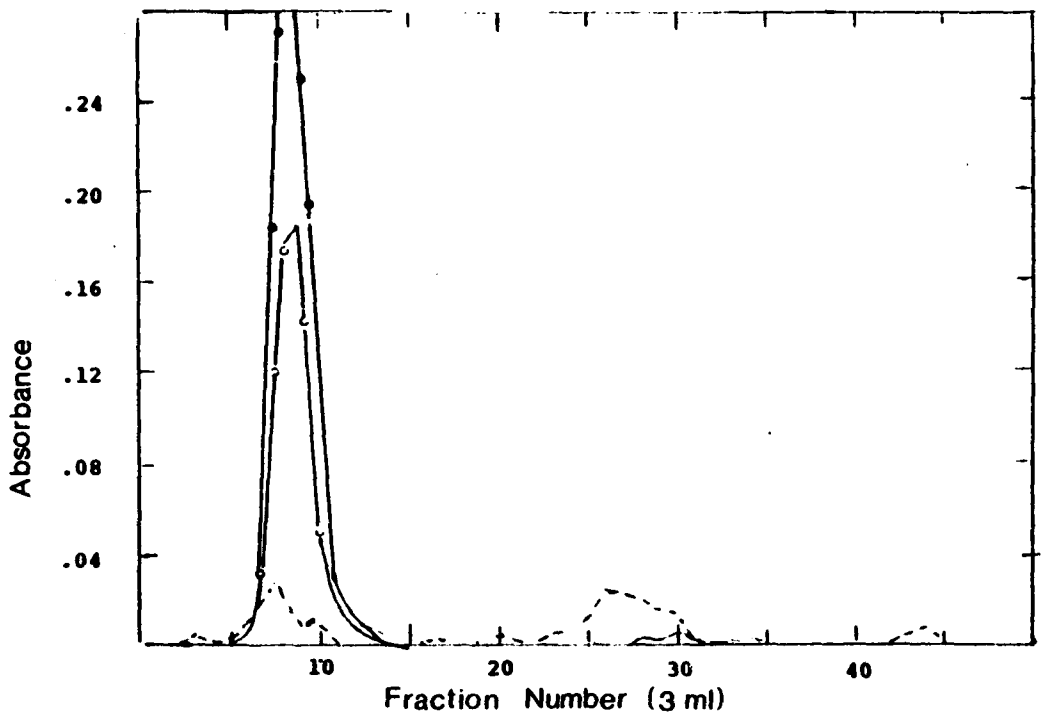


Figure 8. T_1-Resistant rRNA and rRNA-MC Complex. Samples in dialysis bags were incubated with T_1 while being dialyzed vs. DSC. Material remaining in bags after 48 hours was chromatographed on Sephadex G-100 in SSC, 1.5 x 35 cm, and absorbance of fractions determined at 260 nm for control (- -) and complex (•—•), and at 310 nm for complex (○—○).

seemed reasonable to expect that a complex between poly G and MC with a rather low binding ratio could be degraded to GMP plus small guanylate oligomers bound to MC. T_1 digests of poly G-MC, binding ratio 0.2, looked very promising when chromatographed on Sephadex G-25, since the complex showed three distinct peaks, each with absorbance at 310 nm, compared to digests of poly G, which had only two peaks, one at the exclusion volume, and one at the elution volume of GMP. The additional peak from the complex was eluted somewhat earlier than the monomer and might be a small oligo G-MC adduct (Figures 9 and 10). However, it was possible that the 310 nm absorbance in this peak was due to a form of unbound MC eluted in the same volume as small guanylate oligomers, so the next step was chromatography of a similar digest on an anion exchange column. A preliminary control of poly G degraded to oligomers was separated well on DEAE-cellulose in 7 M urea, pH 7.4, with a linear gradient of 0 to .3 N NaCl (Figure 11), so a similar column was used to chromatograph a T_1 digest of about 26 A_{260} units of a poly G-MC complex (binding ratio 0.2). The major peaks were an early one corresponding to the first material eluted from the control digest, and a double peak, eluted between 0.105 N and 0.115 N NaCl, which contained, after desalting to remove urea and guanidine HCl, approximately 42% of the total A_{260} and 84% of the A_{310} of the digest. (Figure 12)

This material was pooled, concentrated, and rechromatographed on Sephadex G-25 (Fine), where it again split into

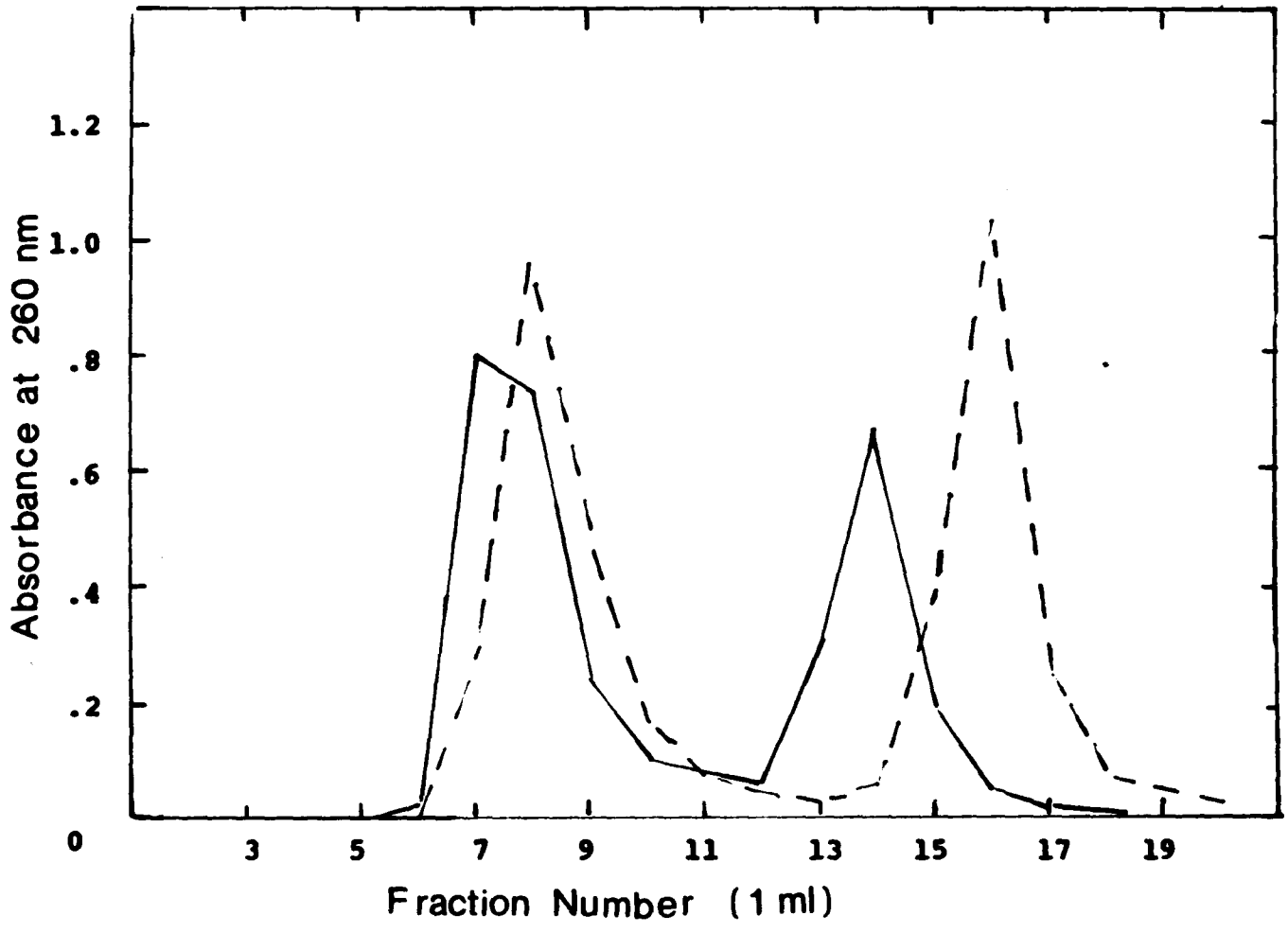


Figure 9. T_1 Ribonuclease Digests of Poly G. Digestion was carried out in H_2O (solid line) or 7 M urea (dashed line), and the digest chromatographed on Sephadex G-25, 1.1 x 20 cm column, in H_2O.

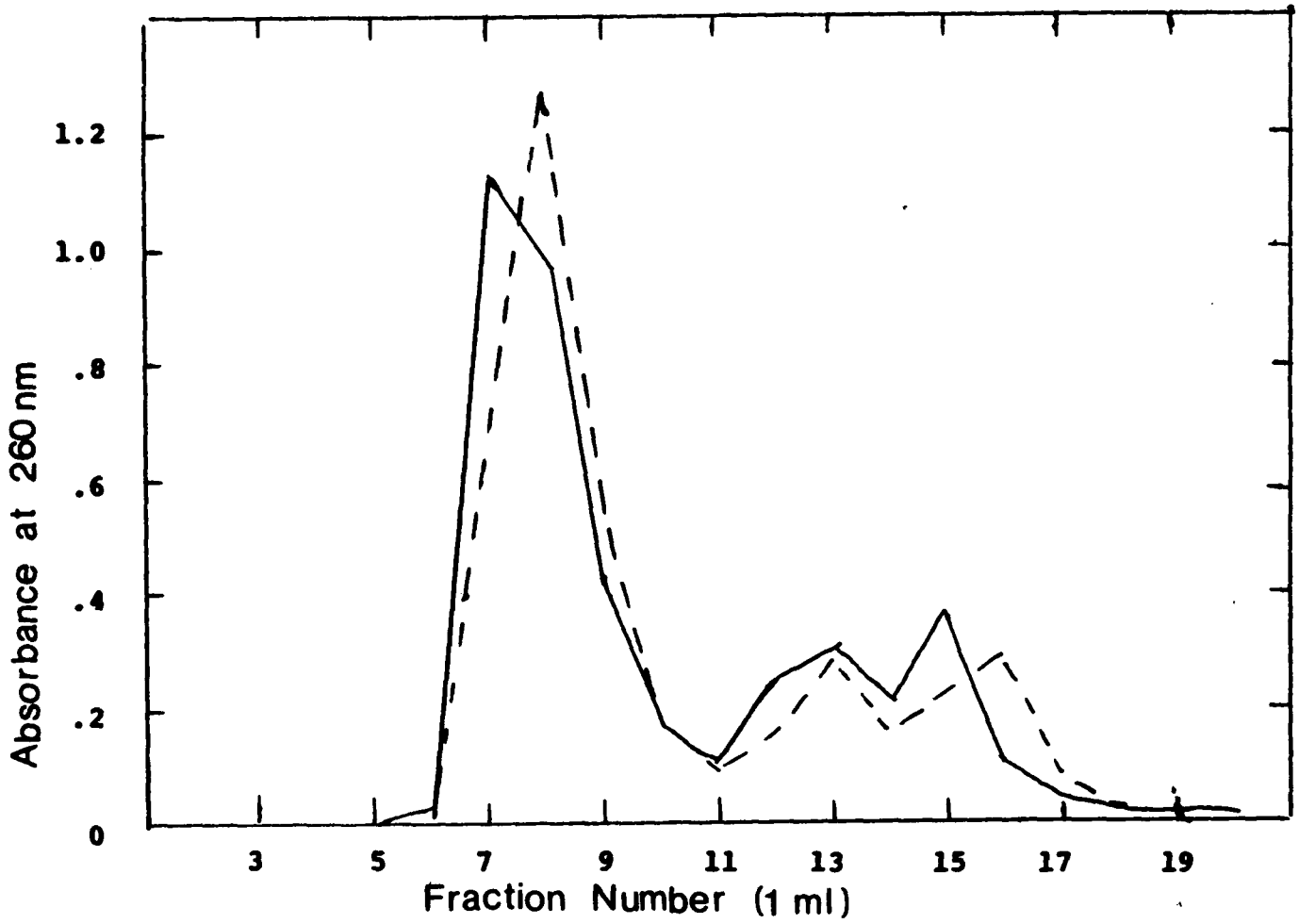


Figure 10. T_1 Ribonuclease Digests of Poly G-MC Complex. Digestion was carried out in H_2O (solid line) or 7 M urea (dashed line), and the digest chromatographed on Sephadex G-25, 1.1 x 20 cm column, in H_2O.

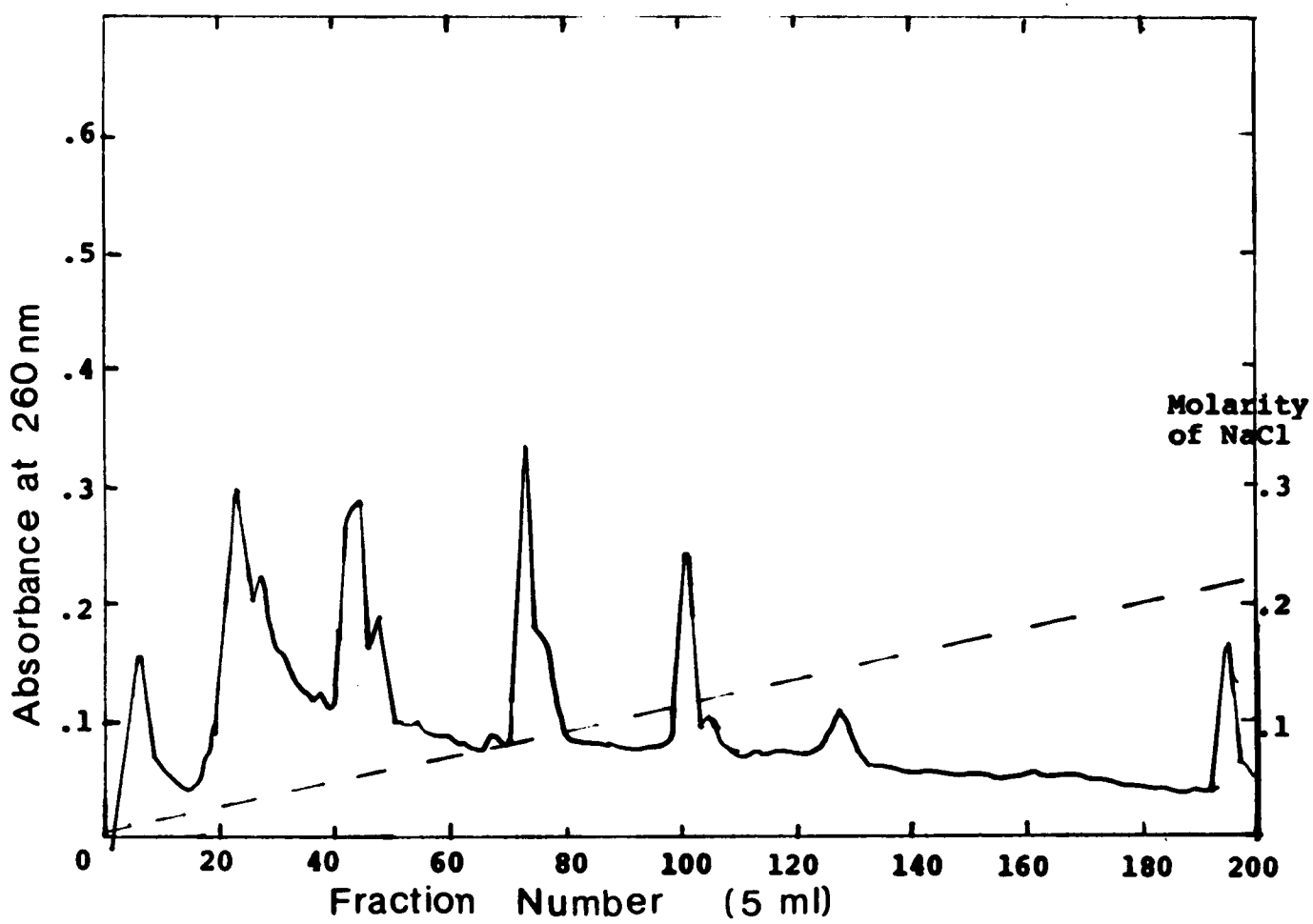


Figure 11. Poly G Partially Degraded by KOH. Digest was chromatographed on DEAE-Cellulose, 1.1 x 15 cm column, in 7 M urea, 0.1 M Tris, pH 7.8, with a linear gradient of 0 to .3 M NaCl.

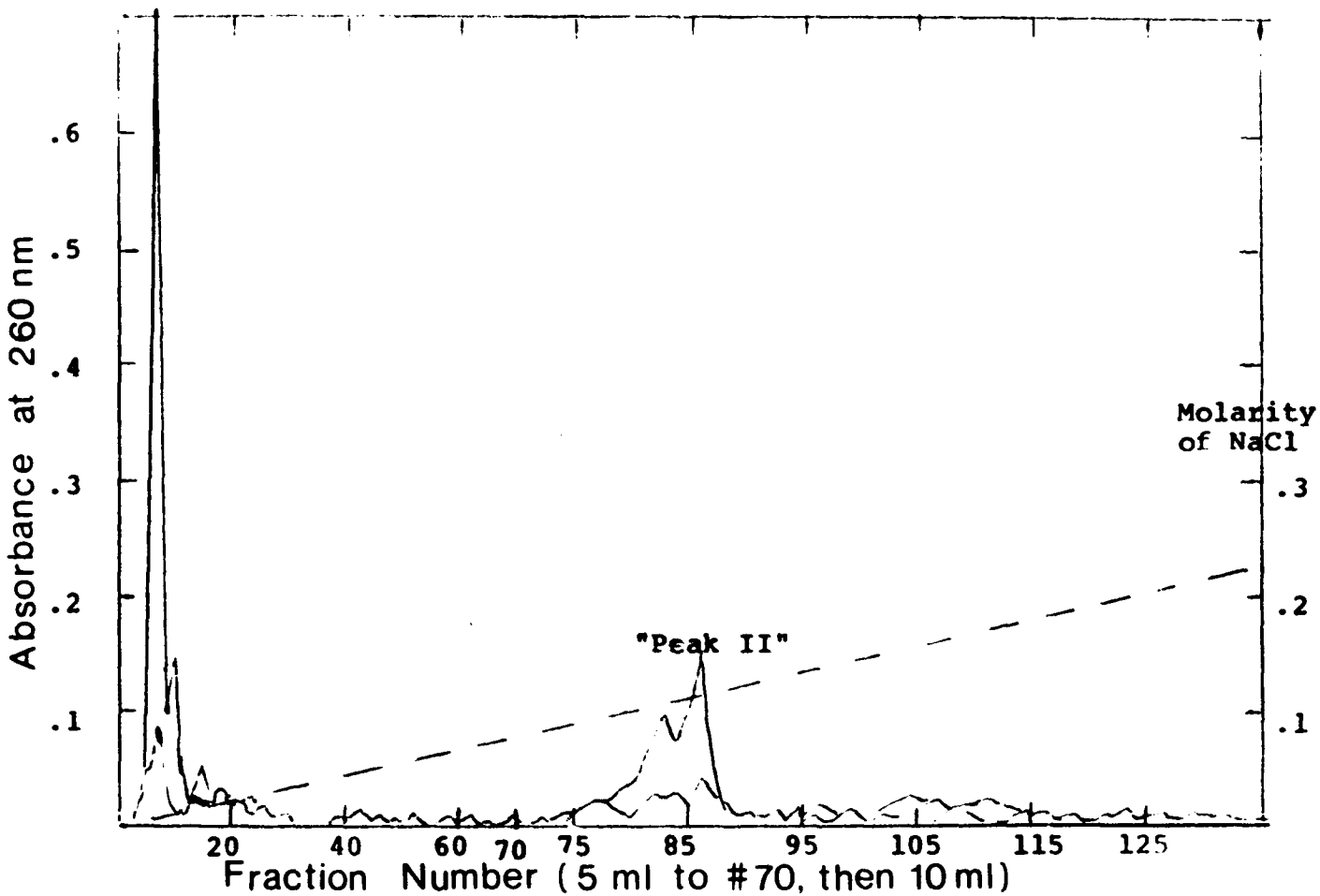


Figure 12. T_1 Digest of Poly G-MC Complex. Digest was chromatographed on DEAE-Cellulose, 1.1 x 15 cm column, in 7 M urea, 0.1 M Tris, pH 7.8, with a linear gradient of 0 to 0.3 M NaCl.

two overlapping peaks (Figure 13). From binding ratios and ratios of total to terminal phosphate, assayed by the procedure of Seaman (1968), it appeared that each peak might contain a small adduct, possibly one an interstrand and the other an intrastrand crosslink.

	Peak I	Peak II
Approximate ratios:		
MC/GMP	1:2	1:2
Total P/Terminal P	1	2
Possible structure		

However, these assays were based on quite small amounts of material, with A_{310} only slightly above background absorbance. Therefore, the experiment was scaled up to about 54 A_{260} units, and [^{3}H]PM was used in place of MC in preparing the next complex for digestion. DEAE-Sephadex was used with a linear gradient of guanidine HCl, since this has been shown to be helpful in preventing aggregation of small guanylate oligomers on the column (Olson and Volkin, 1972). A control digest of poly G by T1 was separated into four major peaks, with the first and largest eluted by .08 M salt. About 40% of the poly G sample appeared to have been resistant to digestion and was eluted by 1.0 M salt.

When the complex digest was chromatographed (Figure 14) about 35% of the [^{3}H]PM apparently had been released from the complex and was recovered in the early fractions as the

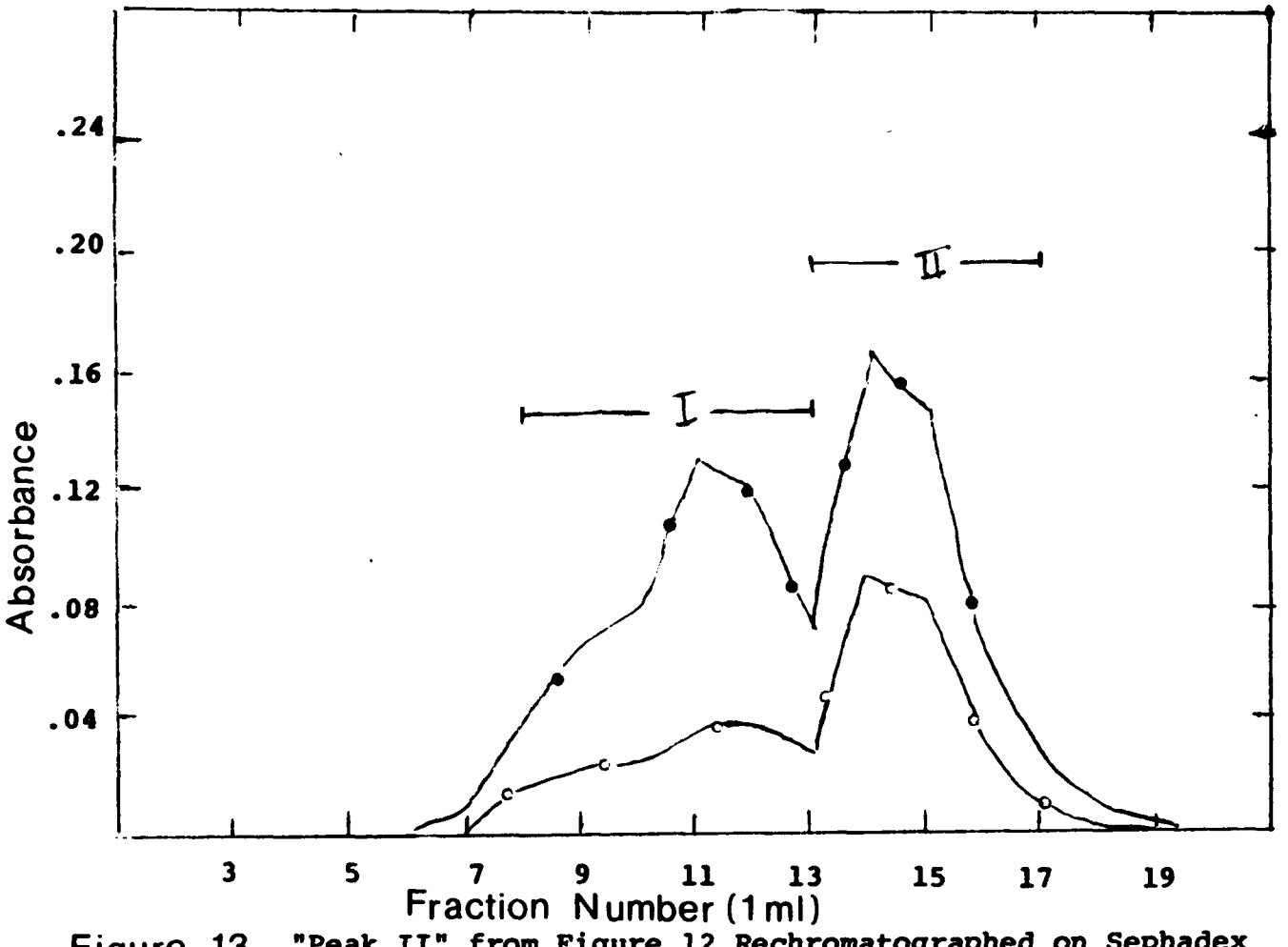


Figure 13. "Peak II" from Figure 12 Rechromatographed on Sephadex G-25. Absorbance at 260 (●—●) and 310 (○—○) nm of material from T_1 digest of poly G-MC complex rechromatographed on 1.1 x 20 cm column of Sephadex G-25 in H_2O.

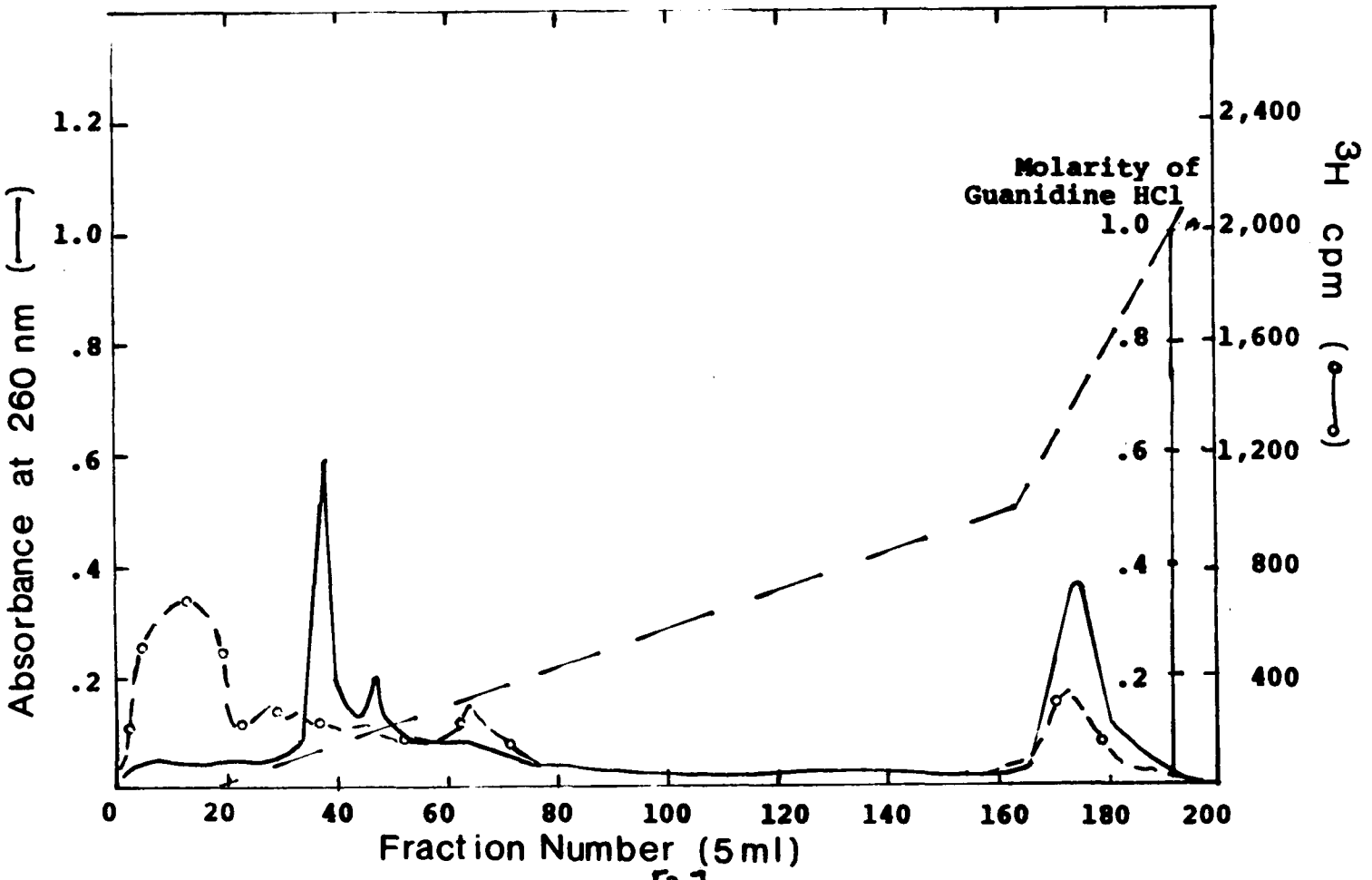


Figure 14. T_1 Digest of Poly G-[^{3}H]Porfiromycin Complex Chromatographed on DEAE-Sephadex. Digest was chromatographed on 1.1 x 26 cm column in 7 M urea, .01 M Tris, pH 7.4 and 0.2 mM EDTA, with a gradient of Guanidine HCl, as shown.

digest was being applied to the column. An additional 24% was recovered in an undegraded portion of the complex which was eluted by 0.7 M salt. Two very small peaks which appeared to contain both nucleotides and [^{3}H]PM were eluted by 0.1 M and 0.15 M guanidine HCl, but again the amounts were so small that the results were unreliable.

C. Poly (U,G)-MC Complexes. Several attempts to achieve more complete digestion of poly G and poly G-MC complex with T1 ribonuclease were unsuccessful. However, T1 in conjunction with pancreatic nuclease was very effective in degrading poly (U,G) to nucleotides, and so this work on degradation of complexes was continued with poly (U,G)-MC complexes (U:G ratio approximately 1:1).

When a poly (U,G)-MC complex, binding ratio 0.1, was digested by T1 and pancreatic RNases, and then chromatographed on a Sephadex G-25 (Fine) column which had been calibrated for GMP, UMP, guanosine and uridine, the elution pattern of Figure 15was obtained. Peak A (calculated binding ratio .4) was eluted close to the exclusion volume of the column. Peaks C, D and E were eluted where one would expect UMP, GMP, and guanosine, respectively, and there was a slight shoulder preceding the GMP peak, where one would expect uridine. An unidentified peak, B, contained a considerable amount of material absorbing at 310 nm, as did the UMP peak. This could have been a form of reduced MC released from the complex, but the pattern of 260 nm absorbance suggested that peak B was a nucleotide-MC adduct.

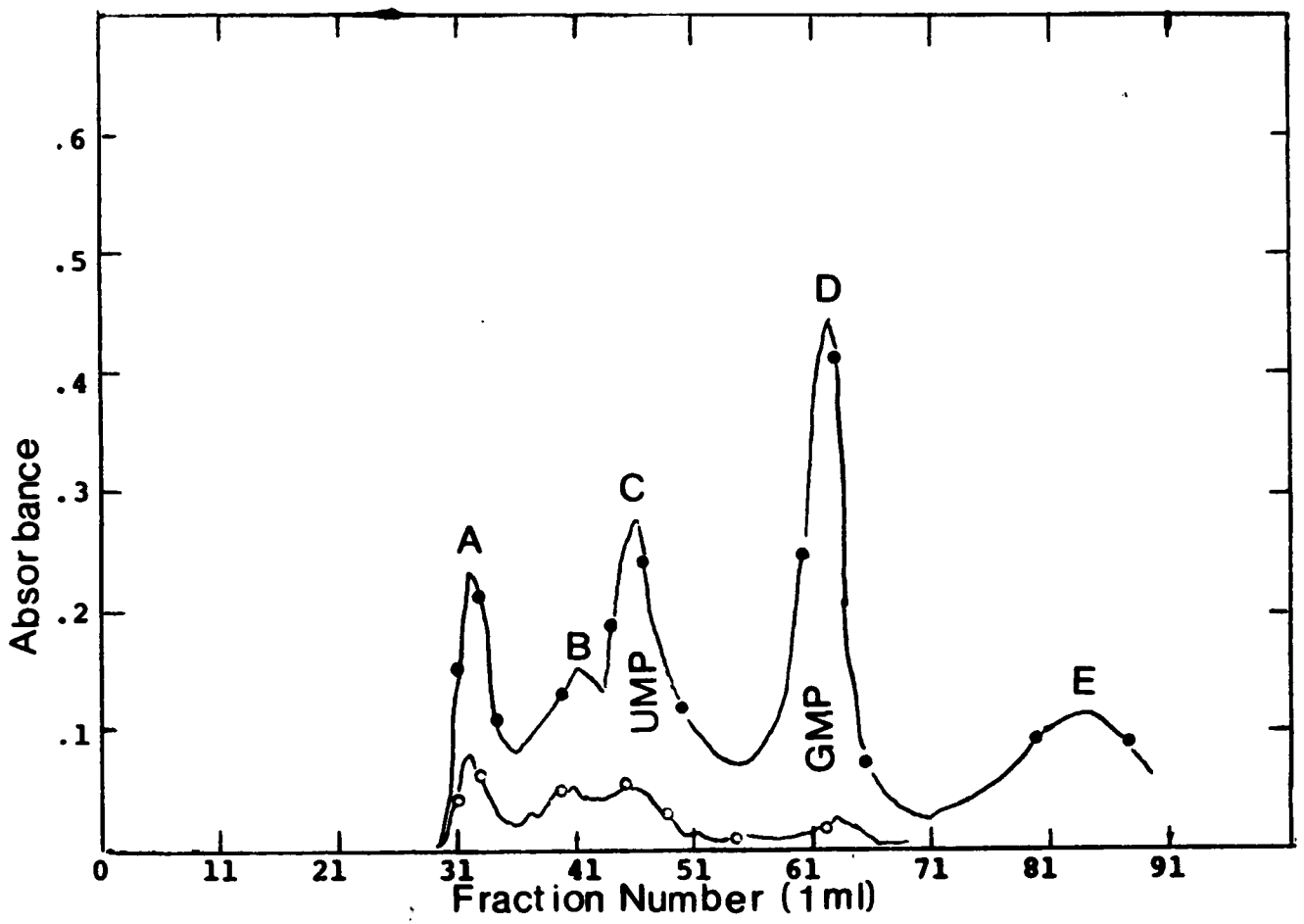


Figure 15. **Enzymatic Digest of Poly (U,G)-MC Complex Chromatographed on Sephadex G-25.** Complex, binding ratio 0.1, was digested by T_1 and pancreatic RNAses, and eluted from a 1.5 x 33 cm column of Sephadex G-25 in 5 mM TEAB, pH 7.5. Graph shows absorbance at 260 (•——•) and 310 (•——•) nm.

Peaks B and C were pooled, and a portion of the combined fractions was treated with bacterial alkaline phosphatase. The digest was then rechromatographed on the same column (Figure 16). Two major peaks were observed: uridine, obtained by removal of phosphate from UMP, and a peak with both 260 nm and 310 nm absorbance (estimated binding ratio 0.6) which was eluted later than the original peak B. This later elution on Sephadex G-25 would be expected if a negatively charged group were removed from a relatively small molecule, for example:

Since this was, again, a very small amount of material, a much larger amount of poly (U,G)-MC complex was prepared for enzymatic degradation and chromatography on DEAE-Sephadex.

D. Large Poly (U,G)-MC Complex Digest. (The following procedures are summarized in Figure 35, page 84.) A poly (U,G)-MC complex, binding ratio 0.11, containing 1659 A_{260} and 251 A_{310} units, was subjected to degradation by T_1 and pancreatic RNases. For this large-scale digest, a preliminary study had been made to determine the optimum time for digestion. This was evaluated by binding ratios and G:U ratios of the portion of the complex which was eluted in the exclusion volume of a Sephadex G-25 column. After four hours at room temperature, although the amount of polymer degraded to nucleotides continued to increase, the binding ratio began to decrease, indicating that

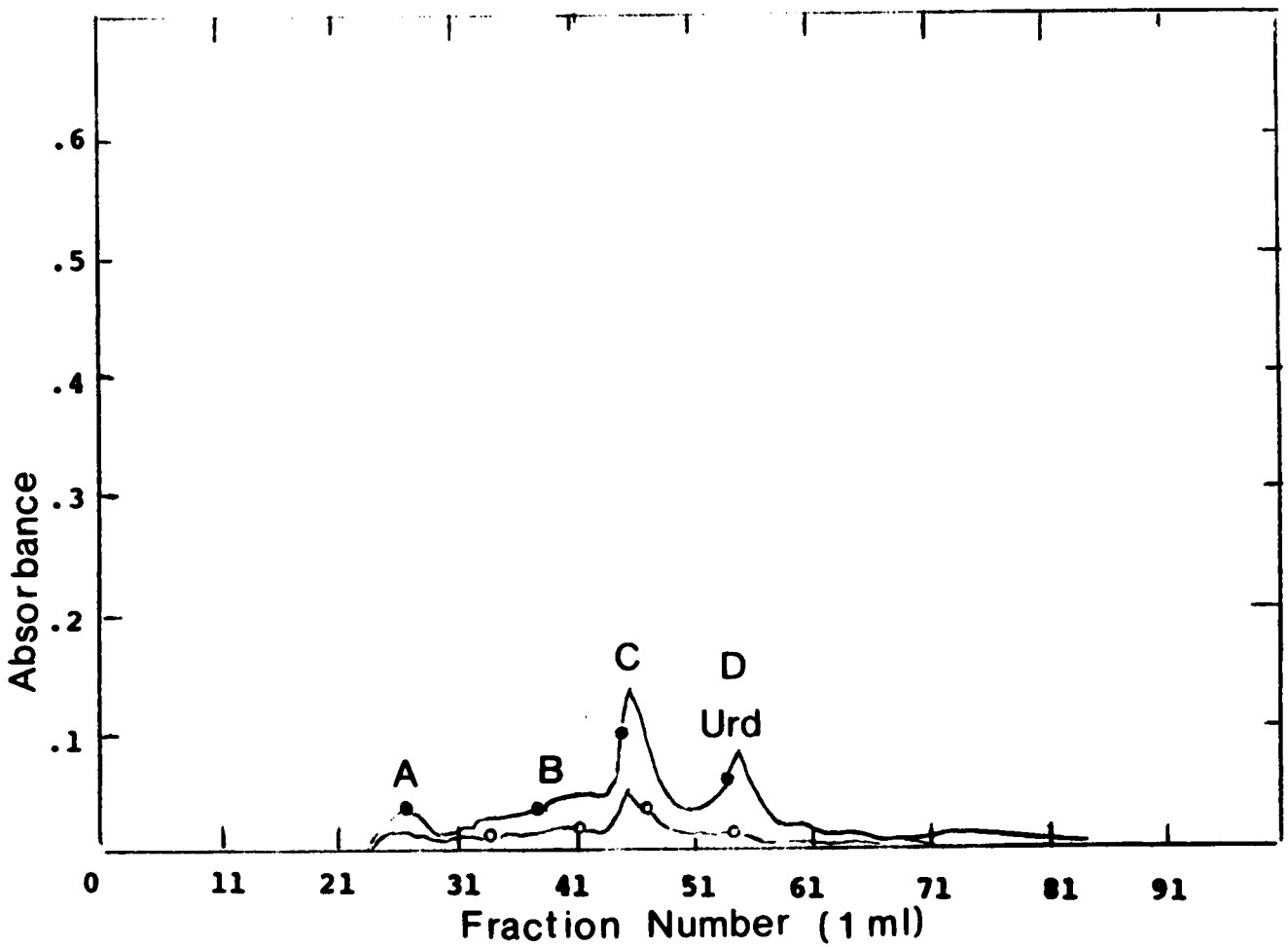


Figure 16. **Alkaline Phosphatase Digest of Peaks B+C, Figure 15.** Material was rechromatographed on same Sephadex G-25 column as in Figure 15, in 5 mM TEAB.

further degradation of the complex included breaking of the bond to MC. G:U ratio was investigated because we believed that, in this random copolymer, if T1 could not cleave the bond at guanine residues which were bound to MC, more UMP than GMP would be released as the complex was degraded. The ratio was determined by alkaline hydrolysis of the material eluted in the exclusion volume of Sephadex G-25, separation of the two nucleotides on Whatman 40 paper in 2-propanol: conc. HCl: H_2^1O (68:17:15, v/v/v), and elution of nucleotides with 0.1 N HCl. The initial G:U ratio of the polymer was 1.06; of the complex, 1.26; and of the complex after 4 hours digestion, about 11. When an additional aliquot of enzyme was added and the digest incubated for four more hours, the ratio was increased to about 13, which was not judged to be a significant increase. Therefore, four hours was selected as the optimum time for digestion.

A purple precipitate was observed in the digest, and this was removed by centrifugation and set aside for later study. The digest supernatant was chromatographed on Sephadex G-50 to simplify ion-exchange chromatography by first separating free nucleotides and free MC from the partially degraded complex. Two peaks were obtained, with most of the A_{310} units in the first (Figure 17). A small portion of the first peak was reserved, and the remainder, containing 347 A_{260} and 83 A_{310} units, was applied to a DEAE-Sephadex column, where it formed a purple band at the top of the column.

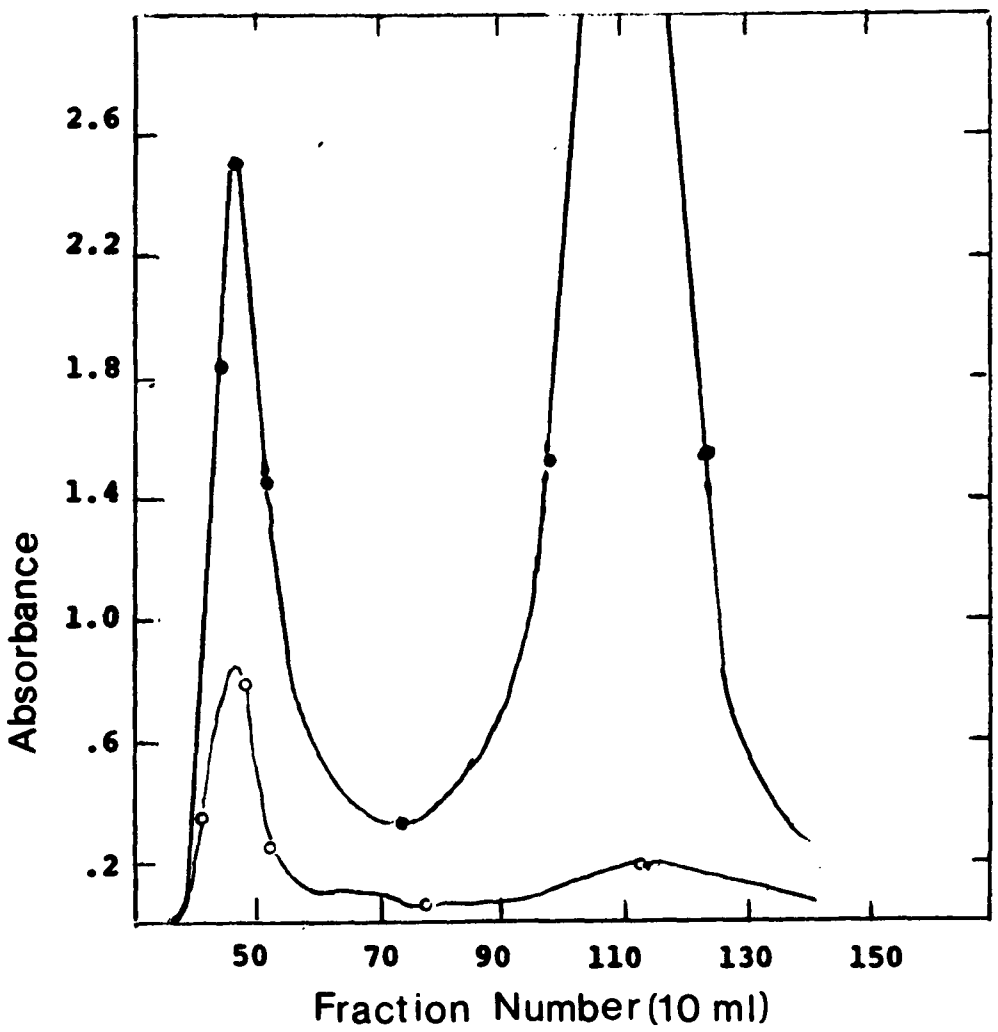


Figure 17. **Enzymatic Digest of Poly (U,G)-MC Complex.** Chromatographed on Sephadex G-50, 5 x 58 cm column, in .05 M Tris, pH 7.4. Graph shows absorbance at 260 (•—•) and 310 (○—○) nm.

With the exception of three small peaks which were eluted at salt concentrations less than 0.1 M and contained about 25% of the total A_{260} and 5% of the total A_{310} units, this material was not removed even by 2 M salt.

The material from the second peak was then separated on DEAE-Sephadex (Figure 18). The two major peaks were identified as UMP and GMP. These were followed by two very small peaks, eluted at about .13 M and .18 M guanidine HCl. After being desalted on DEAE-cellulose, the second peak contained too little material to analyze. The first was rechromatographed on Sephadex G-25, where it split into two peaks (Figure 19). The material in the second had a calculated binding ratio of about .33 and its UV spectrum (Figure 20) has the 310 absorbance characteristic of reduced MC as well as the 254 maximum of GMP. Unfortunately, this peak represented only 0.14% of the total A_{260} and 0.26% of the total A_{310} in the original digest.

The remaining material from the first peak from separation of the total digest on Sephadex G-50, which had been frozen for several months, was thawed and a small amount of precipitate was removed by centrifugation. The supernatant was chromatographed on Sephadex G-100, to find out if it would also be excluded by this gel. It was separated into three peaks (Figure 20), a very small peak at the exclusion volume (calculated binding ratio about 1.0), a larger peak containing both 260 and 310 nm absorbance (calculated binding ratio 0.2), and a third peak eluted in the same volume

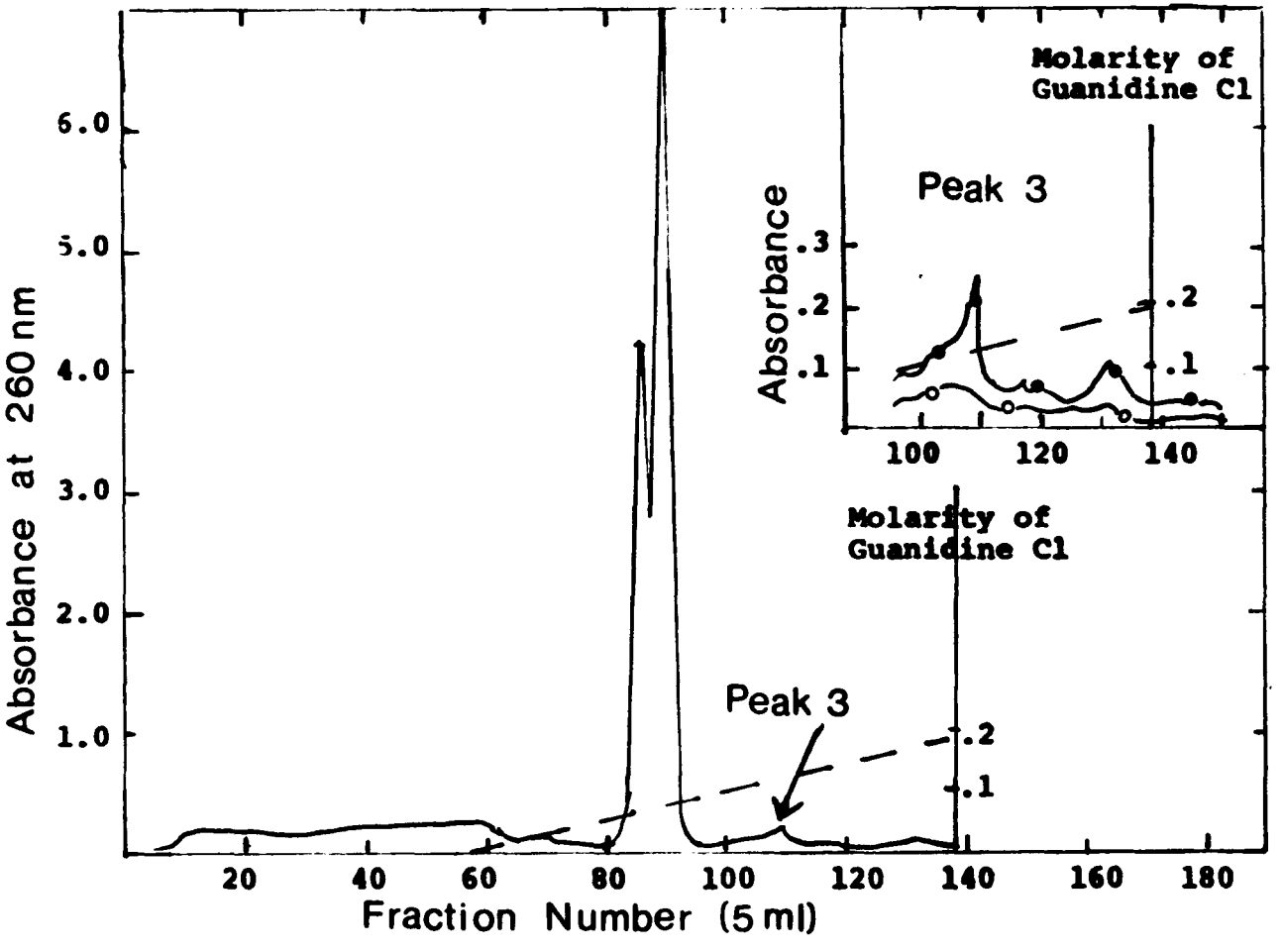


Figure 18. Peak II from Figure 17 Rechromatographed on DEAE-Sephadex. Column, 1.5 x 20 cm, was eluted with 7 M urea, 0.2 mM EDTA, .05 M Tris, pH 7.4, with a linear gradient of 0 to .6 M guanidine HCl. Expanded scale inset shows Peak 3 absorbance at both 260 (●——●) and 310 (○——○) nm.

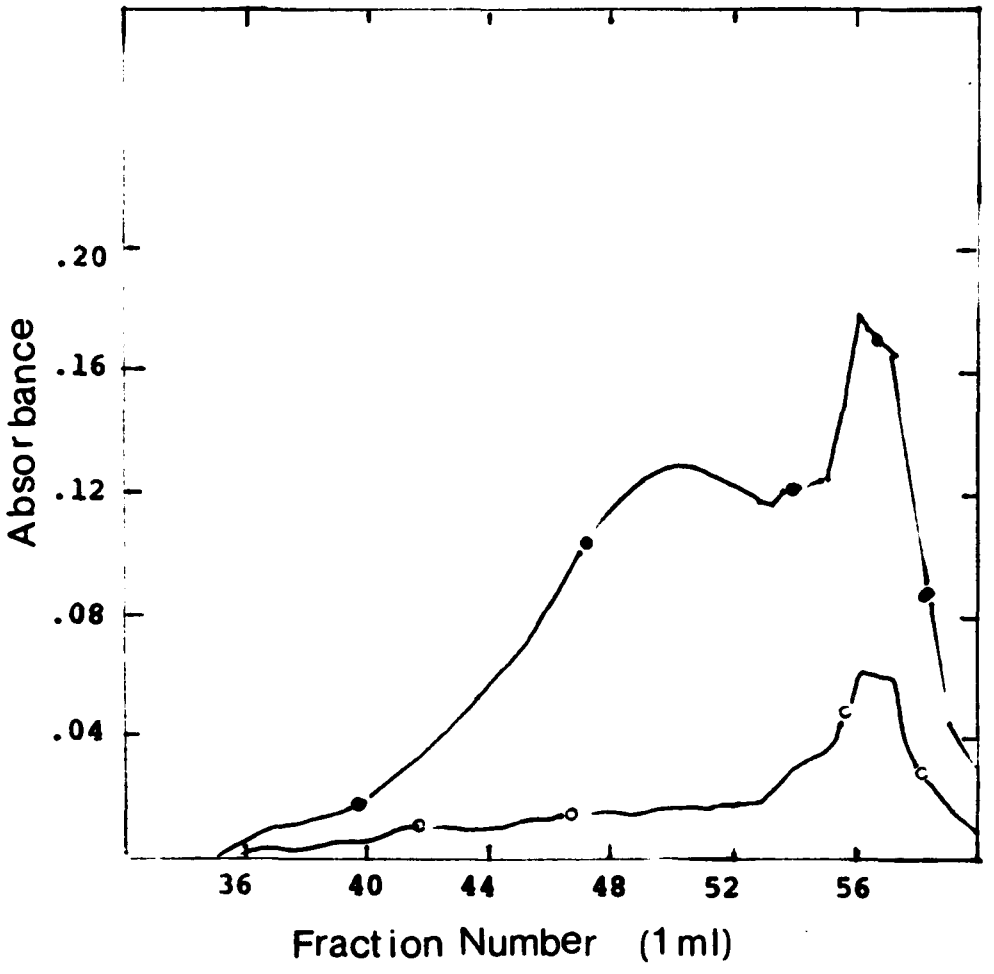


Figure 19. Peak 3 from Figure 18 Rechromatographed on Sephadex G-25, 1.5 x 34 cm column, in .05 M Tris, pH 7.4. Graph shows absorbance at 260 (●—●) and 310 (○—○) nm.

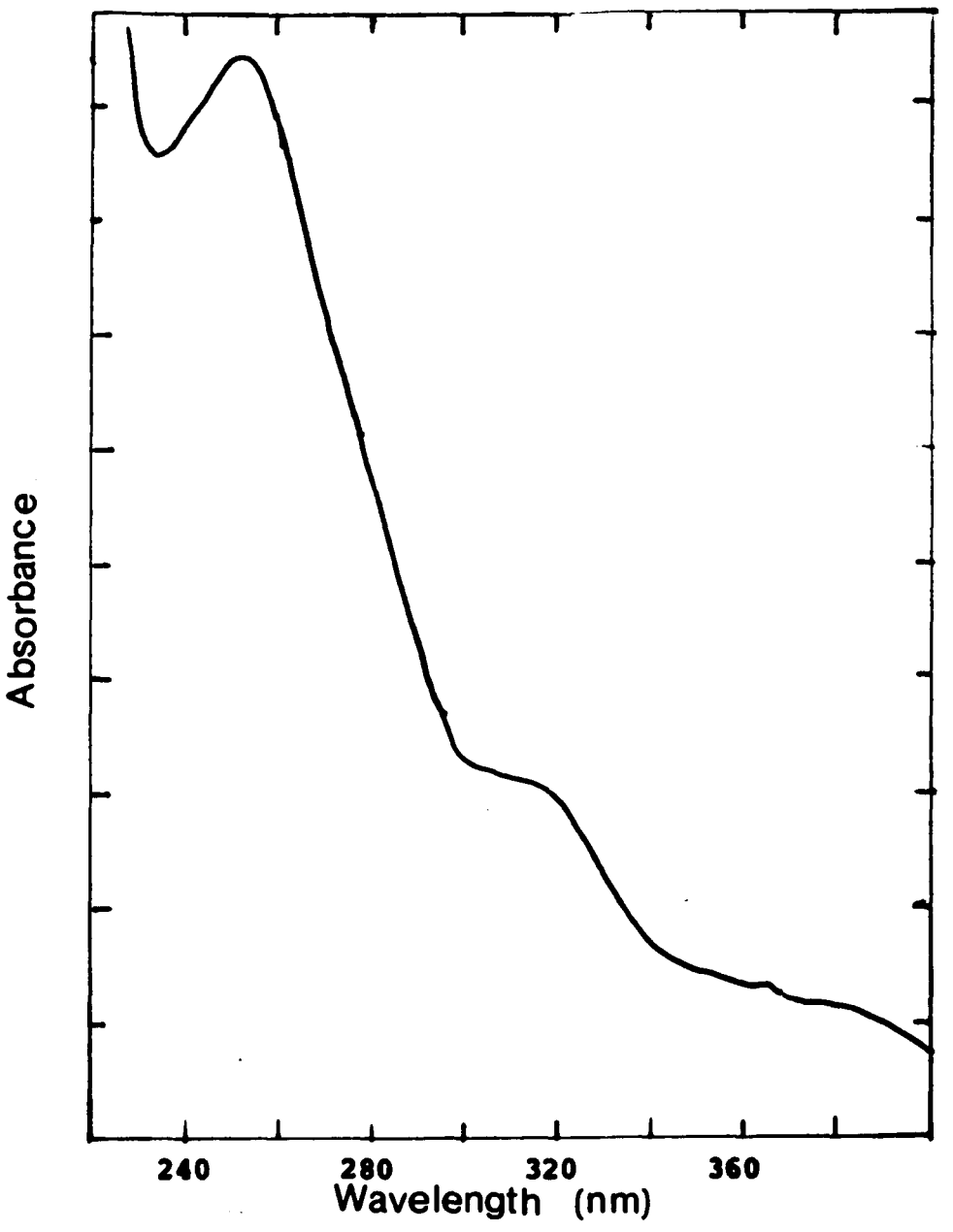


Figure 20. **UV Spectrum of Peak II in Figure 19.**

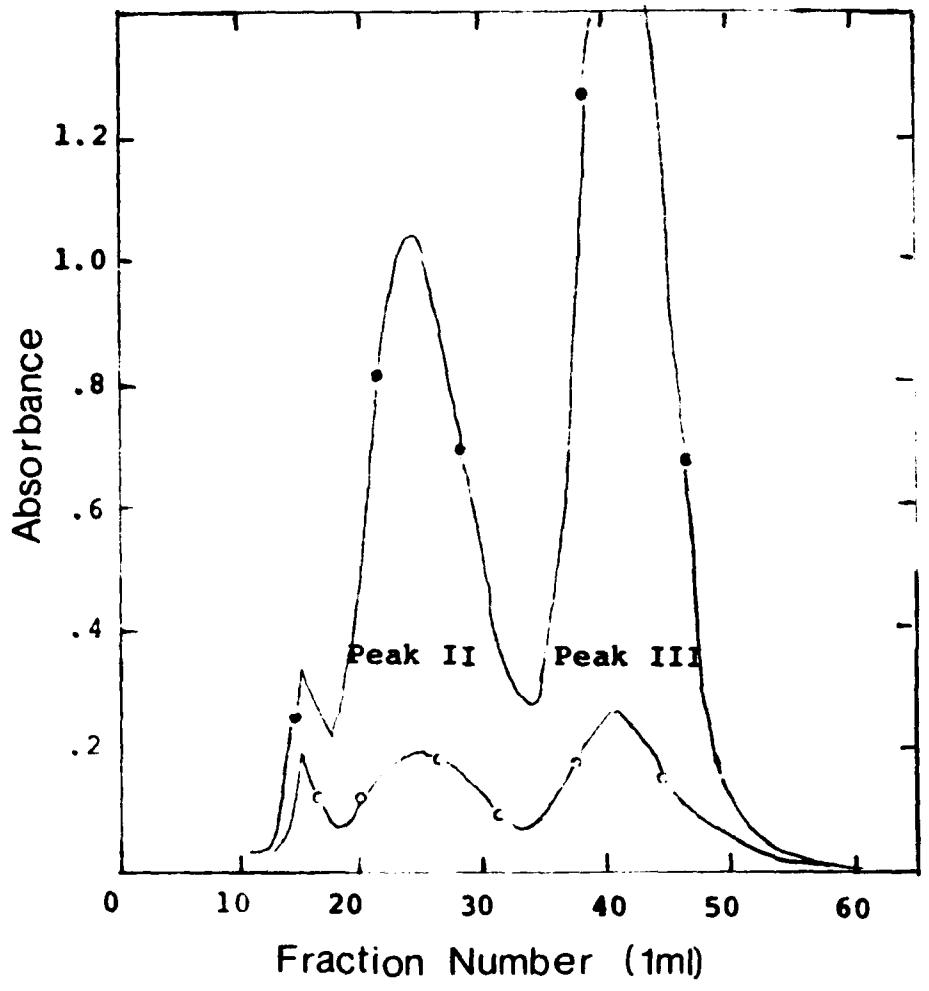


Figure 21. Material from Peak I, Figure 17, Rechromatographed on Sephadex G-100, 1.1 x 40 cm column, in .02 M Tris, pH 7.4. Graph shows absorbance at 260 (●—●) and 310 (o—o) nm.

as nucleotides and free MC. Peak 2, rechromatographed on Sephadex G-25, was eluted in the exclusion volume and therefore did not seem to contain small nucleotide-MC adducts. Peak 3, apparently the result of further breakdown of the complex, when chromatographed on Sephadex G-25, contained guanosine and uridine, rather than the GMP and UMP expected if the degradation were due only to T_1 and pancreatic RNases (Figure 22).

A second poly (U,G)-MC complex was then prepared and the above procedures repeated, with a few modifications. The complex contained 1155 A_{260} and 147 A_{310} units, binding ratio 0.13. Before digestion, a sample of the complex was allowed to stand at room temperature overnight and then rechromatographed on Sephadex G-25 to determine if nucleotides or nucleosides were being released into the medium, possible through some reaction with bound MC. Also, a sample of the MC fraction from the Sephadex G-100 separation of complex from unbound MC was tested on Sephadex G-25 for the presence of nucleosides or nucleotides which might have been released during complex formation. Both tests were negative.

The digest was centrifuged to remove the purple precipitate which was again evident. The supernatant was chromatographed on Sephadex G-100, rather than on G-50, and three peaks were obtained: a very small one at the exclusion volume, and two others.

The third peak from Sephadex G-100 was chromato-

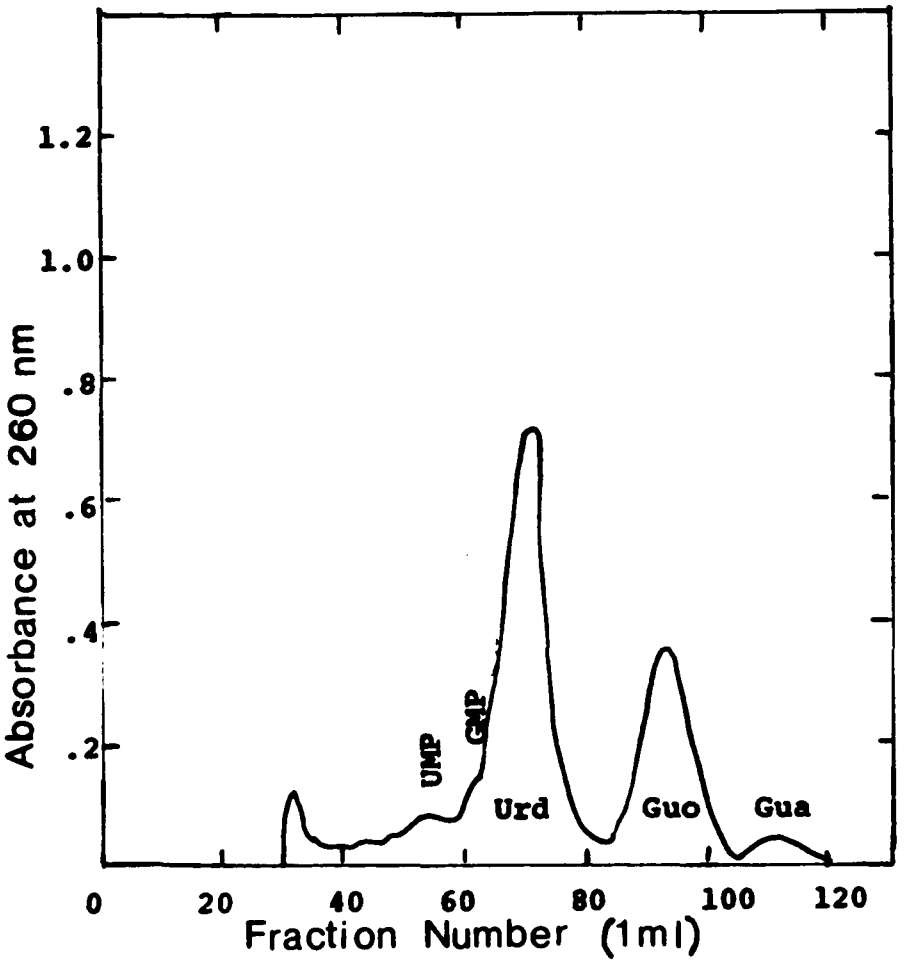


Figure 22. Peak III, Figure 21, Rechromatographed on Sephadex G-25, 1.5 x 41 cm column, in .02 M Tris, pH 7.4.

graphed on DEAE-Sephadex, with results very similar to the chromatography of the second Sephadex G-50 peak of the earlier digest (Figure 23). A small peak with the UV spectrum of GMP, believed to be cyclic (2',3')GMP, and two large peaks, UMP and GMP, were eluted first, followed by two much smaller peaks. The latter were observed as two adjacent narrow purple bands on the column, and were eluted at about 0.16 M guanidine HCl, as two overlapping peaks. After being desalted, they were rechromatographed on Sephadex G-25, and a fraction containing both 260 and 310 nm absorbance eluted at about 55 ml on this column. From its UV spectrum (Figures 24, 25), the peak could be a GMP-MC adduct. One-half of this material was treated with alkaline phosphatase and rechromatographed on the same column, and was eluted later, in about 68 ml. The other half was treated with a combination of snake venom phosphodiesterase (SVPD) and alkaline phosphatase. For a dinucleotide from a T1 plus pancreatic RNase digest, the result of this treatment should be:

$$\text{GpGp} \xrightarrow[-\ P_i]{\text{alk. P-ase}} \text{GpG} \xrightarrow{\text{SVPD}} \text{G} + \text{pG}$$

However, the product of this reaction had the same elution pattern on Sephadex G-25 as the material treated with alkaline phosphatase alone, so it appears that the action of the venom phosphodiesterase was blocked.

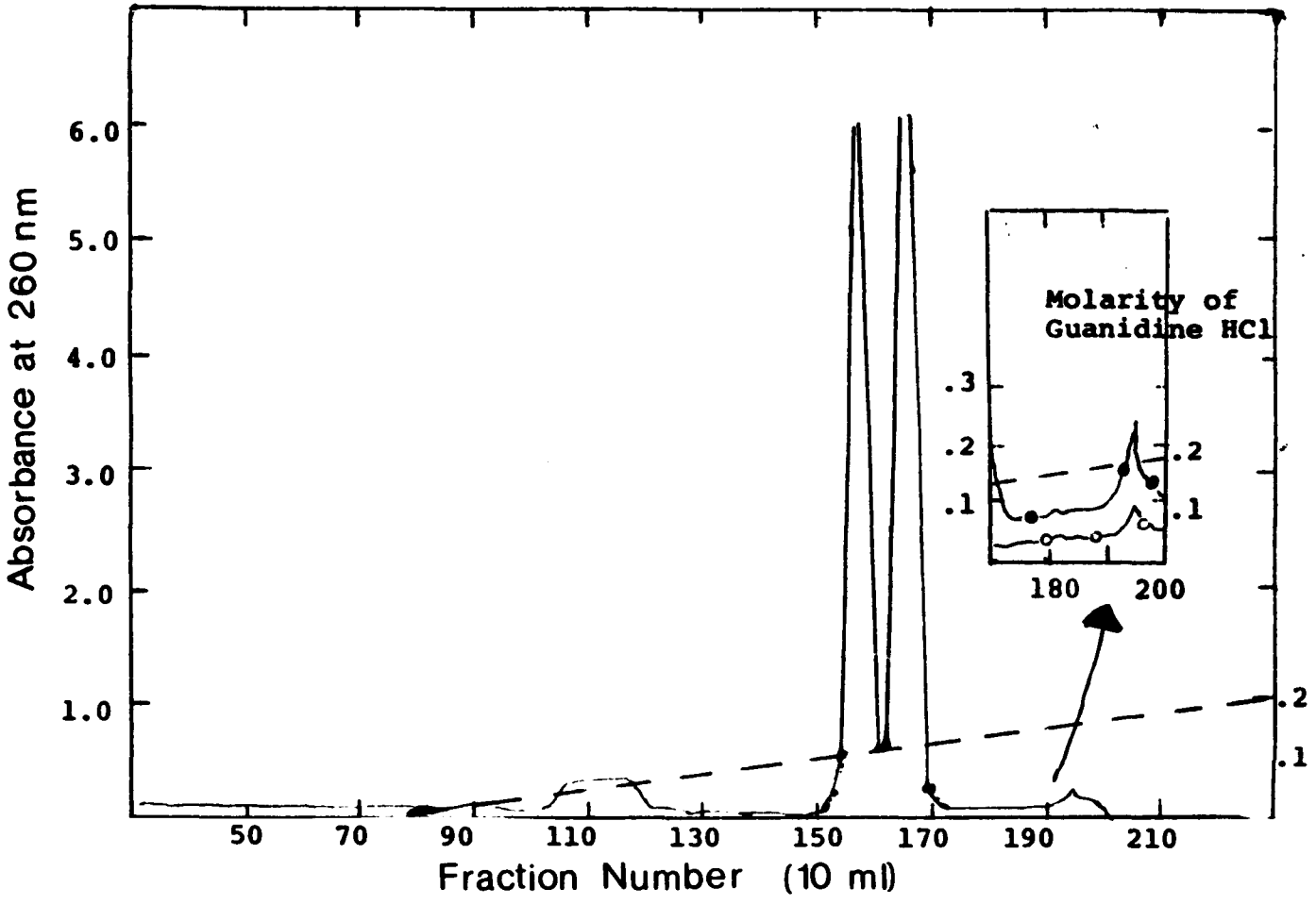


Figure 23. Third Peak from G-100 Chromatography of Poly (U,G)-MC Complex Digest, Rechromatographed on DEAE-Sephadex, in 7 M urea, .05 M Tris, pH 7.4, 0.2 mM EDTA, 2.5 x 28 cm column. Expanded scale inset shows absorbance at both 260 (●—●) and 310 (○—○) nm.

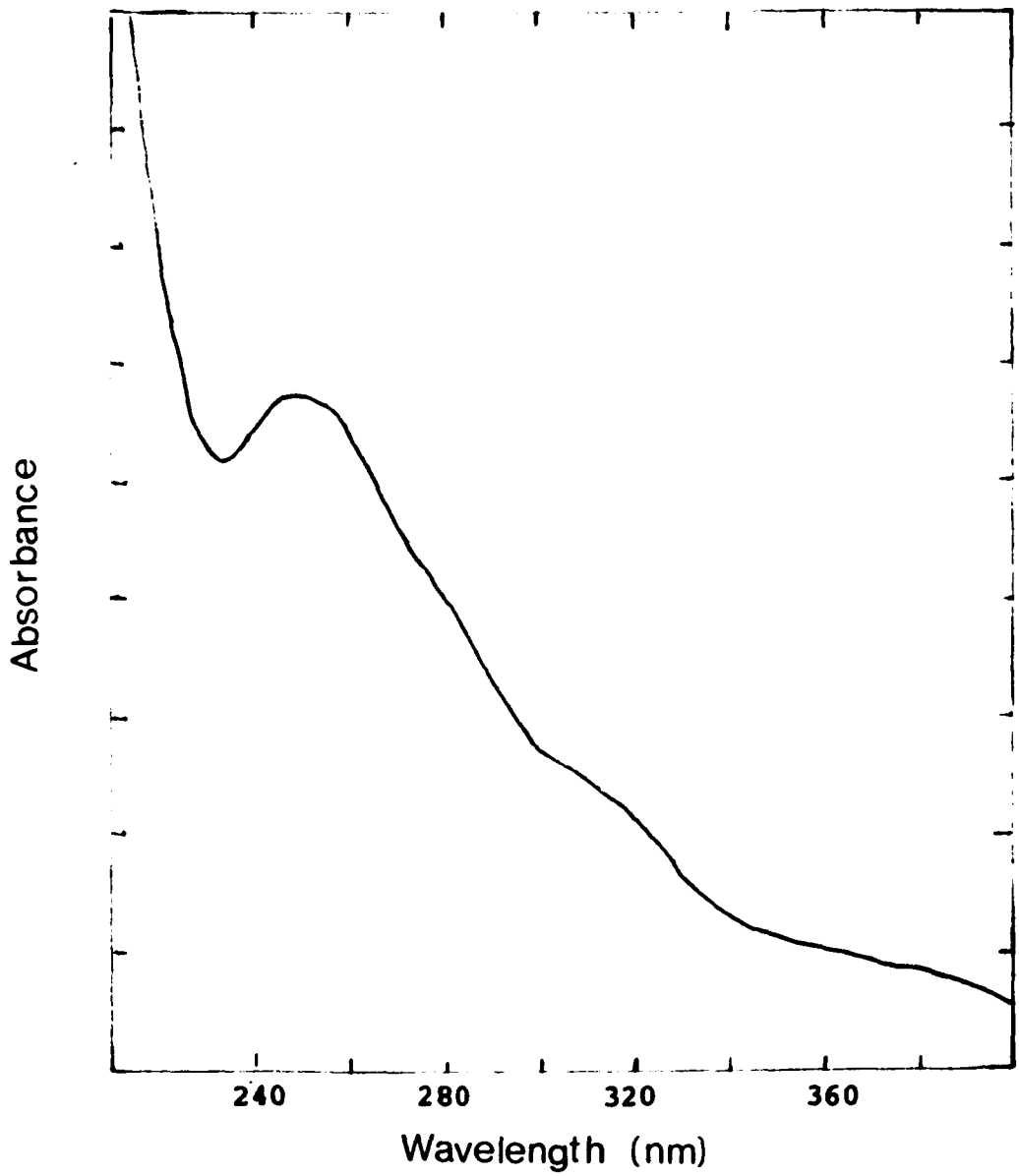


Figure 24. UV Spectrum of Material in Fractions 192-196, Figure 23, after Desalting on DEAE-Cellulose.

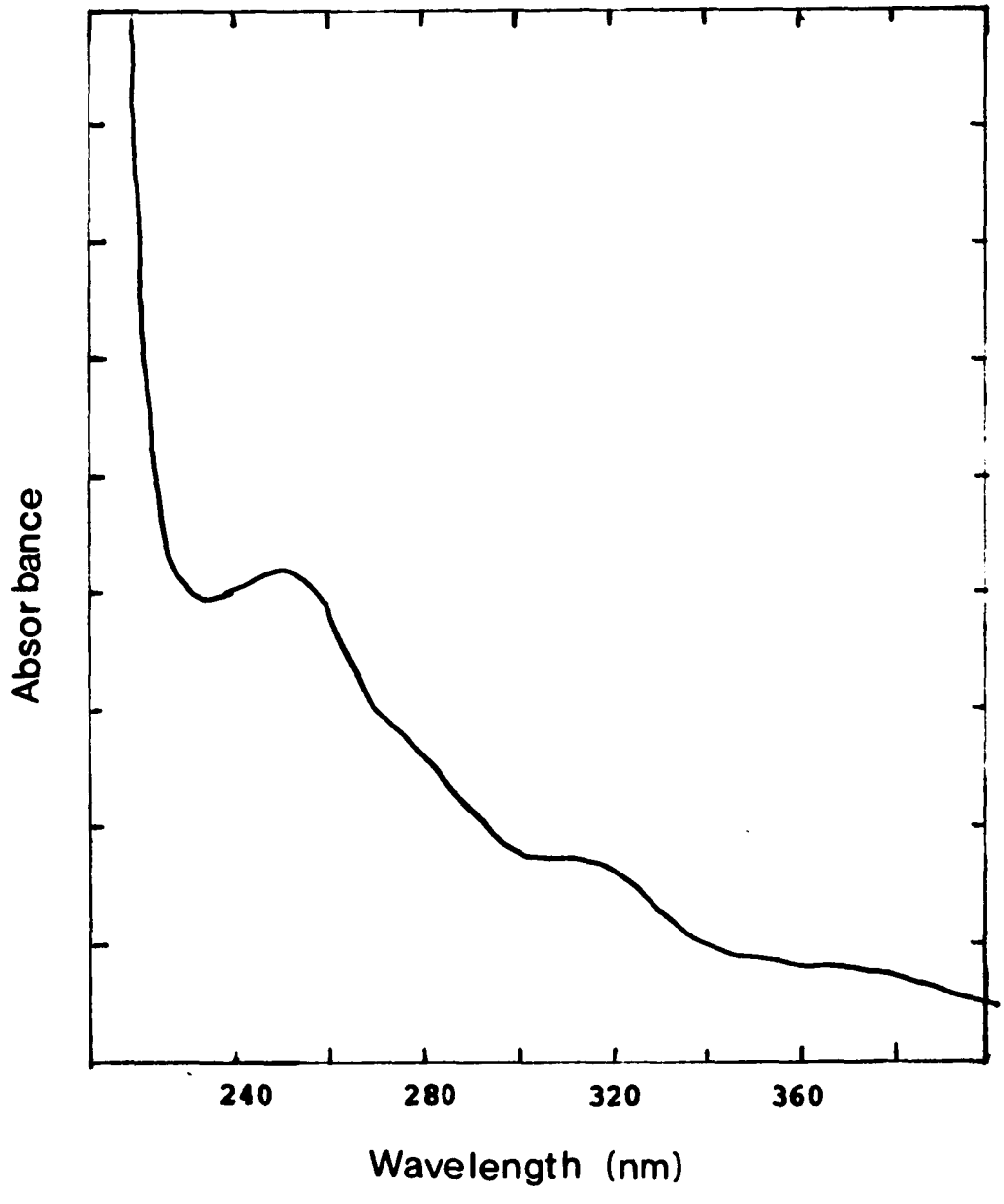


Figure 25. UV Spectrum of Material of Figure 24, after Rechromatography on Sephadex G-25 in .02 M Tris, pH 7.4.

Peak 2 from the Sephadex G-100 chromatography of the digest was rechromatographed on DEAE-Sephadex (Figure 26), with little more success than in the DEAE-Sephadex chromatography of the first peak from Sephadex G-50 of the previous digest. Some material with the UV absorbance characteristic of MC, accounting for 36% of the total A_{310} units applied to the column, was not retained and flowed through as the material was being applied. Five peaks were eluted, containing about 95% of the A_{260} units attributable to nucleotides, but none contained a significant amount of 310 nm absorbance.

E. Purple Precipitate from Enzymatic Digest of Poly (U,G)-MC Complex. The precipitate from the enzymatic digests was insoluble in 50% aqueous methanol, which dissolves MC, and in dimethylformamide, but soluble in dimethylsulfoxide (DMSO) with gentle heating. The solution in DMSO could then be diluted with aqueous buffer without reprecipitation.

Figures 27 and 28 show the UV spectrum of the precipitate compared to the spectra of poly G and of a mitosene compound, "Y", which has the spectral characteristics of MC bound to nucleic acid.

A small amount of the precipitate was chromatographed on Sephadex LH-20 in 50% aqueous methanol, on a column which had been calibrated for poly G and for compound Y. The sample was eluted in approximately the same volume as poly G, but appeared to be in two overlapping peaks, so the chromatography was repeated on a longer column in an attempt at better

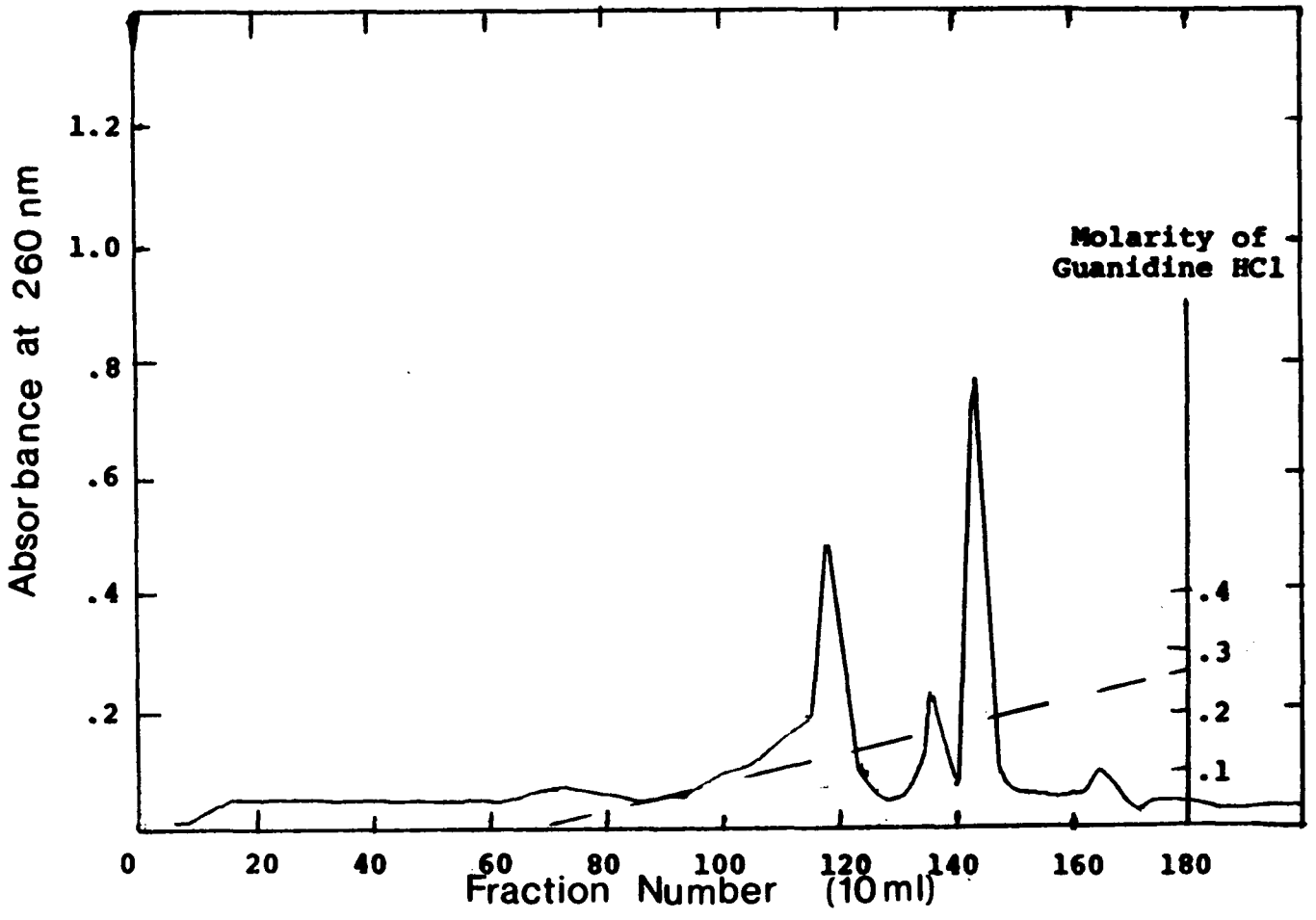


Figure 26 (page 1). **Peak II from Sephadex G-100 Chromatography of Enzymatic Digest of Poly (U,G)-MC Complex, Rechromatographed on DEAE-Sephadex, 2.5 x 28 cm column, in 7 M urea, .05 M Tris, pH 7.4, 0.2 mM EDTA, with Guanidine HCl gradient as shown.**

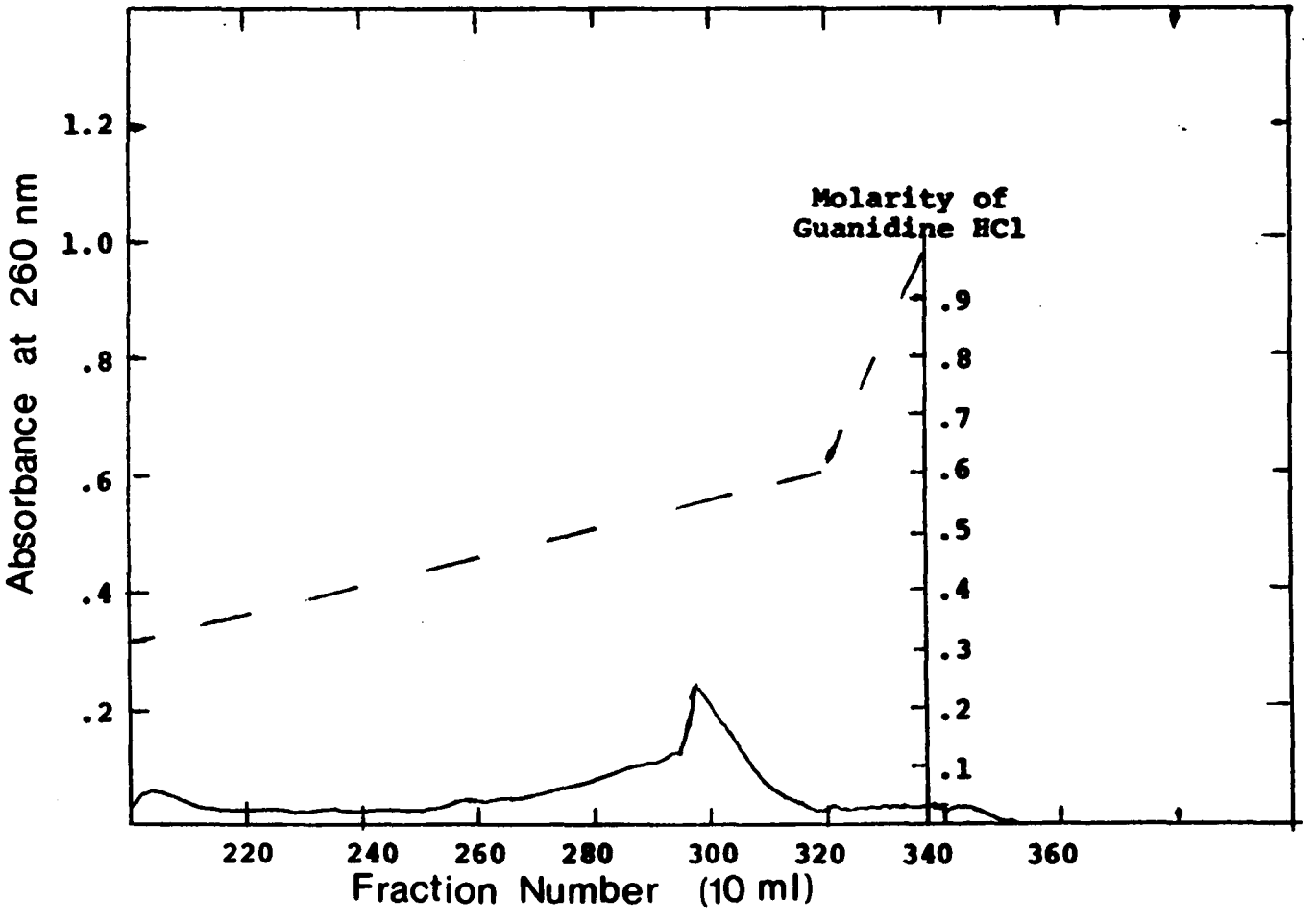


Figure 26 (page 2)

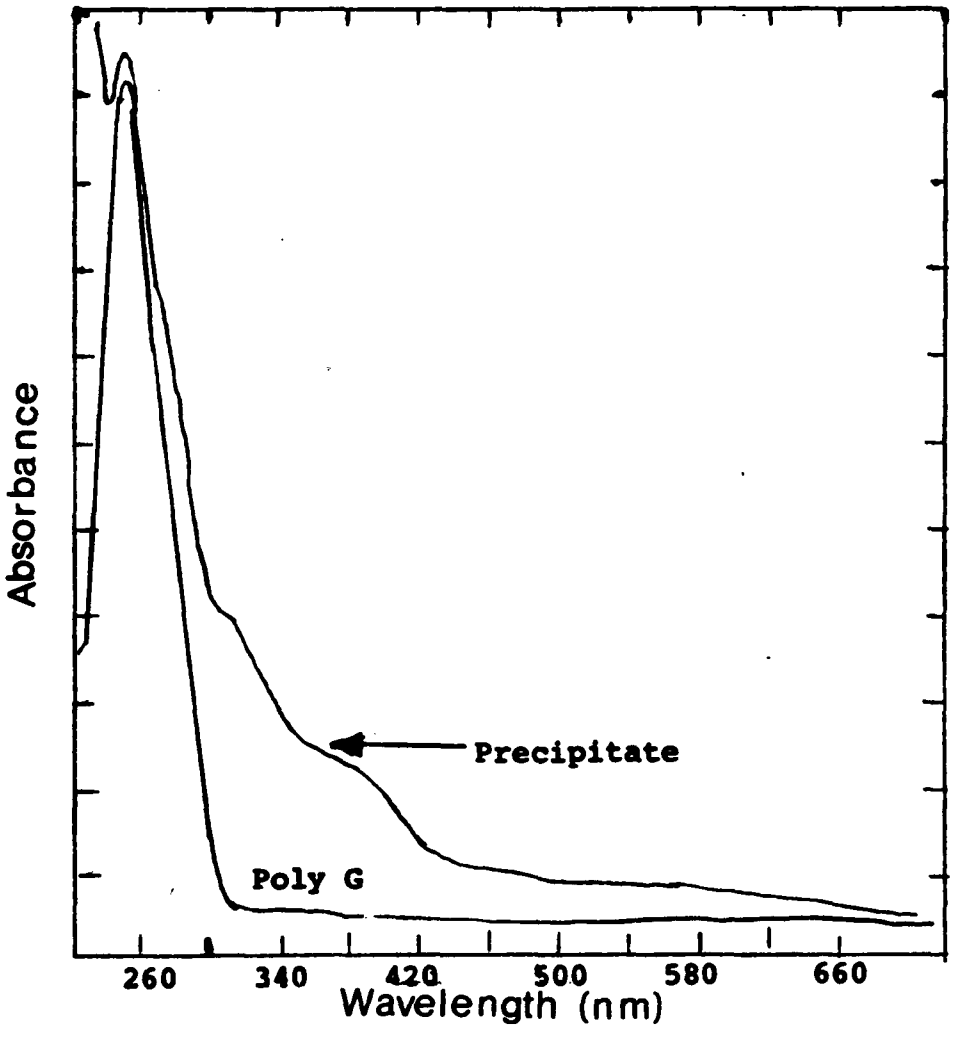


Figure 27. UV Spectra of Poly G and of Purple Precipitate from Enzymatic Digest of Poly (U,G)-MC Complex.

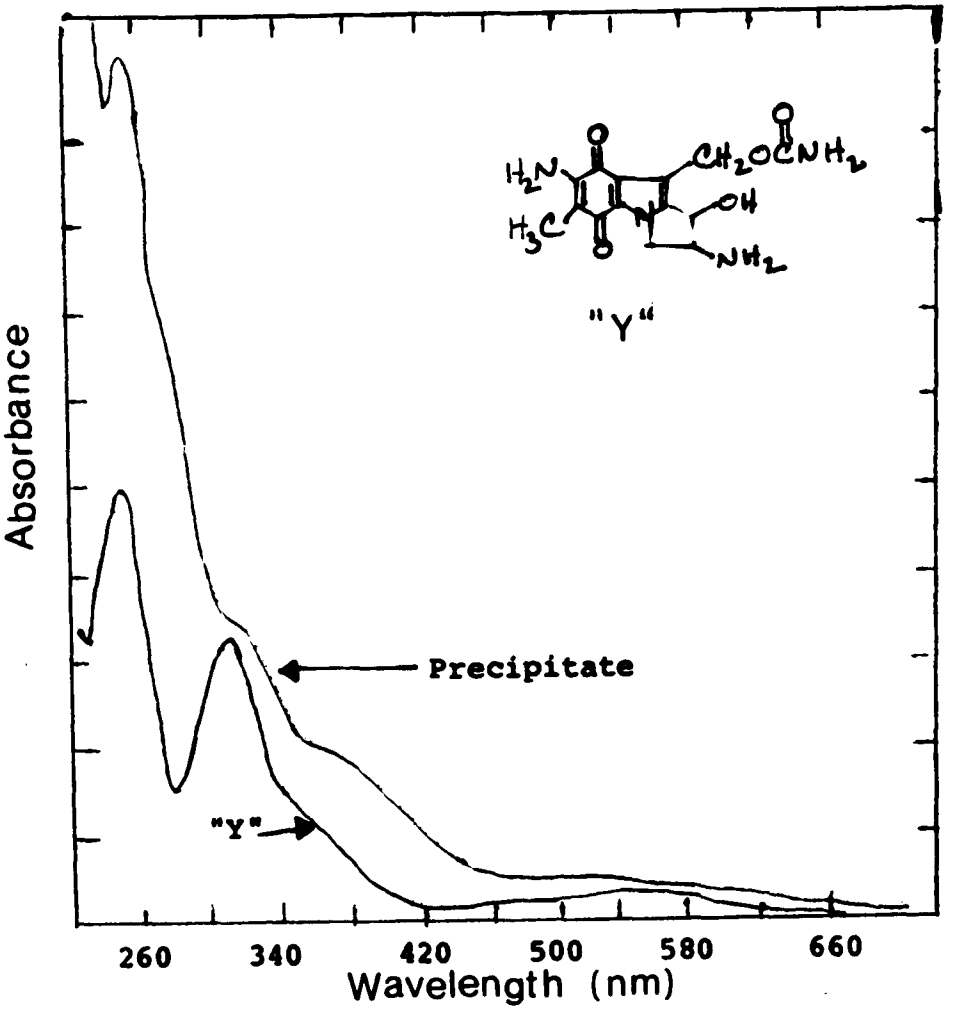


Figure 28. UV Spectra of Mitosene Compound "Y" and of Purple Precipitate from Enzymatic Digest of Poly (U,G)-MC Complex.

separation. However, almost all of the material precipitated on top of the column. The small amount which did not was eluted in the same volume as poly G and had no 310 nm absorbance. Similarly, when a sample of the material was applied to a Sephadex G-25 column in .05 M Tris, pH 7.4, most precipitated on the column and the material absorbing at 310 nm was not recovered. The material eluted from this column, about 30% of the A_{260}, appeared to be guanosine.

Figure 29 shows the results of thin-layer chromatography in several solvents and of paper electrophoresis of the precipitate, compared to guanosine and compound Y. Except for some DMSO and a trace of material with the fluorescence of a guanine derivative and mobility similar to that of guanosine, the complex remained at the origin.

A portion of the precipitate was subjected to alkaline hydrolysis, followed by chromatography on Sephadex G-25. (Figure 30). As a control, compound Y was incubated in KOH overnight and chromatographed on the same column. (Fig. 31) The digest turned yellow-brown overnight, as is usually the case with alkaline digests of MC complexes. On Sephadex G-25 the digest separated into two bands, a fast-moving yellow band which was eluted in the exclusion volume, and a slower pink band which became very diffuse. There were a number of peaks: first, the yellow material, then UMP, then a large amount of GMP, and finally a small peak in the

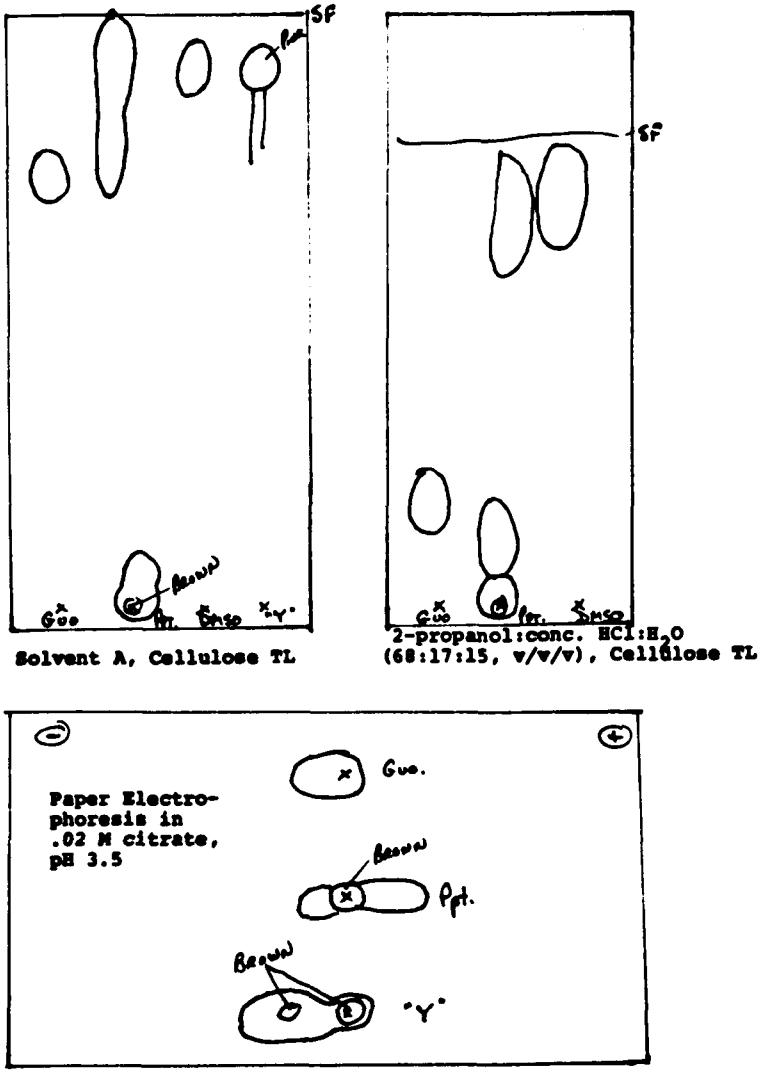


Figure 29. **TLC and Paper Electrophoresis of Purple Precipitate from Enzymatic Digest of Poly (U,G)-MC Complex**

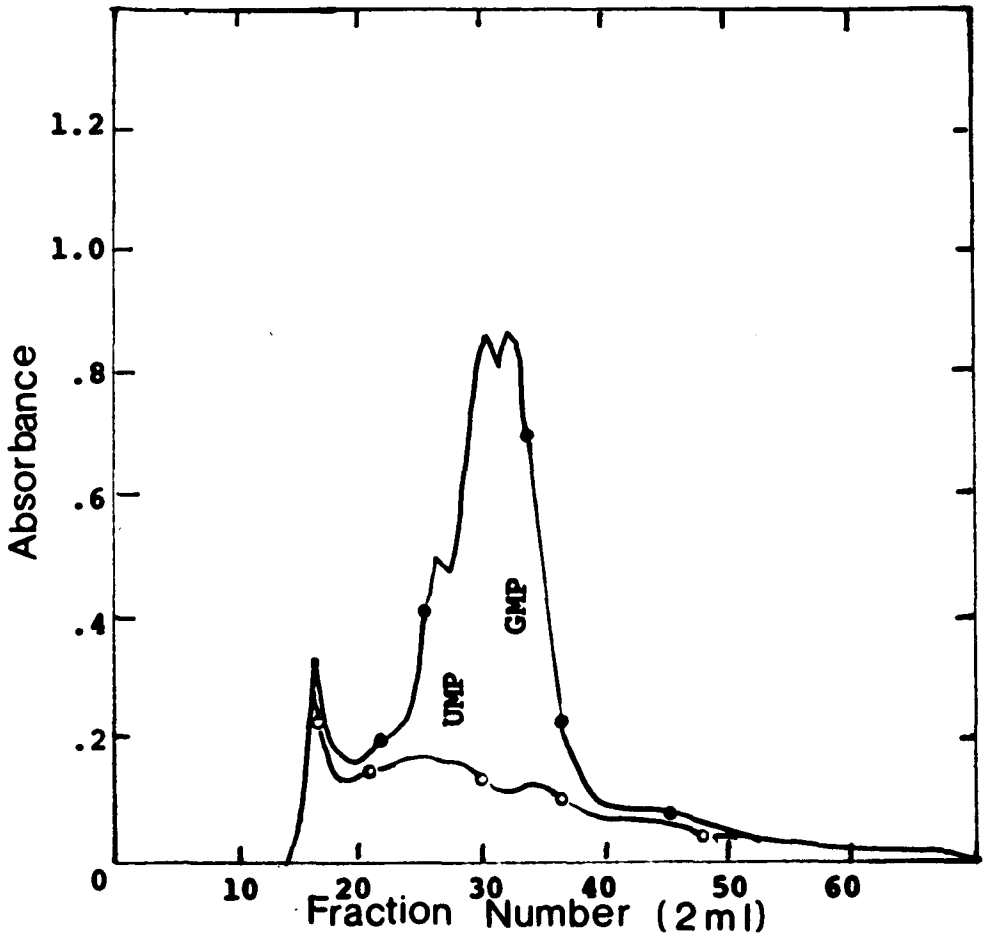


Figure 30. KOH Digest of Purple Precipitate from Enzymatic Digest of Poly (U,G)-MC Complex, Chromatographed on Sephadex G-25, 1.5 x 41 cm column, in .02 M Tris, pH 7.4. Graph shows absorbance at 260 (●—●) and 310 (○—○) nm.

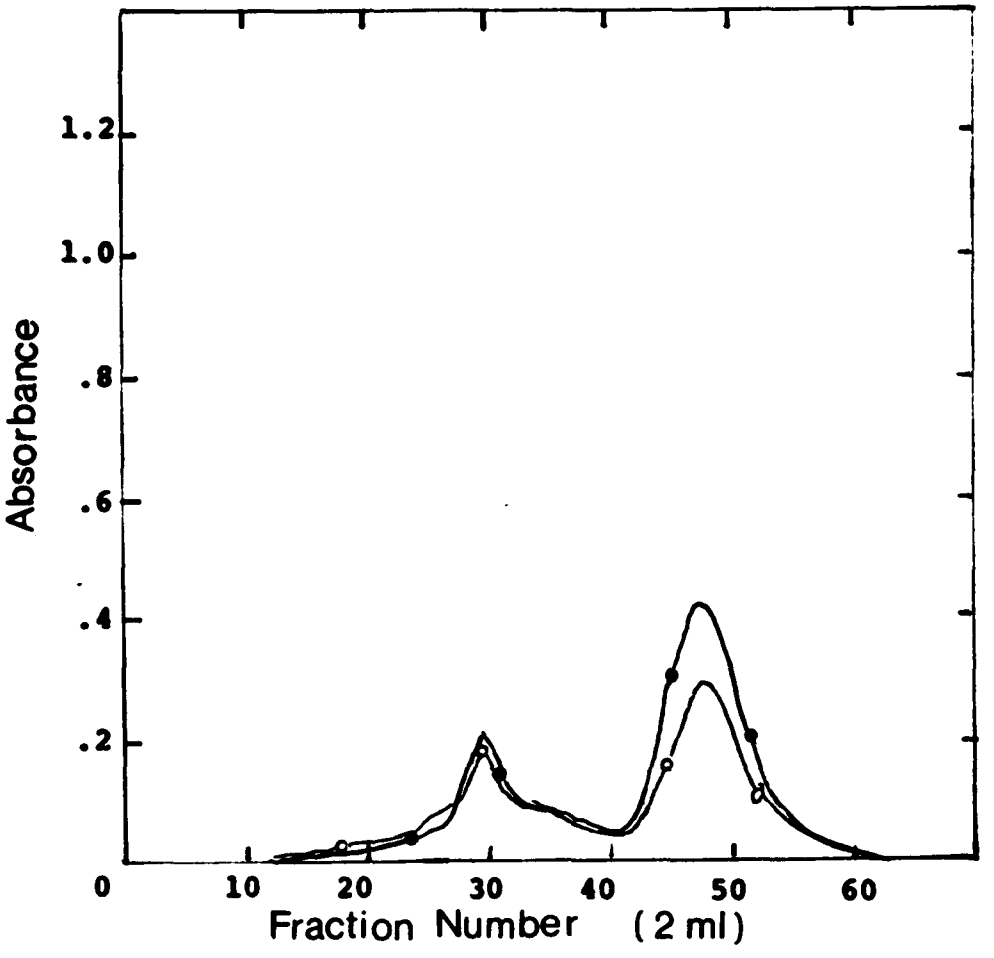


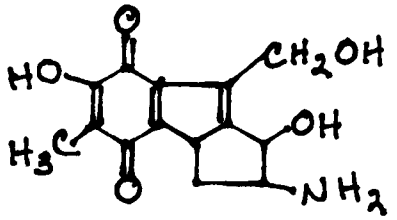
Figure 31. KOH Digest of Compound "Y" Chromatographed on Sephadex G-25, 1.5 x 41 cm column, in .02 M Tris, pH 7.4. Control for Figure 30. Graph shows absorbance at 260 (●—●) and 310 (○—○) nm.

area where the major peak of the control was eluted. The UV spectra of the first and last peaks were very similar, and resembled the spectrum of the control superimposed on the spectrum of a chromophore with strong absorbance at 280 nm.

The yellow material eluted in the exclusion volume was resistant to a second treatment with KOH and was eluted from the column in the same volume.

The GMP:UMP ratio in the precipitated material, based on the amount eluted in each peak on G-25, is approximately 6.

A portion of the precipitate was also subjected to acid hydrolysis in 2 M HCl, 60°, for 2 hours, conditions which convert free MC to 7-OH-decarbamoyl-Y:



(Stevens, et al., 1965)

When this hydrolysate was chromatographed on Sephadex G-25 (Figure 32), the components appeared to be UMP, GMP, guanine, and 7-OH-decarbamoyl-Y (spectra, Figures 33,34). The UMP and GMP fractions both contained some material which absorbed at 310 nm, so they were treated with alkaline phosphatase and rechromatographed. For the UMP fraction most of the 260 nm absorbing material moved to the uridine position, except for a small amount of material also absorbing at 310 nm, which remained in the same position, suggesting that the fraction had contained both UMP and a derivative of MC.

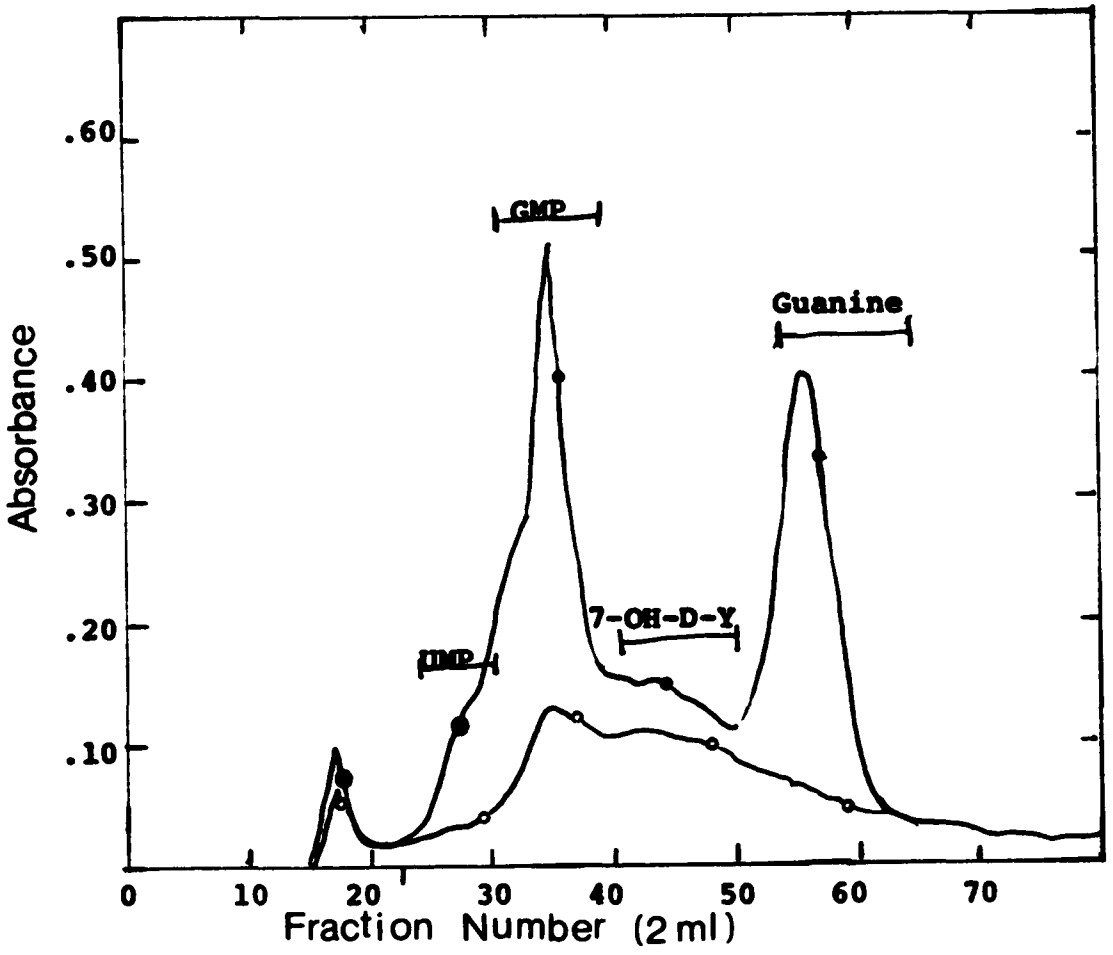


Figure 32. Acid Hydrolysate of Precipitate from Enzymatic Digest of Poly (U,G)-MC Complex, Chromatographed on Sephadex G-25, 1.5 x 41 cm column, in .02 M Tris, pH 7.4. Graph shows absorbance at 260 (●—●) and 310 (○—○) nm.

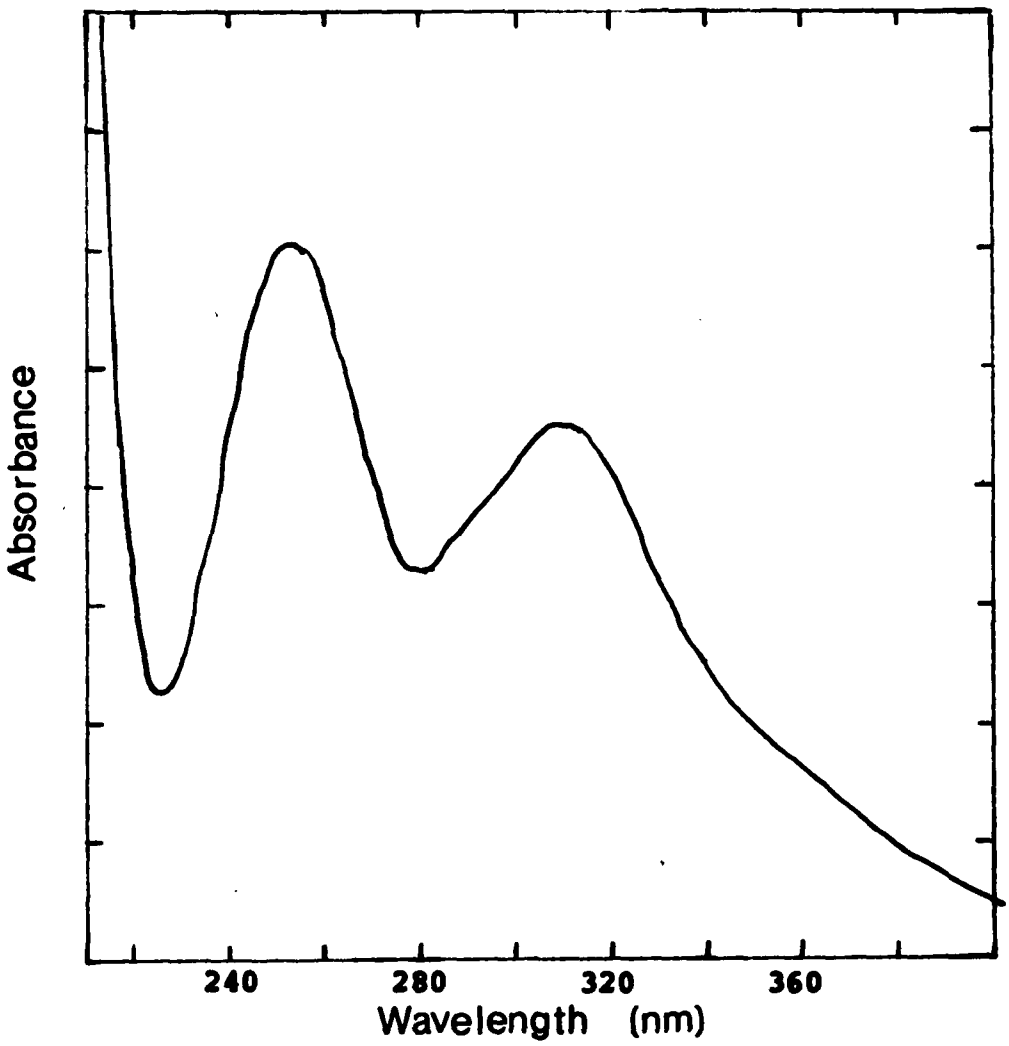


Figure 33. UV Spectrum of Material in Fraction 45, Figure 32.

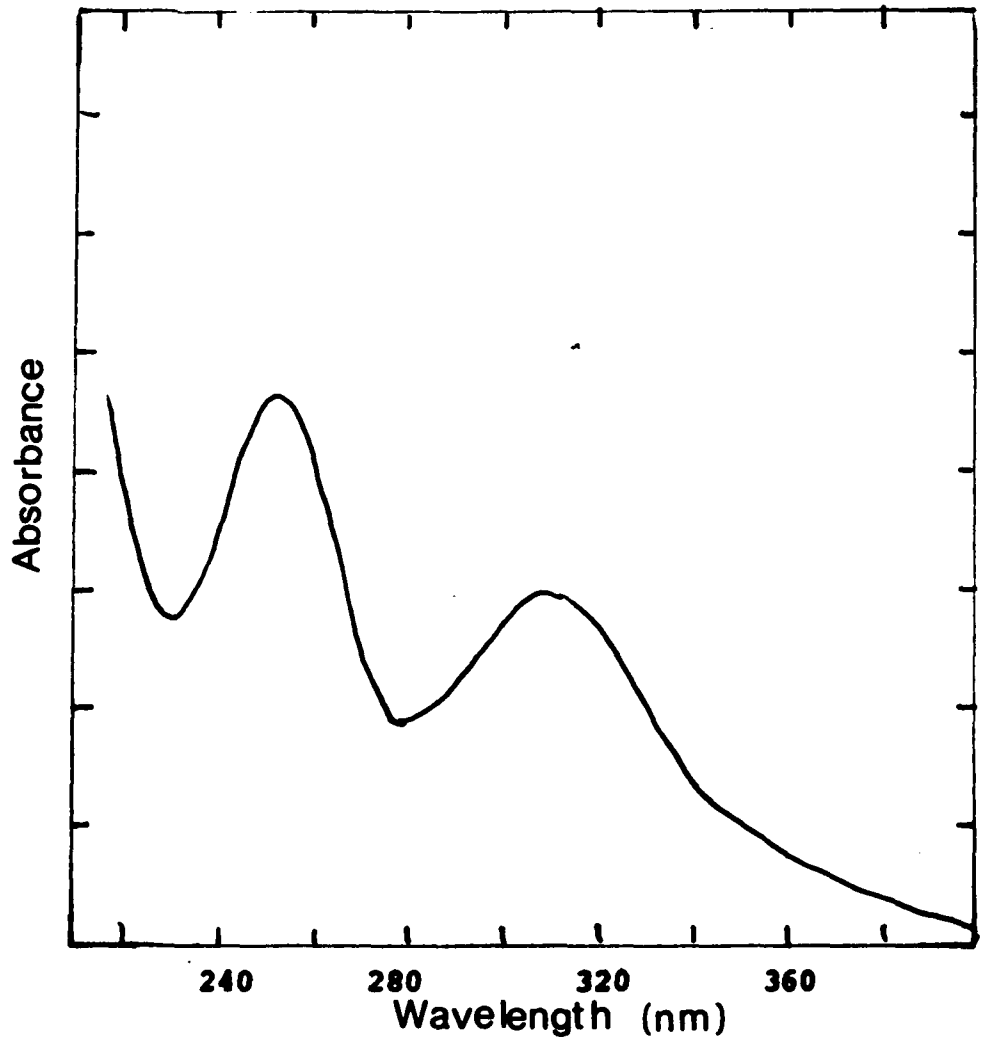


Figure 34. UV Spectrum of 7-OH-Decarbamoyl-Y

Similar results were obtained with the GMP peak. Generally, a small amount of 7-OH-decarbamoyl-Y elutes at this volume when chromatographed on a G-25 column of this size (R. Lipman, personal communication).

Finally, a sample of the precipitate dissolved in DMSO and then diluted with .05 M Tris, pH 7.4, was chromatographed on Sephadex G-100. It was eluted as a single peak in the exclusion volume of the column with an apparent binding ratio of about 1.0.

The precipitate accounted for about 7% of the total A_{260} and 17% of the total A_{310} of the complex before digestion.

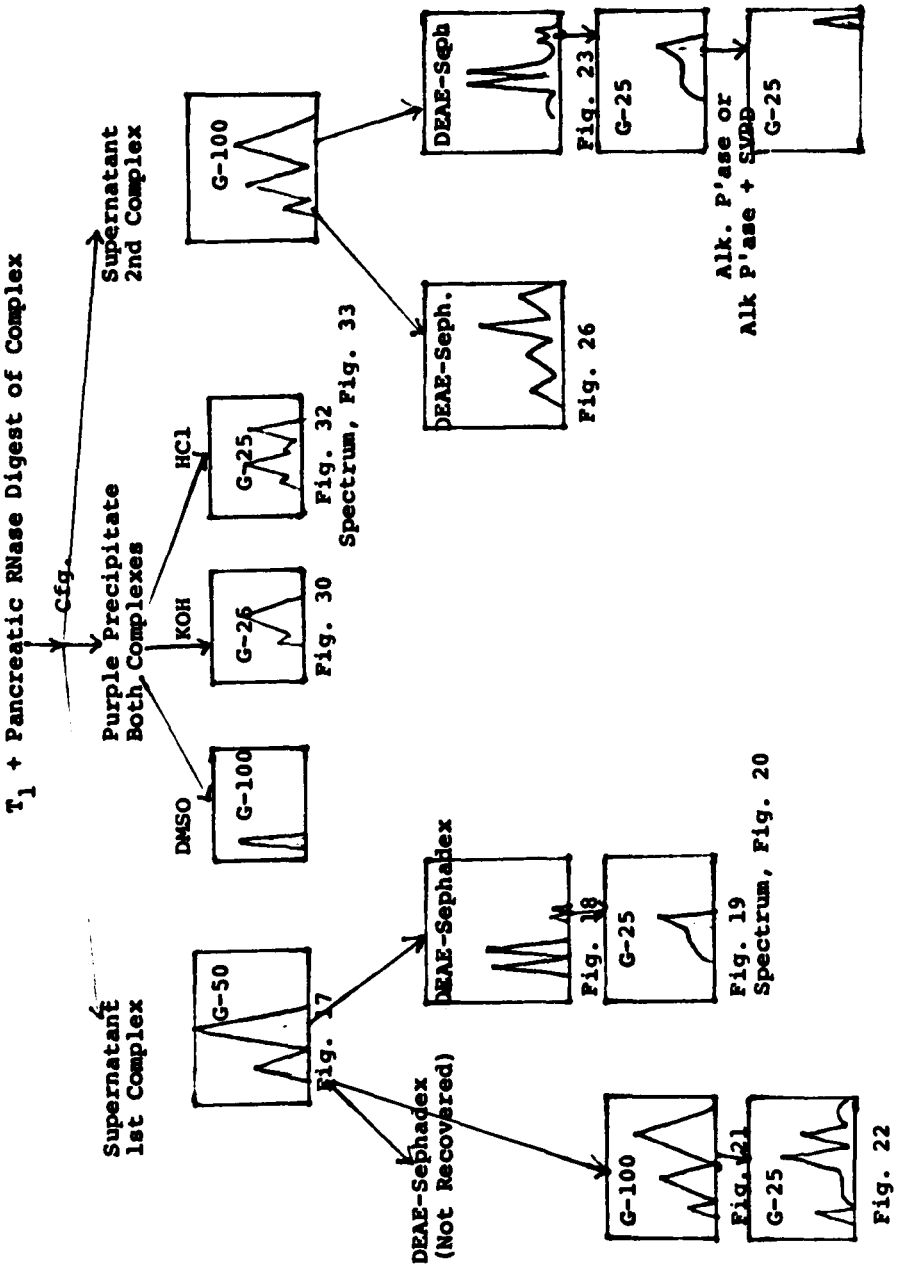


Figure 35. Summary of Analysis of Enzymatic Digests of Poly (U,G)-MC Complexes.

DISCUSSION

This study indicates that the binding of Mitomycin C to RNA closely parallels its binding to DNA in terms of preference for single-stranded nucleic acid, stability to heat and denaturing agents, and specificity for guanine. Double stranded RNA is not cross-linked by MC, but this may be due to steric requirements or a need for flexibility not satisfied by the dsRNA helical structure (Mercado and Tomasz, 1977).

The stability of the complex and the decided preference for guanine suggested formation of a covalent bond between MC and guanine. Therefore, the failure of MC to bond covalently to GMP or to GpC after reductive activation was surprising. However, non-bonding to mononucleotides or dinucleotides under these conditions has also been reported by Horneman, et al. (1976). More recently, binding between mononucleotides and MC has been reported, but under different conditions (Tomasz and Lipman, 1979).

Also, since the bond had appeared to be stable to alkali in DNA, it was disappointing that no GMP-MC adduct could be isolated after alkaline hydrolysis of RNA-MC, just as no guanine-MC adduct was found after depurination of DNA-MC.

Taken together, these results suggest that the MC-G bond is stabilized by the overall structure of the polymer.

and is quite labile in the absence of this stabilization.

The binding study with [^{3}H]-PM and synthetic polymers of ribonucleotides was in some respects a repetition of the work of Lipsett and Weissbach (1965). However, their reaction conditions were quite different, and the highest level of binding they reported was 0.74 micromole ^{3}H -PM to 100 micromoles guanine in a 1:1 GU copolymer. With the new, stepwise method of reductive activation, it was possible to obtain much higher levels of binding, approaching a 1:1 ratio of MC to nucleotide in transfer RNA. Obviously, this was more binding than could be accounted for if MC bound exclusively to guanine residues in RNA. This additional, "nonspecific" binding might be due to electrostatic attraction between the phosphate backbone of the polynucleotide and reduced MC, probably at the aziridine N. Therefore, each reaction in this series was done in both 0.01 M and 0.20 M Tris. The small amount of binding to all the polymers in low salt was not observed at the higher salt concentration, suggesting that it was indeed due to ionic attractions.

Electrostatic binding of MC to polyphosphate and polyvinylsulfate under the standard reductive reaction conditions has now been demonstrated. This binding is stable to heat and dialysis, but is completely dissociated by incubation in 2 M NaCl (Lipman, et al., 1978). Thus, in RNA-MC complexes made with a high MC:RNA reaction ratio and/or at low salt may be a combination of covalent binding to guanine residues and electrostatic binding to phosphate groups.

For the covalent bond, the guanine molecule provides a number of nucleophilic sites. The highly reactive N-7 position had been ruled out by steric considerations as a crosslinking site, and by the absence of thermal instability or C-8 tritium exchange as a site of monofunctional binding in DNA. 7-alkyl guanine is not spontaneously lost from RNA (Shooter, 1975) as it is from DNA, so the stability of the polymer to depurination would not preclude N-7 of guanine as the binding site in RNA-MC complexes. However, binding to poly G which was 40% methylated at this position was greatly increased over binding to unmodified poly G. This increase in binding may have been caused by disruption of the stable secondary structure of poly G by the methylation. Poly N-7 methyl GMP also has some secondary structure, but less than poly G (Pochon and Michelson, 1967; Michelson, et al., 1967). One model for a 4-stranded structure of poly G has guanine residues at approximately 90^{o} angles to each other, with hydrogen bonds from the N-1 and N-2 of one to the O-6 and N-7, respectively, of the other. Methylation at N-7 would weaken this structure.

As confirmation that N-7 is not the alkylated atom, it was found that an RNA-MC complex was methylated at N-7 to almost exactly the same extent as was RNA alone.

Since MC would not bind to monomers, we were very fortunate to receive a small amount of poly O^{6} methyl GMP from Dr. David Ludlum to test MC binding. The O-6 of guanine residues had been suggested as a participant in crosslinking

of DNA, and therefore it was somewhat surprising that binding to this polymer in low salt was higher than to poly G and only slightly less than binding to guanine in poly (U,G). The increase in binding over binding to poly G may be due to the low melting temperature of poly O^6-methyl GMP. Its secondary structure is disrupted at 37° (Mehta and Ludlum, 1976), the temperature at which the reaction was run. We did not have enough of this polymer to run a reaction in 0.20 M Tris. However, further evidence that O-6 of guanine is not the principal MC binding site is the stability of tRNA-MC complexes at pH 1. These data do not preclude, however, the possibility that this position is involved in the minor, crosslinking reaction.

To test binding to the 2-amino group of guanine, a comparison was made between binding to poly G and to poly I. For these two polymers, binding was almost identical at both low and high buffer concentrations. Binding to a 1:1 copolymer of UG was nearly doubled at both buffer concentrations, and this was attributed to disruption of the secondary structure of poly G, making the guanine residues more accessible to the drug. On the other hand, binding to a 1:1 copolymer of UI was again almost identical to binding to poly G in low salt, but in high salt was reduced almost to the level of binding to poly U. In this case, as with the other poly G analogs, it appears that electrostatic binding is enhanced in polymers with a high content of guanine or guanine-like bases. However, the covalent binding to

guanine observed at higher salt concentrations is missing in the absence of the 2-amino group. This strongly suggests that the 2-amino group of guanine is the site of covalent binding of MC, as it is for a number of polycyclic molecules.

This kind of covalent binding could account for the high molecular weight adducts between MC and G-rich sequences of poly (U,G) which were precipitated from the enzymatic digests. However, another mode of binding must be postulated to explain tRNA-MC complexes made at high MC:RNA ratios in .01 M salt, in which some of the tRNA molecules were bound to MC at a ratio of about 1 micromole nucleotide/1 micromole MC.

A number of cationic polycyclic molecules, for example, ethidium bromide and acridine orange, form stable, non-covalent complexes with linear biopolymers (Waring, 1965; Schwarz and Balthaser, 1970). The binding is cooperative and salt-sensitive, and may reach a ratio of one molecule/polymer binding site. After a number of primary binding sites have been occupied, usually by intercalation of the planar molecule, secondary binding to the saturation level occurs, due to stacking of the molecules along the polymer backbone. Electrostatic attraction between the polymer and the cationic molecules tends to reduce charge repulsion between the small molecules and permit stacking interactions.

A large number of aromatic, cationic antibiotic and antitumor compounds, including the antibiotics distamycin and netropsin, the trypanocidal drug berenil, and the bis-quaternary ammonium heterocycles (Denny, et al., 1979) exhibit similar biphasic binding, but do not intercalate in DNA (Braithwaite and Baguley, 1980). High binding ratios observed in 0.01 M salt are attributed to cooperative stacking of the cationic drug molecules on a polyanionic surface. At 0.20 M salt this type of binding is suppressed, and binding is selective for polymers of high dA+dT content. In this case, it is believed that binding is to the minor groove of the DNA helix, and is selective for dA-dT base pairs because depth of the groove is greater in the absence of the guanine 2-amino group.

An analogy could be made to binding of MC to tRNA in 0.01 M salt, but perhaps with the stacked MC molecules stabilized by an occasional MC covalently bound to the guanine N-2 group.

The search for a small guanylate oligomer bound to MC met with very little success. From each enzymatic digest a very small amount of material was isolated which appeared from its spectrum to be such an adduct, and which retained the bound drug after treatment with alkaline phosphatase and venom phosphodiesterase. However, the amount of material was extremely small, and further testing was not feasible.

Except for this, the digests yielded only large, highly bound fragments, or smaller adducts which released the bound MC when chromatographed on DEAE-Sephadex. The loss of MC may indicate that it had been electrostatically bound and that degradation of the polymer had destroyed the possibility for stacking of drug molecules which might have stabilized the bond, so that it was easily displaced by the ion exchange column. Alternatively, it may be that covalent MC-RNA bonds are quite labile and that isolated molecules, covalently bound, are easily released without the reinforcement of stacking interactions.

Finally, it is possible that some of the MC binding is in the form of phosphotriesters which are slowly hydrolyzed. There is some evidence that molecules which can alkylate the guanine N-2 can also form phosphotriesters (Koreeda, et al., 1976). Although these triesters are known to cause strand breaks in RNA, the stability of the bond seems to depend to some extent on the nature of the esterified group, and hydrolysis may favor release of the MC moeity rather than breaking of the phosphodiester bond (Shooter, 1975; Singer and Fraenkel-Conrat, 1975). Therefore, a small amount of phosphotriester formation might not lead to immediate breakdown of the complex or the polymer, but could perhaps account for the eventual breakdown of the complex observed in Figures 21 and 22.

CONCLUSIONS

Complexes formed between RNA and mitomycin C reductively activated by $Na_2S_2O_4$ are stable to repeated chromatography on gel exclusion columns and to heat, mild acid, and 7 M urea. They are also protected from degradation by pancreatic and T_1 ribonucleases.

The binding is selective for guanine, but some binding also occurs to polymers which do not contain guanine. The latter nonspecific binding is suppressed by increasing the salt concentration of the reaction mixture.

Experiments with polymers of modified guanylic acid show that binding to MC is not inhibited when the O-6 or N-7 of guanine is blocked, but that it is drastically reduced in the absence of the 2-amino group, indicating that the 2-amino group of guanine residues is the major MC binding site in nucleic acids.

Binding of MC to GMP or other monomers is not observed under conditions leading to complex formation with polynucleotides. Binding to polymers appears to have some degree of cooperativity, and in low salt may be a combination of covalent binding to guanine plus stacking of the drug molecules along the phosphate backbone of the polynucleotide.

REFERENCES

Ames, B. N. and Dubin, D. T. (1960) J. Biol. Chem. 235, 769.

Bannon, P. and Verly, W. (1972) Eur. J. Biochem. 31, 1.

Billeter, M. A. and Weissmann, C. (1966) in Procedures in Nucleic Acid Research (Cantoni and Davis, Eds.) Harper and Row, New York, p. 508.

Braithewaite, A. W. and Baguley, B. C. (1980) Biochem. 19, 1101.

Brimacombe, R. L. C., Griffin, B. E., Haines, J. A., Haslam, W. J., and Reese, C. B. (1965) Biochem. 4, 2452.

Brookes, P. and Lawley, P. D. (1963) Biochem. J. 89, 127.

Cech, C. L. and Tinaco, I., Jr. (1976) Nucleic Acids Res. 3, 399.

Denny, W. A., Atwell, G. J., Baguley, B. C. and Cain, B. F. (1979) J. Med. Chem. 22, 134.

Dipple, A., Brookes, P., Mackintosh, D. S. and Rayman, M. P. (1971) Biochem. 10, 4323.

Feldman, G., Remsen, J., Wang, T. V. and Cerutti, P. (1980) Biochem. 19, 1095.

Gerchman, L. L., Dombrowski, J. and Ludlum, D. B. (1972) Biochim. Biophys. Acta 272, 672.

Goldberg, I. H. and Friedman, P. A. (1971) Ann. Rev. Biochem. 40, 775.

Grunberger, D. and Weinstein, I. B. (1979) Progress in Nucleic Acid Research and Molecular Biology 23, 105.

Hornemann, U., Ho, Y.-K., Mackey, J. K., Jr. and Srivastava, S. C. (1976) J. Am. Chem. Soc. 98, 7069.

Hornemann, U., Keller, P. J. and Kozlowski, J. F. (1979) J. Am. Chem. Soc. 101, 7121.

Horwitz, S. B. (1971) in Progress in Molecular and Subcellular Biology, Vol. 2 (Hahn, F. E., Ed.) Springer-Verlag, Berlin, Heidelberg, New York, p. 40.

Hsuing, H., Lown, J. W., and Johnson, D. (1976) Can. J. Biochem. 54, 1047.

Iyer, V. N. and Szybalski, W. (1963) Proc. Natl. Acad. Sci. U.S.A. 50, 355.

Iyer, V. N. and Szybalski, W. (1964) Science 145, 55.

Jeffrey, A. M., Blobstein, S. H., Weinstein, I. B., Beland, F. A., Harvey, R. G., Kasai, H., and Nakanishi, K. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2311.

Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Weinstein, I. B., Beland, F. A., Harvey, R. G., Kasai, H., Muira, I., and Nakanishi, K. (1976) J. Am. Chem. Soc. 98, 5714.

Jennette, K. W., Jeffrey, A. M., Blobstein, S. H., Beland, F. A., Harvey, R. G. and Weinstein, I. B. (1977) Biochem. 16, 933.

Jensen, D. E. and Reed, D. J. (1978) Biochem. 17, 5098.

King, H., Osborne, M., and Brookes, P. (1979) Chem.-Biol. Interact. 24, 345.

Koreeda, M., Moore, P. D., Yagi, H., Yeh, H. J. C. and Jerina, D. M. (1976) J. Am. Chem. Soc. 98, 6720.

Kriek, E. (1972) Cancer Res. 32, 2042.

Lawley, P. D. (1966) Progress in Nucleic Acid Research and Molecular Biology 5, 89.

Lawley, P. D. and Brookes, P. (1963) Biochem. J. 89, 127.

Lawley, P. D., Orr, D. J. and Shah, S. A. (1972) Chem.-Biol. Interact. 4, 431.

Lawley, P. D. and Shah, S. A. (1972) Biochem. J. 128, 117.

Lawley, P. D. and Thatcher, C. J. (1970) Biochem. J. 116, 693.

Lieberman, M. W. and Dipple, A. (1972) Cancer Res. 32, 1855.

Lijinsky, W. (1976) Progress in Nucleic Acid Research and Molecular Biology 17, 247.

Lipman, R., Weaver, J. and Tomasz, M. (1978) Biochim. Biophys. Acta 521, 779.

Lipsett, M. N. and Weissbach, A. (1965) Biochem. 4, 206.

Lown, J. W. and Weir, G. (1978) Can. J. Biochem. 56, 296.

Ludlum, D. B. (1969) Biochim. Biophys. Acta 174, 773.

March, J., Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, McGraw-Hill, New York, 1968, p. 290.

Mehta, J. R. and Ludlum, D. B. (1976) Biochem. 15, 4329.

Mercado, C. M. and Tomasz, M. (1972) Antimicrob. Ag. Chemother. 1, 73.

Mercado, C. M. and Tomasz, M. (1977) Biochem. 16, 2040.

Michelson, A. M., Massoulie, J. and Guschbauer, W. (1967) Progress in Nucleic Acid Research and Molecular Biology 6, 83.

Michelson, A. M. and Pochon, F. (1966) Biochim. Biophys. Acta 114, 469.

Min Jou, W. and Fiers, W. (1969) J. Mol. Biol. 40, 187.

Nishimura, S., Harada, F., Narushima, U. and Seno, T. (1967) Biochim. Biophys. Acta 142, 133.

Olson, A. C. and Volkin, E. (1972) Biochim. Biophys. Acta 277, 290.

Osborne, M. R., Thompson, M. H., Tarmy, E. M., Beland, F. A. Harvey, R. G. and Brookes, P., (1976) Chem.-Biol. Interact. 13, 343.

Otsuji, N. and Murayama, I. (1972) J. Bacteriol. 109, 475.

Pace, N. R., Pato, M. L., McKibbin, J., and Radcliffe, C. W. (1973) J. Mol. Biol. 75, 619.

Parish, J. H. (1972) Principles and Practice of Experiments with Nucleic Acids, Halsted Press, John Wiley & Sons, New York, p. 55.

Pochon, F. and Michelson, A. M. (1967) Biochim. Biophys. Acta 145, 321.

Pulkrabek, P., Leffler, S., Weinstein, I. B. and Grunberger, D. (1977) Biochem. 16, 3127.

Randerath, K. and Randerath, E. (1967) in Methods in Enzymology, Vol. 12A (Grossman, L. and Moldave, K., Eds.) Academic Press, New York, pp. 332, 339, 336.

Rhaese, H.-J. and Freese, E. (1969) Biochim. Biophys. Acta 190, 418.

Rushizky, G. W. and Sober, J. H. (1962) Biochim. Biophys. Acta 55, 217.

Sarker, N. K. (1967) Biochim. Biophys. Acta 145, 174.

Schwarz, G. and Balthasar, W. (1970), Eur. J. Biochem. 12, 461.

Seaman, E. (1968) in Methods in Enzymology, Vol. 12B (Grossman, L. and Moldave, K., Eds.), Academic Press, New York, p. 218.

Shapiro, R., Cohen, B. I., Shian-Jan, S. and Maurer, H. (1969) Biochem. 8, 238.

Shiba, S., Terawaki, A., Toguchi, T. and Kawamata, J. (1959) Nature 183, 1056.

Shooter, K. V. (1975) Chem.-Biol. Interact. 11, 575.

Shooter, K. V., Edwards, P. A. and Lawley, P. D. (1971) Biochem. J. 125, 829.

Shooter, K. V., Howse, R., Shah, S. A. and Lawley, P. D. (1974a) Biochem. J. 137, 303.

Shooter, K. V., Howse, R. and Merrifield, R. K. (1974b) Biochem. J. 137, 313.

Singer, B. (1975) Progress in Nucleic Acid Research and Molecular Biology 15, 219.

Singer, B. and Fraenkel-Conrat, H. (1975) Biochem. 14, 772.

Singhal, R. P. (1974) Eur. J. Biochem. 43, 245.

Stevens, C. L., Taylor, K. G., Morton, E. M., Marshall, W. S., Noll, K., Shah, G. D., Shah, L. G. and Uzu, K. (1965) J. Med. Chem. 8, 1.

Sun, L. and Singer, B. (1975) Biochem. 14, 1795.

Tener, G. M. (1967) in Methods in Enzymology, Vol. 12A, (Grossman, L. and Moldave, K., Eds.), Academic Press, New York, p. 218.

Tomasz, M. (1970a), Biochim. Biophys. Acta 199, 18.

Tomasz, M. (1970b), Biochim. Biophys. Acta 213, 288.

Tomasz, M., Mercado, C. M., Olson, J. and Chatterjie, N. (1974) Biochem. 13, 4878.

Tulinsky, A. (1962) J. Am. Chem. Soc. 84, 3188.

Uchida, T. and Egami, F. (1967) in Methods in Enzymology, Vol. 12A (Grossman, L. and Moldave, K., Eds.) Academic Press, New York, p. 237.

Uziel, M., Chong, K. K. and Cohn, W. E. (1968) Anal. Biochem. 25, 77.

Vandenbussche, P. and Fiers, W. (1966) Biochim. Biophys. Acta 114, 182.

Waring, M. J. (1965) J. Mol. Biol. 13, 269.

Warrington, R. C. (1974), Biochim. Biophys. Acta 353, 63.

Webb, J. S., Cosulich, D. B., Mowat, J. H., Patrick, J. B., Broschard, R. W., Meyer, W. E., Williams, R. P., Wolf, C. F., Fulmor, W. Pidacks, C. and Lancaster, J. E. (1962) J. Am. Chem. Soc. 84, 3185, 3187.

Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H. and Nakanishi, K. (1976) Science 193, 592.

Weissbach, A. and Lisio, A. (1965) Biochem. 4, 196.

Wyatt, G. R. (1955) in The Nucleic Acids, Vol. I (Chargaff, E. and Davidson, J. N., Eds.) Academic Press, New York, p. 243.

Zimmerman, S. B., Cohen, G. H. and Davis, D. R. (1975), J. Mol. Biol. 92, 181.