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THE UNIVERSITY OF OKLAHOMA, PH.D., 1979

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GRADUATE COLLEGE

THE BINDING OF DEOXYGUANOSINE AND ADENOSINE TO CHEMICALLY MODIFIED POLYCYTIDYLIC ACID

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

PHILLIP A. BOYD

Norman, Oklahoma

1979

THE BINDING OF DEOXYGUANOSINE AND ADENOSINE TO CHEMICALLY MODIFIED POLYCYTIDYLIC ACID

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Preface

"Two principles are necessary so that life shall succeed: One consists of proteins, the other of nucleic acids." These statements were made by Dr. J. Theorell while introducing the Nobel Prize winners in Physiology and Medicine in 1959. They appropriately characterize the significance of these cell constituents which must be present for any cell to function. Because of their vital importance to life, any probe into the further understanding of their properties and functional characteristics is quite justified. The importance of proteins as related to living systems has long been appreciated, while the importance of the nucleic acids has only been appreciated in recent times.

Nucleic acids were discovered in 1869 by Friedrich Miescher, who isolated a material from pus-containing bandages which consisted largely of nuclei. He named the substance, "nuclein". In his studies, he found that the material contained large amounts of phosphate and was acidic in character. He also found that nuclein was soluble in alkali but insoluble in acid.

The term "nucleic acids" was introduced by Altman in 1889 and in subsequent years several of the nucleic acid constituents were isolated and identified. The most distinguishing feature

which seemed to separate two different types of nucleic acids which were isolated was the sugar moiety. In 1909, Levene and Jacobs identified the sugar in nucleic acids to be the aldopentose, ribose. In further studies they found that the sugar, deoxyribose, was present in some nucleic acids which were isolated. The sugar moiety was thus used to classify these types of nucleic acids, RNA (nucleic acids containing ribose) and DNA (nucleic acids containing deoxyribose).

It was first believed that RNA was found only in plants, while DNA was found only in animals. This was shown to be incorrect as it was firmly established that both types of nucleic acids occur in all living cells.

In 1940, Caspersson developed a procedure whereby the presence of nucleic acids could be detected by observing an absorbance in the ultraviolet range of the light spectrum (around 260nm). This proved to be a valuable analytical tool in some of the continued studies of nucleic acid chemistry.

Further research on the nucleic acids showed that DNA was the important type of nucleic acid with regard to information carrying ability and that it was an integral part of the chromosome. RNA was found to play a decisive role in protein biosynthesis (Caspersson & Brachet, 1974). Numerous studies on viruses further substantiated the vital biological role of DNA.

As biological studies were being carried out, several chemical investigations by such workers as Chargaff, Todd, and

Wilkins, laid the groundwork for the structural model of DNA proposed by Watson and Crick in 1953.

This dissertation is written to present some of the investigations which I have carried out in search of a better understanding of a small, although significant (at least academically), area of nucleic acid chemistry. The dissertation is divided into three parts, each part closely related to the others. The first part deals with the polynucleotide around which the entire study revolves, the conformational and physical properties of polycytidylic acid. The second part deals with the complexes formed between Poly C and deoxyguanosine, and between Poly U and adenosine as a function of pH. Part three deals with the affects of chemical modification of Poly C (with the mutagenic agents hydroxylamine and nitrous acid) on deoxyguanosine and adenosine binding properties. These studies were carried out in order to get a better understanding of the action of mutagens in a defined polynucleotide system.

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PART I

THE PHYSICAL AND CONFORMATIONAL PROPERTIES OF POLYCYTIDYLIC ACID

PART I

THE PHYSICAL AND CONFORMATIONAL PROPERTIES OF POLYCYTIDYLIC ACID

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The biopolymer around which this entire research project revolves, is the homoribopolynucleotide, polycytidylic acid. Polycytidylic acid (Poly C) is a synthetic RNA molecule which is composed solely of cytosine residues as the constituent pyrimidine nitrogen bases. Synthetic polynucleotides are of considerable interest because of their close relation to the naturally occurring nucleic acids. Both the natural and synthetic polymers are often capable of assuming more than one conformation.[1] The structures and hydrogen bonding schemes of the homohelices formed by Poly C and Poly U (polyuridylic acid) are of great interest in view of the fact that they are both biochemically active in the synthesis of polypeptides by the ribosomal system of *E. coli*.[2-4]

Poly C has been shown to be a good model for the study of the conformations of polynucleotides in solution.[5] This homopolymer has been shown to exist in one of two principal conformational states under certain physiological conditions. At acid pH (pH 3.5-5.5) Poly C has been shown to exist in a double helical conformation stabilized by cytosine base stacking

interactions and by the formation of three hydrogen bonds between each cytosine base pair.[4-13] At pH values near neutrality, Poly C has been shown to exist as a single-stranded helix stabilized primarily by cytosine base stacking interactions along the polymer chain.[14] Under conditions of extreme alkaline or acid pH, Poly C assumes no regularity in its conformation.[15,16]

The Double Helical Form of Poly C

The double stranded helical form of Poly C exists in solution under conditions of acid pH (pH 3.5-5.5) as evidenced by hydrodynamic,[29] polarimetric,[30] optical rotatory dispersion,[8,12] circular dichroism,[6] ultraviolet absorption,[7,9,13] acid-base titration,[11,21] infrared absorption,[11] and Raman spectral studies.[10,13] The evidence supports the model of Poly C at acid pH as a double helical polynucleotide in which two strands are stabilized by the formation of three hydrogen bonds between each adjacent pair of cytosine residues existing in a parallel configuration. The suggested binding scheme for a cytosine base pair under conditions of acid pH is shown in Figure 1. The single most important factor in bringing about this conformation is the protonation of the N3 endocyclic nitrogen.

X-ray Diffraction Evidence Supporting Duplex Poly C

Langridge and Rich[1] reported an x-ray diffraction study of Poly C at acid pH which strongly supports its existence as a double helical polynucleotide. The diffraction pattern has

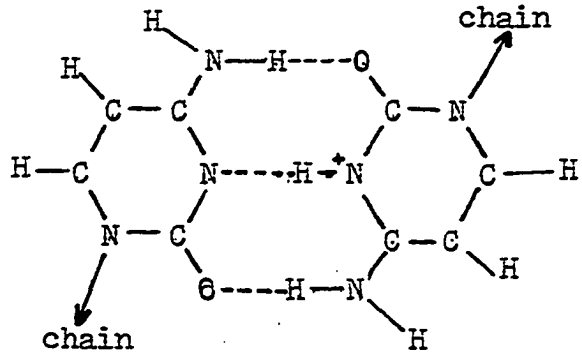


Figure 1. Suggested base-pairing scheme between two cytosine bases in polycytidylic acid under conditions of acid pH. [1]

characteristic features which suggest a helical conformation[17] and there appear to be 12 cytosine residues per turn of the helix. The diameter of the helix is approximately 13.5 Å, suggesting a duplex structure (based on the known diameter of double-stranded DNA, having a purine-pyrimidine base-pair diameter of ca. 20 Å). The strands appear to be parallel to each other and are related by a two-fold axis of rotation. The cytidylic acid residues are approximately 3.1 Å apart and same-strand neighboring residues are rotated by 30 degrees. The data strongly supports the proposed cytosine base-pairing scheme as shown in Figure 1. This type of base pairing has been observed in the structure of cytosine-5-acetic acid.[20]

The addition of protons apparently is the single most important factor in bringing about this ordered form of the polymer. Three hydrogen bonds result as a consequence of the addition of one proton for every two cytosine residues. This type of hydrogen bonding explains the need for an increased level of protonation in the formation and stabilization of the double helical form of Poly C. It was found that altering the pH from

5.5 to 3.0 resulted in a drastic disorder in the x-ray patterns, suggesting that protonation of both cytosine residues in each base pair of the double helix caused a break-up of the two-fold rotation axis of symmetry due to like-charge repulsion. The resulting structure probably exists as a disordered coil.

The Single-Helical Form of Poly C

In 1976 Arnott et al.[14] presented x-ray fibre diffraction data that shows Poly C to exist as a single-stranded, right-handed 6-fold helical structure at pH 7.0. There appears to be no hydrogen bonding between bases and the molecular structure is apparently stabilized primarily by base-stacking interactions. In this single helical form of Poly C, the ring nitrogen of one base lies over the center of the preceeding ring. There appear to be no direct interactions between bases of neighboring molecules. The only intermolecular interactions are seen in the hydrogen bonding potential of ribose hydroxyl groups on alternate cytidine residues.

Fasman et al.[8] deduced from optical rotatory dispersion measurements that the conformation of Poly C at neutral pH is highly ordered. These workers proposed a single-stranded helical structure which was stabilized by cytosine stacking interactions along the polymer chain.

C.D., O.R.D., and UV Absorption Studies on Poly C

In 1967, Brahms et al.[5] presented circular dichroism and ultraviolet absorption data on Poly C solutions at pH 4.5 and 7.0.

At pH 4.5, the C.D. curve is composed of a positive and a negitive band with a λmax at 287nm and a λmin at 265nm (see Figure 2). The inflection point is at λ274, which agrees with the wavelength of the UV absorption max of the main band of Poly C at this pH. At pH 7.0, the C.D. spectrum is composed of one main band centered at 276nm. It can be readily seen that the spectrum of Poly C at pH 7.0 is quite different from that seen at pH 4.5. The UV spectrum at neutral pH can also be easily distinguished from the spectrum of Poly C at the acid pH value. At pH 7.0, the UV absorption spectrum has one main band centered at 268nm with a shoulder at 225nm. As can be seen, the position of the maximum of the C.D. band is shifted to shorter wavelengths parallel with the shift of the absorption band maxima.

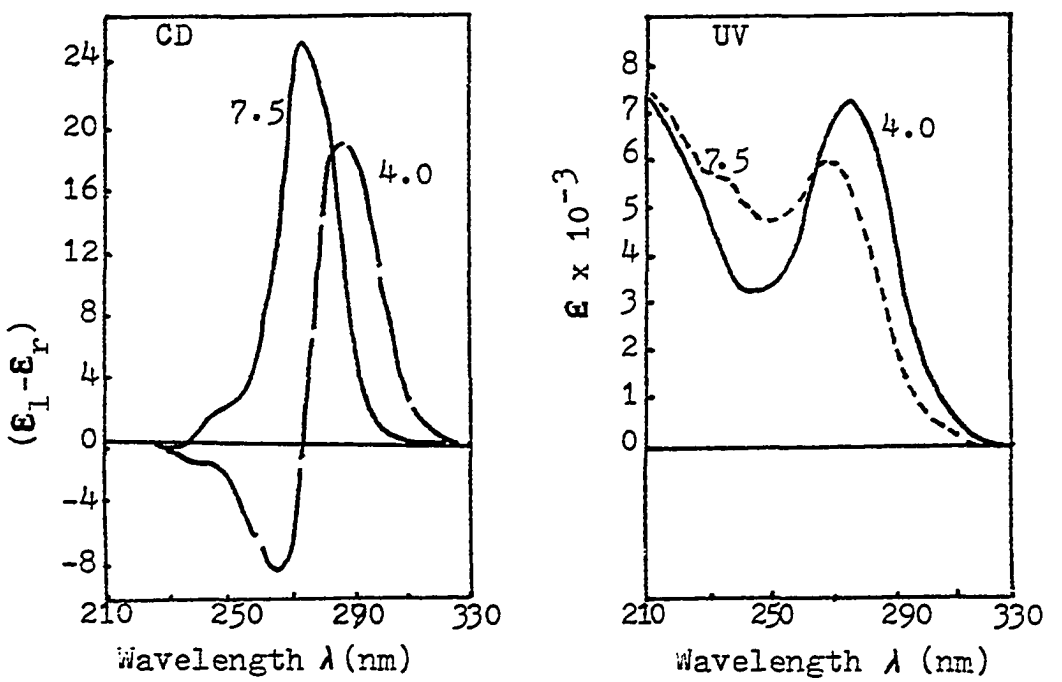


Figure 2. Circular dichroic and UV absorption spectra of Poly C at pH 4.0 (in 0.1 M NaCl, 0.05 M acetate) and at pH 7.5 (in 0.1 M KF, 0.01 M TRIS); C.D. spectra at 0°C, UV absorption spectra at 25°C. (Brahms 1967).

One can generally use the shape of the UV and/or C.D. spectra to predict the major form of Poly C present in solution. The C.D. spectrum shifted to the longer wavelengths is characteristic of the protonated form of cytidine compounds, and the presence of adjacent positive and negitive circular dichroic bands reflect the formation of the double-stranded helical form of Poly C. The UV spectra at the two pH values are characteristically different in λmax, λmin, amplitude, and in the presence or absence of the shoulder at λ225.

These circular dichroic spectra are in general agreement with the optical rotatory dispersion data observed for Poly C by Fasman et al.,[8] Sarocchi et al.,[22] and Guschlbauer.[23] These studies suggest that Poly C at pH 7.1 and 4.1 exist as highly ordered asymmetric helical structures. The helical structure at the neutral pH is apparently maintained in the absence of hydrogen bonding.

The anomalous rotatory dispersion (the Cotton effect) is known to occur near (optically active) absorption bands; thus any chromophore may exhibit a Cotton effect when located in a disymmetric environment.[24] Helical polymers have been shown to display Cotton effects which are lost when the helix is denatured or destroyed.[25-27] Similar Cotton effects are observed with Poly C solutions, suggesting a high degree of asymmetry and a highly ordered secondary structure both at pH 4.1 and 7.1.[8] The optical rotatory dispersion of Poly C and CMP are shown in Figure 3. A positive Cotton effect is observed at both pH 4.1 and 7.1, indicating highly ordered asymmetric structures. However,

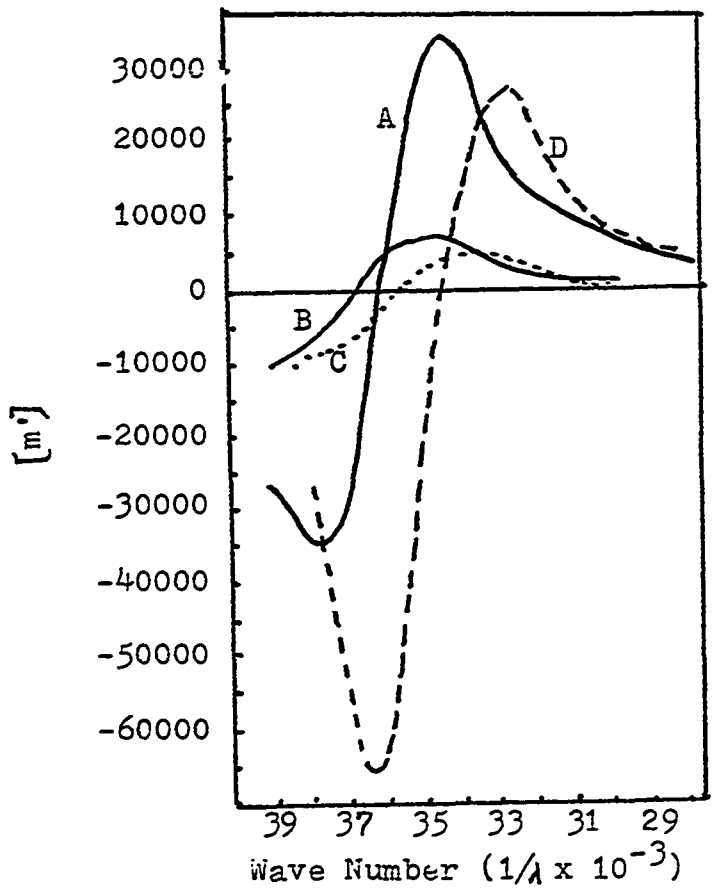


Figure 3. Optical rotatory dispersion on Poly C and CMP. Poly C: at pH 7.1 (citrate buffer), curve A; at pH 4.1 (0.1 M acetate buffer), curve D. CMP: at pH 7.1 (citrate buffer), curve B; at pH 4.1 (0.1 M acetate buffer), curve C. [m'] is the mean residue rotation. (Fasman et al. 1964).

the ORD spectral patterns are indeed different at the two pH values, indicating that the two samples contain differing conformations of Poly C. These studies suggest that solutions of Poly C, under conditions of neutral and acid pH, exist as highly ordered asymmetric structures (probably helical, although different helical species). These findings support the x-ray diffraction studies which show double helical and single helical conformations of Poly C at pH 4.5 and 7.0, respectively.

Hypochromism in Poly C

The change in light absorption which occurs when a polynucleotide such as Poly C, undergoes a conformational change from an ordered (native) state to a less ordered (denatured) state, is a result of dipole-dipole interactions induced in the chromophores (nitrogen bases) by the incident light.[31] The decrease in absorbance (per chromophore) observed in an ordered polynucleotide as compared to that exhibited by its constituent chromophores, is known as hypochromism. Conversely, the increase in absorbance (per chromophore) observed upon denaturation of an ordered polymer is known as hyperchromism.

The hypochromism phenomenon in polynucleotides has been explained in terms of dipole interactions among the stacked nitrogen bases,[35] overlap of pi orbitals of the stacked bases,[36] tautomeric base shifts,[37-38] physical shielding of the bases from one another,[39] and geometrical orientation of the bases.[40-41]

Hypochromism in Poly C is thought to be primarily the result of cytosine base stacking interactions along the polymer chain. The UV spectrum of Poly C shows the absorption at the main absorption band (270nm) to be considerably less than the sum of the absorptions of the constituent nucleotides (CMP's). Upon structuring an ordered polymer from a group of randomly ordered monomer molecules, there is a decrease in the oscillator strength of the $pi \rightarrow pi^*$ electronic transition.[9] In a solution of unordered monomeric units, the transition moments are randomly oriented with respect to each other and consequently, there is no effect on the spectrum. If these transition moments happened to be

colinear, the result would be an increase in absorption (hyperchromism). However, in polynucleotides, parallel stacking of the moments causes a decrease in absorption (hypochromism).

Hypochromism in Poly C has been reported to be as large as 47%. Disruption of the ordered polymer by various denaturation procedures (i.e. thermal denaturation, organic solvent denaturation, alkaline hydrolysis, etc.)[42] is performed in ordered to determine such values. It has been reported that thermal denaturation of polynucleotides seldom results in total denaturation.[7] Therefore, accurate hypochromism data must be obtained using alternate procedures (such as acid or alkaline hydrolysis which hydrolyzes the polynucleotide to mononucleotide units). In this way, the ordered polymer spectrum can be directly compared to the spectrum of the hydrolyzed polymer (constituent nucleotide spectrum), and accurate hypochromism data can then be obtained.

Thermal Denaturation Studies on Poly C

Ultraviolet absorption-temperature profiles of Poly C at various pH values and ionic strengths have been reported by several investigators.[4,8,11,21-23] The results of these studies lend evidence to further support the existence of the two different helical forms of Poly C under certain physiological conditions. The thermal denaturation of Poly C from an ordered helical structure to a less ordered structure can be seen in both UV and CRD spectra as the temperature is elevated. The UV

absorption vs temperature profile of Poly C at pH 4.1 (in 0.1 M Na acetate) presented by Fasman et al.[8] shows a cooperative-type melting (denaturation) with a Tm~81°C, while the melting curve at pH 7.0 (in 0.1 M Na citrate) shows a gradual, non-cooperative type transition with a Tm~41°C.

Brahms et al.[5] have shown that at pH 4.0, cooperative changes, indicating an ordered temperature-dependent secondary structure, are quite evident at the level of the heptamer (Tm=20° C) as can be seen in the UV absorption vs temperature profiles of a series of oligocytidylates and Poly C. These melting profiles are shown in Figure 4. The proposed double helical conformation of Poly C at pH 4.0 suggests the stability of the polymer at this pH is due to contributions not only from vertical cytosine base stacking interactions, but also from horizontal hydrogen bonding interactions between the two strands.[4,8,29] This three dimensional network of interactions greatly hinders denaturation

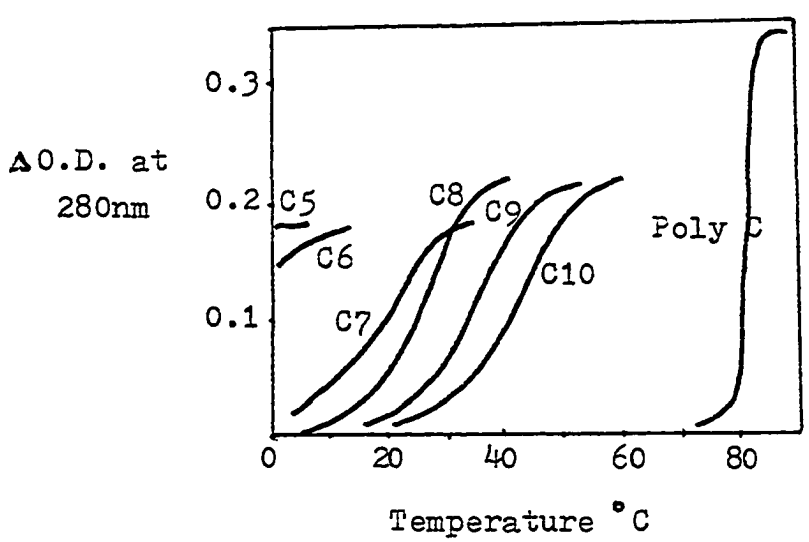
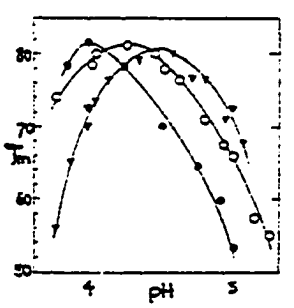


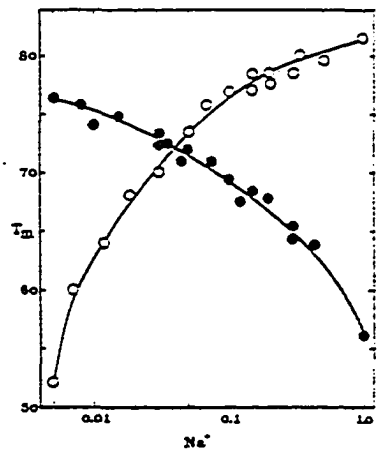
Figure 4. Ultraviolet absorption-temperature profiles of 3'→5' oligocytidylates in 0.1 M NaCl, 0.05 M Na acetate (pH 4.0) Changes in optical density recorded at 280nm for solutions ca. 2×10^{-4} M with respect to nucleoside residue. (Brahms et al.)[5]

of the double helix until a critical temperature is reached. The three hydrogen bonds formed between cytosine residues adds a significant stabilization force both in multiple-hydrogen bond strength and in the alignment of cytosine bases so as to allow for maximum stacking interactions. When the thermal energy is great enough, hydrogen bonds break cooperatively, cytosine bases unstack, and a cooperative melting profile is observed.

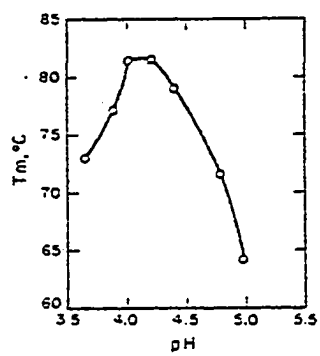
Studies showing the influence of pH and ionic strength on the Tm of protonated Poly C has been demonstrated by several workers.[4,22,23] The stability of the homopolymer under a given set of conditions is reflected in the melting temperature (Tm). The changes in Tm as a function of pH and ionic strength as reported by Guschlbauer,[23] Akinrimisi et al.,[4] and Sarocchi et al.[22] are shown below in Figures below. Based on these data, the most stable form of Poly C appears to exist at~pH 4.1 and~0.1 M Na^+. The Tm under these conditions is ca. 82°C.



Dependence of Tm of Poly C on pH at different ionic strengths. ○, 0.9 M Na^+; ●, 0.13 M Na^+; ×, 0.04 M Na^+. All solutions in sodium acetate buffer. (Guschlbauer) and (Sarocchi et al.)



Dependence of Tm of Poly C on ionic strength. o=pH 4; •=pH 5. (Guschlbauer).



Variation of Tm of Poly C with pH. 0.1 M Na acetate buffer used over a pH range of 3.6-4.9. (Akinrimisi et al.).

All the findings reported here from the various investigators lend evidence to support the two principal helical conformations of polycytidylic acid. To verify and expand these results, experiments were set up and carried out in our labora-

tory. The results of some of these studies are reported in the following chapter.

CHAPTER II

EXPERIMENTAL METHODS AND MATERIALS

UV Absorption Spectra of Polycytidylic Acid

The potassium salt of polyribocytidylic acid was obtained from Miles Laboratories (Elkhart, IN). UV absorption spectra of Poly C at various pH values (in 0.1 M Na^+) were obtained by preparing aqueous solutions of Poly C (ca. 1 mM) and scanning the UV spectral range, 200-320nm using the Perkin-Elmer Double Beam Spectrophotometer (Coleman, Model 124). In most cases 1 mm pathlength cuvettes were used so that relatively high (1-10 mM) concentrations of the polymer could be detected. Extinction coefficient values of Poly C under various conditions are on the order of ca. $7x10^3$.

Poly C spectra at pH 4.1 (in 0.1 M Na acetate buffer) and 7.1 (in 0.1 M Na phosphate buffer) are shown in Figure 5. Figure 6 shows Poly C spectra at four acid pH values (3.1, 4.1, 5.1, and 6.0). Figure 7 shows spectral patterns of Poly C at three alkaline pH values (7.1, 8.0, and 9.0).

Cytidine, CMP Spectra; Extinction Coefficient Determinations

Cytidine, 5'-CMP, and 2'&3'-CMP (mixed isomers) were purchased from Sigma Chemical Company (St. Louis, MO). 1.0 mM aqueous solutions of these compounds were prepared at pH 2.5, 4.5, and 7.0 in 0.1 M Na^+ buffers for UV spectral analysis. These spectra are shown in

Figures 8-10. Extinction coefficient values of these compounds were determined by carefully preparing known concentrations of each at the various pH values . The absorbance value at a given wavelength for a specific concentration was used to calculate $\mathcal{E}$ using the Beer-Lambert relationship ($A=\mathcal{E}Cl$). The results of this work is shown in Tables 1-3. In each case, 1 mm cuvettes were used. The extinction coefficient values determined for 2'&3'-CMP at the various pH values are used to calculate Poly C concentrations (expressed in CMP units), since upon alkaline hydrolysis, Poly C is degraded to monomeric 2'&3'-CMP isomers.

Alkaline hydrolysis of Poly C is carried out by incubating Poly C in 0.3 N KOH for 22 hours at 37°C. The spectra of four such Poly C hydrolysates at different pH values are shown in Figure 11.

Poly C Hypochromism Studies

The hypochromism of Poly C was determined via alkaline hydrolysis of the polynucleotide to monomeric CMP units as described earlier. The absorbance of the solution before (Poly C) and after (CMP) treatment with KOH reveals the hypochromic effect of the structured polymer. Generally, the greater the increase in absorbance of the hydrolysate over the initial polymer solution, the greater the secondary structure of the polymer.

The scheme used for Poly C hypochromism studies is shown in the following chapter (Results). Two sets of hypochromism data on Poly C at pH 4.1 and 7.1 are shown in Figures 12-15. Appropriate controls were run in each case. In addition, treatment of 2'&3'-CMP

with 0.3 N KOH was carried out to insure a non-hypochromic effect of the mononucleotide solution.

Thermal Denaturation

Thermal denaturation profiles of Poly C at pH 4.1 and 7.0 (in 0.1 M Na^+ buffers) were obtained by determining the change in absorbance (at a given wavelength) with increasing temperature. The Cary 118 Spectrophotometer, equiped with a jacketed curvette holder was utilized. Temperature control was accomplished by water flow through the jacket using an external variable-temperature water bath. Temperatures were taken directly in the reference cell and were recorded to the nearest degree. Data reported is at five degree intervals. At the pH values near neutrality, absorbance readings were recorded at 268nm (λmax), while at pH 4.1 and 4.5 optical density readings were recorded at 273nm (λmax). The midpoint of the changes of the optical properties was taken as the Tm (melting or denaturation temperature). Tm is presumably the temperature at which 100% of the polymer is 50% denatured.

Acid-Base Titration

A 1.0 mM solution of Poly C (expressed in CMP units) was prepared in 0.1 M NaCl at pH 7.0. Into exactly 5.0 ml of this solution was titrated, drop by drop, a 0.001 N HCl solution. After each drop, the pH of the solution was recorded, using the digital Corning pH meter (Model 125). Titration was terminated after 5.0 ml of the diluted HCl had been added. Acid titration data and the curve to which they correspond, are shown in Table 6 and Figure 18.

CHAPTER II

RESULTS

Figure 5. Absorption Spectra of Poly C at pH 4.1 and 7.1.

Conditions: Temp. = 24°C; Poly C in 0.1 M Na acetate (pH 4.1) and in 0.1 M Na phosphate (pH 7.1).

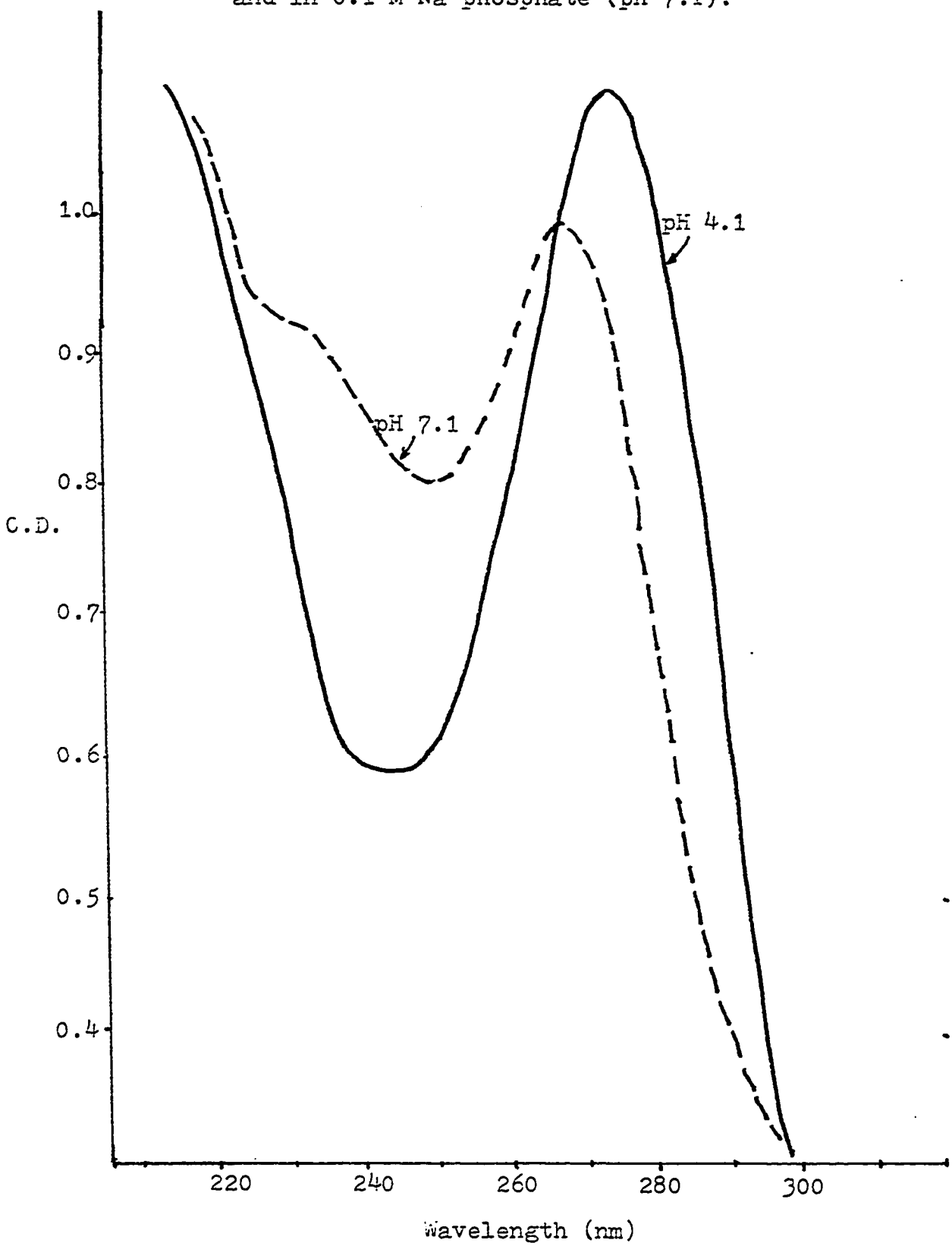


Figure 6. Absorption Spectra of Poly C at Acid pH Values

Conditions: Temp. = 24° C; Poly C in 0.1 M Na acetate buffer.

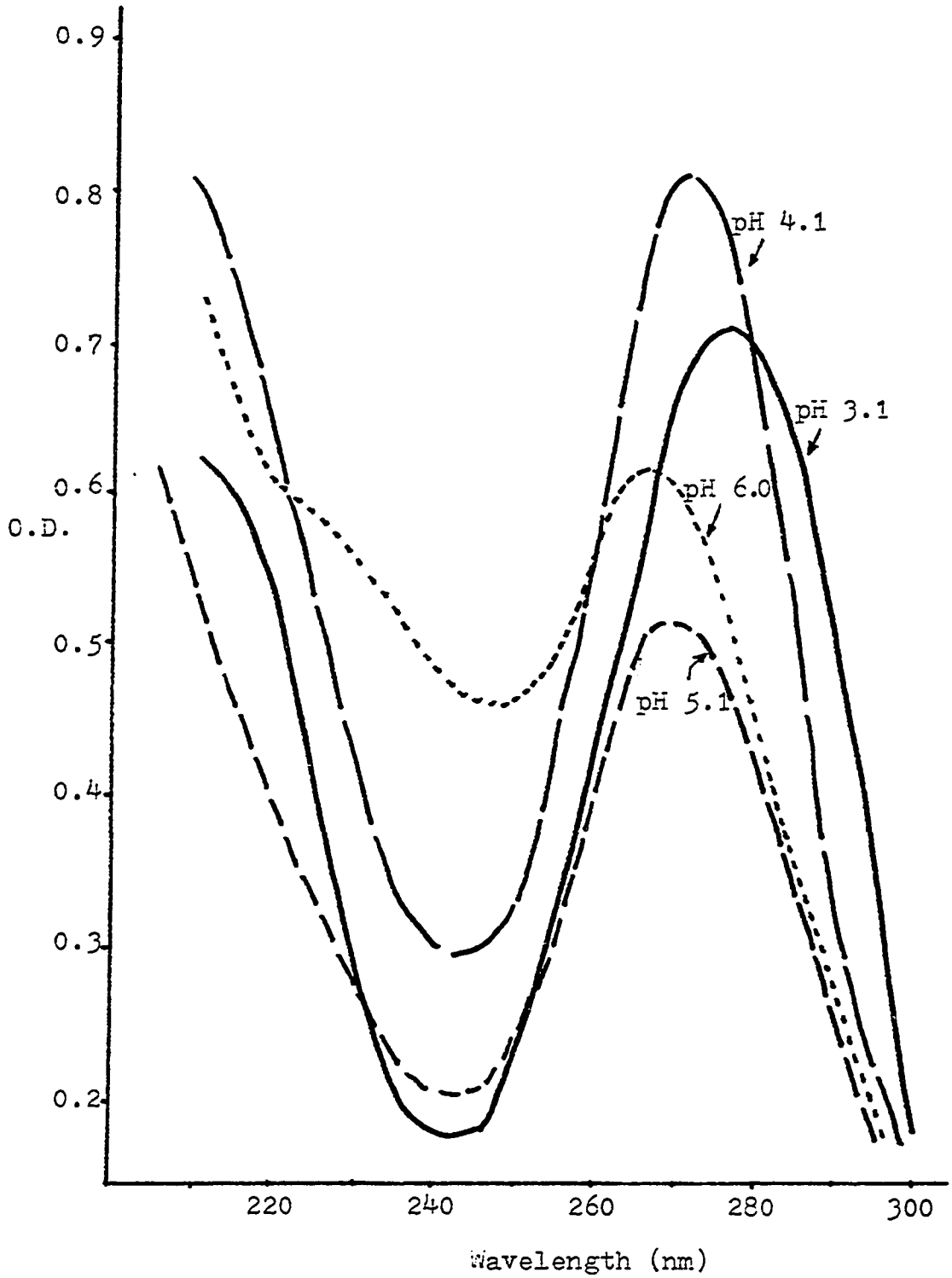


Figure 7. Absorption Spectra of Poly C at Alkaline pH Values

Conditions: Temp = 24°C; Poly C in 0.1 M Na phosphate buffer.

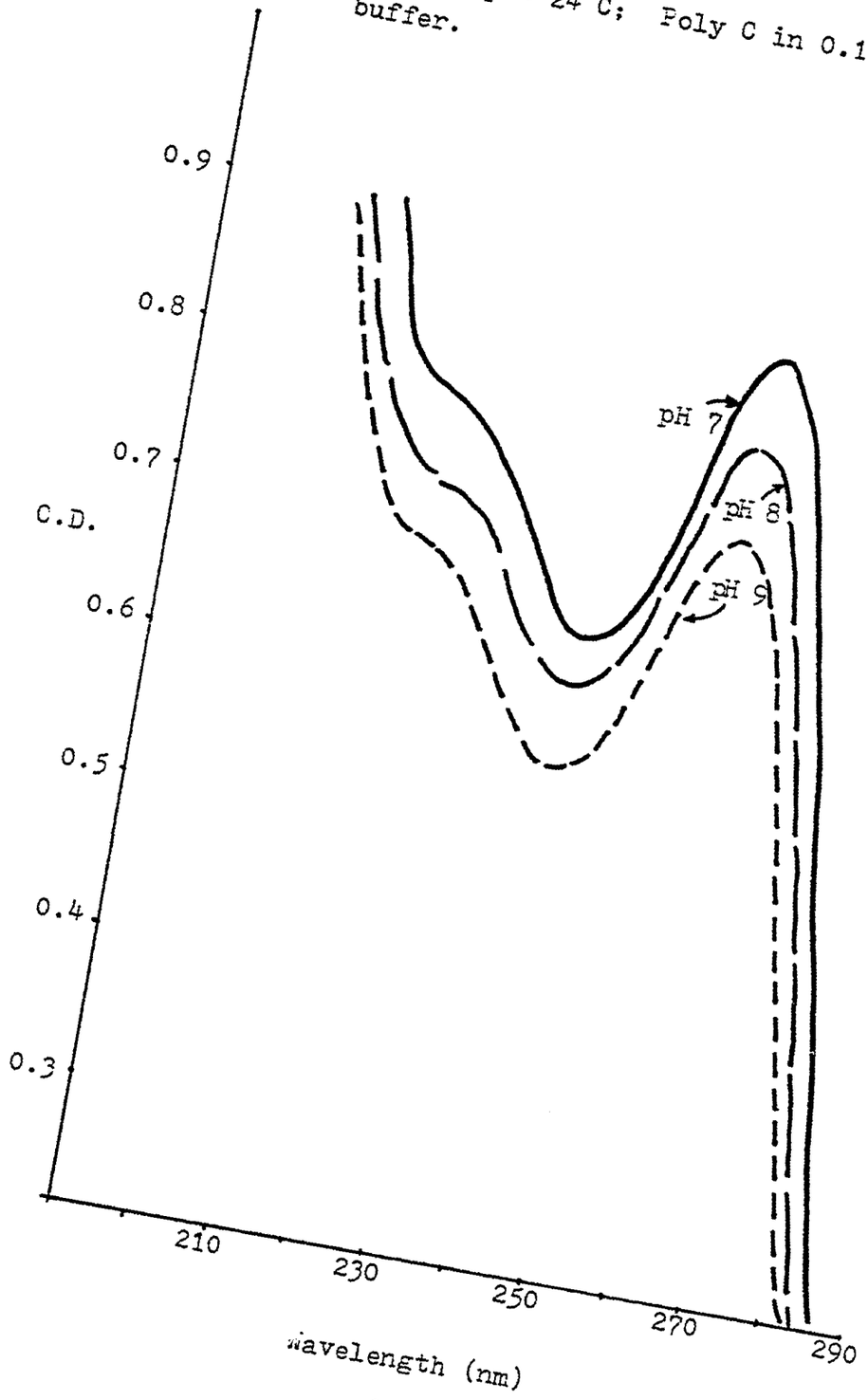


Figure 8. UV Absorption Spectra of 1.0 mM Cytidine.

pH 2.5: Cytidine in 0.1 M NaCl, adjusted to pH 2.5 with 1 N HCl.
pH 4.5: Cytidine in 0.1 M Na acetate buffer.
pH 7.0: Cytidine in 0.1 M phosphate buffer.

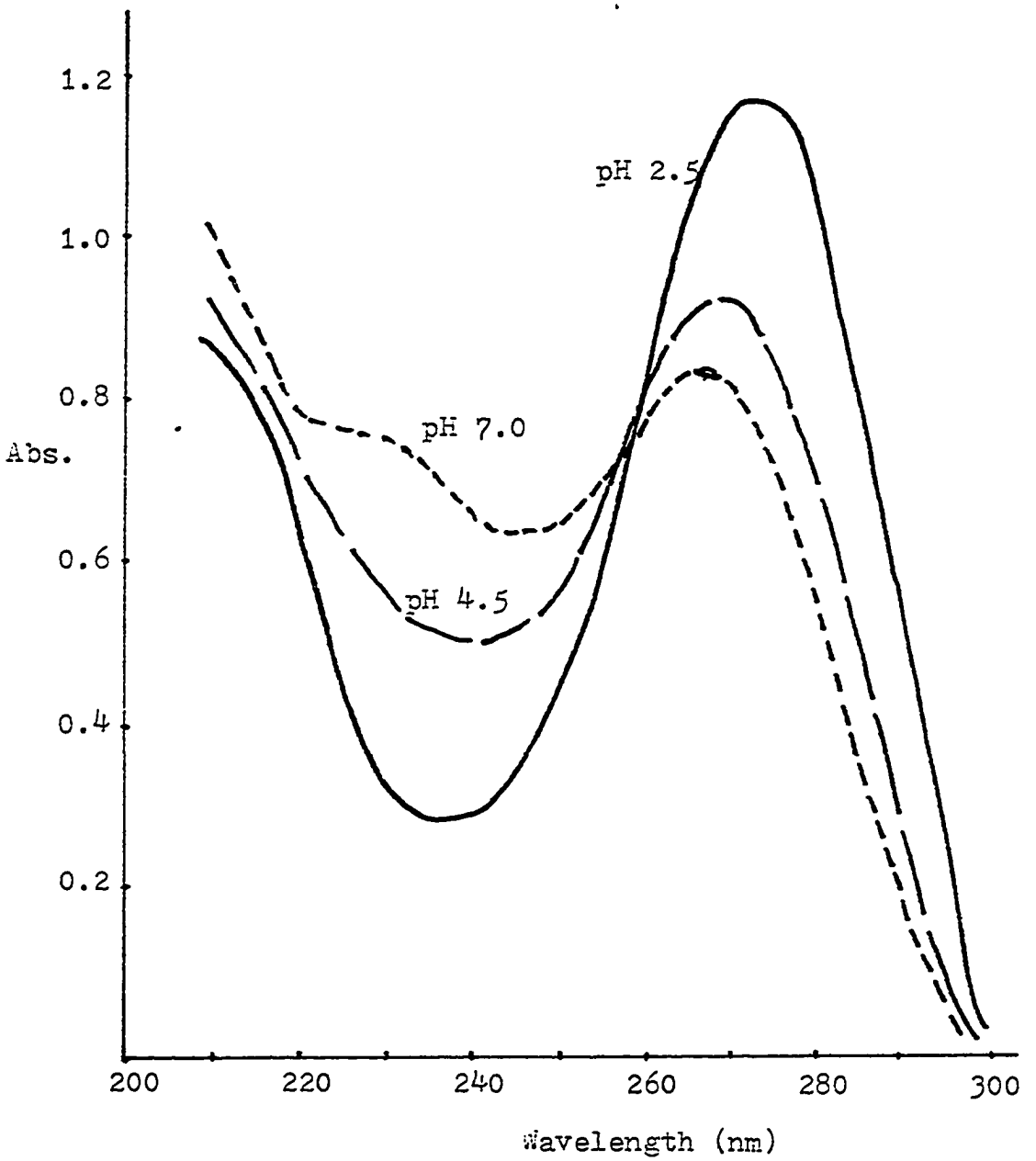


Table 1. Extinction Coefficient Determinations for Cytidine.

pH 2.5: Cytidine in 0.1 M NaCl, adjusted to pH 2.5 with 1 N HCl.

[Cytidine]	A_{277}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	1.19	11.90
7×10^{-4} M	0.83	11.85
5×10^{-4} M	0.60	12.00
3×10^{-4} M	0.35	11.66

Spectral Data: λmax=277nm; λmin=239nm; ε=11,900

pH 4.5: Cytidine in 0.1 M Na acetate buffer.

[Cytidine]	A_{272}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	0.96	9.60
7×10^{-4} M	0.67	9.56
5×10^{-4} M	0.48	9.60
3×10^{-4} M	0.29	9.66

Spectral Data: λmax=272 nm; λmin=241 nm; ε=9,600

pH 7.0: Cytidine in 0.1 M Na phosphate buffer.

[Cytidine]	A_{268}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	0.86	8.60
8×10^{-4} M	0.69	8.62
5×10^{-4} M	0.43	8.60
3×10^{-4} M	0.26	8.66

Spectral Data: λmax=268 nm; λmin=246 nm; ε=8,600

Figure 9. UV Absorption Spectra of 1.0 mM 5'-CMP.

pH 2.5: CMP in 0.1 M NaCl, adjusted to pH 2.5 with 1 N HCl.
pH 4.1: CMP in 0.1 M Na acetate buffer.
pH 7.0: CMP in 0.1 M Na phosphate buffer.

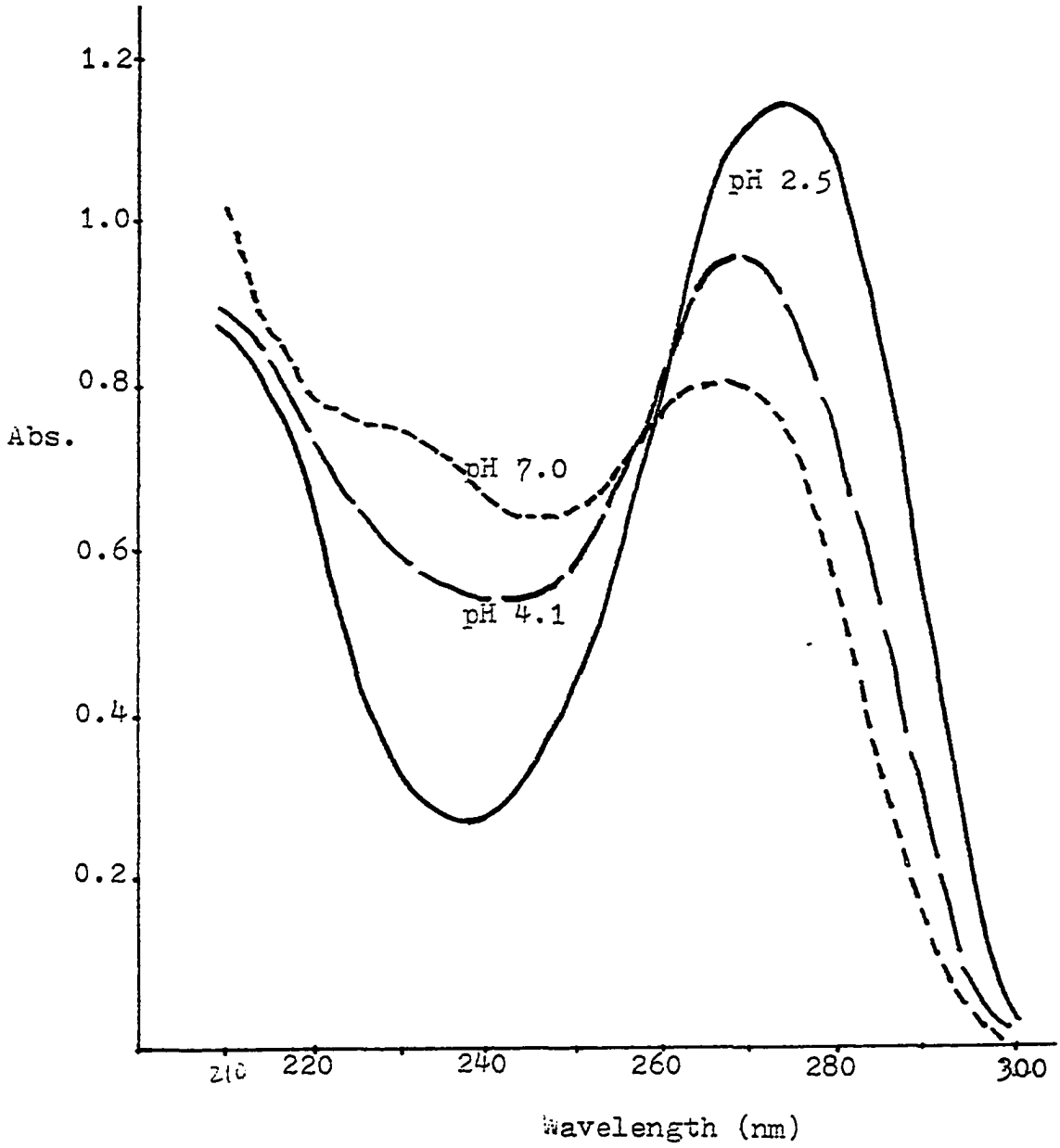


Table 2. Extinction Coefficient Determinations for 5'-CMP.

pH 2.5: 5'-CMP in 0.1 M NaCl, adjusted to pH 2.5 with 1 N HCl.

[5'-CMP]	A_{277}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	1.17	11.70
7×10^{-4} M	0.82	11.71
5×10^{-4} M	0.58	11.60
3×10^{-4} M	0.35	11.66

Spectral Data: λmax=277 nm; λmin=239 nm; ε=11,700

pH 4.1: 5'-CMP in 0.1 M Na acetate buffer.

[5'-CMP]	A_{273}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	0.99	9.90
7×10^{-4} M	0.69	9.85
5×10^{-4} M	0.50	10.00
3×10^{-4} M	0.29	9.66

Spectral Data: λmax=273 nm; λmin=241 nm; ε=9,900

pH 7.0: 5'-CMP in 0.1 M Na phosphate buffer.

[5'-CMP]	A_{268}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	0.83	8.30
8×10^{-4} M	0.66	8.25
5×10^{-4} M	0.41	8.20
3×10^{-4} M	0.25	8.33

Spectral Data: λmax=268 nm; λmin=247 nm; ε=8,300

Figure 10. UV Absorption Spectra of 1.0 mM 2'&3'-CMP (Mixed Isomers)

pH 2.5: CMP in 0.1 M NaCl, adjusted to pH 2.5 with 1 N HCl.
pH 4.5: CMP in 0.1 M Na acetate buffer.
pH 7.0: CMP in 0.1 M Na phosphate buffer.

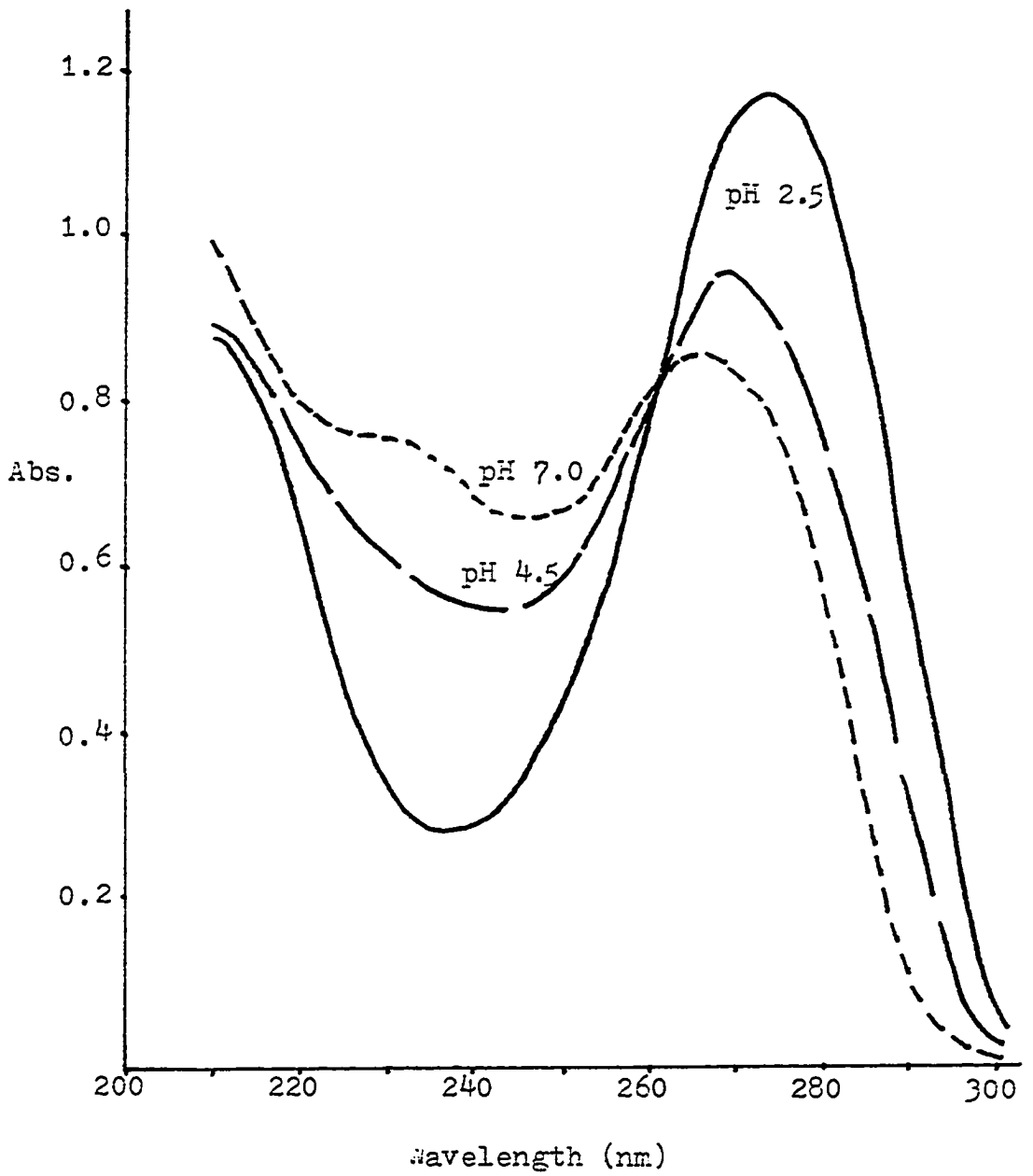


Table 3. Extinction Coeffecient Determinations for 2'&3'-CMP.

pH 2.5: 2'&3'-CMP in 0.1 M NaCl, adjusted to pH 2.5 with 1 N HCl.

[2'&3'-CMP]	A_{276}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	1.19	11.90
7×10^{-4} M	0.83	11.85
5×10^{-4} M	0.60	12.00
3×10^{-4} M	0.35	11.66

Spectral Data: λmax=276 nm; λmin=238 nm; ε=11,900

pH 4.5: 2'&3'-CMP in 0.1 M Na acetate buffer.

[2'&3'-CMP]	A_{272}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	0.98	9.80
7×10^{-4} M	0.69	9.85
5×10^{-4} M	0.49	9.80
3×10^{-4} M	0.29	9.66

Spectral Data: λmax=272 nm; λmin=242 nm; ε=9,800

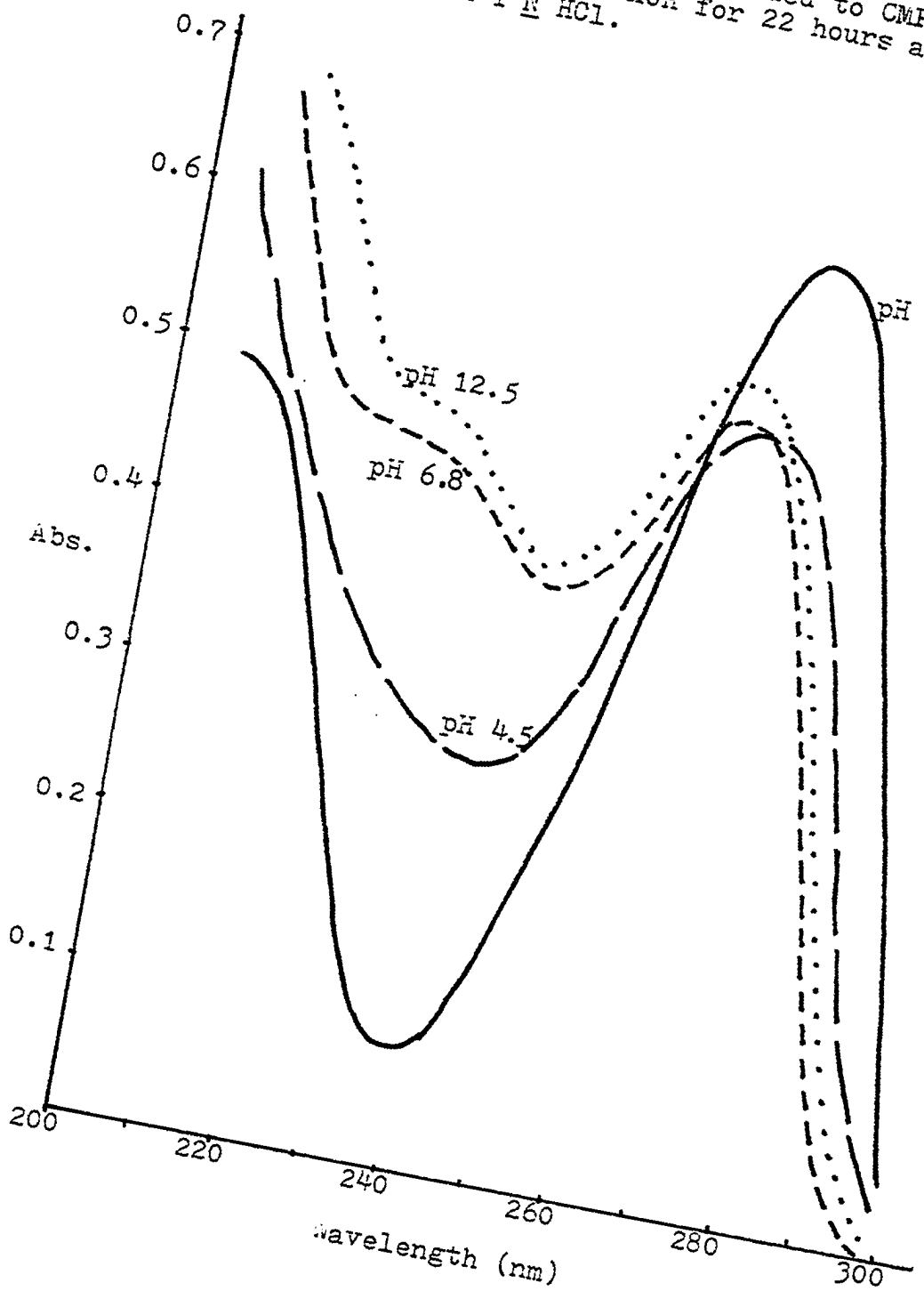
pH 7.0: 2'&3'-CMP in 0.1 M Na phosphate buffer.

[2'&3'-CMP]	A_{268}	$\varepsilon \times 10^{-3}$
1×10^{-3} M	0.88	8.80
8×10^{-4} M	0.70	8.75
5×10^{-4} M	0.44	8.80
3×10^{-4} M	0.27	9.00

Spectral Data: λmax=268 nm; λmin=247 nm; ε=8,800

Figure 11. Ultraviolet Absorption Spectra of Poly C Hydrolysate

Poly C in 0.1 M NaCl degraded to CMP units via alkaline hydrolysis (0.3 N KOH for 22 hours at 37°C). pH adjustments made with 1 N HCl.



General Experimental Scheme For Poly C Hypochromism Study

Poly C Solution
(under given conditions of pH and ionic strength)



1:1 (V:V) Poly C :0.6 N KOH

incubate for
22 hours at
37°C

Adjust solution to initial pH with volume, V, of 0.1 N HCl (in 0.1 M NaCl).

Hydrolyzed Poly C = CMP

1:1 (V:V) Poly C :0.6 M KCl

incubate for
22 hours at
37°C

Add volume, V, of 0.1 M Na^+ buffer at initial pH value.

Poly C

$$\frac{\text{Abs at } \lambda\text{max of CMP}}{\text{Abs at } \lambda\text{max of Poly C}} = X-1 = \frac{\% \text{ Hypochromism of}}{\text{Poly C}}$$

Poly C Hypochromism Data at pH 4.5

---Hypochromism of Poly C in 0.1 M Na acetate buffer at pH 4.5---

(1) Initial Poly C: λmax=273nm A_{273}=1.02

(2) 2 ml Poly C + 2 ml 0.6 N KOH (pH 12.5): λmax=267 A_{267}=0.49

(3) 2 ml Poly C + 2 ml 0.6 M KCl (pH 4.5): λmax=273 A_{273}=0.51

incubation of (2) and (3) for 22 hours at 37°C.

adjustment of (2) to pH 4.5 with 0.1 N HCl (in 0.1 M NaCl):
λmax=273 A_{273}=0.28

added equal volume of 0.1 M Na acetate buffer to (3):
λmax=273 A_{273}=0.20

$$\frac{\text{CMP}}{\text{Poly C}} = \frac{0.28}{0.20} = 1.40 - 1.00 = 0.40 \text{ or } \underline{40\% \text{ Hypochromicity}}$$

(see Figure 16)

---Hypochromism of Poly C in 0.1 M Na acetate buffer at pH 4.5---

(1) Initial Poly C: λmax=272 A_{272}=0.97

(2) 2 ml Poly C + 2 ml 0.6 N KOH (pH 12.5): λmax=268 A_{268}=0.47

(3) 2 ml Poly C + 2 ml 0.6 M KCl (pH 4.5): λmax=272 A_{272}=0.49

incubation of (2) for 22 hours at 37°C (pH 12.5):
λmax=268 A_{268}=0.67

$$\frac{\text{CMP}}{\text{Poly C}} = \frac{0.67}{0.47} = 1.42\text{-}1.00 = 0.42 \text{ or } \underline{42\% \text{ hypochromicity}}$$

(see Figure 17)

Figure 12. Hypochromism of Poly C in 0.1 M Na^+, pH 4.5

Poly C in 0.1 M Na acetate buffer, pH 4.5.
Initial Poly C: λmax=273 A_{273}=1.02

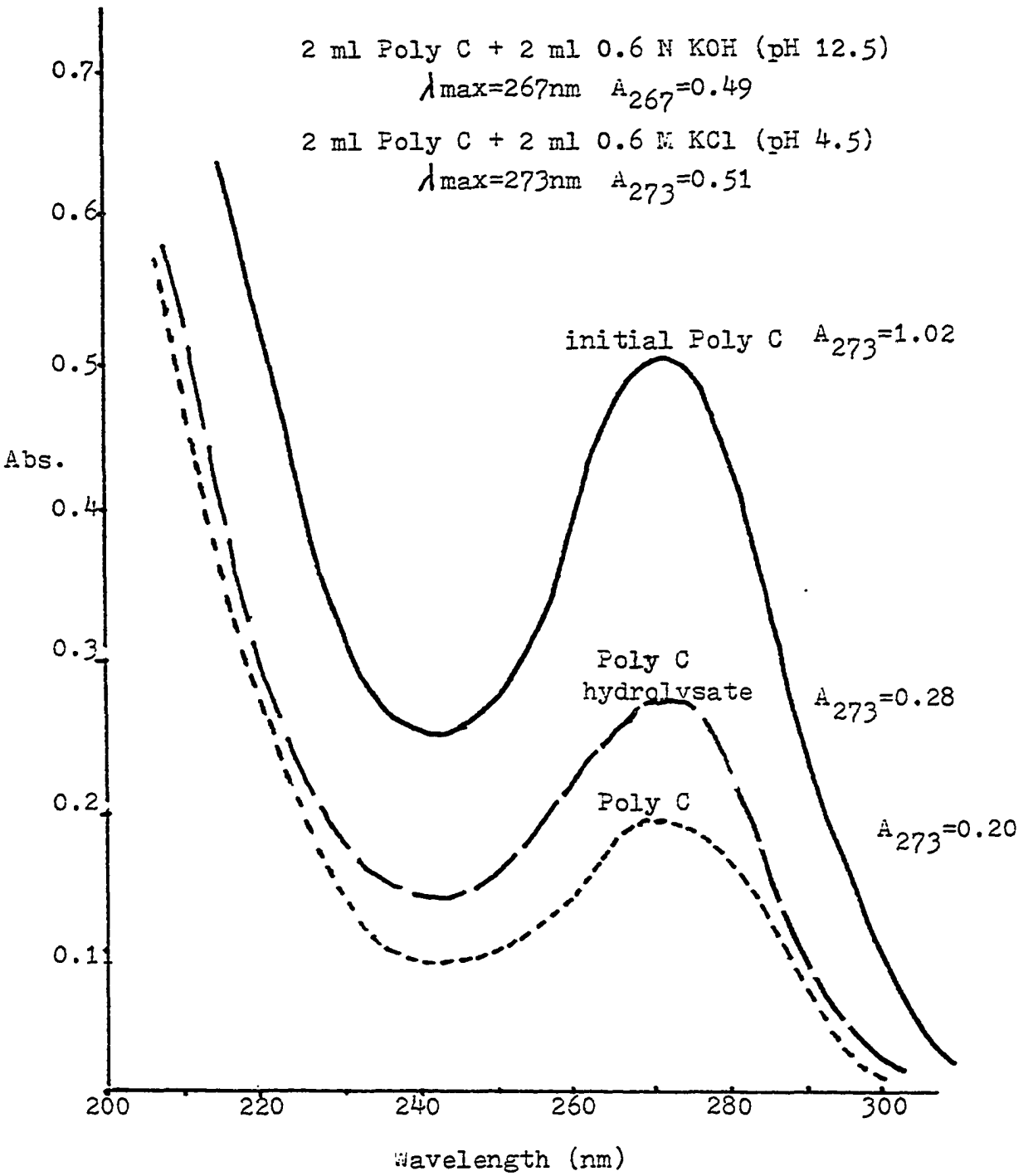
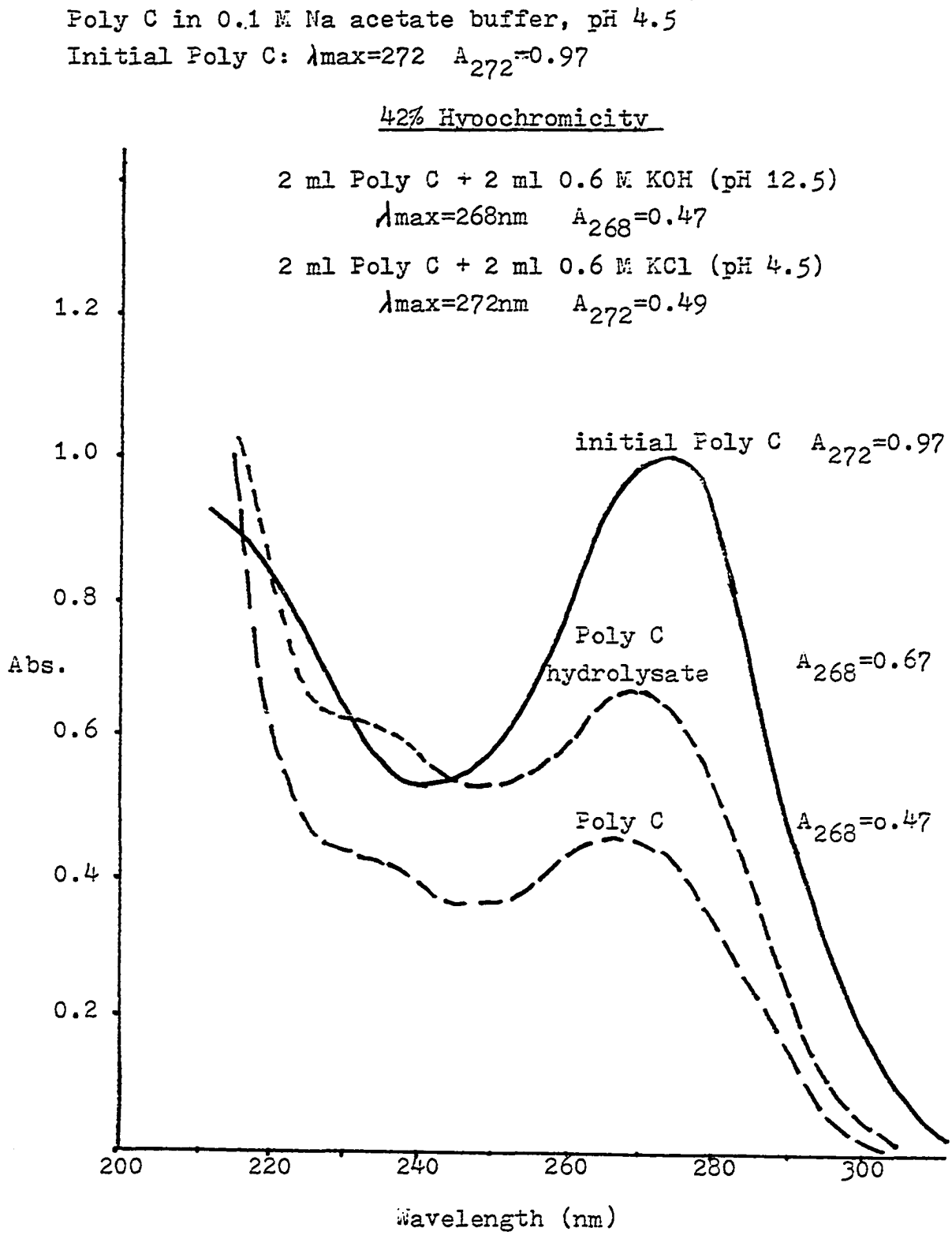


Figure 13. Hypochromism of Poly C in 0.1 M Na^+, pH 4.5



Poly C Hypochromism Data at pH 7.1 and 6.8

---Hypochromism of Poly C in 0.1 M Na phosphate buffer at pH 7.1---

(1) Initial Poly C: λmax=268nm A_{268}=0.65

(2) 2 ml Poly C + 2 ml 0.6 N KOH (pH 12.5): λmax=267 A_{267}=0.33

(3) 2 ml Poly C + 2 ml 0.6 M KCl (pH 7.1): λmax=268 A_{268}=0.32

incubation of (2) and (3) for 22 hours at 37°C.

adjustment of (2) to pH 7.1 with 0.1 N HCl (in 0.1 M NaCl):
λmax=268 A_{268}=0.28

added equal volume of 0.1 M Na phosphate buffer to (3):
λmax=268 A_{268}=0.21

$$\frac{\text{CMP}}{\text{Poly C}} = \frac{0.28}{0.21} = 1.33 - 1.00 = 0.33 \text{ or } \underline{33\% \text{ Hypochromicity}}$$

(see Figure 18)

---Hypochromism of Poly C in 0.1 M Na phosphate buffer at pH 6.8---

(1) Initial Poly C: λmax=268nm A_{268}=0.64

(2) 2 ml Poly C + 2 ml 0.6 N KOH (pH 12.5): λmax=267 A_{267}=0.32

(3) 2 ml Poly C + 2 ml 0.6 M KCl (pH 6.8): λmax=268 A_{268}=0.32

incubation of (2) and (3) for 22 hours at 37°C.

After incubation, (2): λmax=267nm A_{267}=0.41

(3): λmax=268nm A_{268}=0.32

$$\frac{\text{CMP}}{\text{Poly C}} = \frac{0.41}{0.32} = 1.29\text{-}1.00=0.29 \text{ or } \underline{29\% \text{ Hypochromicity}}$$

(see Figure 19)

Figure 14. Hypochromism of Poly C in 0.1 M Na^+, pH 7.1

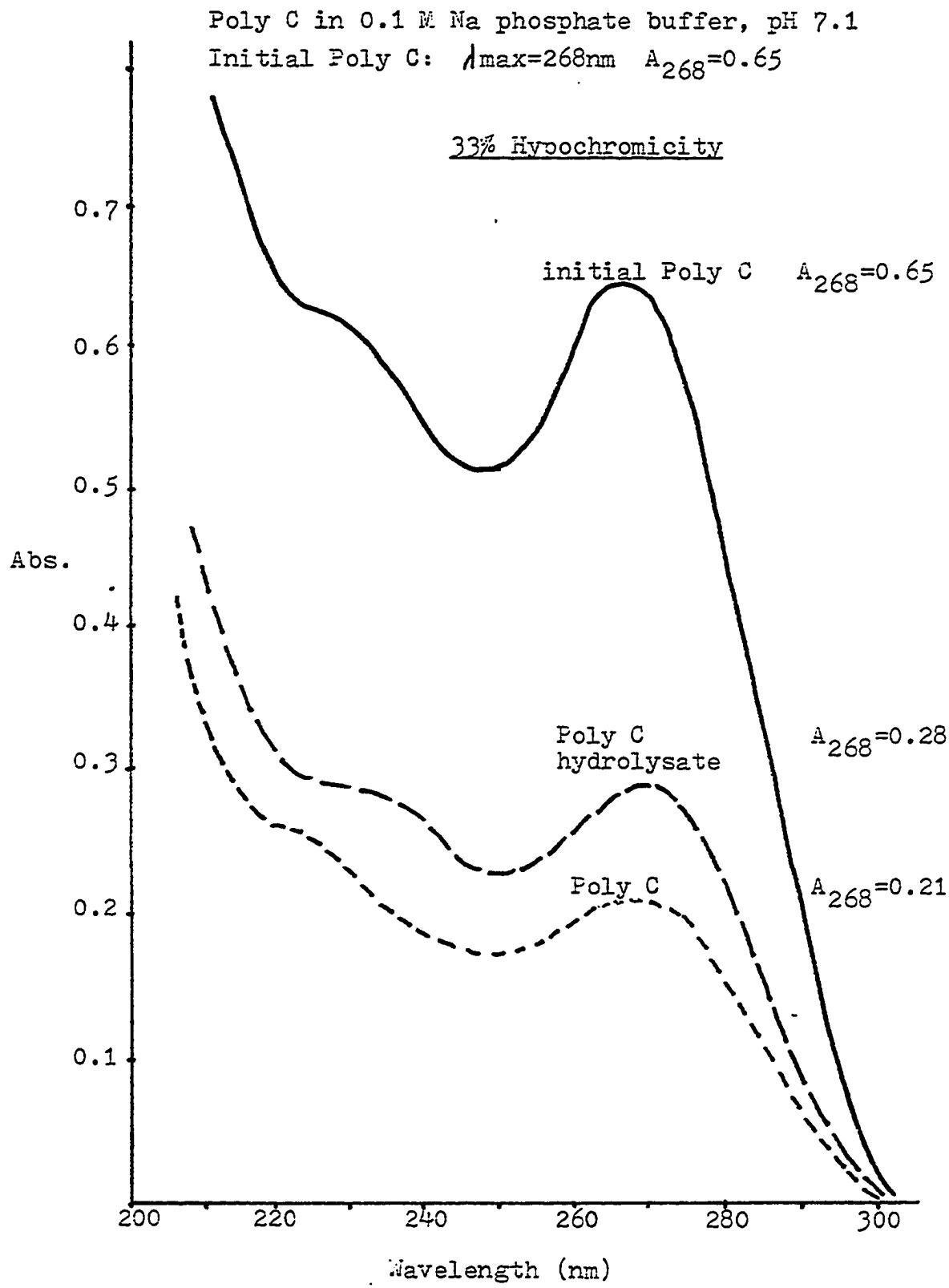
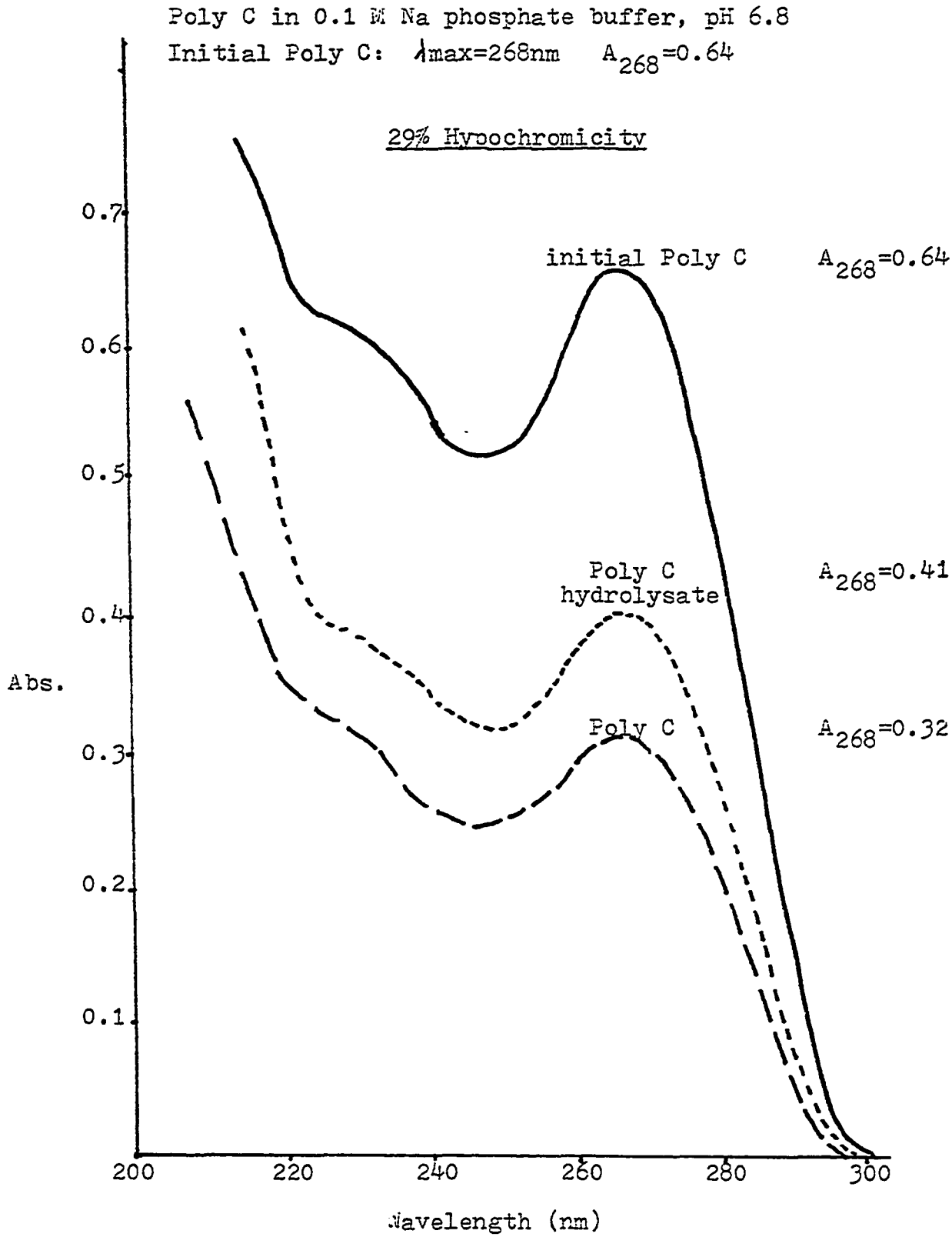


Figure 15. Hypochromism of Poly C in 0.1 M Na^+, pH 6.8



2' & 3'- CMP Hypochromism Data at pH 4.5 and 7.1

--Hypochromism of 2'&3'-CMP in 0.1 M Na acetate buffer at pH 4.5--

(1) Initial CMP: λmax=272nm A_{272}=0.76

(2) 2 ml CMP + 2 ml 0.6 N KOH (pH 12.5): λmax=267nm A_{267}=0.36

(3) 2 ml CMP + 2 ml 0.6 M KCl (pH 4.5): λmax=272nm A_{272}=0.38

incubation of (2) and (3) for 22 hours at 37°C.

Adjustment of (2) to pH 4.5 with 0.1 N HCl (in 0.1 M NaCl):
λmax=272nm A_{272}=0.25

added equal volume of 0.1 M Na acetate buffer to (3):
λmax=272nm A_{272}=0.25

No Hypochromism

--Hypochromism of 2'&3'-CMP in 0.1 M Na phosphate buffer at pH 7.1--

(1) Initial CMP: λmax=268nm A_{268}=0.71

(2) 2 ml CMP + 2 ml 0.6 N KOH (pH 12.5): λmax=267nm A_{267}=0.35

(3) 2 ml CMP + 2 ml 0.6 M KCl (pH 7.1): λmax=268nm A_{268}=0.36

incubation of (2) and (3) for 22 hours at 37°C.

Adjustment of (2) to pH 7.1 with 0.1 N HCl (in 0.1 M NaCl):
λmax=268nm A_{268}=0.26

added equal volume of 0.1 M Na phosphate buffer to (3):
λmax=268nm A_{268}=0.26

No Hypochromism

Table 4. Melting Curve Data on Poly C at pH 6.8 and 7.0.

Poly C in 0.1 M Na phosphate buffer at pH 6.8 and 7.0.

Temperature, °C	pH 6.8 A_{268}	pH 7.0 A_{268}
15	0.70	0.52
20	0.70	0.52
25	0.70	0.53
30	0.71	0.54
35	0.72	0.56
40	0.73	0.57
45	0.74	0.58
50	0.75	0.59
55	0.76	0.59
60	0.77	0.60
65	0.77	0.60
70	0.77	0.61
75	0.78	0.62
80	0.79	0.62
85	0.79	0.63

Figure 16. Melting Curves of Poly C at pH 6.8 and 7.1

Poly C in 0.1 M Na phosphate buffer pH 6.8 and 7.1.

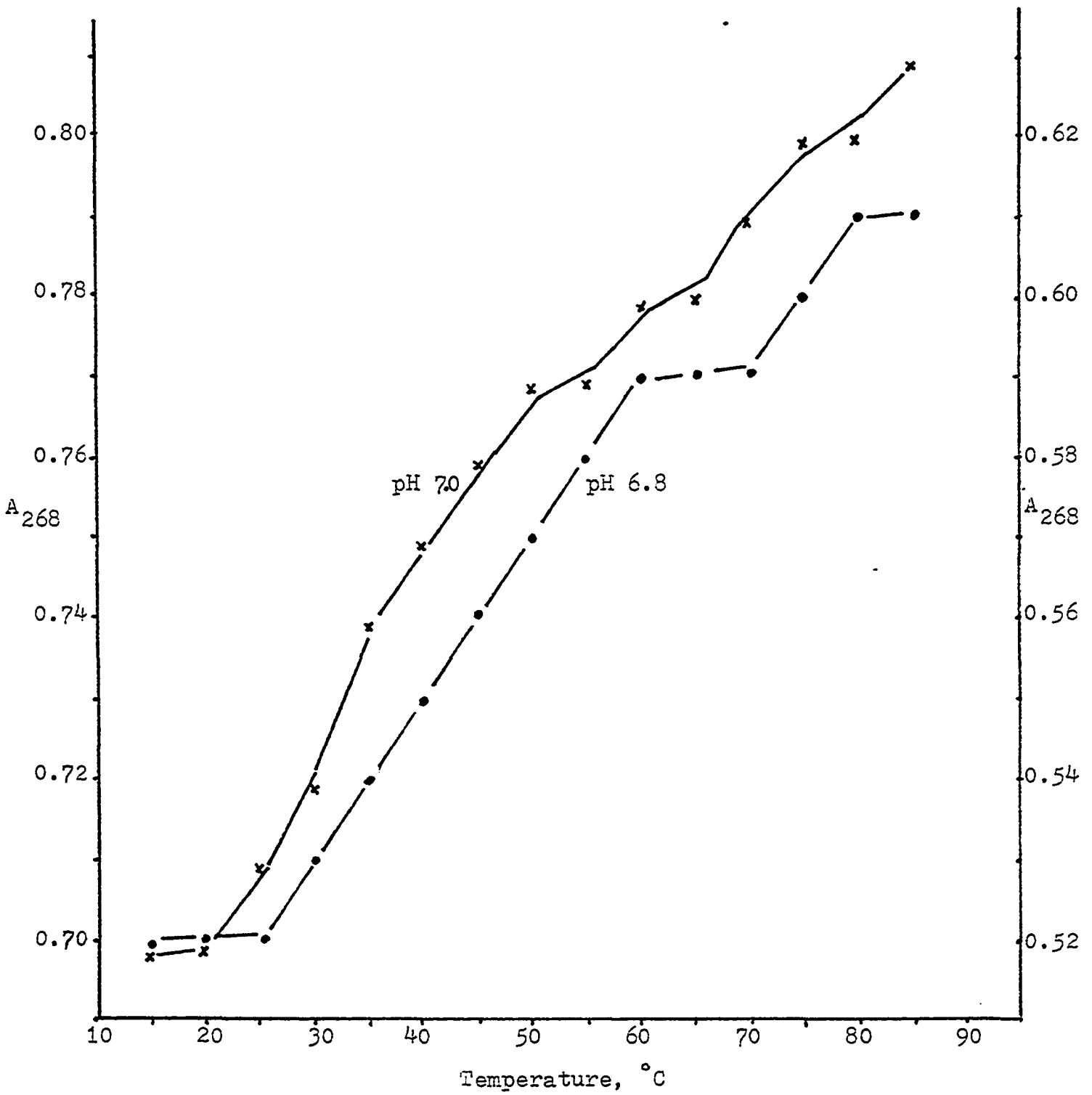


Table 5. Melting Curve Data on Poly C at pH 4.1 and 4.5.

Poly C in 0.1 M Na acetate buffer at pH 4.1 and 4.5

Temperature, °C	pH 4.1 A_{273}	pH 4.5 A_{273}
15	0.724	1.31
20	0.721	1.31
25	0.719	1.30
30	0.715	1.30
35	0.711	1.30
40	0.710	1.30
45	0.706	1.30
50	0.702	1.30
55	0.698	1.30
60	0.698	1.30
65	0.698	1.30
70	0.698	1.30
75	0.841	1.30
80	0.842	1.41
85	0.842	1.43
90	0.843	1.43

Figure 17. Melting Curves of Poly C at pH 4.1 and 4.5

Poly C in 0.1 M Na acetate buffer pH 4.1 and 4.5.

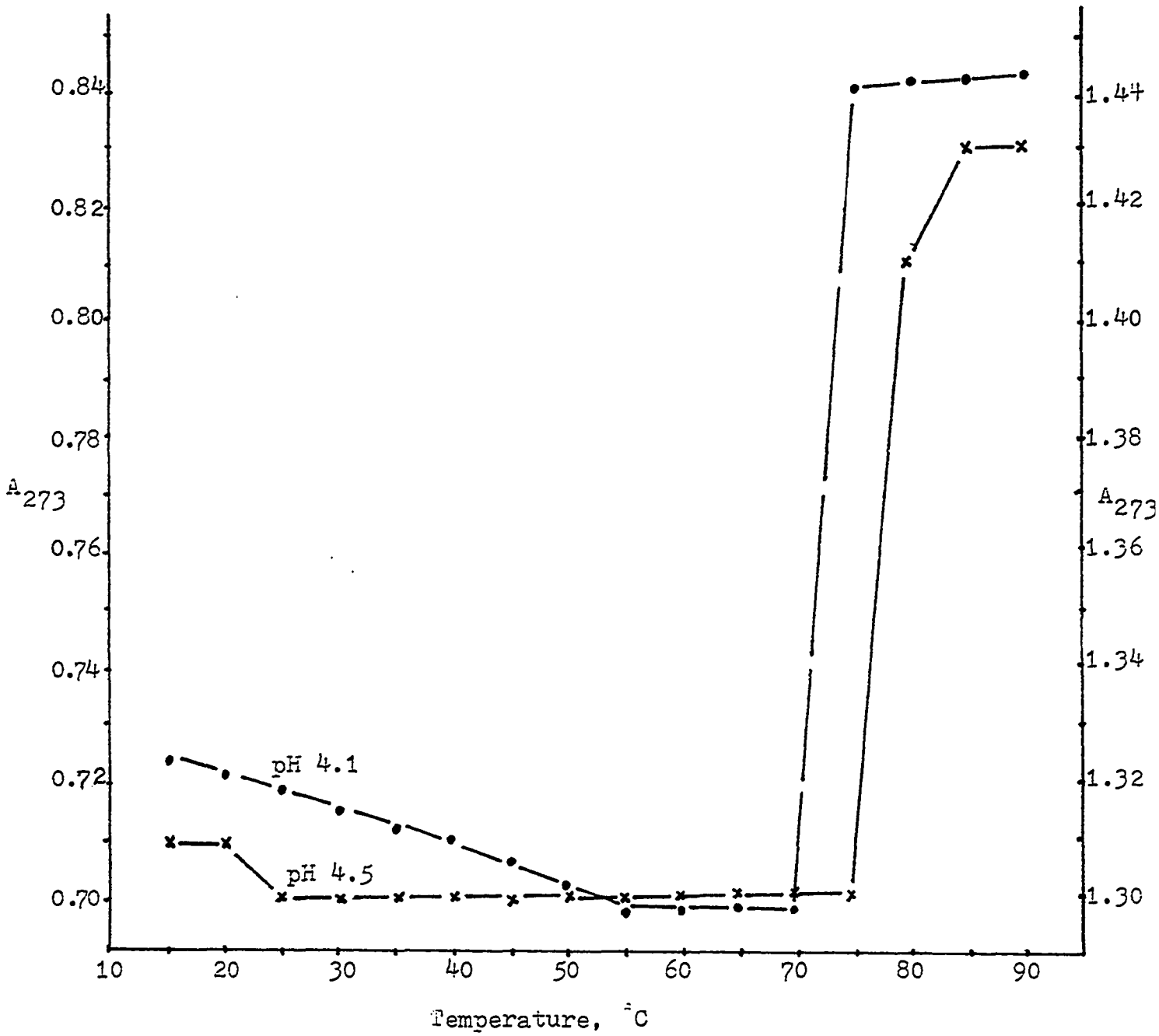
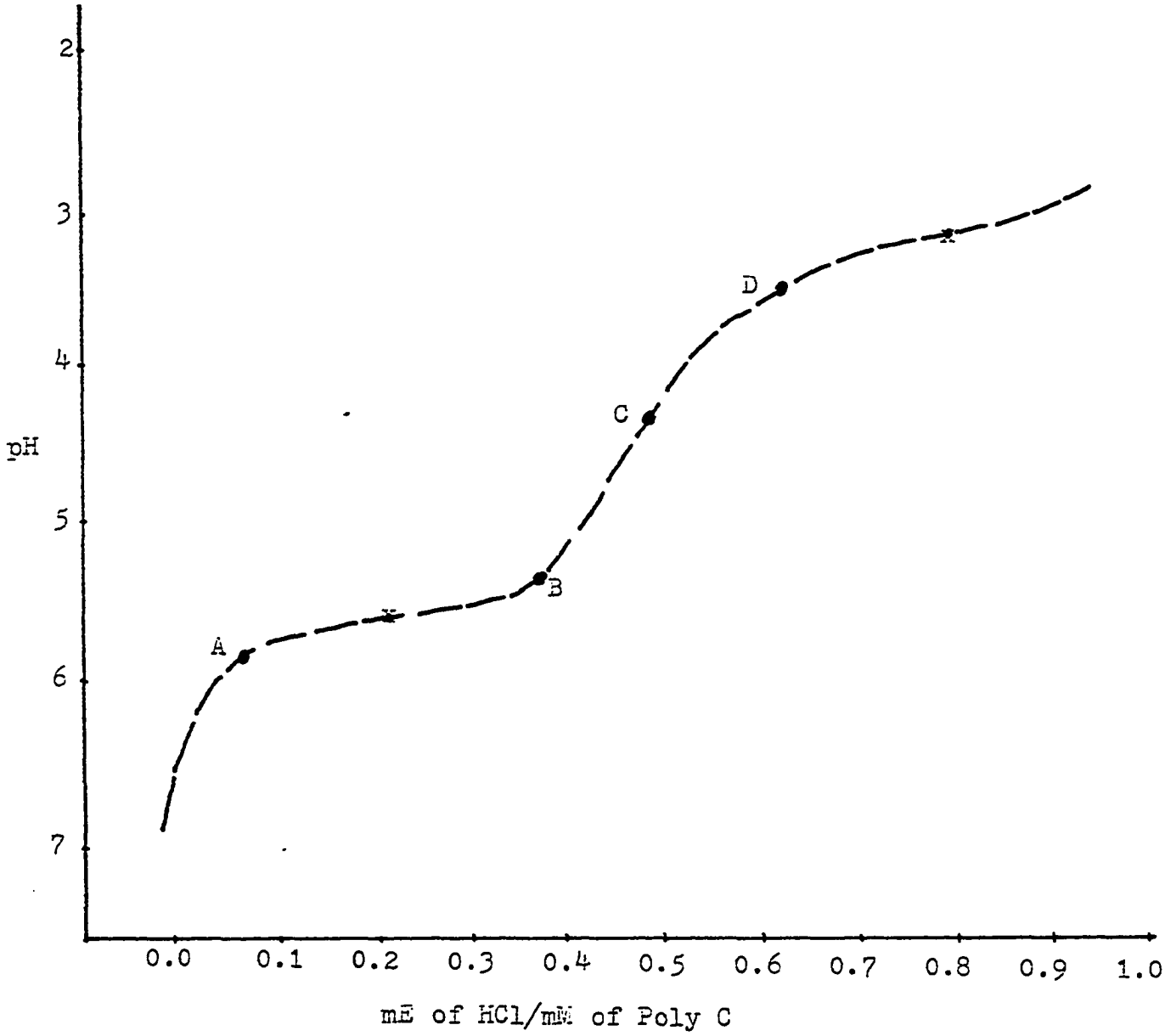


Table 6. Acid-base Titration Data on Poly C.

mE HCl/CMP	pH of solution	mE HCl/CMP	pH of solution
0.00	7.0	0.48	4.5
0.02	6.3	0.50	4.2
0.04	6.1	0.52	4.0
0.06	6.0	0.54	3.9
0.08	5.9	0.56	3.9
0.10	5.9	0.58	3.8
0.14	5.9	0.62	3.7
0.16	5.8	0.68	3.6
0.18	5.8	0.72	3.5
0.22	5.7	0.74	3.4
0.24	5.7	0.78	3.3
0.28	5.7	0.82	3.3
0.30	5.7	0.86	3.3
0.34	5.7	0.90	3.3
0.38	5.7	0.92	3.3
0.40	5.6	0.94	3.2
0.42	5.4	0.96	3.2
0.44	5.0	0.98	3.2
0.46	4.7	1.00	3.1

Figure 18. Acid-base Titration Curve of Poly C.

1.0 mM Poly C in 0.1 M NaCl at 25°C.



CHAPTER III

CONCLUSIONS AND DISCUSSION

Absorption Spectra of Poly C

The absorption spectra of Poly C solutions at various pH values show two major spectral patterns. The pH values at which these are observed correspond to the two principal conformations of Poly C suggested by other investigators. The transition from one major spectral pattern to the other occurs at about pH 5.7. At pH values between 3.1 and 5.7 (the pH range in which Poly C has been shown to exist primarily as a double stranded helix), Poly C UV spectral patterns show a λmax near 274nm and a λmin near 240nm. At pH 3.0 Poly C precipitation is observed. Above pH 5.7 (near neutrality where Poly C has been shown to exist in a single stranded conformation), the spectral patterns show a λmax near 267nm, a λmin near 247nm, and a prominent shoulder at about 228nm. The amplitude observed in the spectra of Poly C under acidic conditions is more than twice that observed at pH values near neutrality. Knowing these UV spectral characteristics of Poly C, one can easily identify the major form of the polymer present in solution. Spectra at pH 4.1 and 7.1 (in 0.1 M Na^+), exemplifying these characteristics, are shown in Figure 5.

Absorption spectra of Poly C at four different acid pH values are seen in Figure 6. The transition from one conformation to the other can be seen in the comparison of the spectrum at pH 5.1 with that at pH 6.0. Poly C spectra at three alkaline pH values are shown in Figure 7. No significant difference in the spectra can be seen, suggesting that the single stranded conformation is the prominent conformation at each of the three pH values.

Cytidine, CMP Spectra: Extinction Coefficient Determinations

Cytidine, 5'-CMP, and 2'&3'-CMP UV absorption spectra were obtained in order that a comparison might be made between the nucleoside and the nucleotides. In each case, a 1.0 mM solution was carefully prepared in 0.1 M Na^+ at pH 2.5, 4.5, and 7.1. It can be seen in Figures 8-10 that the spectra of the nucleoside (Figure 8) compares very closely with both the 5'-CMP (Figure 9) and 2'&3'-CMP (Figure 10). All three spectra are very similar to Poly C spectra at comparable pH values. These data suggest that neither the ribose sugar moiety nor the phosphate group contribute to, or significantly alter the spectral pattern. Apparently the cytosine pyrimidine base chromophore is not significantly affected by the presence or absence of the phosphate group.

Extinction coefficient values for cytidine, 5'-CMP, and 2'&3' CMP were determined at pH 2.5, 4.5, and 7.0. For cytidine: ε_{277} at pH 2.5 was found to be 11,900; ε_{272} at pH 4.5 = 9,600; ε_{268} at pH 7.0 = 8,600. For 5'-CMP: ε_{277} at pH 2.5 = 11,700; ε_{273} at pH 4.1 = 9,900; ε_{268} at pH 7.0 = 8,300. For 2'&3'-CMP (mixed isomers)

ε_{276} at pH 2.5 = 11,900; ε_{272} at pH 4.5 = 9,800; and ε_{268} at pH 7.0 = 8,800.

The extinction coefficient values determined for 2'&3'-CMP are used to estimate Poly C concentrations (expressed in monomeric CMP units) since polyribonucleotides are hydrolyzed, via treatment with mild alkali, to a mixture of 2'&3'-mononucleotide isomers. In each case an aliquot of a particular Poly C solution is hydrolyzed with 0.3 N KOH for 22 hours at 37°C. The resulting hydrolysate is adjusted to pH 7.0, 4.5, or 2.5 and the extinction coefficient value of 2'&3'-CMP at the proper pH value is used to express the Poly C concentration in monomeric CMP units.

Poly C Hypochromism Studies

The degradation of Poly C via alkaline hydrolysis is not only a useful method for the spectrophotometric determination of Poly C concentrations (expressed in monomeric units), but is also a useful method for hypochromism studies on the homopolymer. Hypochromism in Poly C was found to be slightly variable from sample to sample. This variation was minimal with samples at comparable pH values. In all cases, the hypochromic effect was found to be greatest at pH values near 4.0 where hypochromism values approached 45%. It is at this pH where Poly C exists primarily as a double-stranded helix and hydrogen bonding between cytosine residues allows for maximum base stacking interactions. The overlap of pi orbitals of the stacked bases has been suggested to be the primary hypochromism factor in polynucleotides.

At pH values near neutrality, the hypochromism was found to be lower (ca. 30%), though still quite significant, suggesting a great deal of secondary structure at neutral pH. The primary conformation of Poly C at this pH is a single-stranded helix stabilized by cytosine base stacking interactions along the polymer chain.

Figures 12-15 show UV spectra of various Poly C solutions before and after alkaline hydrolysis with appropriate controls of diluted Poly C. The increase in absorbance of the hydrolysate over the diluted polymer reveals the hypochromism effect of the polymer. The hydrolysate (2'&3'-CMP) was examined and found to show no hypochromic effects due to non-interaction of the CMP residues.

Thermal Denaturation Studies

UV absorption-temperature profiles of Poly C were performed in order that melting (denaturation) characteristics and Tm (melting temperature) values could be evaluated. These melting profiles were carried out at pH values near neutrality and near pH 4.1 in 0.1 M Na^+ aqueous buffer solutions. The melting curve data on Poly C at pH 6.8 and 7.0 (in 0.1 M Na phosphate buffer) is shown in Table 4. The melting profile (see Figure 16) shows a gradual non-cooperative denaturation of the polymer with a Tm = 50°C. This data lends evidence to support the proposed single helical conformation of Poly C at this pH. This is shown by the gradual, constant, increase in absorption (at 268nm) with

increasing temperature, suggesting non-cooperative base-unstacking actions as would be expected of such a single-stranded structure.

The denaturation data at pH 4.1 and 4.5 show a very different melting profile. The results (see Table 5 and Figure 17) show an initial decrease in absorption at λmax (273nm), a stabilization, then a sudden increase in absorption near 75°C. This melting profile is indicative of cooperative-type melting and can be explained on the basis of the proposed double-helical conformation of the polymer at these pH values. The initial decrease in absorption at 273nm is apparently due to the formation of a more perfect helix (a more ordered conformation). The structure then apparently enters a phase of stabilization as no optical property changes are observed. This stabilization phase is followed by a sudden increase in absorbance at 273nm, indicative of a nucleation event which involves cooperative hydrogen bond breakage with the concomitant unstacking of cytosine bases.

It should be noted that in none of the denaturation profiles is there observed an increase in absorption which can be compared to that seen upon alkaline hydrolysis. This indicates that even at very high temperatures a certain degree of secondary structure still exists. This should not be surprising for homopolymers such as Poly C where total denaturation (no base stacking interactions) would be highly unlikely.

Acid-Base Titration of Poly C

Poly C exhibits two pK values in the acid range, one at approximately 5.7 and the other at about 3.3. Each of these involves the protonation of the cytosine endocyclic nitrogen. The acid-base titration curve, shown in Figure 18, portrays a two-step process. The first step occurs at about pH 5.9 (point A). At this pH there is an abrupt uptake of protons by the polymer (endocyclic nitrogen protonation) without any significant change in the pH of the solution. It is at this pH where the proposed conformational transition from the single-helical form to the double-helical form is thought to occur. When approximately 0.38 protons/CMP are present (point B) the pH of the solution gradually decreases with a concomitant gradual uptake of protons by the polymer. At point C, 0.5 protons/CMP are present. At this point, the hemiprotonated duplex form of Poly C should, theoretically, be in its most stable form, with regard to the proposed hydrogen bonding scheme. The pH at point C is about 4.5.

At point D, a second transition occurs in which an increased rate of proton uptake by the polymer occurs with little change in the pH of the solution. Finally, when the proton:CMP ratio equals one, it is assumed that all the endocyclic nitrogens of the cytosine residues have been protonated. This occurs at about pH 3.1. Continued addition of HCl results in the precipitation of the Poly C.

These results agree fairly well with those reported by Hartman et al.[11] and are consistent with the suggestion that the double

helical conformation of Poly C begins to form upon protonation of the cytosine endocyclic nitrogen (which begins at approximately pH 5.7). This conformation is stabilized by the addition of one proton per base pair as evidenced by the curve which shows a gradual pH change in the region around 0.5 proton/CMP. The continued protonation of the endocyclic nitrogen between points B and D (pH 5.6 and 3.7) is resisted somewhat until, at point D, the rate of protonation increases. Upon further protonation the double helix probably splits apart due to like-charge repulsion and precipitation finally occurs at ca. pH 3.0.

PART II

BASE PAIRING BETWEEN POLYNUCLEOTIDES AND COMPLEMENTARY NUCLEOSIDES

PART II

BASE PAIRING BETWEEN POLYNUCLEOTIDES AND COMPLEMENTARY NUCLEOSIDES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The property of nucleic acids which is directly involved in the storage, translation and expression of genetic information in living systems, is the specific interaction between the purine and pyrimidine bases.[43-44] This property, first proposed by Watson and Crick,[45] is explained by the specific hydrogen bonding complementarity of adenine with thymine or uracil and of guanine with cytosine. In this manner, the base sequence of DNA is replicated "semiconservatively" and the genetic information (expressed as the base sequence) is thereby conserved and can ultimately be translated into protein via RNA mediation.[46-47]

Evidence for complementary base pairing has been demonstrated in many nucleic acid model systems. Among the studies reported, complementary homopolynucleotide systems have received the most attention. Complexes formed between homopolynucleotides and their complementary nucleosides, however, have received little attention in past years, but seem to provide simple and flexible systems for investigations of base pairing and complex formation. Of major

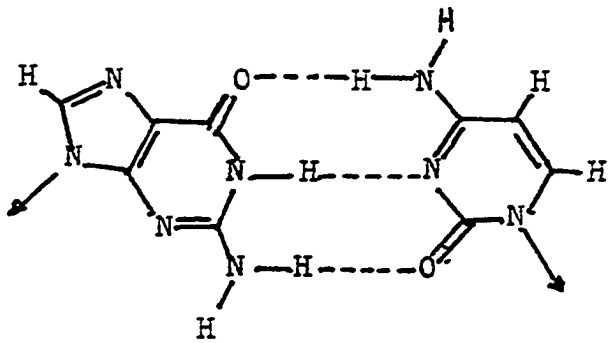
interest to this study are the base pairing interactions between guanosine and Poly C, and between adenosine and Poly U. A variety of investigations regarding these interactions have been reported in the literature.[46, 48-66]

Interactions between Poly U and adenosine were studied in considerable detail by Huang and Ts'o.[59,65] Using the technique of equilibrium dialysis, they showed a critical threshhold concentration of adenosine was essential for initial complexing to Poly U. Upon nucleation, binding of the nucleoside to the polymer was found to occur in a cooperative fashion. At saturation of binding sites (when complex formation failed to increase) the stoichiometry of the complex was found to be ca. 2U:1A. Further investigations of this system by Burr et al.[66] were carried out in order to study certain thermodynamic parameters of complex formation. In their study a thermodynamic model was developed to represent the entire binding isotherm for the cooperative binding of adenosine to Poly U.

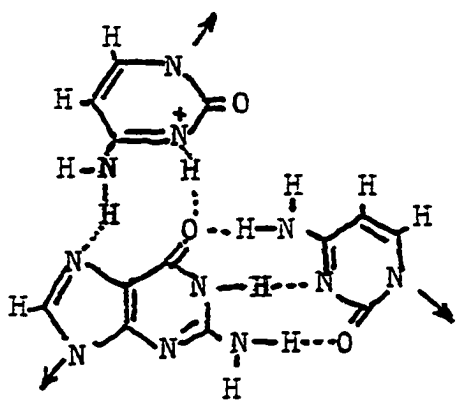
The studies of the Poly U:Adenosine system encouraged investigations into the analogous Poly C:Guanosine system. Investigations in this specific area have been reported by Sarocchi et al.[63] and Davies et al.[64] Lipsett[57,60] reported a 1:1 G:C complex between guanine trinucleotide and Poly C at dilute concentrations of reactants (0.1 mM) and pH 7.4. The stability of the complex (evidenced by Tm studies) was found to be highly dependent upon the concentration of the trimer. An increase in the GpGpG concentration markedly stabilized the complex. A 2:1 Poly C:oligo G complex was found when pH conditions were in the range 5.0-6.0. The degree of proton-

ation of the polymer which occurs in this pH range seems important in the formation of the C+G+C complex. The Tm of the 2:1 complex, as reported by Lipsett,[57] was ca. 42°C, considerably greater than that observed for the 1:1 complex (Tm = 23°C), indicating greater stability of the triplex over the duplex. Below are shown the proposed structures of the complexes as suggested by Lipsett.

(I)



(II)



→ SUGAR PHOSPHATE CHAIN

Figure 19. Poly C:Guanine complexes as suggested by Lipsett.[57] (I)-1:1 complex; (II)-2:1 C:G complex.

In 1968, Huang and Ts'o reported the formation of insoluble complexes of Poly C with guanine mono- and triphosphates under conditions of high nucleotide concentration (0.01-0.02 M), low Mg^{++} (4.0 mM) and low temperature. As the complex formed the nucleosides were said to become "polymer-like" and a phase transition occurred which resulted in the precipitation or gelation of the

complex near 0°C. The stoichiometry of the complex was found to be ca. 1C:1G. Guanine base stacking interactions[67-69] were suggested as the primary factors involved in the cooperative complexing effect which was observed.

Pochon et al.[56] found that a stable 1:1 Poly C:Poly G complex forms rapidly at low temperature, in 0.1 M NaCl at pH 7.1. Further studies showed that when Poly G was treated with alkali to cause partial degradation to oligo G and short chain Poly G, a 2:1 complex was formed with Poly C. The stoichiometry of this complex was 2G:1C and was assumed to be triple stranded with G+G+C-type interactions.

Howard and coworkers[61] reported investigations of complexes formed between Poly C and a variety of guanyl monomers. It was found that complex formation between a homopolymer and its complementary monomer (be it the nucleotide, nucleoside, or the lone nitrogen base) occurs in a well-defined stoichiometry under certain conditions. These studies show that helix formation involving specific pairing between bases is possible even when one of the components lacks both internucleotide linkages (phosphate groups) and sugar moieties. The comparable stabilities of the complexes formed in each case provide information into forces responsible for stabilization of these and other polynucleotide helices.

The formation of monomer:polymer complexes apparently occurs at the expense of the polymer (Poly C) self-structure which would otherwise be quite stable. At slightly acid pH, the 2C:1G complex is generally preferred (more stable) over the 1C:1G complex (pH 7.4).[53] In these triplexes, it appears necessary for the monomeric

component (guanyl monomer) to occupy the central strand and thereby be bonded on two sides by polymer (Poly C) chains. This is probably the case since pyrimidine nucleosides lack an N-7 acceptor position and thus cannot receive a third strand; nor do they appear to be able to serve as outside strands. It also appears likely that the smaller planar area of a pyrimidine would result in less overlap of the bases and as a consequence, less stabilization by base stacking interactions. The 2C:1G complexes were found to have a Tm = 36°C (in 0.1 M Na^+, acid pH), while the 1:1 Poly C:5'-GMP complex at pH 7.4 was found to have a Tm = 14°C.[61] The 1:1 complex was explained on the basis of lack of protonation of half the C's in Poly C which is necessary for the attachment of the third strand.

Miles[70] has suggested that these monomer:polymer interactions may represent one possible mechanism for the control of the expression of genetic information. Such control could occur at the level of translation of mRNA to protein where certain portions of the mRNA might be converted to helical regions and thus be unavailable for binding to the ribosome.

Complex Formation Between Poly C and Guanosine

Complex formation between Poly C and the guanyl monomers, guanosine and deoxyguanosine has been investigated by Sarocchi.[63] Results of these studies showed that binding of the monomer to the polynucleotide was concentration dependent, i.e. complex formation did not occur until a critical concentration of the monomer was reached. Upon initiation of complex formation, cooperative-type

binding followed until saturation was reached. At pH 4.5, in 0.1 M Na^+ acetate buffer, a 2C:1G stoichiometry was found to exist at the saturation point, the complex having a Tm = 36°C. At pH 6.6, in 0.1 M Na acetate, a 1:1 complex was formed. In the latter case the presence of magnesium ion and higher monomer concentration was found necessary for stable complex formation. The 1C:1G complex was found to have a Tm = 14°C.

In order to investigate the sites involved in the 2C:1G complex, chemical substitutions were made on guanosine monomers in selected locations. It was found that substituting at positions supposedly involved in the triplex interaction resulted in no complex formation. This was found to be the case in the interaction of Poly C with 7-methyl guanosine, 6-chloropurine riboside, 6-thio guanosine, 2,6-diaminopurine riboside, and adenosine.[63]

Complex formation between Poly C and guanosine demonstrates that these mononucleosides can stack and form pseudo-polymers upon the template (Poly C). The ORD and CD spectra of these Poly C:guanosine complexes closely resemble those spectra reported for Poly C:Poly G complexes at acid pH.[71-72]

The triple stranded complex (2C:1G) at acid pH values presents the possibility for two different binding schemes between the three bases. Morgan and Wells[73] in their studies of hybrid triple helices favor a structure shown in Figure 20A, implying a Watson-Crick and Hoogsteen pair with a second cytosine residue complexed via a shared proton to N7 of guanine. Lipsett,[57,60] however, favors a triplex involving a reverse Hoogsteen pair. This complex initially appears less probable for steric reasons. However, the

structure suggested by Lipsett (Figure 20B) would better account for the denaturation and renaturation of the complex, since in this arrangement the two Poly C strands are parallel as in the acid form of Poly C.

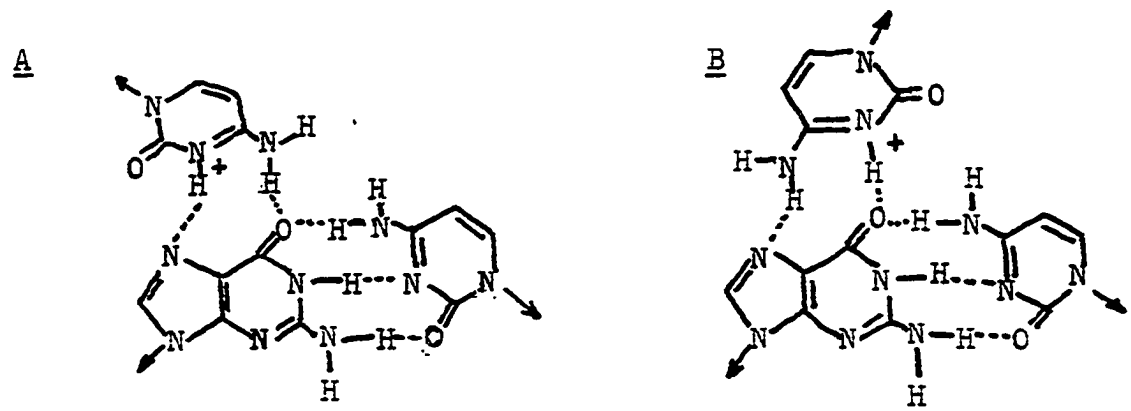


Figure 20. Possible binding schemes for the 2C:1G triplex. A-proposed by Morgan and Wells; B-proposed by Lipsett.

The method of equilibrium dialysis has been used to measure the actual degree of binding of a monomer to a homopolymer at equilibrium (by measuring the amount of uncomplexed monomer present at equilibrium). This method was used in the Poly U: Adenosine system[65] and in the Poly C:Guanosine[63] and Poly C:Deoxyguanosine systems.[64] This technique, combined with melting profiles help provide detailed information about the stoichiometry and stability of polynucleotide:monomer complexes.[64]

The binding isotherms for the Poly U:Adenosine and Poly C: Deoxyguanosine systems, as reported by Davies and Davidson[64] are shown in Figure 25. These binding isotherms show three principal things: (1) a concentration dependence of the monomer for binding, (2) a strongly cooperative binding process which occurs upon nucleation, and (3) an approximate 2:1 polymer:monomer stoichiometry

which is evident at saturation. In contrast to those reports of other investigators, they reported a 2:1 polymer:monomer stoichiometry at pH 7.1 which had a Tm of 17°C. The stability of the complex was found to increase (higher Tm) at lower pH values.

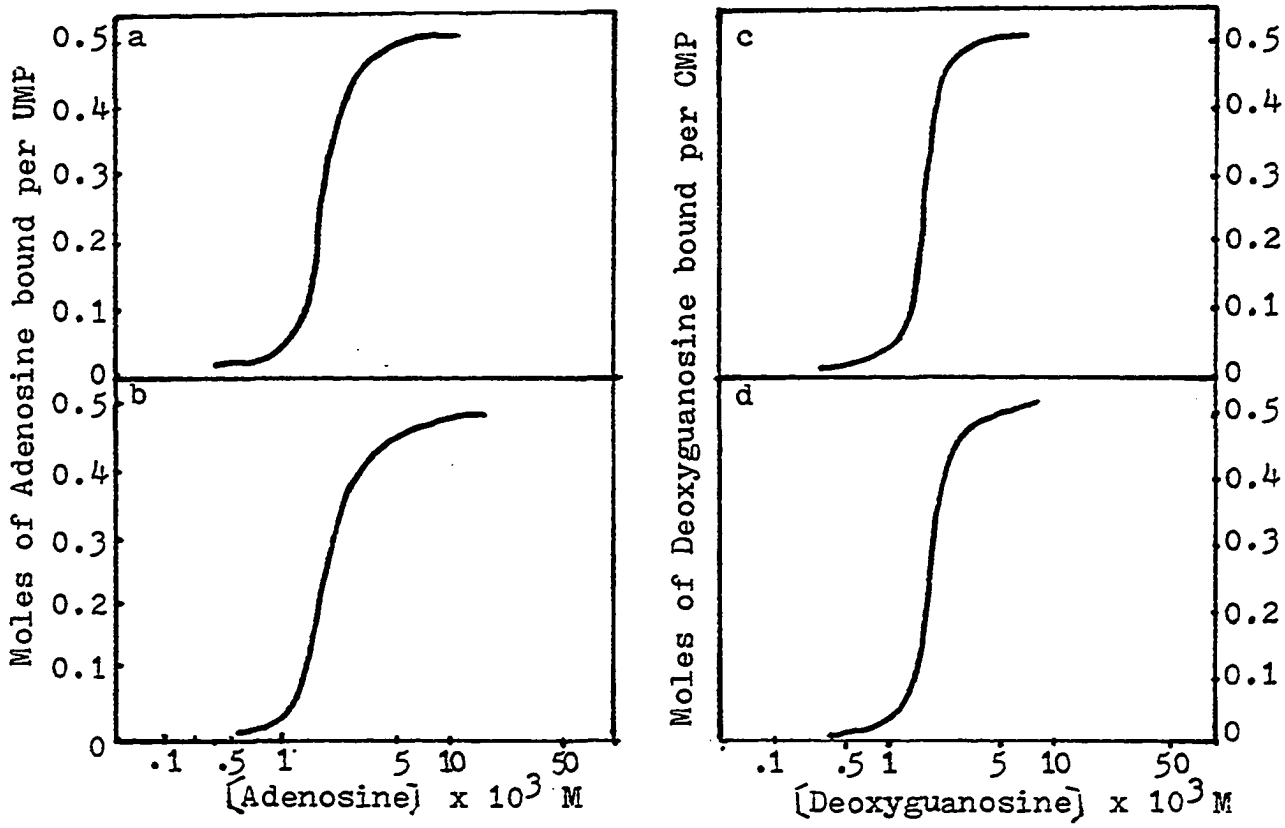


Figure 21. Binding isotherms for formation of the Poly U:Adenosine complex at 5 C, pH 7.1, 0.15 M Na^+ (a) 0.0033 M, (b) 0.128 M Poly U; and Poly C:Deoxyguanosine complex at 4 C, pH 7.1, 0.15 M Na^+ (c) 0.0028 M, (d) 0.0112 M Poly C. (from Davies and Davidson- reference 64).

This part of the study was performed in order that the Poly C: Deoxyguanosine complex could be clearly defined experimentally. Upon finding the appropriate conditions for complex formation, the thermodynamic model used to describe the binding of the Poly U:

Adenosine system (as described by Burr et al.[66]) will be applied to the Poly C:Deoxyguanosine system for thermodynamic parameter analysis.

CHAPTER II

EXPERIMENTAL

METHODS AND MATERIALS

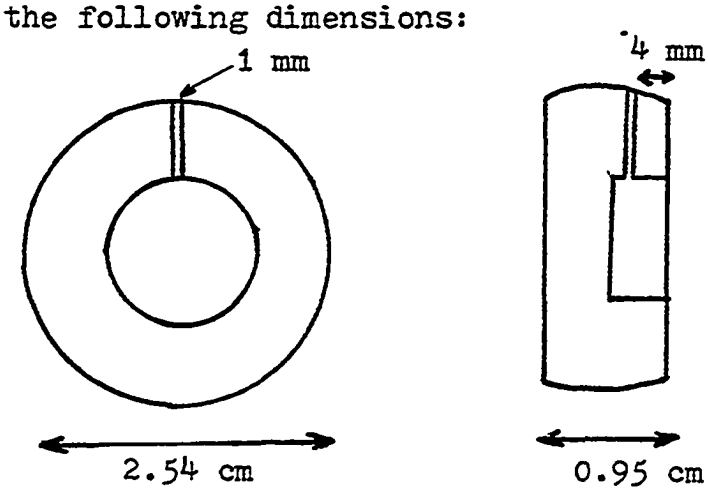
Extinction Coefficient Determinations for Nucleosides

Molar extinction coefficients were experimentally determined for the nucleosides, deoxyguanosine, guanosine, adenosine, 6-N methyl adenosine, and 1-methyl adenosine. These compounds were purchased from Sigma Chemical Company and P&L Laboratories. The Perkin-Elmer UV Spectrophotometer (Coleman Model 124) was used to measure optical density values of precise concentrations of these nucleosides. The absorbance data were then used to calculate extinction coefficient values (using Beer's Law) at a given wavelength (λmax) under specific conditions of pH, ionic strength, and temperature. In most cases a 1 mm OD pathlength curvette was utilized.

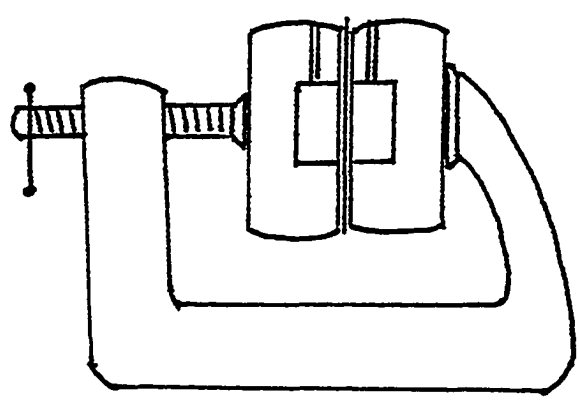
Equilibrium Dialysis

Equilibrium dialysis experiments were performed with Plexiglas acrylic plastic discs. Each disc (dialysis cell) had a small cylindrical depression of volume approximating 200 µl. A narrow

orafice leading from the side of the depression to the outside surface of the dialysis cell allowed access to each depression chamber with the use of a hypodermic syringe. These dialysis cells were constructed in the Physics Department Workshop (O.U.), each having the following dimensions:



The apparatus for dialysis experiments was assembled by placing two of the dialysis cells face to face, separated by a prepared dialysis membrane (see next section). The two cells were held together with a "C" clamp. The apparatus arrangement is shown below. The dialysis cells were readied for use by washing with ethanol followed by complete drying in a warm oven for ca. 0.5 hour.



-Equilibrium Dialysis Apparatus-

Dialysis Membrane Selection and Preparation

For the equilibrium dialysis experiments which are to be described shortly, a dialysis membrane had to be selected which had certain characteristics. It had to be semipermeable, allowing relatively small molecules (nucleosides) to pass through freely, while not allowing larger molecules (polynucleotides) to pass through at all. The membrane had to allow relatively fast equilibration time (24-48 hours) and be easily manipulated. After several attempts to find a dialysis membrane to meet these criteria, one was found--Technicon Dialysis Membrane (Type C). This particular type of membrane is most often used in auto-analyzer experiments for the separation of small polypeptides in protein purification dialysis work. This dialysis membrane has a molecular weight cutoff of approximately 3000 daltons.

Dialysis membranes were prepared as described by Huang and Ts'o.[65] The preparation is initiated by cutting the dialysis sheet to the circular dimensions of the flat surface of the dialysis cell. The circular membranes are then placed in cold distilled water and allowed to swell for 24 hours. The membranes are then rinsed in a 1% EDTA solution for ca. 0.5 hour; placed in boiling distilled water for about 10 minutes; transferred to a boiling solution of 5% $NaHCO_3$ for about 10 minutes; washed briefly in distilled water; washed briefly in 50% ethanol; then finally placed in cold distilled water and kept covered until ready for use. This membrane preparation was performed in order to remove trace amounts of UV-absorbing materials which may have been in contact with the membrane, which could cause interferences in the UV spectral data.

Equilibrium Dialysis Experiments

Equilibrium Dialysis experiments were carried out by injecting 180 µl of a known concentration of the homopolymer (Poly C or Poly U) in one chamber and 180 µl of a known concentration of nucleoside (deoxyguanosine or adenosine) in the opposite chamber. The injection ports were then sealed and the dialysis apparatus was then emmersed in a constant temperature water bath (Forma Scientific Model 2095) which was initially allowed to cool from room temperature to 2°C. The temperature was then held constant at 2°C for 48 hours. Equilibrium was generally established after ca. 24 hrs. at this temperature (2°C), but dialysis was continued for 48 hrs. to insure that equilibrium had been reached. Equilibration time was determined by injecting a solution of known concentration of nucleoside (adenosine or deoxyguanosine) in one chamber and buffer in the opposite chamber of a dialysis apparatus. After 24 or 48 hours, samples from each chamber were extracted and a spectrum of each was recorded. The matching of the two spectra (especially the O.D. reading at λmax) was indication that equilibration had been reached.

This technique of equilibrium dialysis was utilized in order to investigate the binding of deoxyguanosine to Poly C and of adenosine to Poly U under varing conditions of pH. In each experiment controls were run to ensure equilibration of the nucleoside (nucleoside vs buffer) and to ensure that no migration of the polynucleotide across the membrane occurred (polymer vs buffer). For a given experiment, a polynucleotide solution was prepared

and placed in a dialysis sac (Spectropor #3; Markson Science Inc.) and dialyzed in identical buffer solution at 5°C. The polynucleotide in the dialysis sac was placed in fresh buffer solution twice a day for three days. This procedure was performed in order to remove small oligonucleotides from the polymer solution. To insure that the removal of the small fragments was complete, the polymer solution was dialyzed against buffer in an equilibrium dialysis apparatus (as described earlier) for 48 hours at 2°C. After 48 hours, a UV spectrum was recorded from the solution in each chamber. The migration of polymer fragments across the membrane could be easily detected by observing absorption near 250nm on the buffer side and/or a decrease in the absorption near 250nm on the polymer side (as compared with the initial absorption readings).

Concentrations of polymer solutions were determined via alkaline hydrolysis (polynucleotide in 0.6 N KOH for 22 hours at 37°C). A series of nucleoside solutions (all of known concentration) were dialyzed against a prepared polynucleotide solution for 48 hours at 2°C. At equilibrium, UV spectra were taken from solutions of nucleoside present in the chamber which was initially injected with a known concentration of nucleoside. This solution represents the concentration of nucleoside which is "free" or unbound and in equilibrium with itself on both sides of the membrane. This concentration of free nucleoside, multiplied by two (assuming equilibrium) and subtracted from the initial concentration of nucleoside will be a direct measure of the concentration of nucleoside bound or complexed to the polymer. See equation (1).

(1) $[N_i] - 2[N_f] = [N_b]$, where $[N_i]$ = initial nucleotide con-

centration, $[N_f]$ = free or uncomplexed nucleoside concentration at equilibrium, and $[N_b]$ = bound or complexed nucleoside concentration.

Data regarding the binding of deoxyguanosine to polycytidylic acid was fitted to a model developed by Burr et al.[66] for the analogous adenosine:Poly uridylic acid complex. This model views the association of the purine nucleoside with the pyrimidine polynucleotide, as a series of small complexes which expand until the polymer reaches a binding saturation point.

Using mass action relationships, the total concentration of bound deoxyguanosine (G_b) (i.e. deoxyguanosine bound to Poly C) can be written in terms of the equilibrium constants in each of these smaller regions of complex formation based on the concentration of free (unbound) deoxyguanosine (G_f) in the bulk solution. From this the following expression can be written:

$$\theta = \frac{G_b}{S} = \frac{(K_1 G_f)^n \left[\frac{n}{(1-K_1 G_f)} + \frac{K_1 G_f}{(1-K_1 G_f)^2}\right]}{1 + (K_1 G_f)^n \left[\frac{n}{(1-K_1 G_f)} + \frac{K_1 G_f}{(1-K_1 G_f)^2}\right]}$$

where θ = fraction of binding sites on Poly C to which deoxyguanosine is bound; K_1 is the equilibrium constant for the binding of one purine nucleoside to one site on the polymer; n is the number of purine residues stacked together in interaction with the polynucleotide for stable complex formation. The values of G_b (bound deoxyguanosine) and G_f (free deoxyguanosine) are experimentally determined values. S represents the concentration of polymer binding sites occupied by dG on saturation; thus it too

is an experimentally determined value. The best values of K_1 and n are fitted to these experimental data via a non-linear least squares program.[66] From the value of K_1 the value of the free energy of binding, ΔG can be calculated. From the value of the slope of the isotherm at the midpoint the base stacking free energy of the dG residues in the complex can be estimated. From the difference between the total free energy and the free energy of base stacking, the value for the hydrogen bonding energy in the complex can be approximated.

Chapter III

RESULTS

Figure 22. UV Absorption Spectra of Deoxyguanosine, Guanosine, 3'-GMP, and 5'-GMP.

conditions: 0.1 M Na acetate buffer, pH 4.5, 24°C.
-only spectral patterns shown; concentrations not determined.

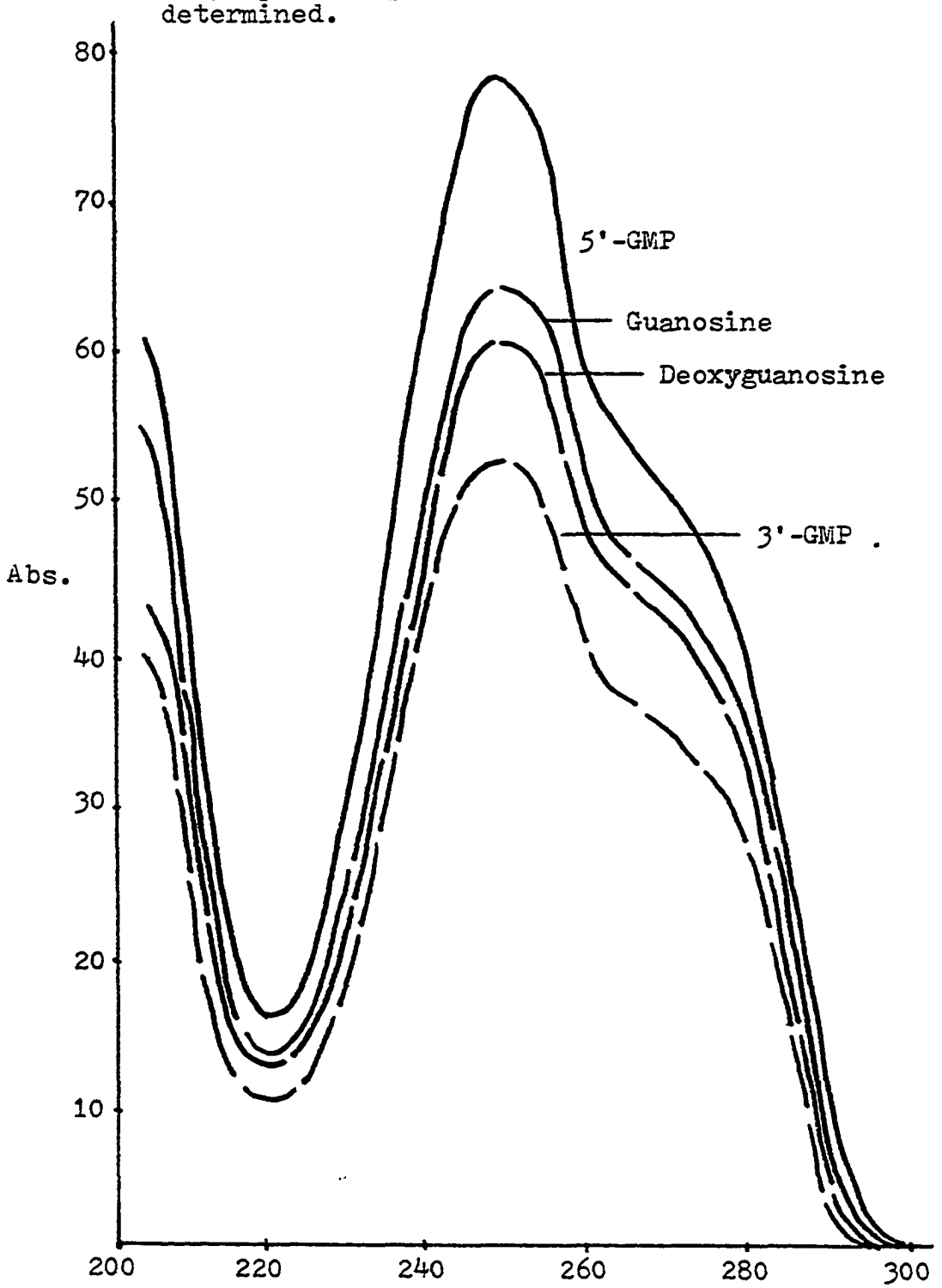
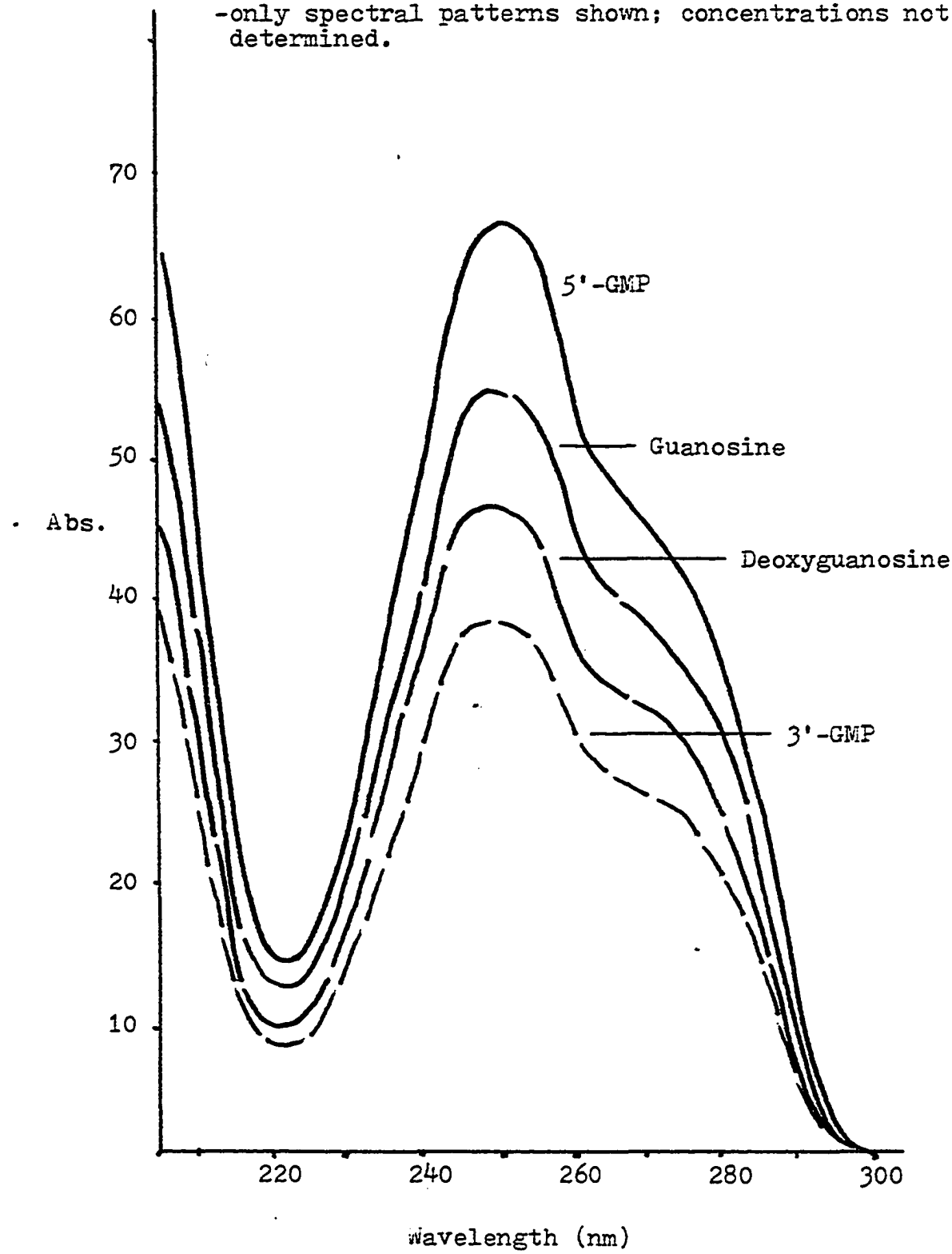


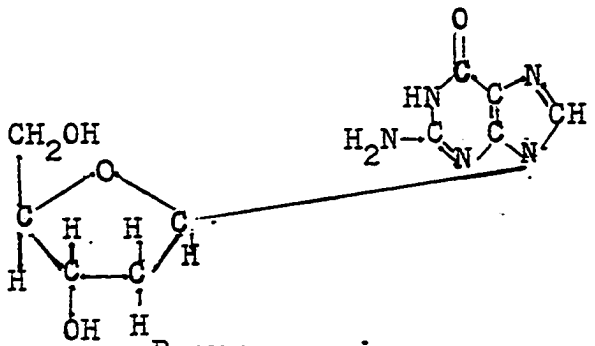
Figure 23. UV Absorption Spectra of Deoxyguanosine, Guanosine 3'-GMP, and 5'-GMP.

conditions: 0.1 M Na phosphate buffer, pH 7.0, 24°C.
-only spectral patterns shown; concentrations not determined.



Extinction Coefficient Determination of Deoxyguanosine at pH 4.5 and 6.9.

Deoxyguanosine in 0.1 M Na acetate buffer, pH 4.5, 24°C; and in 0.1 M Na phosphate buffer, pH 6.9, 24°C.

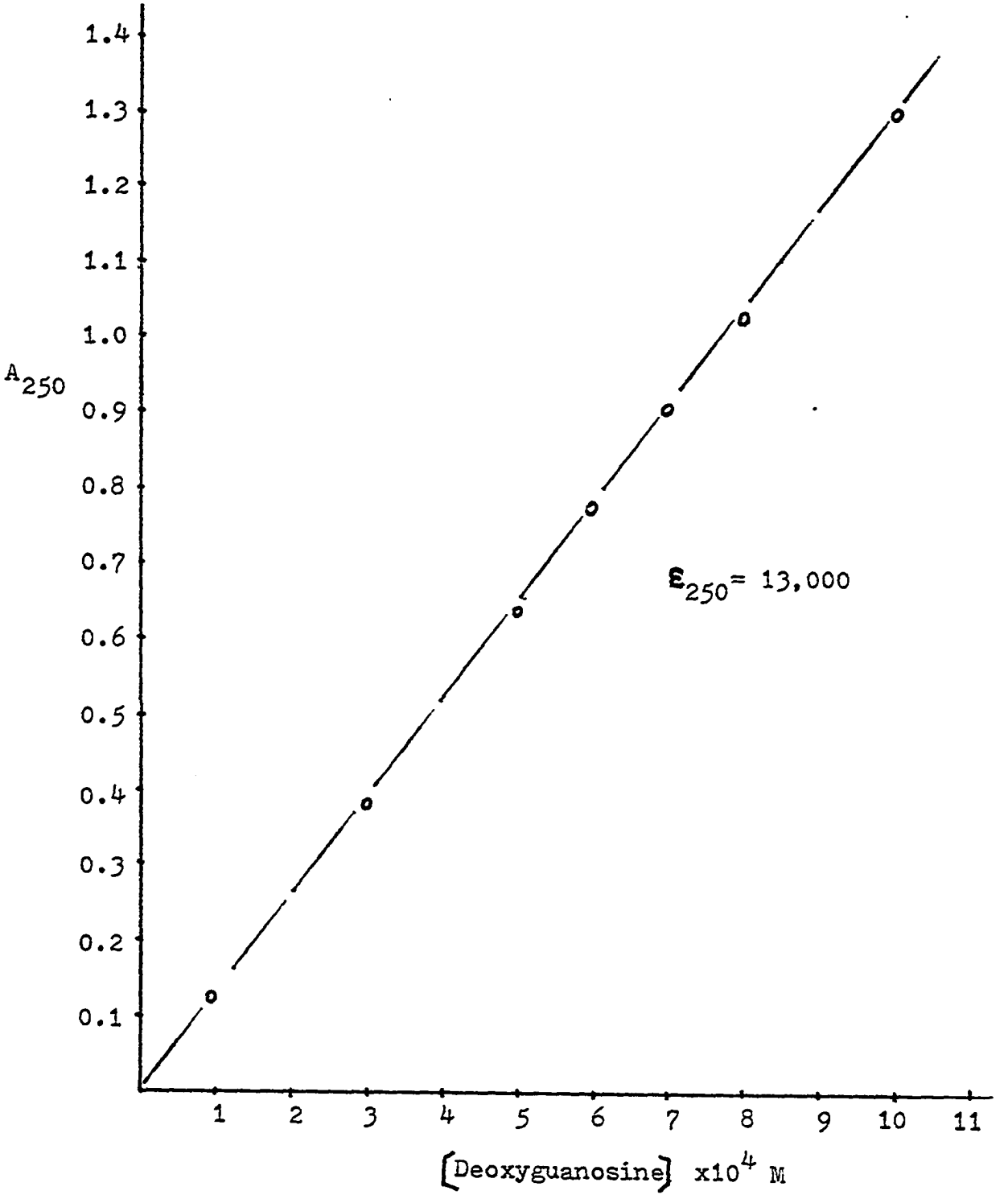


Deoxyguanosine

{Deoxyguanosine}	A_{250}	$\varepsilon_{250} \times 10^{-3}$
pH 4.5: λmax = 250nm λmin = 219nm		ε_{250} = 13,000
1×10^{-4} M	0.13	13.0
3×10^{-4} M	0.39	13.0
5×10^{-4} M	0.65	13.0
7×10^{-4} M	0.91	13.0
8×10^{-4} M	1.03	12.9
1×10^{-3} M	1.30	13.0
pH 6.9: λmax = 250nm λmin = 219nm		ε_{250} = 13,000
1×10^{-4} M	0.13	13.0
3×10^{-4} M	0.38	12.7
5×10^{-4} M	0.64	12.8
7×10^{-4} M	0.91	13.0
8×10^{-4} M	1.03	12.9
1×10^{-3} M	1.29	12.9

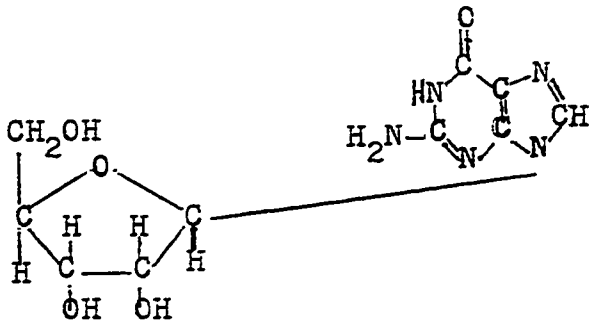
Figure 24. Extinction Coefficient Determination of Deoxyguanosine.

conditions: in 0,1 M Na acetate buffer, pH 4.5, 24°C;
and in 0.1 M Na phosphate buffer, pH 6.9, 24°C.



Extinction Coefficient Determination of Guanosine at pH 4.5 & 6.8.

conditions: in 0.1 M Na acetate buffer, pH 4.5, 24°C;
and in 0.1 M Na phosphate buffer, pH 6.8, 24°C.



Guanosine

[Guanosine]	A_{250}	$\varepsilon_{250} \times 10^{-3}$
pH 4.5: λmax = 250nm	λmin = 220nm	ε_{250} = 12,800
1×10^{-4} M	0.13	13.0
3×10^{-4} M	0.38	12.6
5×10^{-4} M	0.64	12.8
7×10^{-4} M	0.90	12.8
8×10^{-4} M	1.02	12.7
1×10^{-3} M	1.29	12.9
pH 6.8: λmax = 251nm	λmin = 220nm	ε_{251} = 12,800
1×10^{-4} M	0.125	12.5
3×10^{-4} M	0.38	12.6
5×10^{-4} M	0.635	12.7
7×10^{-4} M	0.89	12.7
8×10^{-4} M	1.03	12.8
1×10^{-3} M	1.28	12.8

Figure 25. Extinction Coefficient Determination for Guanosine.

conditions: in 0.1 M Na acetate buffer, pH 4.5, 24°C;
and in 0.1 M Na phosphate buffer, pH 6.8, 24°C.

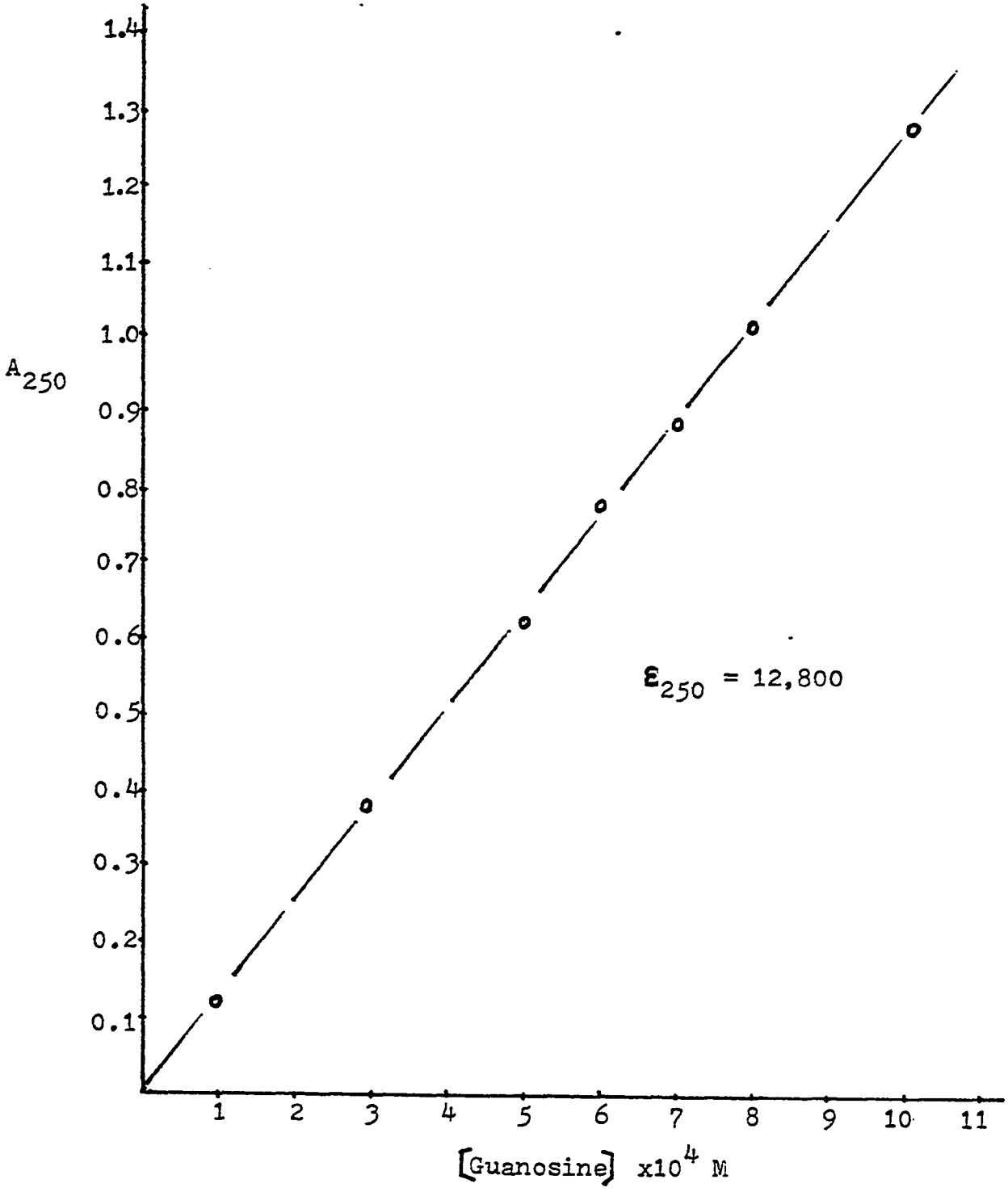


Figure 26. UV Absorption Spectra of Adenosine and 5'-AMP.

conditions: 0.1 M Na acetate buffer, pH 4.5, 24°C.

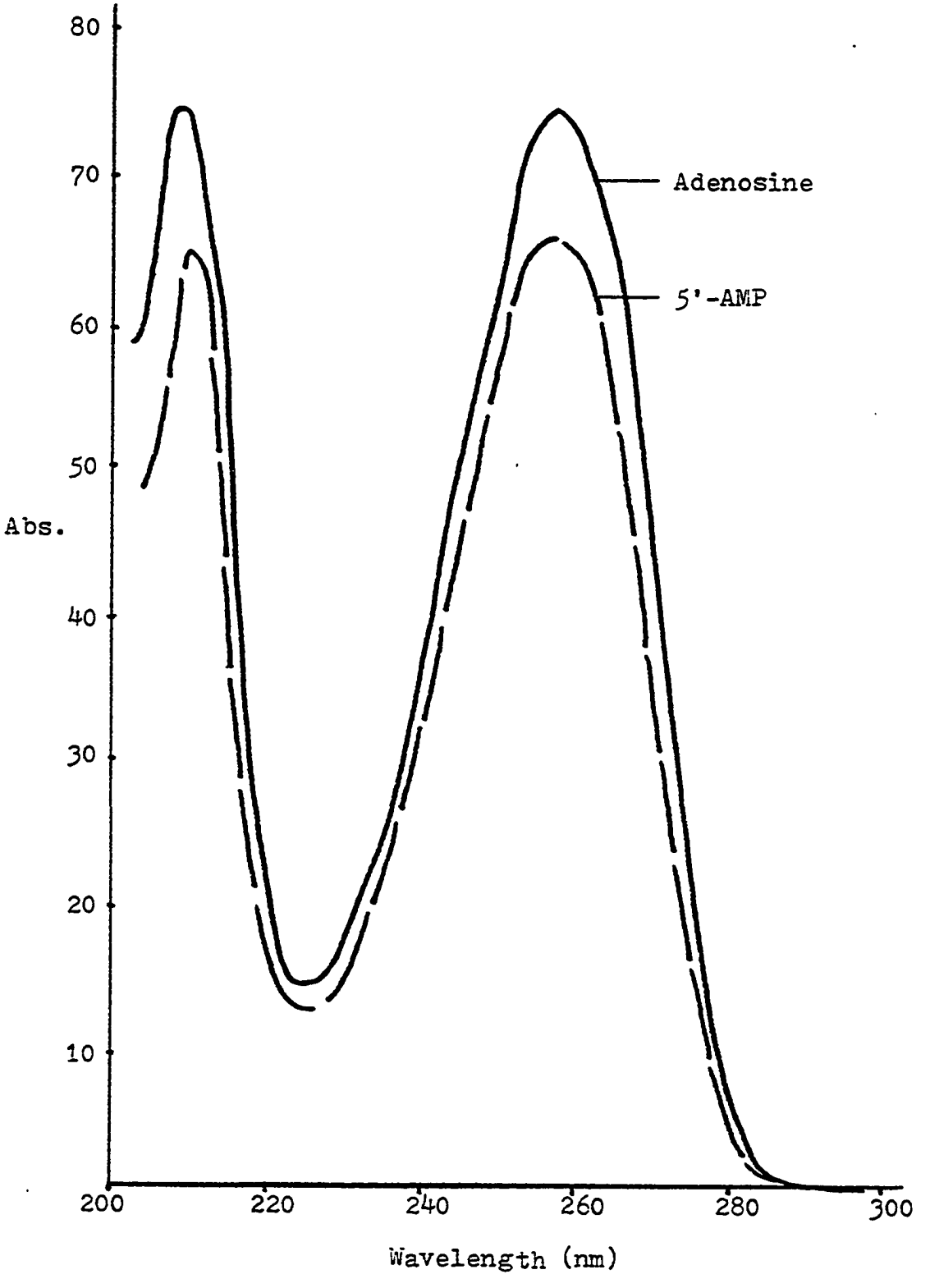
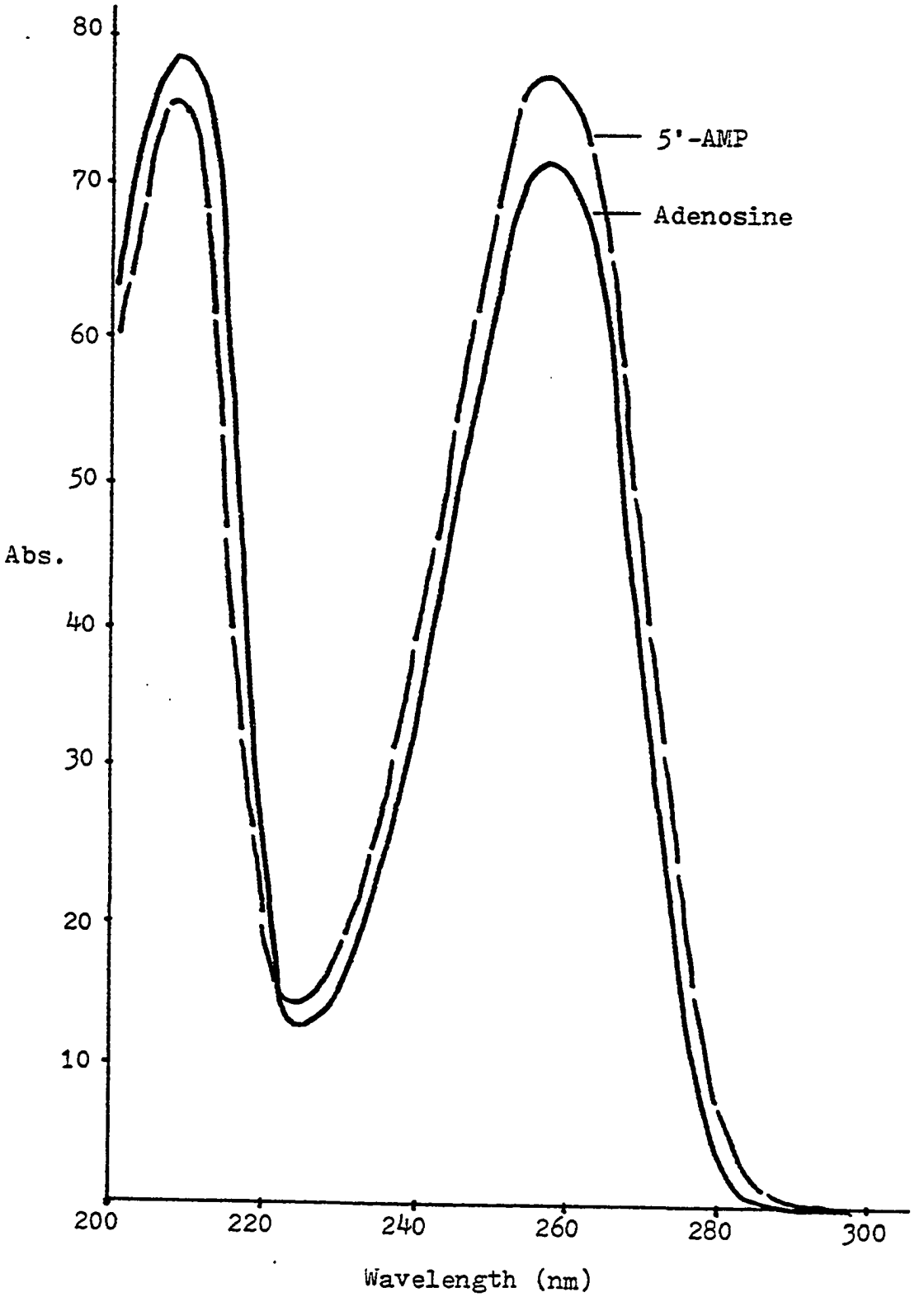


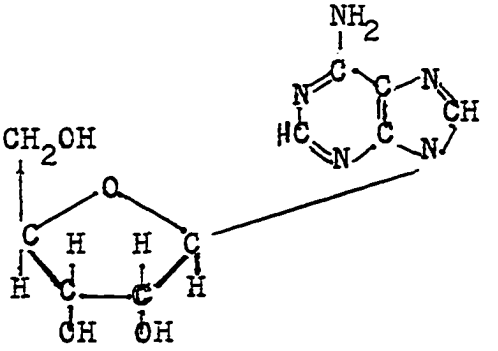
Figure 27. UV Absorption Spectra of Adenosine and 5'-AMP.

conditions: 0.1 M Na phosphate buffer, pH 7.0, 24°C.



Extinction Coefficient Determination of Adenosine at pH 4.5 & 6.8.

Adenosine in 0.1 M Na acetate buffer, pH 4.5, 24°C and in 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, pH 6.8, 24°C.



Adenosine

[Adenosine]	A_{257}	$\varepsilon_{257} \times 10^{-3}$
pH 4.5: λmax = 257nm λmin = 221nm ε_{257} = 14,600		
1×10^{-4} M	0.14	14.0
3×10^{-4} M	0.44	14.6
5×10^{-4} M	0.75	15.0
6×10^{-4} M	0.88	14.6
7×10^{-4} M	1.03	14.7
8×10^{-4} M	1.16	14.5
1×10^{-3} M	1.43	14.3
pH 6.8 : λmax = 258nm λmin = 220nm ε_{258} = 14,800		
1×10^{-4} M	0.15	15.0
3×10^{-4} M	0.45	15.0
5×10^{-4} M	0.75	15.0
6×10^{-4} M	0.87	14.5
8×10^{-4} M	1.17	14.6
1×10^{-3} M	1.47	14.7

Figure 28. Extinction Coefficient Determination of Adenosine.

conditions: in 0.1 M Na acetate buffer, pH 4.5, 24°C;
and in 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.025 M NaH_2PO_4,
0.025 M Na_2HPO_4, pH 6.8, 24°C.

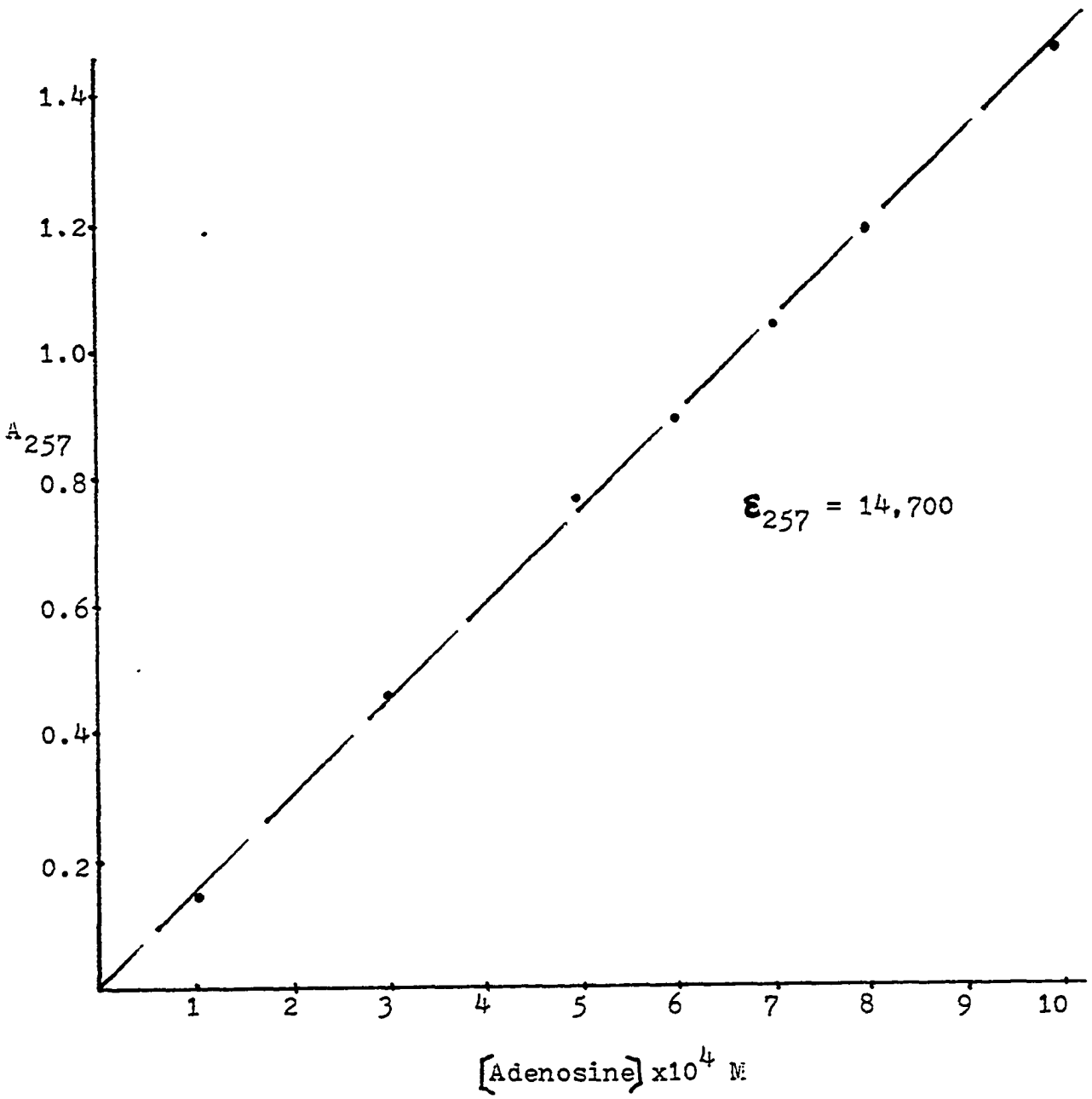


Figure 29. UV Absorption Spectra of Deoxyguanosine, Guanosine, and Adenosine.

conditions; 0.1 M Na acetate buffer, pH 4.5, 24°C.

-spectral patterns for comparison purposes-

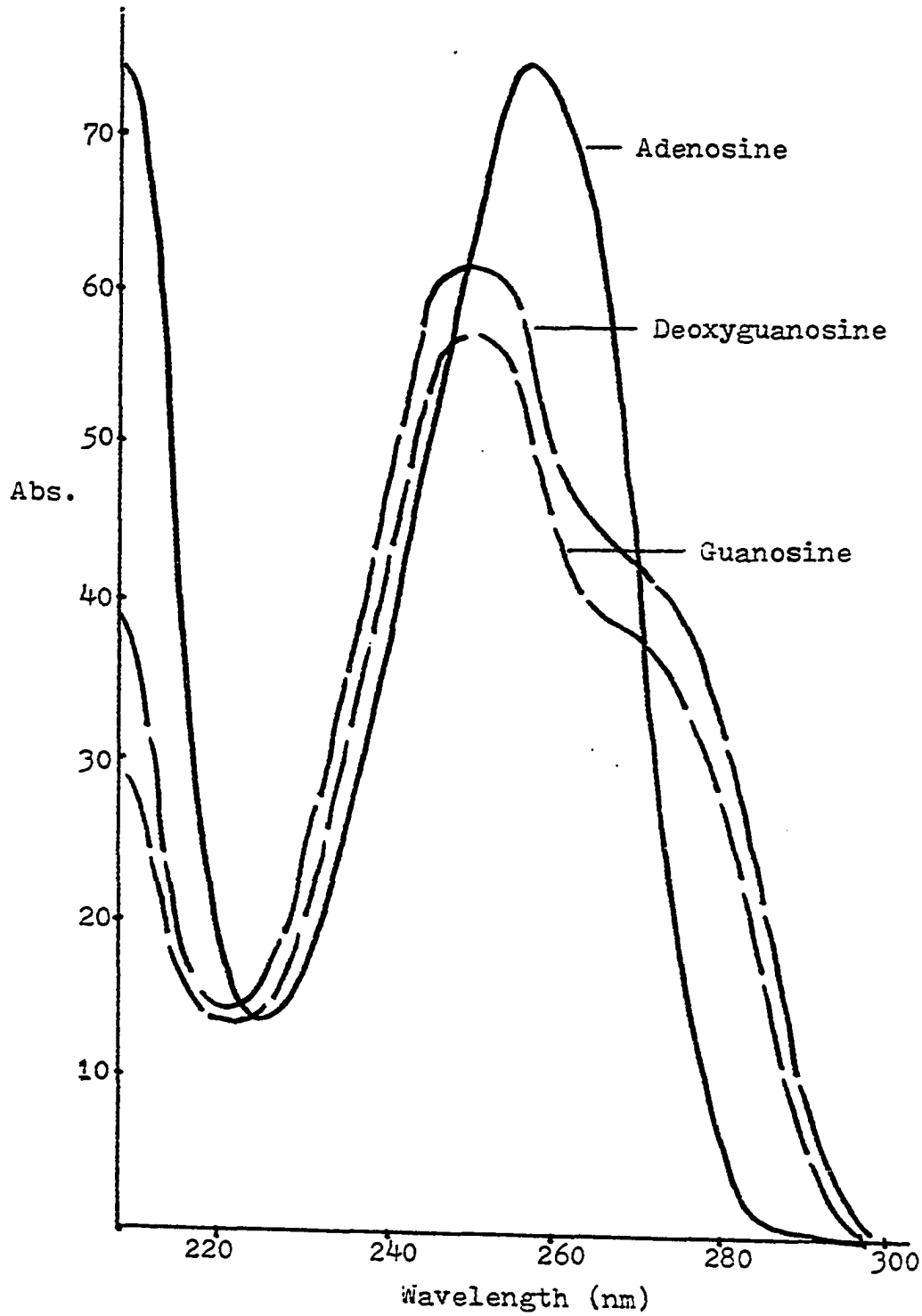
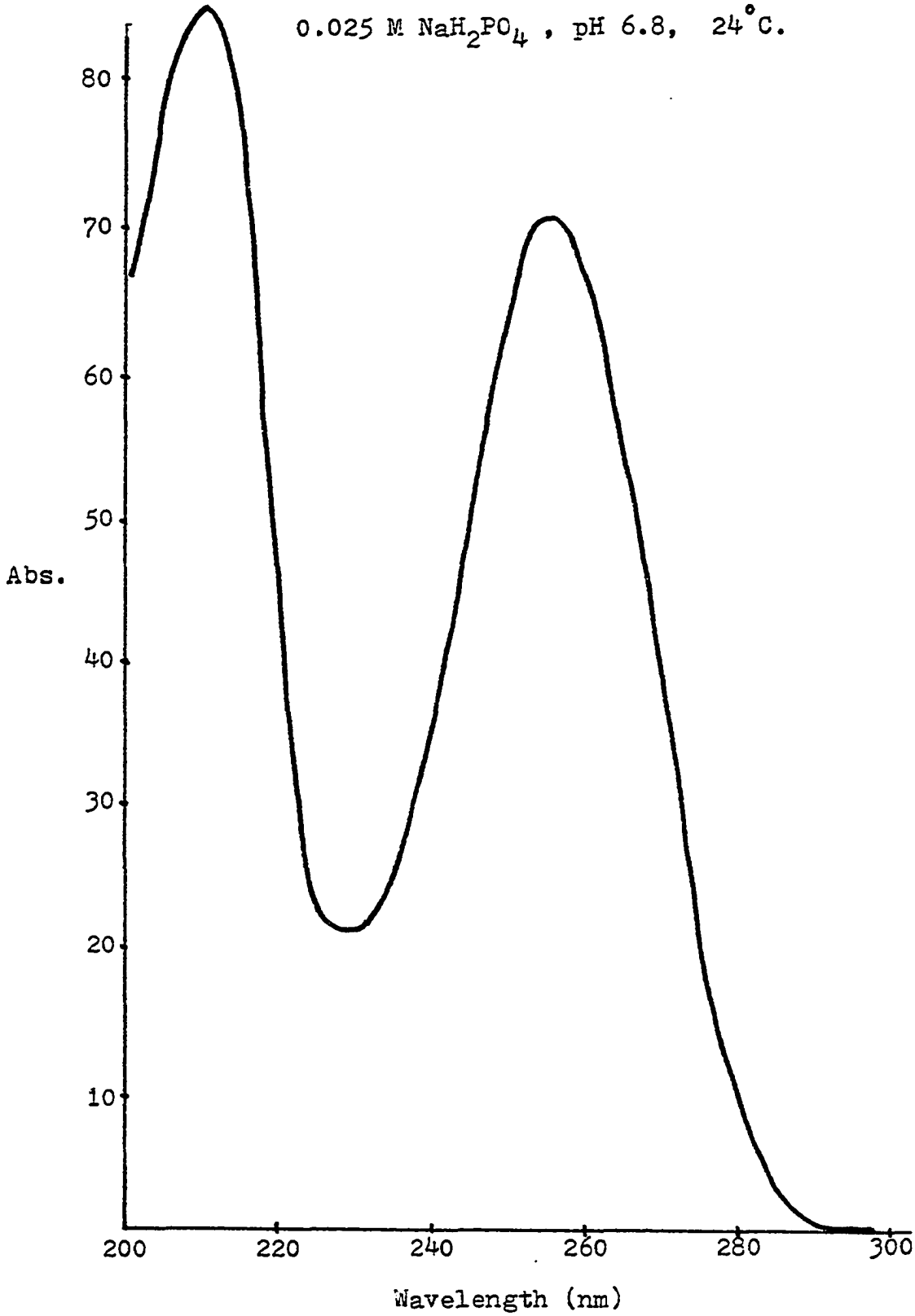


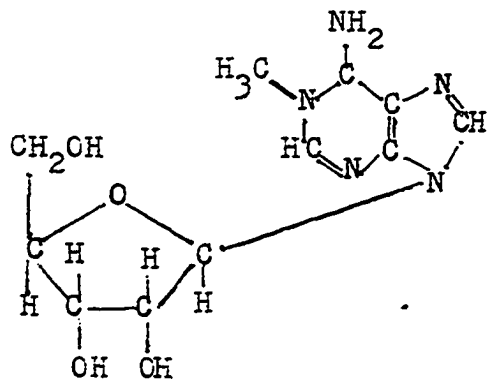
Figure 30. UV Absorption Spectrum of 1-Methyl Adenosine.

conditions: in 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4 , pH 6.8, 24°C.



Extinction Coefficient Determination of 1-Methyl Adenosine.

1-Methyl Adenosine in 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, pH 6.8, 24°C.



1-Methyl Adenosine

(1-Methyl Adenosine)	A_{255}	$\varepsilon_{255} \times 10^{-3}$
3×10^{-4} M	0.32	10.7
5×10^{-4} M	0.53	10.6
7×10^{-4} M	0.74	10.5
8×10^{-4} M	0.84	10.5
1×10^{-3} M	1.07	10.7

λmax = 255nm λmin = 228nm

ε_{255} for 1-Methyl Adenosine = 10,700

Figure 31. Extinction Coefficient Determination of 1-Methyl Adenosine.

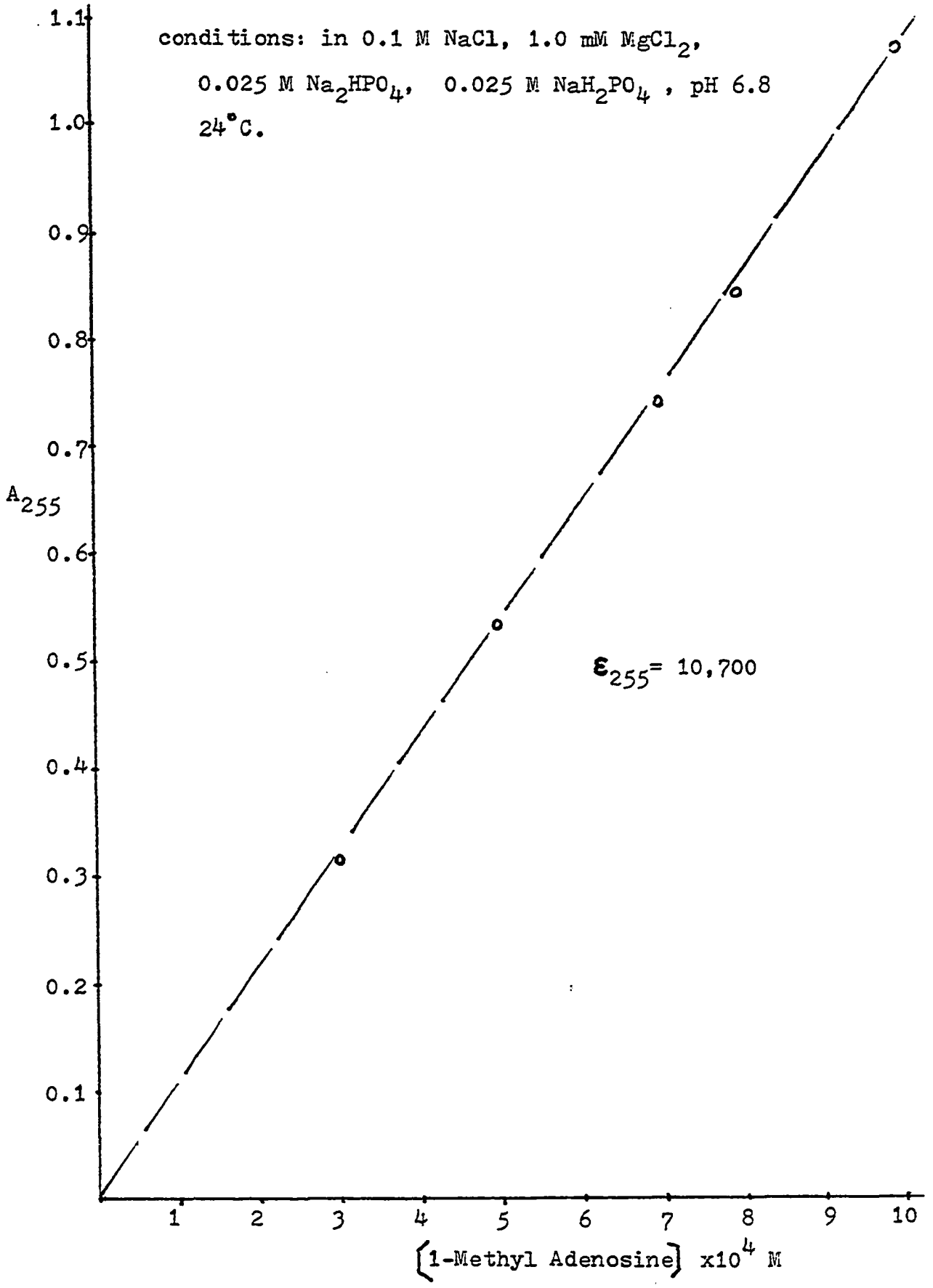
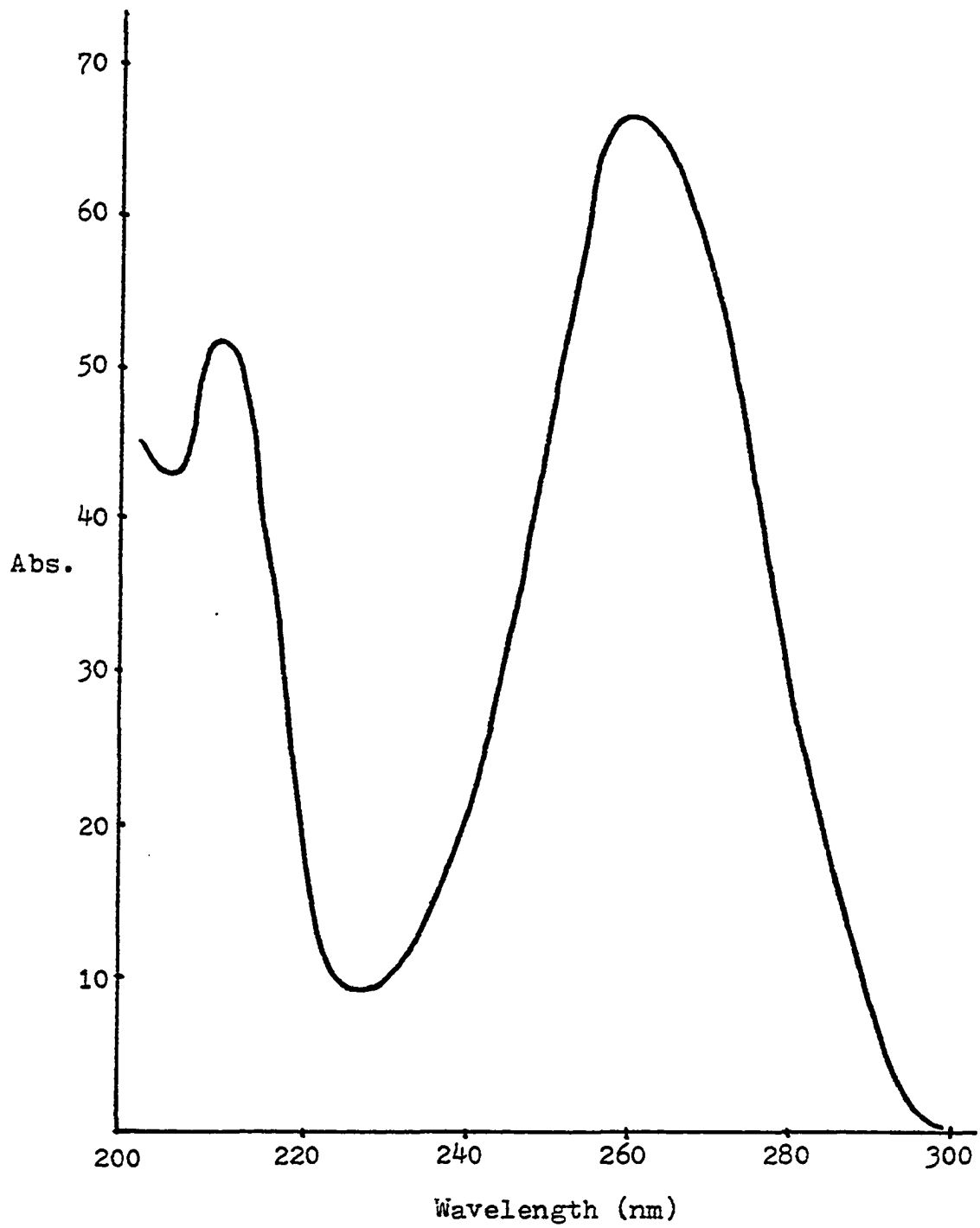


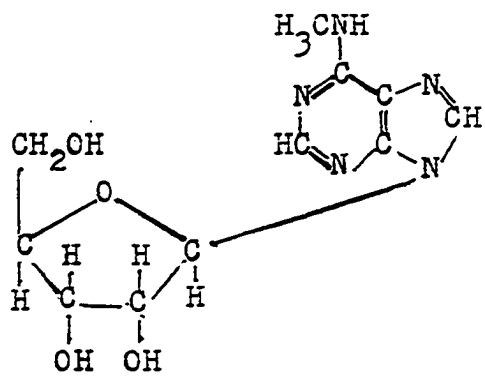
Figure 32. UV Absorption Spectrum of 6-N-Methyl Adenosine.

conditions: in 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, pH 6.8, 24°C.



Extinction Coefficient Determination of 6-N-Methyl Adenosine.

6-N-Methyl Adenosine in 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, pH 6.8, 24°C.



6-N-Methyl Adenosine

(6-N-Methyl Adenosine)	A_{263}	$\varepsilon_{263} \times 10^{-3}$
3×10^{-4} M	0.46	15.3
5×10^{-4} M	0.77	15.4
6×10^{-4} M	0.94	15.6
7×10^{-4} M	1.07	15.2
8×10^{-4} M	1.26	15.7
1×10^{-3} M	1.53	15.3

λ max = 263nm λ min = 227nm

ε_{263} for 6-N-Methyl Adenosine = 15,300

Figure 33. Extinction Coefficient Determination for 6-N-Methyl Adenosine.

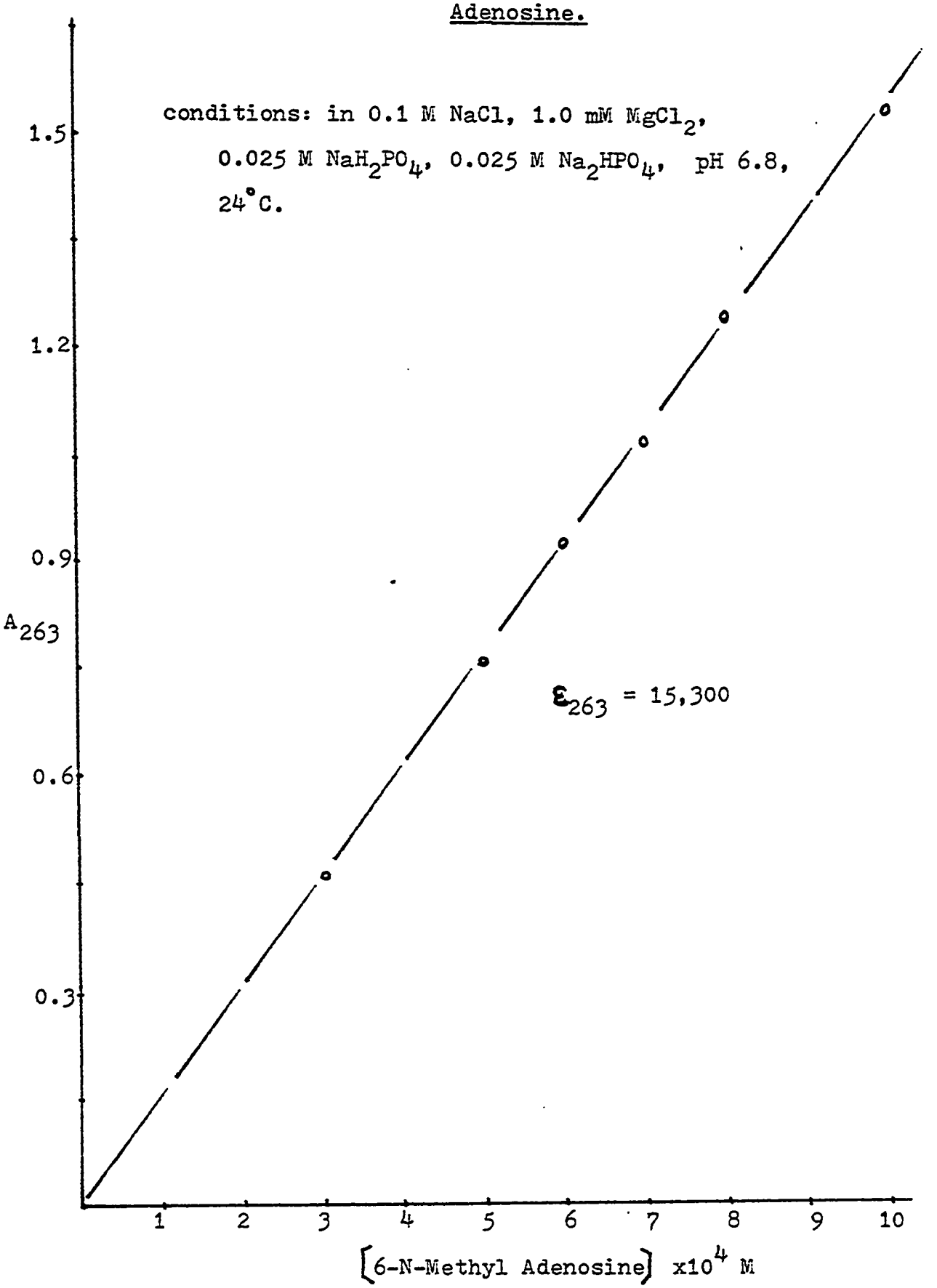


Figure 34. UV Absorption Spectrum of Polyuridylic Acid

in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 0.1 mM $MgCl_2$ at pH 6.8. ε_{258} for UMP = 9,100

Poly U shows 8% hypochromicity under these conditions (23°C).

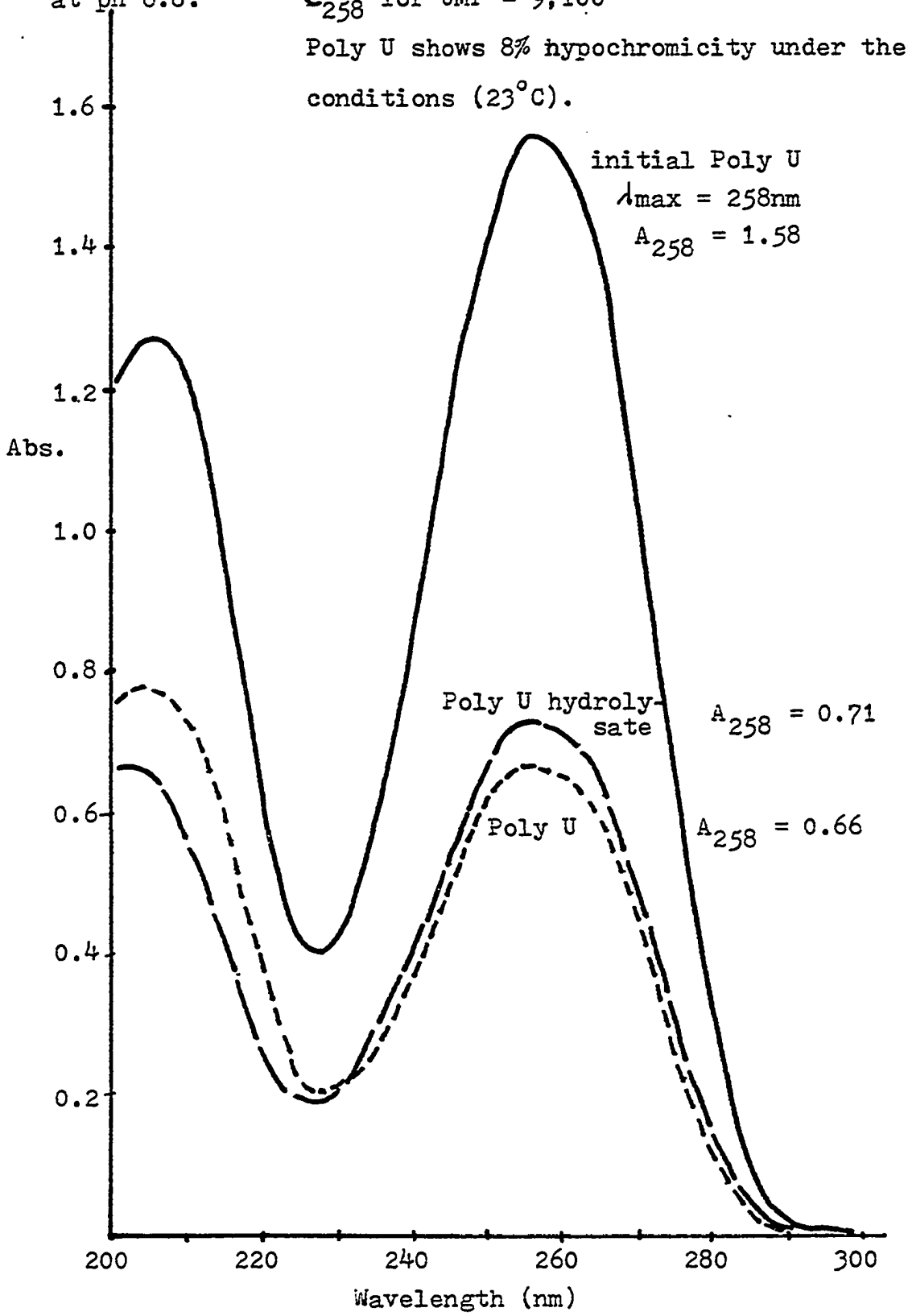


Table 7. Binding of Deoxyguanosine to Polycytidylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M Na acetate buffer, pH 4.1

equilibration after 48 hrs. at 2°C.

initial [Poly C] = 1.7×10^{-3} M A_{273}=1.11

E_{250} for deoxyguanosine = 13,000

Poly C showed 41% hypochromicity

dG_i-A_{250}	$[dG_i] \times 10^4$ M	dG_f-A_{250}	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.38	2.92	0.19	1.46	0.00
0.68	5.23	0.27	2.07	1.08
0.81	6.23	0.31	2.38	1.47
0.92	7.07	0.33	2.53	2.00
1.06	8.15	0.35	2.69	2.77
1.33	10.23	0.41	3.15	3.93
1.83	14.07	0.49	3.76	6.54
2.61	20.07	0.82	6.30	7.47
3.68	28.30	1.34	10.30	7.69

Migration Check: Poly C vs buffer A_{273}= 1.11 : 0.00

Equilibration Check: 2.0 mM dG vs buffer A_{250} = 1.31 : 1.29

dG_i = initial deoxyguanosine

dG_f = free or unbound deoxyguanosine

dG_b = bound or complexed deoxyguanosine

Table 8. Binding of Deoxyguanosine to Polycytidylic Acid

Computer Data

Conditions: in 0.1 M Na acetate buffer, pH 4.1

n	S	K_1	RMSD
3	7.85×10^{-4} M	1691	8.03×10^{-5}
4	7.78×10^{-4} M	1852	6.24×10^{-5}
**5	7.66×10^{-4} M	1986	5.47×10^{-5}
6	7.53×10^{-4} M	2098	5.51×10^{-5}
7	7.41×10^{-4} M	2195	6.01×10^{-5}

** best value of n based on lowest RMSD

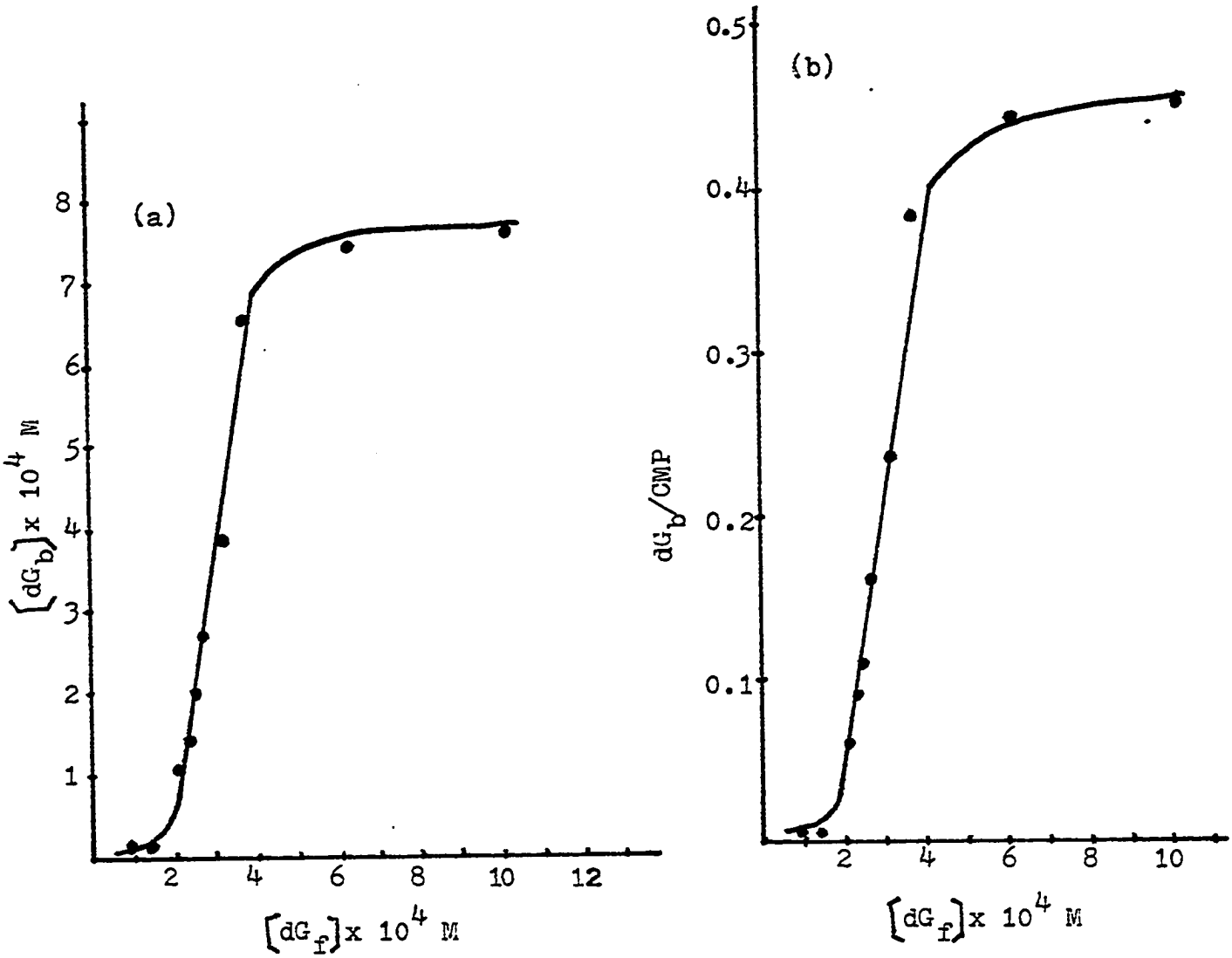
RMSD = root mean square deviation

for n=5

$[dG_b]$exp x 10^4 M	$[dG_b]$calc x 10^4 M	dG_b/CMP
0.00	0.00	0.000
0.00	0.01	0.000
1.08	0.78	0.063
1.47	1.60	0.086
2.00	2.13	0.117
2.77	2.79	0.163
3.93	4.83	0.231
6.54	6.74	0.384
7.47	7.66	0.439
7.69	7.66	0.452

Figure 35. Binding Isotherms for Deoxyguanosine:Poly C Complex

conditions: in 0.1 M Na acetate buffer, pH 4.1



(a) binding isotherm showing bound deoxyguanosine vs free deoxyguanosine at equilibrium

(b) binding isotherm showing ratio of bound deoxyguanosine per CMP residue vs free deoxyguanosine at equilibrium

Table 9. Binding of Deoxyguanosine to Polycytidylic Acid

Equilibrium Dialysis Data

conditions: in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$

equilibration after 48 hrs. at 2°C.

initial [Poly C] = 1.2×10^{-3} M A_{273}=0.67

E_{250} for deoxyguanosine = 13,000

Poly C showed 42% hypochromicity

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.38	2.92	0.18	1.38	0.16
0.68	5.23	0.28	2.15	0.93
0.81	6.23	0.31	2.38	1.47
0.92	7.07	0.34	2.61	1.84
1.06	8.15	0.39	2.84	2.46
1.33	10.23	0.47	3.30	3.62
1.83	14.07	0.60	4.38	5.31
2.61	20.07	0.93	7.15	5.77
3.68	28.30	1.46	11.23	5.84

Migration Check: Poly C vs buffer A_{273} = 0.66 : 0.00

Equilibration Check: 1.0 mM dG vs buffer A_{250} = 0.63 : 0.62

Table 10. Binding of Deoxyguanosine to Polycytidylic Acid

Computer Data

conditions: in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$

n	S	K_1	RMSD
2	5.94×10^{-4} M	1420	4.06×10^{-5}
3	5.86×10^{-4} M	1636	1.36×10^{-5}
**4	5.75×10^{-4} M	1819	8.15×10^{-6}
5	5.65×10^{-4} M	1970	2.18×10^{-5}
6	5.57×10^{-4} M	2093	3.33×10^{-5}

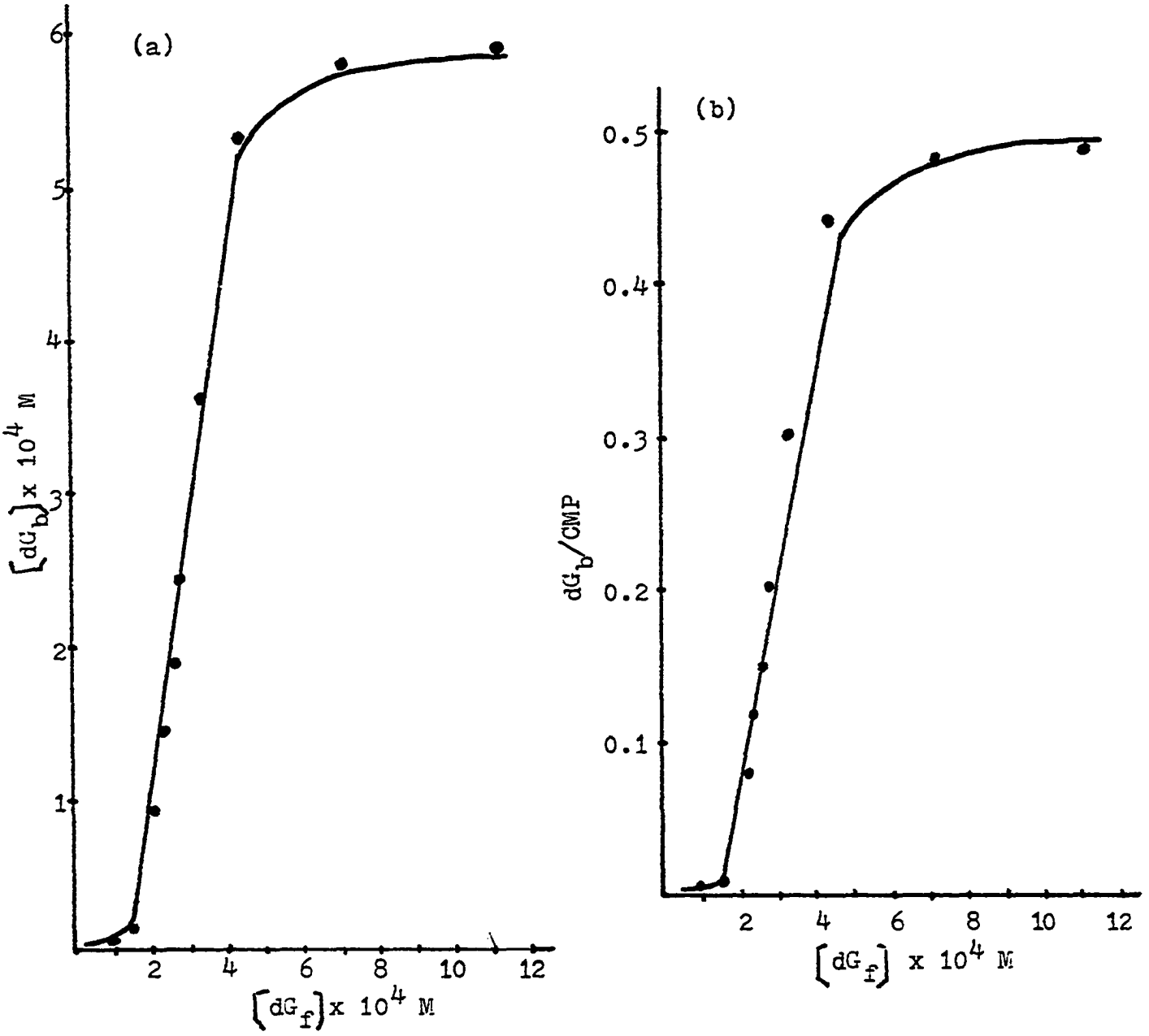
** best value of n based on lowest RMSD

for n=4:

$[dG_b]$ exp x 10^4 M	$[dG_b]$ calc x 10^4 M	dG_b/CMP
0.00	0.03	0.000
0.16	0.12	0.013
0.93	0.87	0.077
1.47	1.31	0.122
1.84	1.85	0.153
2.46	2.46	0.205
3.62	3.69	0.301
5.31	5.41	0.442
5.77	5.75	0.480
5.84	5.75	0.486

Figure 36. Binding Isotherms for Deoxyguanosine:Poly C Complex

conditions: in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$



(a) binding isotherm showing bound deoxyguanosine vs free deoxyguanosine at equilibrium

(b) binding isotherm showing ratio of bound deoxyguanosine per CMP residue vs free deoxyguanosine at equilibrium

Table 11. Binding of Deoxyguanosine to Polycytidylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M Na acetate buffer, pH 4.6
equilibration after 48 hrs at 2°C.

initial [Poly C] = 1.4×10^{-3} M A_{272}=1.01

E_{250} for deoxyguanosine = 13,000

dG_i-A_{250}	$[dG_i] \times 10^4$ M	dG_f-A_{250}	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.41	3.15	0.21	1.61	0.00
0.68	5.23	0.28	2.15	0.97
0.82	6.30	0.31	2.38	1.54
0.93	7.15	0.36	2.76	1.63
1.07	8.23	0.38	2.92	2.39
1.20	9.23	0.40	3.07	3.09
1.33	10.23	0.41	3.15	3.93
1.88	14.46	0.59	4.53	5.40
2.64	20.30	0.95	7.30	5.69
3.44	26.46	1.37	10.53	5.39

Migration Check: Poly C vs buffer A_{272} = 1.00 : 0.01

Equilibration Check: 2.0 mM dG vs buffer A_{250} = 1.30 : 1.32

Table 12. Binding of Deoxyguanosine to Polycytidylic Acid.

Computer Data

conditions: in 0.1 M Na acetate buffer, pH 4.6.

n	S	K_1	RMSD
4	5.88×10^{-4} M	1833	3.42×10^{-5}
**5	5.53×10^{-4} M	1968	3.31×10^{-5}
6	5.50×10^{-4} M	2073	3.54×10^{-5}

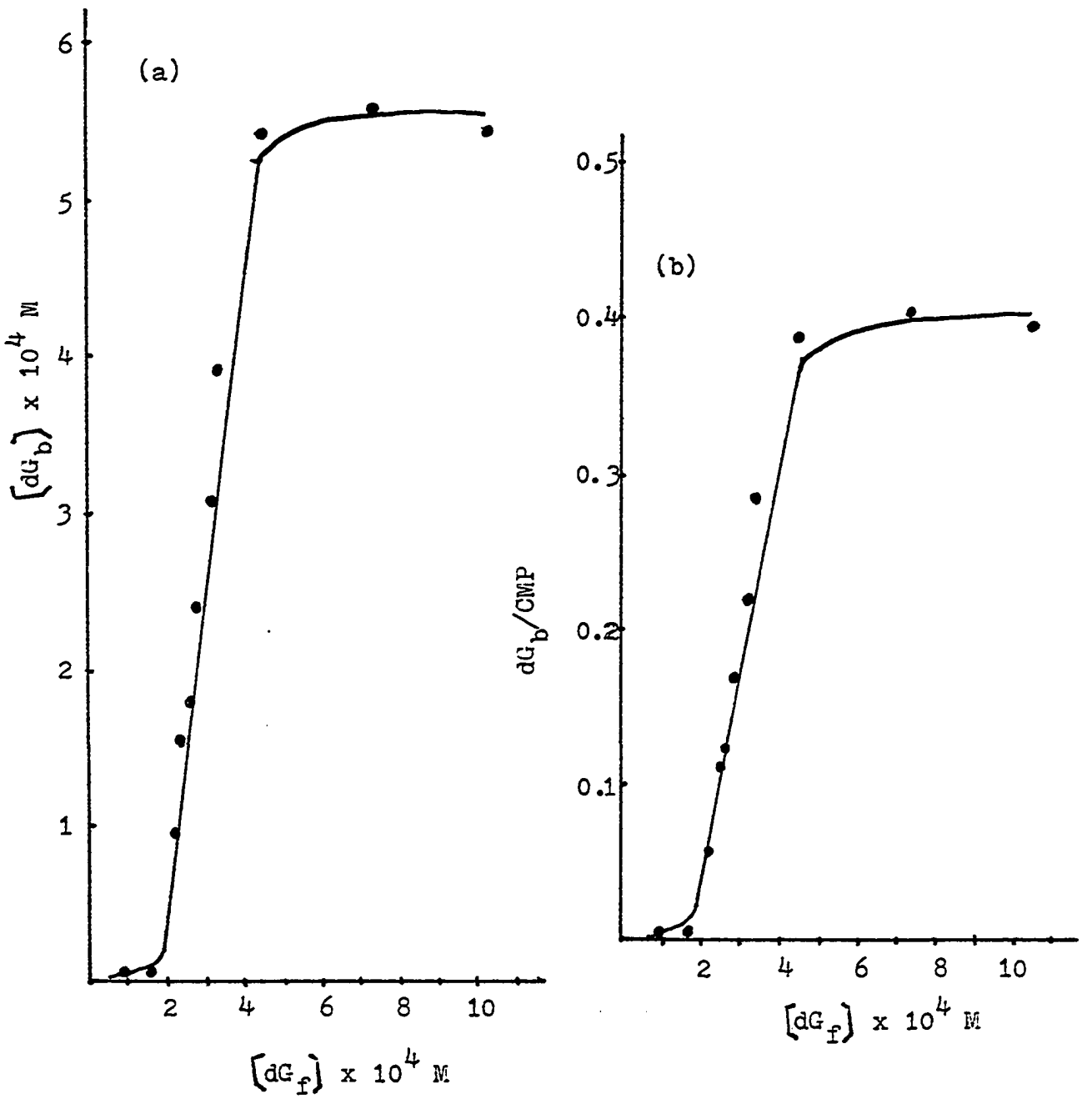
** best value of n based on lowest RMSD

for n = 5

$[dG_b]$exp x 10^4 M	$[dG_b]$calc x 10^4 M	dG_b/CMP
0.00	0.00	0.000
0.00	0.13	0.000
0.93	0.65	0.069
1.54	1.10	0.110
1.62	2.16	0.116
2.39	2.67	0.170
3.08	3.15	0.220
3.93	3.40	0.280
5.39	5.45	0.385
5.69	5.53	0.406
5.39	5.53	0.385

Figure 37. Binding Isotherms for Deoxyguanosine:Poly C Complex.

conditions: in 0.1 M Na acetate buffer at pH 4.6.



(a) binding isotherm showing bound deoxyguanosine vs free deoxyguanosine at equilibrium

(b) binding isotherm showing ratio of bound deoxyguanosine per CMP residue vs free deoxyguanosine at equilibrium

Table 13. Binding of Deoxyguanosine to Polycytidylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M NaCl at pH 4.6

equilibration after 48 hours at 2°C.

initial [Poly C] = 1.5×10^{-3} M $\quad A_{273}=1.02$

E_{250} for deoxyguanosine = 13,000

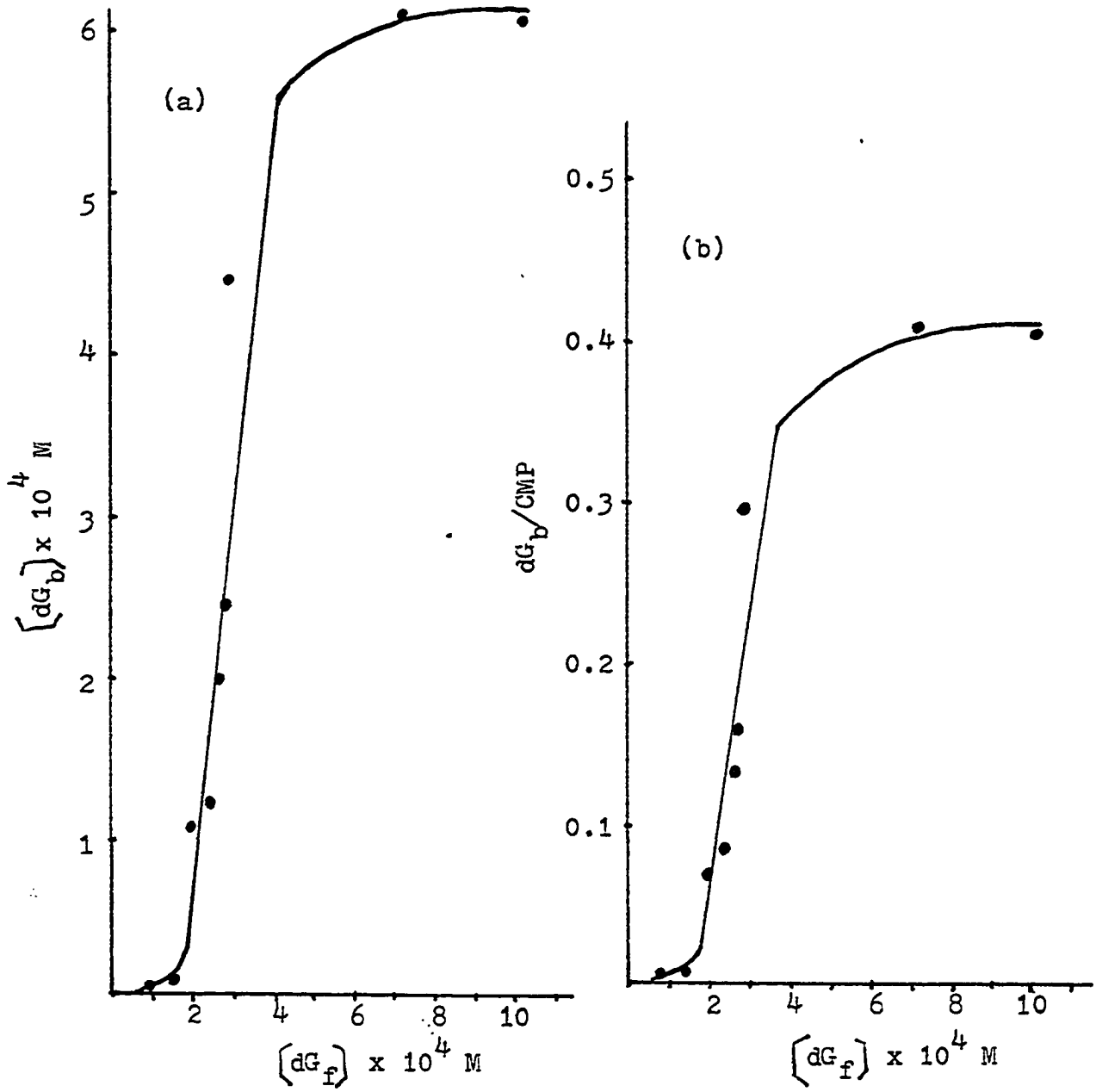
dG_i-A_{250}	$[dG_i] \times 10^4$ M	dG_f-A_{250}	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.40	3.07	0.19	1.46	0.16
0.67	5.15	0.26	2.00	1.15
0.80	6.15	0.32	2.46	1.23
0.92	7.07	0.33	2.53	2.00
1.02	7.84	0.35	2.69	2.46
1.30	10.00	0.36	2.76	4.47
2.64	20.30	0.92	7.07	6.15
3.46	26.61	1.34	10.30	6.01

Migration Check: Poly C vs buffer A_{273} = 1.02: 0.00

Equilibration Check: 2.0 mM dG vs buffer A_{250} = 1.31:1.30

Figure 38. Binding Isotherms for Deoxyguanosine:Poly C Complex.

conditions: in 0.1 M NaCl at pH 4.6.



(a) binding isotherm showing bound deoxyguanosine vs free deoxyguanosine at equilibrium

(b) binding isotherm showing ratio of bound deoxyguanosine per CMP residue vs free deoxyguanosine at equilibrium

Table 14. Binding of Deoxyguanosine to Polycytidylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M NaCl, 0.025 N NaH_2PO_4, 0.025 M Na_2HPO_4, 1.0 mM $MgCl_2$ at pH 6.8.

equilibration after 48 hrs. at 2°C.

initial [Poly C] = 1.5×10^{-3} M A_{267}=0.99

E_{250} for deoxyguanosine = 13,000

dG_i-A_{250}	$[dG_i] \times 10^4$ M	dG_f-A_{250}	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.40	3.07	0.20	1.53	0.00
0.68	5.35	0.34	2.61	0.00
0.82	6.30	0.41	3.51	0.00
0.88	6.76	0.44	3.38	0.00
1.02	7.84	0.51	3.92	0.00
1.24	9.53	0.61	4.69	0.15
1.82	14.00	0.85	6.53	0.93
2.52	19.38	0.99	7.61	4.15
3.68	28.30	1.34	10.30	7.70
5.52	42.46	1.88	14.46	13.54
6.46	49.69	2.30	17.69	14.31

Migration Check: Poly C vs buffer A_{267} = 0.98:0.00

Equilibration Check: 3.0 mM dG vs Poly C A_{250} = 1.81:1.83

Poly C showed 36% hypochromicity under these conditions

Table 15. Binding of Deoxyguanosine to Polycytidylic Acid.

Computer Data

conditions: in 0.1 M NaCl, 0.025 M NaH_2PO_4, 0.025 M Na_2HPO_4, 1.0 mM $MgCl_2$ at pH 6.8.

n	S	K_1	RMSD
2	15.71×10^{-4} M	382	1.05×10^{-4}
3	14.95×10^{-4} M	492	5.79×10^{-5}
**4	14.27×10^{-4} M	555	4.88×10^{-5}
5	13.98×10^{-4} M	599	6.41×10^{-5}

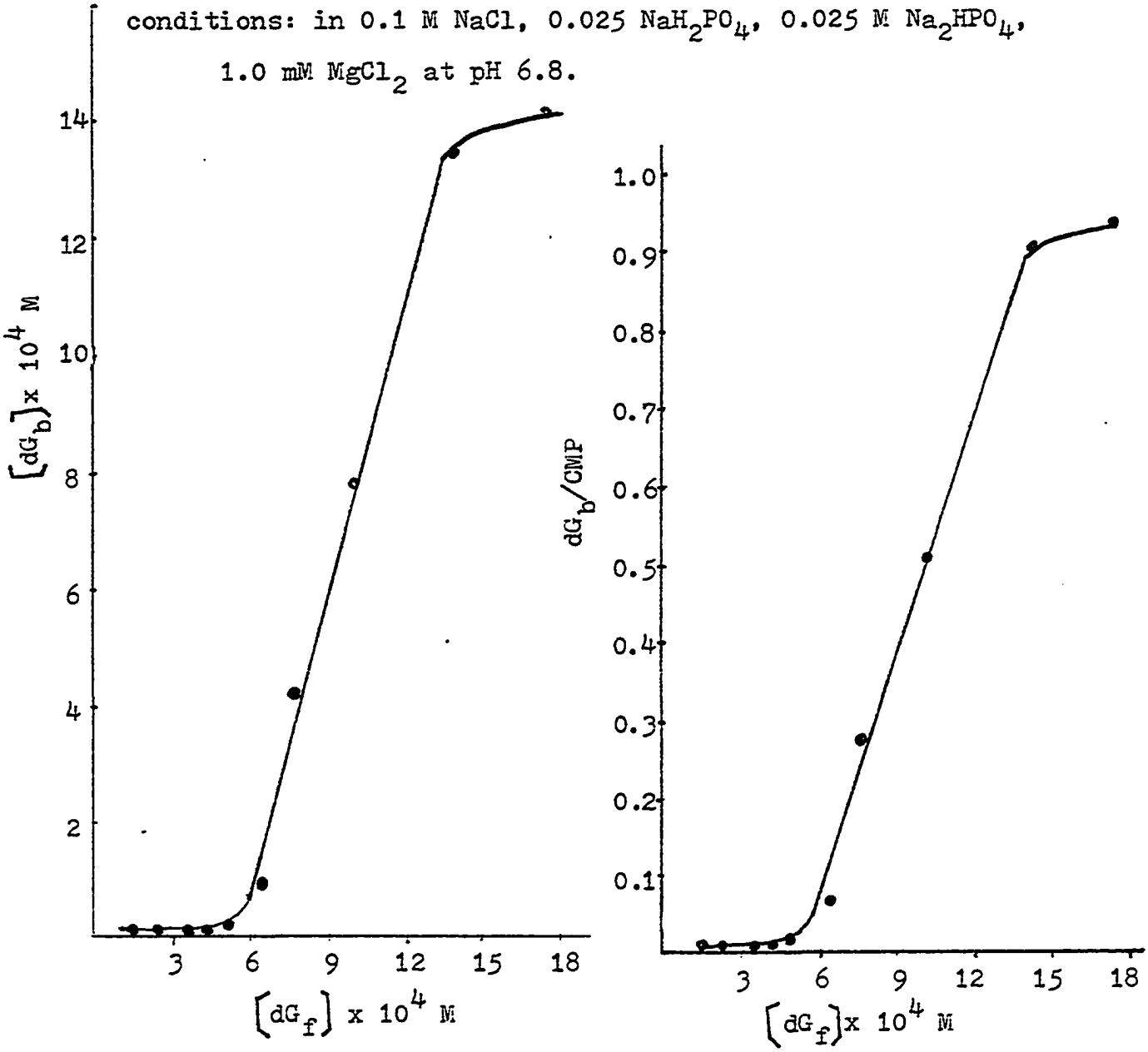
**best value of n based on lowest RMSD

for n=4

$[dG_b]$exp x 10^4 M	$[dG_b]$calc x 10^4 M	dG_b/CMP
0.00	0.00	0.000
0.00	0.03	0.000
0.00	0.06	0.000
0.00	0.09	0.000
0.00	0.17	0.000
0.15	0.37	0.010
0.93	1.57	0.062
4.15	2.96	0.276
7.70	8.16	0.513
13.54	13.48	0.902
14.31	14.27	0.954

Figure 39. Binding Isotherms for Deoxyguanosine:Poly C Complex.

conditions: in 0.1 M NaCl, 0.025 NaH_2PO_4, 0.025 M Na_2HPO_4, 1.0 mM $MgCl_2$ at pH 6.8.



(a) binding isotherm showing bound deoxyguanosine vs free deoxyguanosine at equilibrium

(b) binding isotherm showing ratio of bound deoxyguanosine per CMP residue vs free deoxyguanosine at equilibrium

Table 16. Binding of Adenosine to Polycytidylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M Na acetate buffer at pH 4.1.

equilibration after 48 hrs. at 2°C.

initial [Poly C] = 1.7×10^{-3} M $A_{273} = 1.11$

E_{257} for Adenosine = 14,200

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.43	3.02	0.21	1.51	0.00
0.71	5.00	0.35	2.50	0.00
0.99	6.97	0.50	3.52	0.00
1.35	9.50	0.68	4.78	0.00
2.66	18.73	1.33	9.36	0.00
3.74	26.33	1.87	13.16	0.00

Migration Check: Poly C vs buffer $A_{273} = 1.10:0.00$

Equilibration Check: 2.0 mM A vs buffer $A_{257} = 1.31:1.34$

Table 17. Binding of Adenosine to Polyuridylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$

at pH 4.1; equilibration after 48 hrs. at 2°C.

initial [Poly U] = 2.1×10^{-3} M A_{258}=1.63

E_{257} for adenosine = 14,200

Poly U showed 8% hypochromicity

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.43	3.02	0.22	1.54	0.00
0.72	5.07	0.36	2.53	0.00
0.86	6.05	0.43	3.02	0.00
0.99	6.97	0.49	3.45	0.07
1.12	7.88	0.55	3,87	0.14
1.35	9.50	0.66	4.64	0.21
2.66	18.73	1.22	8.59	1.55
3.74	26.33	1.67	11.76	2.81
5.44	38.30	2.16	15.21	7.88
6.70	47.18	2.70	19.01	9.16
7.80	54.92	3.24	22.81	9.29
13.60	95.77	6.10	42.95	9.86

Migration Check: Poly U vs buffer A_{258} = 1.63:0.00

Equilibration Check: Adenosine vs buffer A_{257} = 1.84:1.83

A_i = initial adenosine

A_f = free or unbound adenosine

A_b = bound or complexed adenosine

Table 18. Binding of Adenosine to Polyuridylic Acid.

Computer Data

conditions: in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at pH 4.1

n	S	K_1	RMSD
3	9.88×10^{-4} M	388	5.23×10^{-5}
4	9.69×10^{-4} M	423	4.15×10^{-5}
**5	9.58×10^{-4} M	451	3.85×10^{-5}
6	9.50×10^{-4} M	473	4.00×10^{-5}
7	9.43×10^{-4} M	493	4.36×10^{-5}

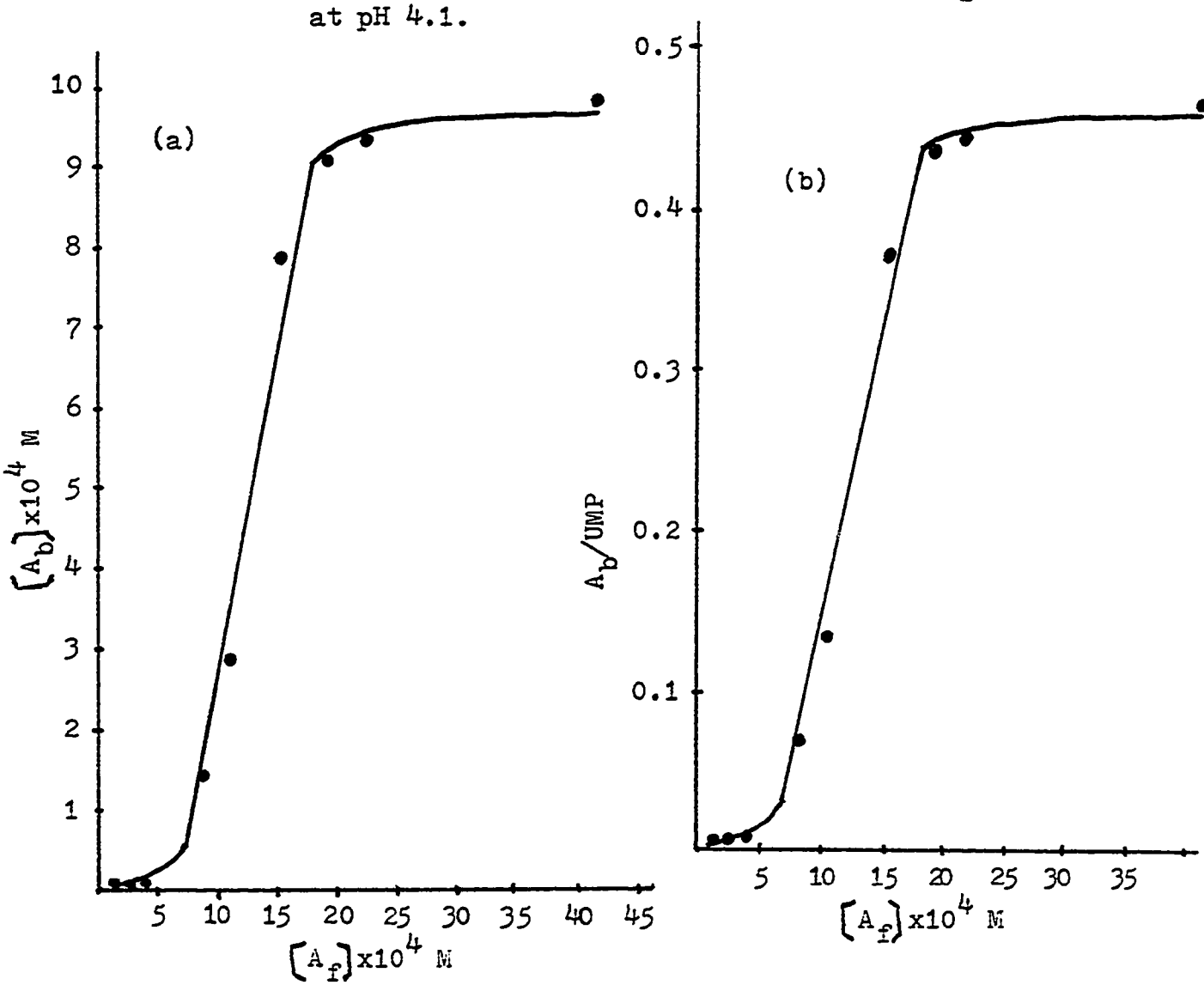
**best value of n based on lowest RMSD

for n=5

$[A_b]$exp x 10^4 M	$[A_b]$calc x 10^4 M	A_b/UMP
0.00	0.00	0.000
0.08	0.00	0.000
0.00	0.00	0.000
0.07	0.00	0.003
0.14	0.00	0.007
0.21	0.02	0.010
1.55	0.71	0.073
2.81	3.39	0.133
7.88	7.45	0.375
9.16	9.32	0.436
9.29	9.58	0.442
9.86	9.58	0.469

Figure 40. Binding Isotherms for Adenosine:Poly U Complex.

conditions: in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at pH 4.1.



(a) binding isotherm showing bound adenosine vs free adenosine at equilibrium.

(b) binding isotherm showing ratio of bound adenosine per UMP residue vs free adenosine at equilibrium.

Table 19. Binding of Adenosine to Polyuridylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 1.0 mM $MgCl_2$ at pH 6.8.

equilibration after 48 hrs. at 2°C.

initial [Poly U] = 2.0×10^{-3} M A_{258}=1.54

E_{257} for Adenosine = 14,200

Poly U showed 7% hypochromicity

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.43	3.02	0.21	1.51	0.00
0.72	5.07	0.36	2.53	0.00
0.86	6.05	0.43	3.02	0.00
0.97	6.83	0.48	3.38	0.07
1.12	7.88	0.55	3.87	0.14
1.38	9.71	0.67	4.71	0.28
2.76	19.43	1.28	9.01	1.41
3.74	26.33	1.66	11.69	2.95
5.44	38.30	2.36	16.61	5.07
6.80	47.88	2.85	20.07	7.74
8.10	57.04	3.36	23.66	9.72
13.60	95.77	6.11	43.02	9.72

Migration Check: Poly U vs buffer A_{258} = 1.52:0.01

Equilibration Check: 3.0 mM Aden A_{257} = 1.86:1.84

Table 20. Binding of Adenosine to Polyuridylic Acid.

Computer Data

conditions: in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 1.0 mM $MgCl_2$ at pH 6.8.

n	S	K_1	RMSD
2	10.19×10^{-4} M	289	4.45×10^{-5}
**3	9.94×10^{-4} M	322	3.69×10^{-5}
4	9.71×10^{-4} M	348	5.44×10^{-5}
5	9.57×10^{-4} M	366	7.10×10^{-5}

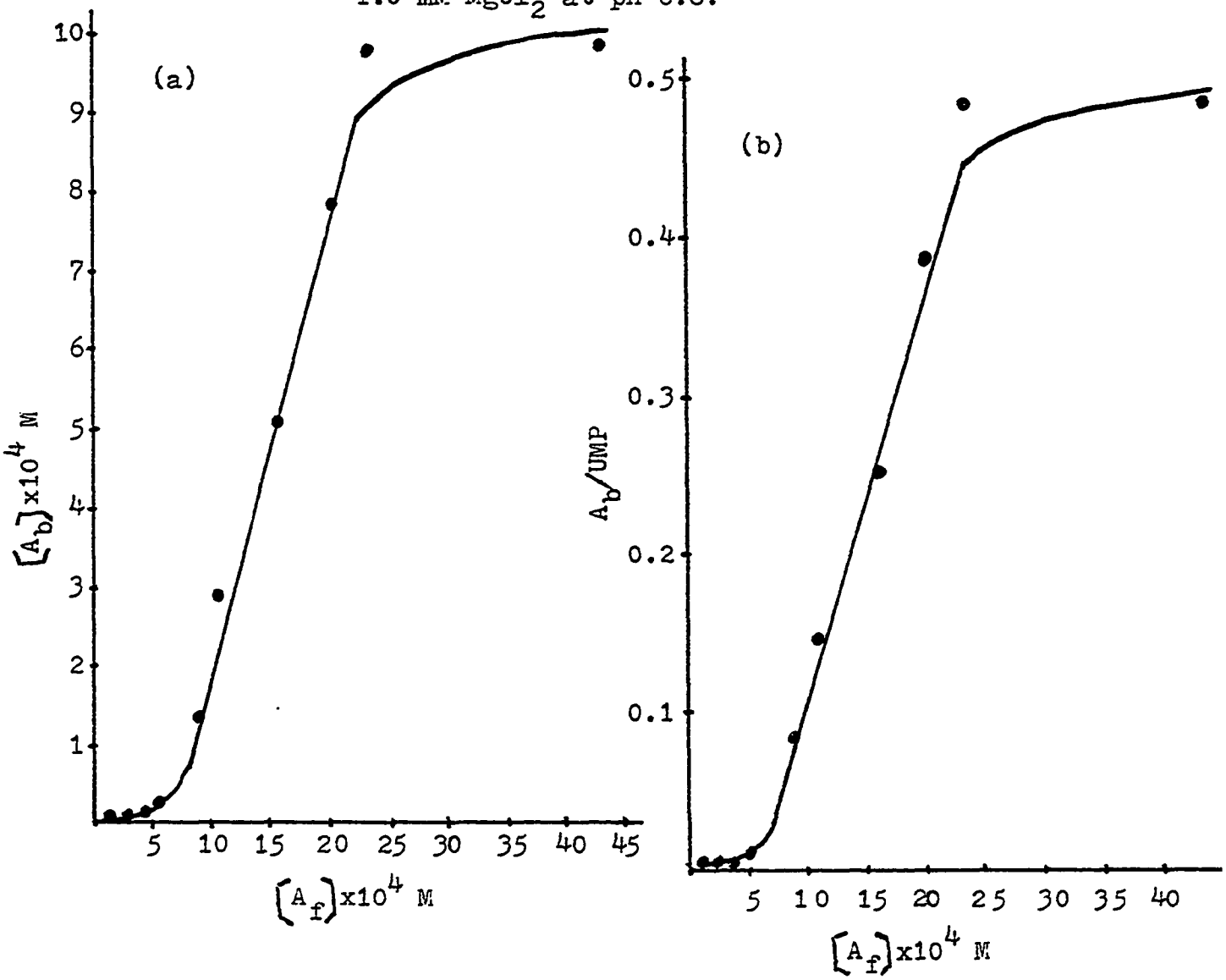
**best value of n based on lowest RMSD

for n=3

$[A_b]$exp x 10^4 M	$[A_b]$calc x 10^4 M	A_b/UMP
0.00	0.00	0.000
0.00	0.01	0.000
0.00	0.03	0.000
0.07	0.04	0.003
0.14	0.06	0.006
0.28	0.12	0.014
1.41	1.04	0.070
2.95	2.35	0.147
5.07	5.75	0.253
7.74	7.83	0.387
9.72	9.15	0.486
9.72	9.94	0.486

Figure 41. Binding Isotherms for Adenosine:Poly U Complex.

conditions: in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 1.0 mM $MgCl_2$ at pH 6.8.



(a) binding isotherm showing bound adenosine vs free adenosine at equilibrium.

(b) binding isotherm showing ratio of bound adenosine per UMP residue vs free adenosine at equilibrium.

Table 21. Binding of Deoxyguanosine to Polyuridylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 1.0 mM $MgCl_2$ at pH 6.8.

equilibration after 48 hrs. at 2°C.

Initial [Poly U] = 2.0×10^{-3} M A_{258}=1.54

E_{250} for deoxyguanosine = 13,000

dG_i-A_{250}	$[dG_i] \times 10^4$ M	dG_f-A_{250}	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.38	2.92	0.19	2.92	0.00
0.67	5.15	0.34	2.62	0.00
0.92	7.07	0.46	3.53	0.00
1.30	10.00	0.65	5.00	0.00
2.64	20.30	1.32	10.15	0.00
3.46	26.61	1.73	13.30	0.00

Migration Check: Poly U vs buffer A_{258} = 1.53:0.00

Equilibration Check: 2.0 mM dG vs buffer A_{250} = 1.30:1.30

Table 22. Comparison of Binding Data from Two Systems.

Complex	pH	n	K_1	G ($-RT\ln K_1$)
Poly C:dG (triple helix)	4.1	4	1819	-4.08 kcal/mole
	4.1	5	1986	-4.13 kcal/mole
	4.6	5	1968	-4.12 kcal/mole
Poly C:dG (double helix)	6.8	4	555	-3.43 kcal/mole
Poly U:rA (triple helix)	4.1	5	451	-3.32 kcal/mole
	6.8	3	322	-3.14 kcal/mole

Table 23. Binding of 1-Methyl Adenosine to Polyuridylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 1.0 mM $MgCl_2$ at pH 6.8.

equilibration after 48 hrs. at 2°C.

initial [Poly U] = 1.9×10^{-3} M $A_{258} = 1.58$

E_{255} for 1-Methyl Adenosine = 10,700

Poly U showed 8% hypochromicity

$mA_i - A_{255}$	$[mA_i] \times 10^4$ M	$mA_f - A_{255}$	$[mA_f] \times 10^4$ M	$[mA_b] \times 10^4$ M
0.35	3.27	0.17	1.63	0.00
0.56	5.23	0.28	2.61	0.00
0.66	6.15	0.33	3.08	0.00
0.77	7.19	0.38	3.59	0.00
0.87	8.13	0.43	4.06	0.00
1.13	10.56	0.56	5.33	0.00
2.20	20.56	1.09	10.18	0.00
3.10	28.97	1.54	14.39	0.19
4.08	38.13	2.04	19.06	0.00
5.05	47.19	2.52	23.55	0.09
6.10	57.75	3.08	28.78	0.19

Migration Check: Poly U vs buffer A_{258} = 1.57:0.00

Equilibration Check: 3.0 mM mA vs buffer A_{255} = 1.53:1.54

Table 24. Binding of 6-N Methyl Adenosine to Polyuridylic Acid.

Equilibrium Dialysis Data

conditions: in 0.1 M NaCl, 0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, 1.0 mM $MgCl_2$ at pH 6.8.

equilibration after 48 hrs. at 2°C.

initial $[Poly\ U] = 2.25 \times 10^{-3}$ M $\quad A_{258} = 1.90$

E_{263} for 6-N Methyl Adenosine = 15,300

Poly U showed 8% hypochromicity

$NmA_i - A_{263}$	$[NmA_i] \times 10^4$ M	$NmA_f - A_{263}$	$[NmA_f] \times 10^4$ M	$[NmA_b] \times 10^4$ M
0.45	2.94	0.23	1.50	0.00
0.95	6.20	0.47	3.07	0.06
1.27	8.30	0.64	4.18	0.00
1.53	10.00	0.76	4.96	0.08
2.83	18.49	1.42	9.28	0.00
4.35	28.43	2.18	14.24	0.00
6.04	39.47	3.01	19.67	0.13
7.45	48.69	3.69	24.11	0.37
9.10	59.47	4.54	29.67	0.13

Migration Check: Poly U vs buffer A_{258} = 1.90:0.00

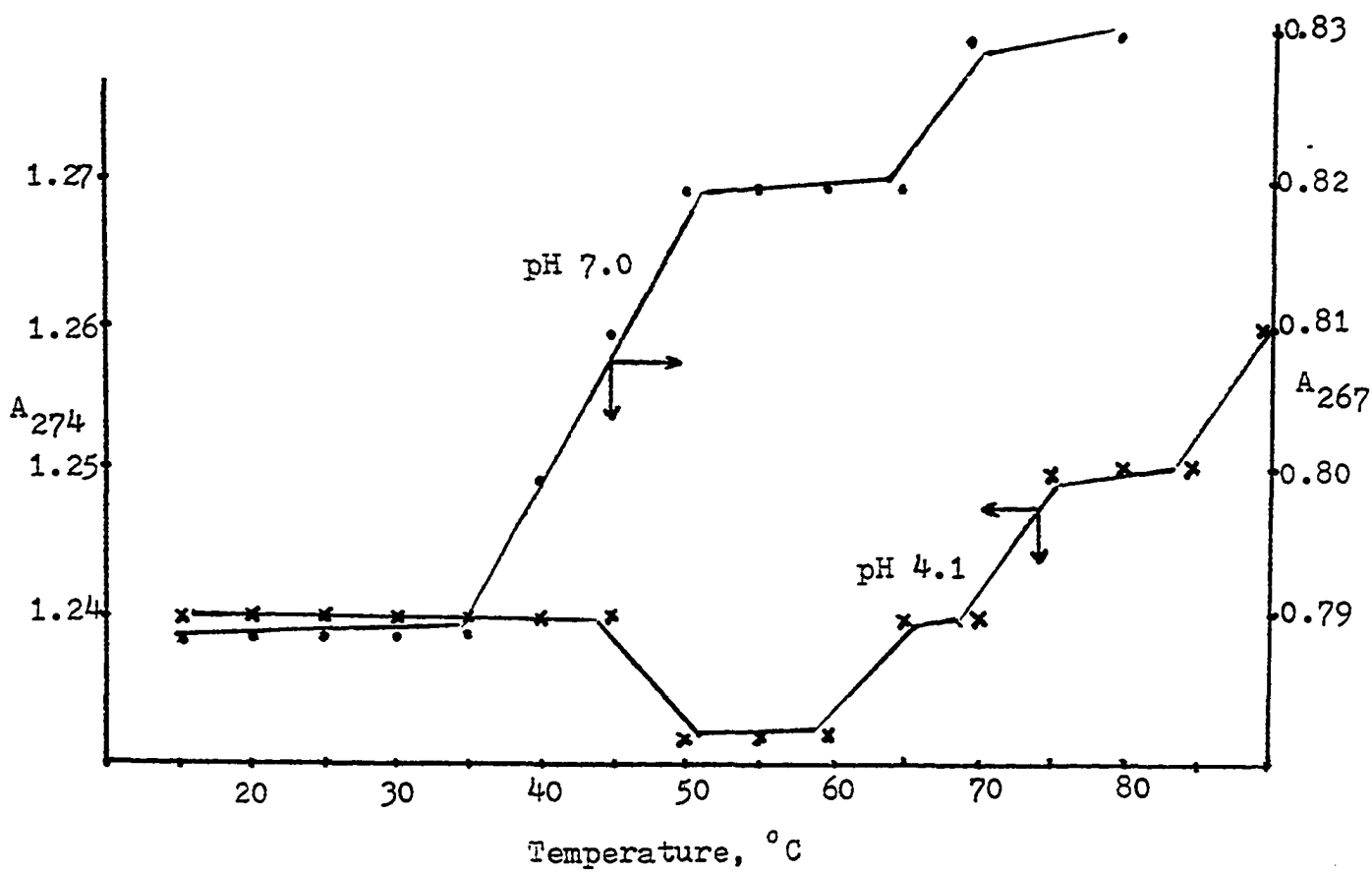
Equilibration Check: 6-N met A vs buffer A_{263} = 1.41:1.43

Table 25. Melting Data on Poly C:Deoxyguanosine Complex

at pH 4.1, in 0.1 M Na acetate buffer
at pH 7.0, in 0.1 M Na phosphate buffer + 1.0 mM $MgCl_2$

1.00 mM deoxyguanosine + 1.3 mM Poly C

Temperature, °C	A_{274} (pH 4.1)	A_{267} (pH.7.0)
15	1.24	0.79
20	1.24	0.79
25	1.24	0.79
30	1.24	0.79
35	1.24	0.79
40	1.24	0.80
45	1.24	0.81
50	1.23	0.82
55	1.23	0.82
60	1.23	0.82
65	1.24	0.82
70	1.24	0.83
75	1.25	0.83
80	1.25	0.83
85	1.25	
90	1.26	



CHAPTER IV

DISCUSSION AND CONCLUSIONS

Extinction Coefficients of Nucleosides

Molar extinction coefficient values for the nucleosides, deoxyguanosine, guanosine, adenosine, 1-methyl adenosine, and 6-N methyl adenosine were found to be 13.0×10^3, 12.8×10^3, 14.7×10^3, 10.7×10^3, and 15.3×10^3, respectively. In each case the ε value was determined by plotting UV absorbance measurements (at a given wavelength) vs concentration, the resulting slope approximating the extinction coefficient value under a specific set of conditions. For deoxyguanosine, guanosine, and adenosine, the ε value (in each case) was essentially unchanged at both pH 4.5 and 7.0. These experimentally determined molar absorptivity values were utilized in nucleoside concentration calculations during equilibrium dialysis experiments.

The extinction coefficient for guanosine was determined at the onset of this research project, when the Poly C:Guanosine system was being investigated. The low solubility of guanosine in aqueous solution (ca. 1.0 mM at 5°C) encouraged a change to the deoxyribonucleoside, deoxyguanosine, which was found to have a seven-fold greater solubility (ca. 7.0 mM) in aqueous solution at 5°C. It has been shown in previous studies[64-66] that in order to

observe complex formation, the solubility of the monomer must exceed the threshold for binding to the polymer. It was therefore desirable to use the monomer derivative with the maximum solubility under the particular experimental conditions employed. Deoxyguanosine was thus used as the monomer nucleoside for investigations of interactions with Poly C.

Hypochromicity in Poly U

Poly U exhibits very little secondary structure at 23°C. Upon treating a prepared solution of Poly U with 0.3 N KOH for 22 hours at 37°C (alkaline hydrolysis to UMP units), only a 7-8% increase in absorption was observed. At low temperatures (<5°C) significant secondary structure in Poly U is apparent as can be seen by the increase of UV absorption of monomer over the polymer.[74] This is probably due to the double stranded conformation of Poly U at temperatures less than 5°C. In 1 M Na^+ the Tm of Poly U is ca. 5.5°C with the observed hypochromicity between nonstructured and structured forms being approximately 23%.

Equilibrium Dialysis

The results of the equilibrium dialysis experiments show an approximate 2:1 (polymer:monomer) stoichiometry for the Poly U: adenosine complex both at pH 4.1 and 6.8 and for the Poly C:deoxyguanosine complex at pH 4.1 and 4.6. In contrast to the results reported by Davies and Davidson[64] a 2:1 Poly C:deoxyguanosine complex was never observed at pH values near neutrality. In fact, at pH 6.8 the Poly C:deoxyguanosine complex showed an approximate 1:1

polymer:monomer stoichiometry upon polymer saturation with a definite dependence of a low concentration of Mg^{++} (1.0 mM) and a higher monomer concentration than that required for complex formation at the lower pH values (see Figure 39). At low concentrations of free nucleoside or in the absence of Mg^{++}, no complex formation was observed at this pH (6.8). The fact that higher concentrations of the monomer is required for 1:1 C:G complex formation (in the presence of 1.0 mM Mg^{++} at pH 6.8) suggests that monomer base stacking interactions may play a major role in the stability of this complex.

All the complexes were found to exhibit a cooperative-type binding and were found to be stable only in the presence of a large excess of free monomer. These properties can be seen in the binding isotherms. It is readily apparent that a minimal threshold concentration of free nucleoside must be reached before complex formation begins. This "nucleation" event, then, allows for subsequent binding of the monomer to the polymer in a cooperative fashion. Upon saturation of the polymer binding sites, the curve levels off. Since the nucleosides are independent moieties (unpolymerized), the interaction of these units with the polynucleotide is most likely governed by the secondary structure of the polymer. A pseudo-polymerization of the nucleoside residues thus occurs upon interaction with the polymer and the sites available for binding on the polymer determine the resulting stoichiometry of the saturated complex.

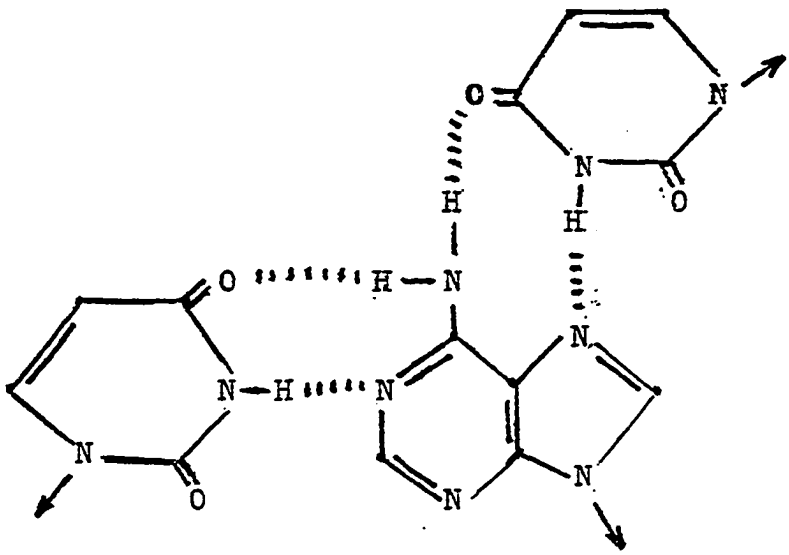
For the Poly C:deoxyguanosine system, the pH and the presence or absence of Mg^{++} are both critical parameters for complex formation. Under conditions of acid pH (4.1-4.6), where Poly

C exists primarily in a double helical conformation, a 2:1 (C:dG) stoichiometry is observed upon polymer saturation. In this system, binding of the monomer to the polymer is initially detected at a free nucleoside concentration of ca. 0.2 mM. Upon formation of this 2C:1dG triplex, the monomer probably occupies a position between cytosine base pairs forming a C+dG+C triple helix, stabilized both by base stacking interactions and by the formation of hydrogen bonds as suggested in the scheme by Lipsett (see Figure 20). This hydrogen bonding scheme involves the protonation of one of the Poly C strands, as in the double helical form of Poly C. This degree of protonation seems important for the formation of this complex.

At pH values near neutrality, where Poly C exists primarily as a single stranded helix, an approximate 1:1 (C:dG) stoichiometry is observed upon saturation of the polymer. This complex formation has a marked dependence on the presence of Mg^{++} and on higher monomer reactant concentration, as binding is not detected until the free nucleoside concentration has exceeded ca. 0.5 mM.

The Poly U:adenosine complexes were found to be seemingly independent of pH (in the presence of 1.0 mM Mg^{++}) as 2U:1A stoichiometry was observed upon saturation at both pH 4.1 and 6.8. For this system, complex formation was initially detected at a free adenosine concentration of approximately 0.5 mM, similar to that seen in the Poly C:deoxyguanosine system at neutral pH. In the Poly U:adenosine triplex, as in the Poly C:deoxyguanosine triplex the purine nucleoside probably occupies a central strand location, bonded on two sides by uracil residues as in the scheme shown

below. The involvement of two of these binding sites were examined by dialyzing Poly U against 1-methyl adenosine and 6-N methyl adenosine. In neither case was significant binding observed.



The results of these studies indicate that purine nucleosides can form complexes with complementary homopolymers in simple stoichiometric ratios. The binding isotherms are typical of a strongly cooperative process which show a sharp transition once a critical threshold concentration of free nucleoside has been reached. These studies show that hydrogen bonding cannot be the sole force responsible for binding since no binding is detected until a certain concentration of monomer is reached, even though hydrogen bonding capacity is still present. The complex formation between homopolynucleotides and complementary nucleosides apparently involves an intrinsic purine base stacking energy, independent of the contribution of the phosphate internucleoside linkages. Hydrogen bonding forces are most important with respect

to base pairing specificity, while base stacking forces are most important for complex stabilization. Both hydrogen bonding and hydrophobic stacking interactions thus cooperate and complement each other in providing the driving forces for complex formation and stability.

Thermodynamic parameters have often been estimated from binding isotherms, such as these reported here. The thermodynamic model developed by Burr et al.[66] to represent the binding isotherm for the cooperative binding of adenosine to Poly U, was applied to the Poly C:deoxyguanosine system at both pH 4.1 (C+dG+C triplex) and pH 6.8 (C+dG duplex). The equilibrium constant, K_1 (see equation on pp. 63) for the formation of the triple helical complex was found to be ca. 1900 with n=5 (where n represents the number of purine nucleoside monomer units which must interact contiguously with Poly C -with hydrogen bonding and base stacking forces- in order to form a stable complex). The K_1 value, related to the total free energy of formation, ΔG ($\Delta G = -RT\ln K_1$) corresponds to a value of ca. -4.1 kcal/mole. The formation constant for the Poly C:deoxyguanosine duplex (pH 6.8) is 555 with n=4. This value corresponds to a ΔG of ca. -3.4 kcal/mole. For the Poly U:adenosine triplex the K_1 values were found to be ca. 400 kcal/mole with n=5, corresponding to a ΔG equal to ca. -3.2 kcal/mole.

The equation model presented by Burr et al. for the Poly U: adenosine system provides an excellent fit of binding data for the Poly C:deoxyguanosine system. In each isotherm, the points represent the experimental data (concentration of bound nucleoside vs concentration of free nucleoside), while the solid line is the

calculated curve based on the experimental data. The best values of K_1 and n (based on the lowest root mean square deviation) are fitted to these experimental data via a non-linear least squares program.

The calculated value of S is always close to the maximum experimental bound nucleoside. K_1 was found to increase and S to decrease as n was increased. Only integral values of n were used in computer calculations. The method could be improved slightly if non-integral values of n were used. The slope of the isotherm was found to depend only on n, i.e. all binding isotherms with the same n value will have the same slope.

The formation constant, K_1, was found to decrease with movement of unbound nucleoside on the x-axis, i.e. if complexing begins at a low free nucleoside concentration, the formation constant will be high, while initiation of complex formation at a higher nucleoside concentration will yield a lower value of K_1. These values of K_1 are related to complex stability, the Poly C: deoxyguanosine triplex being more stable than the Poly C:deoxyguanosine duplex, which is, in turn, slightly more stable than the Poly U:adenosine triplex. Because of the supposed involvement of three hydrogen bonds in the base pairing scheme of the Poly C: deoxyguanosine duplex as opposed to four hydrogen bonds in the base pairing scheme of the Poly U:adenosine triplex, either the three hydrogen bonds are stronger than four, or the stacking energy of guanosine stabilizes that complex more than the stacking energy of adenosine in the other complex. The formation constant values for these complexes agree nicely with the Tm data reported for the complexes.

PART III

THE BINDING OF DEOXYGUANOSINE AND ADENOSINE TO CHEMICALLY MODIFIED POLYCYTIDYLIC ACID

PART III

THE BINDING OF DEOXYGUANOSINE AND ADENOSINE TO CHEMICALLY MODIFIED POLYCYTIDYLIC ACID

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Mutations

A mutation is a change in the genetic information (i.e. DNA and RNA) which remains upon replication in the absence of the physical or chemical agent (i.e. the mutagen) responsible for the mutagenic event. These genetic modification events are considered to be among the most important biological phenomena because they furnish the basis for evolutionary processes.

In simplest terms a mutation is an alteration or modification of a nucleic acid. Two categories of mutations are often mentioned: (1) induced mutations and (2) spontaneous mutations. Induced mutations are those which, as a result of the action of some physical or chemical agent or event, cause a change in the nucleic acid properties in a defined way. Spontaneous mutations, on the other hand, are often undefined as to their mode of action. For example, a mutation may result from a mistake occurring during the replication process; the enol form of thymine may base pair with guanine or the imino form of C may base pair with A.

The most frequent and probably the most biologically important type of mutation is the point mutation. This type of mutation involves action only at the site of the purine or pyrimidine nitrogen base. A chemical or physical modification of a nitrogen base can be of two types: (1) transition point mutation or (2) transversion point mutation. A transition is characterized by a change of one purine to another purine, or of one pyrimidine to another pyrimidine. The outcome of such a mutational event may be the replication or transcription of an "incorrect" complementary base. The mutation need not be an exact chemical change from one known purine or pyrimidine to another known purine or pyrimidine (respectively) to be considered a transition mutation. The modification need only result in a mis-pairing during a complementary binding process. Transition point mutations are the best known and most widely studied of the point mutations.

Transversion point mutations involve a change of a purine to a pyrimidine, or vice versa. These mutations have been shown to occur only in rare cases.[75]

In many instances, mutations may occur at "silent" or inactive points in nucleic acids. In such cases, the mutations are not considered to be significant events insofar as immediate evolutionary processes are concerned. However, if a mutation occurs in an active portion of the nucleic acid (i.e. a gene, mRNA, or tRNA), an insertion of an incorrect amino acid in a protein may be the secondary result. The surest evidence that a mutation has occurred is shown in the permanent change in the amino acid sequence of a protein. Many cases are known (e.g. normal hemoglobin vs. sickle-

cell hemoglobin) where a single amino acid change is attributed to a change of a single base in the genetic material.[76] This can often lead to disease or death in the organism.

Because of the specificity involved in protein structure and function, the miscoding for a single amino acid (the possible outcome of a mutational event) may significantly alter the polypeptide so as to render it inactive or alter its biological activity or primary function. This inactivation or alteration of the protein properties, brought on indirectly by the mutational event, may prove to be a favorable or unfavorable happening. If the protein modification is unfavorable, the organism may disease or die and consequently the mutation will not be allowed to be passed on to future generations. A favorable mutation, however, will continue to be replicated (unless a back mutation or second mutation occurs). In this way the possibility of passing this mutation to future progeny exists. Such is the basis for evolution. Nature selects out the unfavorable genetic sequences, while the favorable genetic combinations survive and remain in the gene pool.

Mutagens

Mutagens are chemical or physical agents or events which cause genetic mutations. Chemical mutagens have been used for many years to produce known base modifications in polynucleotides.[77] Among the known chemical mutagens, nitrous acid and hydroxylamine have commanded special attention because of their apparent specificity and ability to induce point mutations.[78] These mutational events are evidenced upon reported mis-replication of a polynucleotide.[79] Nitrous acid reacts with the nucleic acid nitrogen bases containing

a primary amino group; adenine, guanine, and cytosine, to yield the deamination products, hypoxanthine, xanthine, and uracil, respectively.[80,81] For DNA none of these deamination products are normal DNA bases and therefore mutational events must arise through the hydrogen bonding properties of the new bases. This is thought to occur as hypoxanthine (in the 6-keto form), behaving like guanine, base pairs with cytosine, and uracil, behaving like thymine, base pairs with adenine.[100] Xanthine, the deamination product of guanine, does not seem to resemble any of the known bases in its hydrogen base pairing properties, and therefore is not thought to be mutagenic. Xanthine has, in some cases, been shown to cause an inactivation of the genetic material.[82,111]

Hydroxylamine reacts only with pyrimidine nitrogen bases in nucleic acids. This mutagen has been shown to be a powerful nucleophilic agent which reacts with cytosine optimally at pH 6 and with uracil at pH 10.[83] Only the reaction with the cytosine nucleus is thought to be mutagenic.[84] One or two molecules of hydroxylamine can react with cytosine to form two defined reaction products. The addition of NH_2OH across the 5,6 double bond followed by the replacement of the C4 amino group with the hydroxyamino group forms one product, 6-hydroxyamino-5,6-dihydro-4-hydroxyaminocytosine.. The second product of the reaction is the result of the direct displacement of the C4 amino group by the hydroxyamino, forming 4-hydroxyaminocytosine. It has been shown that the mono-substitution product (4-hydroxyaminocytosine) favors a tautomeric shift and thus displays the binding properties of uracil. This product is, therefore, thought to be the mutational species of the reaction.[85,86]

Mutagenic Action of Nitrous Acid

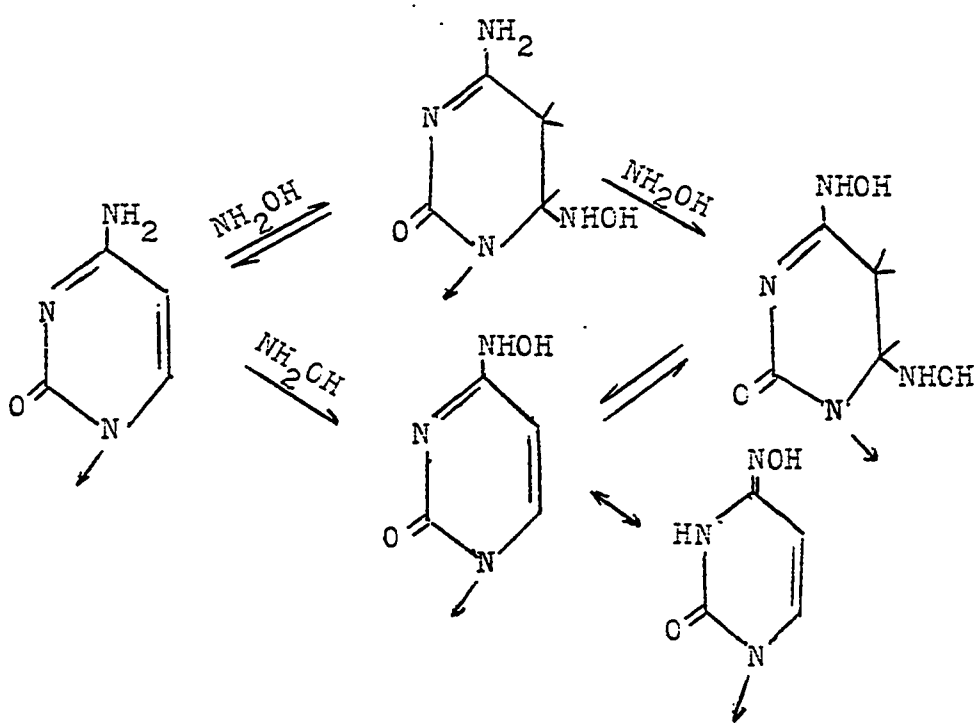
Nitrous acid was the first *in vitro* chemical mutagen whose action seemed to be understandable in simple molecular terms. It became a classical mutagen in 1958 when Schuster and Schramm[87] showed that sodium nitrite, at low pH (below 5), reacted with purified RNA from TMV to yield three deamination products: adenine being converted to hypoxanthine, guanine to xanthine, and cytosine to uracil. Mundry and Gierer[88-89] further demonstrated the mutagenic properties of nitrous acid as they reported the modified TMV RNA preparations to be infective to tobacco plants, producing new properties in succeeding generations. Thus nitrous acid, administered in the form of sodium nitrite at low pH, was shown to be a mutagen which reacted with nucleic acid nitrogen bases in a chemically defined way to produce defined mutations.[90]

The deamination of cytosine to uracil is a self-evident mutagenic event. Wittmann[91] found that C→U transitions brought about by the action of nitrous acid in TMV RNA, resulted in specific amino acid replacements in the coat protein. This finding helped greatly in deciphering the genetic code.[92,102]

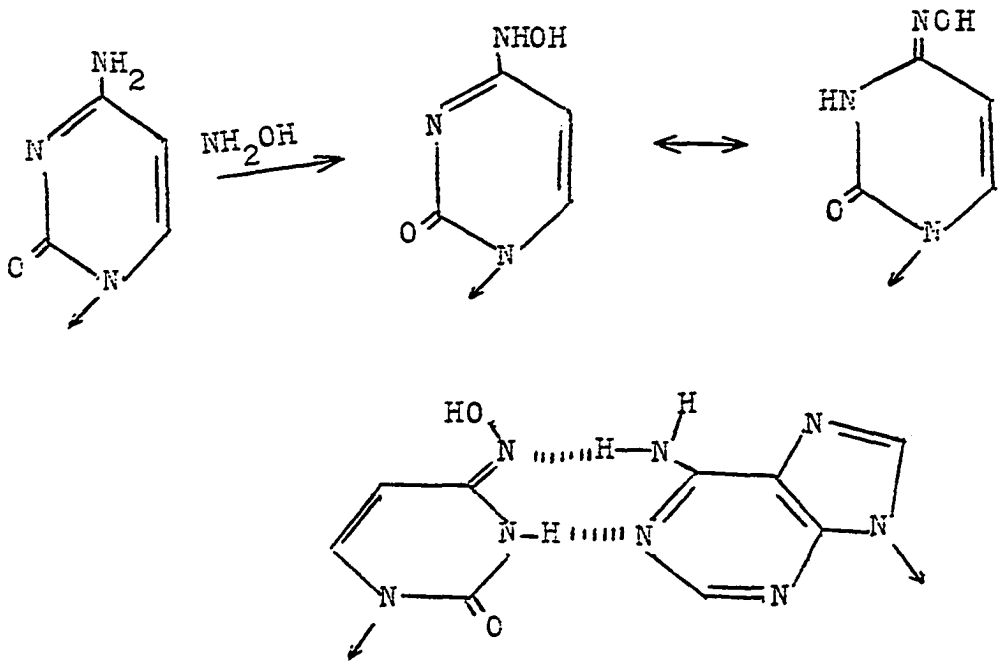
These initial investigations stimulated a great number of related studies regarding the mutagenic effects of nitrous acid on various biological systems and defined polynucleotides. These include studies on the bacteriophages T2[93], T4[99], and S13[94,95,101], *Drosophila melanogaster*[96], *Diplococcus pneumoniae*[97], *Salmonella typhi*[98], yeast alanine tRNA[103], *E. coli* 5s rRNA[104], *Rhizobium lupini*[105], denatured DNA from *Haemophilus influenza*[106], Tobacco Mosaic Virus[107,108], and cultured mouse and hamster cells.[109,110]

Mutagenic Action of Hydroxylamine

Hydroxylamine and certain hydroxylamine derivatives are mutagenic toward a variety of organisms.[111-123] These mutagens have the ability to interact specifically with pyrimidines under specific conditions of pH, temperature, and reagent concentration.[124] At pH near 6.0 and high NH_2OH concentration, the reaction is specific with cytosine residues in polynucleotides, resulting in the formation of two possible cytosine derivatives, 4-hydroxyamino cytosine and 6-hydoxyamino-5,6-dihydro-4-hydroxyaminocytosine.[124-130] Under these conditions the cytosine nucleus readily binds hydroxylamine at the 5,6 double bond, which facilitates nucleophilic substitution of the exocyclic amino group. The mono substitution product, 4-hydroxyaminocytosine forms as a result of a single stage irreversible reaction. The reaction of NH_2OH with the cytosine nucleus is shown below.



4-OH-aminocytosine has been suggested to be the species responsible for mutagenic events in living systems. This cytosine derivative resembles uracil (or thymine) in its hydrogen binding properties and thus causes adenine instead of guanine to bind to the altered cytosine residue. The suggested binding scheme of pseudo U (or T), 4-OH-aminocytosine, with adenine, is shown below.



Mutagenic Action and Polynucleotide Secondary Structure

In the interim between the formation of a modified polynucleotide and its replication, transcription, or translation, there occur a number of biological processes (primarily enzymatic), whose character and intensity produce a marked effect on the genetic consequences of the mutagen's action. In addition to these events, the biological processes of repair and recombination also come into play.

Effects of mutagens on polynucleotides is the result of non-random interactions with certain reactive or "exposed" bases. Thus

the secondary structure of the polynucleotide is of critical importance to the effect or attack of a given mutagenic agent. With duplex DNA, for example, a potential mutation site consists of a pair of bases so that the action of the mutagen is partly obscured due to hydrogen bonding interactions. However, with single stranded polynucleotides individual bases are generally exposed and mutagen attack is much more probable.

Often times the chemistry of base modification with a suspected mutagen is determined by using nucleoside or nucleotide monomer units.[132] This affords, of course, only a general guide as to the sites and extent of mutagen action on polynucleotides. The reactivity of a base in a polynucleotide depends on whether the base is hydrogen bonded in a duplex structure, whether base stacking interactions are significant, and/or whether there is protein interaction with the polymer. It should also be realized that mutation mechanisms that apply to single-stranded polynucleotides might not apply to duplex polynucleotides, since base pairing may effectively block some potentially mutagenic reactions. While mutagenic action on single stranded polynucleotides occurs in a somewhat random fashion, mutagenic action on duplex polynucleotides occurs in a non-random fashion which is governed primarily by "looped-out" or "hot-spot" regions which are more susceptible to mutagenic attack.

The higher structure of polynucleotides is formed and stabilized at the expense of base stacking and hydrogen bonding forces. The distortion of base stacking interaction by the action of certain mutagens (e.g. bis-product of NH_2OH action on cytosine) will

cause a disorder in the structure of both single and double stranded regions of the polynucleotide and often a distortion of complementary hydrogen bonding interactions (affecting duplex regions only). Modification of bases resulting in the loss of aromaticity and violations of planarity are usually accompanied by a general breakdown of polynucleotide secondary structure.

Mutagenic action on nitrogen base sites involved in complementary hydrogen bonding may result in altered hydrogen bonding interactions without affecting base stacking interactions. Such is the case for HNO_2 action on cytosine residues and of unimolecular action of NH_2OH on cytosine. The products of these reactions do not affect the aromaticity or planarity of the cytosine base. However, in both cases, the mutagen acts to change the hydrogen bonding properties of cytosine. Addition of NH_2OH across the 5,6 double bond of cytosine residues results in the loss of aromatic character and diminished polymer secondary structure due to decreased base stacking interactions. Substitution of the hydroxyamino group for the C4 amino group alters the functional specificity of the cytosine and results in a C→pseudo U or T transition mutation, while addition of NH_2OH across the 5,6 double bond causes the loss of functional activity as the secondary structure is adversely modified.

When reacting a chemical mutagen with a polynucleotide, the modification of a small fraction of the nitrogen bases may result in a compounded structural alteration of the polymer, due to changes in base stacking interactions and hydrogen bonding properties.[133]

Mutagenic Action of HNO_2 and NH_2OH on Poly C

The genetic consequences of the action of particular mutagens depends on the functional and physical state and conditions of mutagenesis. All of the many variables involved elucidate the complexity of mutagenic events. For investigative purposes it is desirable to separate the direct action of mutagenic factors on the polynucleotides from the indirect influence on enzymatic processes.[134] To accomplish this we used the homopolynucleotide, polycytidylic acid, as the polynucleotide model system. Poly C may be an excellent choice for mutagen action examination, since this polymer exists in both single stranded and double stranded conformations under certain conditions. Modification of the cytosine units of Poly C by reaction with HNO_2 and NH_2OH may reveal a promising means for studying the structure and function of polynucleotide systems as they relate to mutagenesis. The chemical reagents, NH_2OH and HNO_2, have been shown to react with cytosine residues in polynucleotides and be mutagenic in living systems, causing C→U or pseudo U (T) transition point mutations.

Previous studies have shown that the reaction of Poly C with hydroxylamine is considerably slower than with the cytosine monomer (free base, nucleoside, or nucleotide).[135-136,139-140] It has also been found that the reaction proceeds much more slowly at pH 4 (duplex Poly C) than at pH 6 (single stranded Poly C).[143] At the lower pH there was found to be a greater preference for the substitution reaction at C4 over the 5,6 double bond addition reaction.[85] Concomitant with the reaction of Poly C with NH_2OH

is a loss of secondary structure of the polynucleotide. This is probably the result of the addition reaction across the 5,6 double bond which destroys the aromaticity and planarity of the cytosine residues and consequently disrupts cytosine base stacking interactions.[83,138] Treatment of Poly C with NH_2OH produces an inactivation of its normal template properties as measured by 5'-GMP incorporation.[137] The binding of 5'-AMP was observed in some cases, suggesting that NH_2OH produces in Poly C a cytosine species which binds AMP in place of GMP and a second species which will not base pair with either of the purine nucleotides.[141-142] Salganik and coworkers[122] have shown 4-OH-amino-cytidine to be the product species responsible for the C→pseudo U (T) mutagenic event in E. coli while the bis product was found to cause inactivation of the genetic material.

Few studies on the reaction of Poly C with nitrous acid have been reported. Singer et al.[84,135] reported transitions of C to U in Poly C when acted upon with $NaNO_2$ at pH 4.2. These studies showed that the effectiveness of $NaNO_2$ to act as a mutagen is clearly pH dependent, since the reaction rate is a function of the concentration of the undissociated HNO_2.

The purpose here is to investigate the effects of NH_2OH and $NaNO_2$ on polycytidylic acid as it relates to the binding of deoxyguanosine and adenosine. In the case of nitrous acid treatment, the reaction was carried out at pH 4.1 (giving consideration to the pH dependence of HNO_2 generation from $NaNO_2$ in solution). The reaction of Poly C with hydroxylamine was performed at pH 6.0 due to the extremely slow rate of reaction at pH 4.1. Equilibrium

dialysis experiments are employed to investigate the binding of the nucleosides, deoxyguanosine and adenosine to chemically modified Poly C.

CHAPTER II

EXPERIMENTAL

METHODS AND MATERIALS

Reaction of Poly C With Nitrous Acid

Nitrous acid can easily be generated by dissolving $NaNO_2$ in an aqueous buffer solution below pH 5.5:

$$NaNO_2 \rightleftharpoons Na^+ + NO_2^-$$

$$NO_2^- + H_2O \rightleftharpoons HNO_2 + OH^-$$

In the presence of surplus H^+ (low pH) the equilibrium is pushed to the right, thereby increasing the concentration of the reactive free acid. Undissociated HNO_2 can react with the primary amino group in cytosine residues causing deamination and chemical conversion of cytosine to uracil.

In these experiments, equal volumes of 2 M $NaNO_2$ (at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$) and Poly C sclution (in the same buffer) were allowed to react for various time intervals (1-12 hours) at 37°C. Upon termination of a particular reaction, the reaction mixture was cooled at 5°C for ca. 0.5 hour. The modified polymer was then collected via gel-filtration column chromatography using Sephadex G-25. Eighty, 8-9 ml fractions were collected. The fractions containing the modified Poly C were detected by UV absorption at 270nm. The absence of $NaNO_2$ in the

polymer fractions was insured by the observation of no absorption at 356nm. The fractions containing the modified polymer were combined and precipitated using one volume of 2.0 M potassium acetate buffer (pH 5.0) and two volumes of 100% ethanol at -20° C for 18 hours.

The polymer precipitate was then centrifuged at 15,000 RPM for 30 minutes at 5°C. The filtrate was decanted, the polymer dried with N_2, and finally, reconstituted by dissolving in a small volume (usually 5-7 ml) of 0.1 M NaCl solution. Following this, the modified polymer solution was dialyzed against 0.1 M Na acetate buffer (pH 4.1) + 1.0 mM $MgCl_2$ at 5°C. Fresh dialysis buffer was added five times.

After dialysis, an aliquot of the modified polymer solution was treated with 0.3 N KOH for 22 hours at 37°C to accomplish hydrolysis of the polymer to mononucleotide units. A direct spectrophotometric method was then used to determine the CMP:UMP ratio in the modified Poly C. The nucleotide reaction products were separated using ascending paper or thin-layer chromatography to insure the presence of only C and U monophosphates. The products were detected using a short-wave UV light and compared against known CMP and UMP solutions.

The remaining modified polymer solution was used in equilibrium dialysis experiments to investigate the binding of deoxyguanosine and adenosine to modified Poly C. The dialysis experiments were carried out at 2°C for 48 hours. In each experiment, controls were run to ensure equilibration and non-migration of the modified polymer across the membrane.

Reaction of Poly C With Hydroxylamine

5-^{3}H polycytidylic acid was purchased from Miles Laboratories (Elkhart, IN). The use of the radioactively labeled polymer was found to be the best means for the detection and quantitation of cytidine derivatives after reaction of the polymer with NH_2OH. A direct spectrophotometric method was not found to applicable in this system.

Solutions of ^{3}H-Poly C (in 0.1 M Na acetate buffer, pH 6.0) were mixed with equal volumes of 2 M NH_2OH for various time periods (3-24 hours), after which they were cooled at 5°C for ca. 30 minutes. The modified polymer fractions were separated from NH_2OH by Sephadex G-25 gel filtration. Concentration of the polymer was accomplished via acetate buffer and ethanol precipitation as described earlier. For analytical purposes, an aliquot of the modified polymer solution was treated with 0.3 N KOH for 22 hours at 37°C in order to digest the polymer to nucleotide monomers. The cytidine and NH_2OH-cytidine derivatives were then separated by thin-layer chromatography on silica gel, using n-butanol: ethanol:water (80:10:25) as the solvent.

The extent of reaction (and thus the ratio of the NH_2OH-cytidine reaction products) was established by comparing the radioactivity associated with the chromatographically separated products. A known CMP solution was used as a reference to differentiate between 4-hydroxyaminocytidine and unmodified cytidine, since both were detected via the use of a short-wave UV lamp. 6-hydroxyamino-5,6-dihydro-4-hydroxyaminocytidine was detected by radioactivity association since it does not absorb light in the

UV range. Radioactivity was measured by scraping silica gel strips and counting in a toluene, PPO, POPOP scintillation cocktail (using a Packard Scintillation Counter).

The remaining modified polymer solution at pH 6.0 was dialyzed against 0.1 M Na acetate buffer (pH 4.1) + 1.0 mM $MgCl_2$ at 5°C. Fresh dialysis buffer was added three times. Upon effective pH adjustment of the modified polymer solution, equilibrium dialysis experiments were carried out at 2°C for 48 hours. The binding of deoxyguanosine and adenosine to the modified polymer was thereby investigated. In each case, appropriate controls were run to ensure equilibration and non-migration of the polymer.

CHAPTER III

RESULTS

Figure 42. Poly C Elution on Sephadex G-25 Column

Initial Poly C in 0.1 M Na acetate buffer at pH 4.1 + 1.0 mM $MgCl_2$. A_{273} = 1.49

Loaded 5 ml of Poly C solution on Sephadex G-25 column and collected 80, 8.0 ml fractions using the above buffer solution as the eluent.

Fraction #	A_{273}
1-18	0.00
19	0.40
20	0.37
21	0.06
22-80	0.00

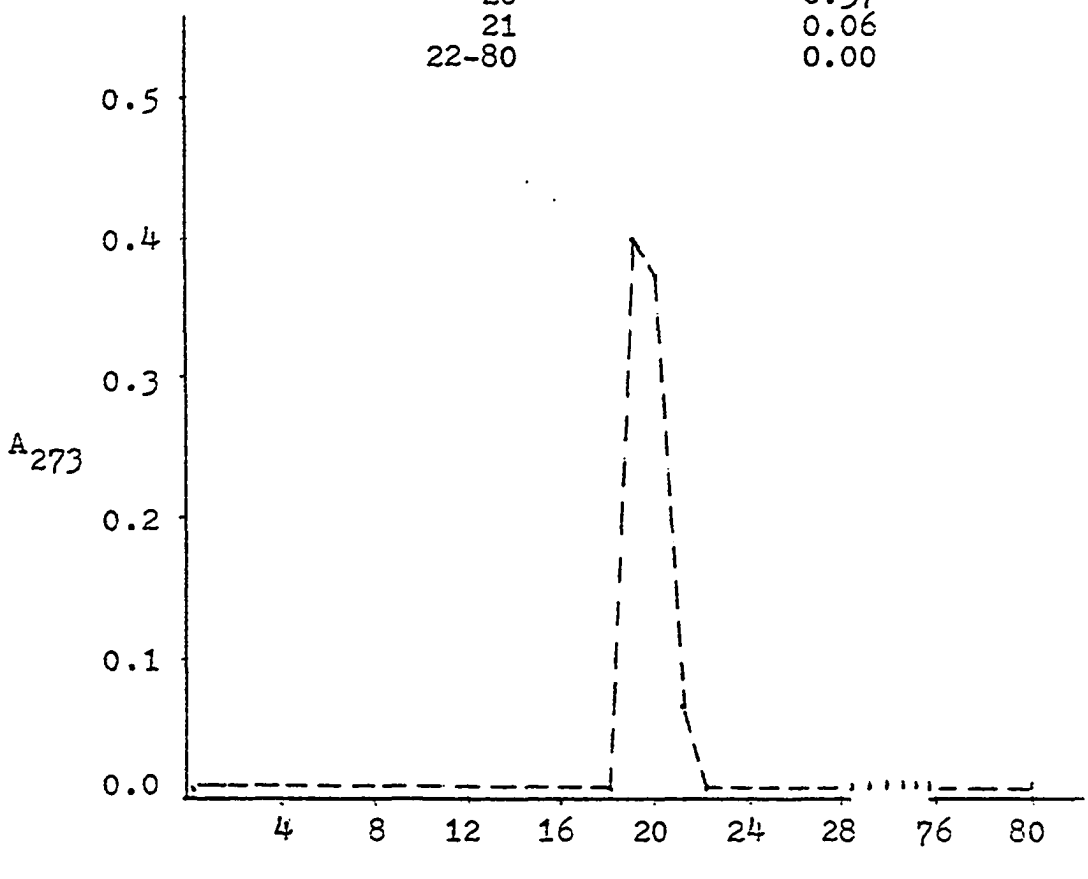
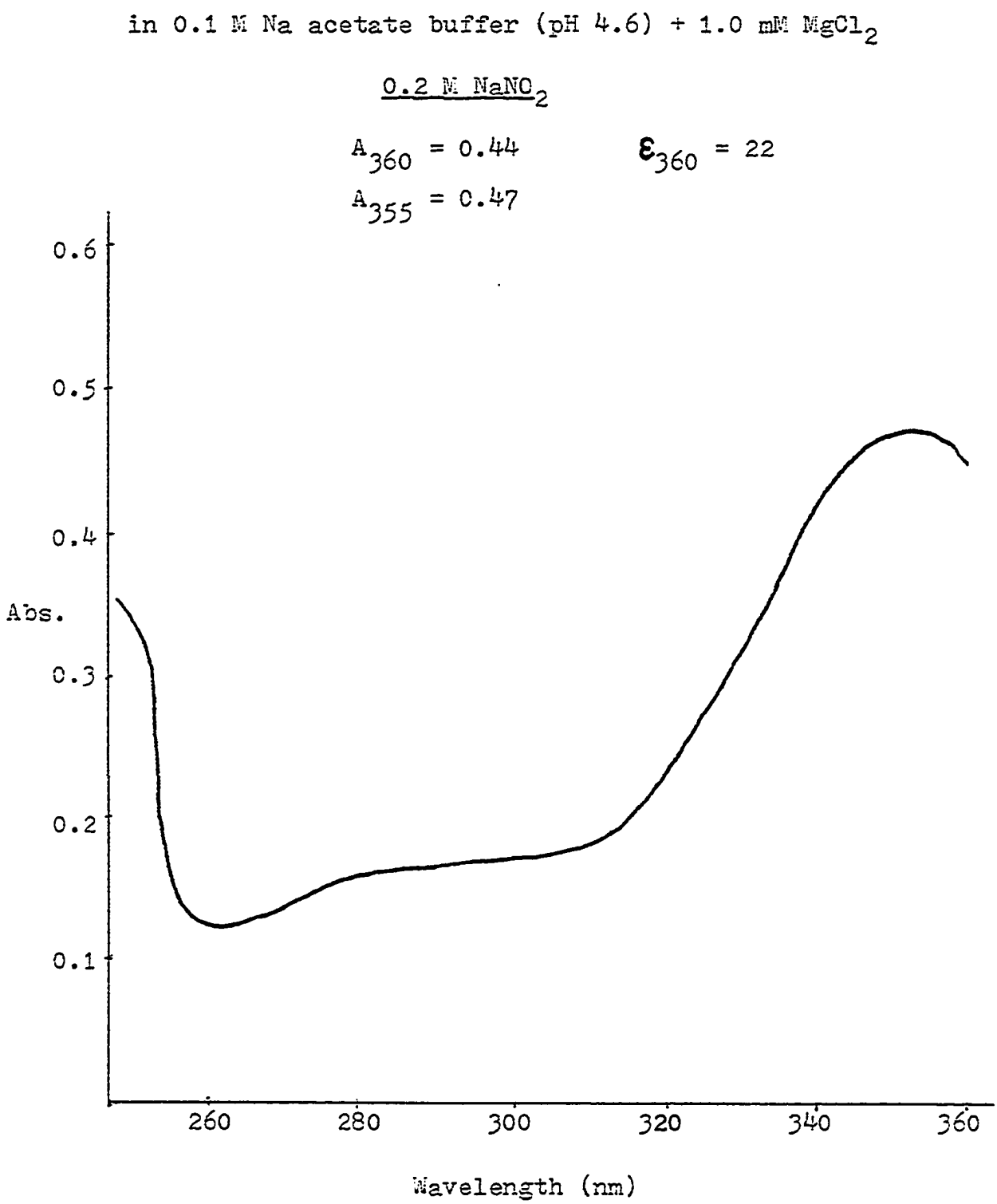


Figure 43. Absorption Spectrum of Sodium Nitrite at pH 4.6



Pyrimidine Nucleotide Concentration Determination By UV Absorption Spectroscopy

Beer-Lambert Relationship $A=\varepsilon Cl$,

where A=Absorbance, E=Molar Extinction Coefficient, C=Concentration, and l=light pathlength.

For a mixture of two components, a and b (CMP and UMP):

$$C_a\varepsilon_a\lambda_1 + C_b\varepsilon_b\lambda_1 = A\lambda_1$$

and $$C_a\varepsilon_a\lambda_2 + C_b\varepsilon_b\lambda_2 = A\lambda_2$$

Therefore, $$C_a = \frac{\varepsilon_b\lambda_1 A\lambda_2 - \varepsilon_b\lambda_2 A\lambda_2}{\varepsilon_a\lambda_2\varepsilon_b\lambda_2 - \varepsilon_a\lambda_1\varepsilon_b\lambda_2}$$

and $$C_b = \frac{\varepsilon_a\lambda_2 A\lambda_1 - \varepsilon_a\lambda_1 A\lambda_2}{\varepsilon_a\lambda_2\varepsilon_b\lambda_2 - \varepsilon_a\lambda_1\varepsilon_b\lambda_2}$$

It is desirable to pick wavelengths (λ_1 and λ_2) where relatively large differences in molar extinctions of the two components occur.

CMP and UMP UV Absorption Spectroscopy Data at pH 12.5.

nucleotides in 0.1 M NaCl + 0.3 N KOH at pH 12.5.

1 mM CMP: λmax = 267nm $A_{267} = 0.88$ Therefore, $\varepsilon_{267} = 8{,}800$

$A_{234} = 0.755$ $\varepsilon_{234} = 7{,}550$

$A_{278} = 0.765$ $\varepsilon_{278} = 7{,}650$

1 mM UMP: λmax = 257nm $A_{257} = 0.76$ Therefore, $\varepsilon_{257} = 7{,}600$

$A_{234} = 0.58$ $\varepsilon_{234} = 5{,}800$

$A_{278} = 0.29$ $\varepsilon_{278} = 2{,}900$

λ_1 = 234nm and λ_2 = 278nm

isobestic points at 260 and 248nm $A_{260} = 0.735$ $\varepsilon_{260} = 7{,}350$

$A_{248} = 0.610$ $\varepsilon_{248} = 6{,}100$

1:1 Mixture of 1 mM CMP + 1 mM UMP: $A_{234} = 0.675$

$A_{278} = 0.53$

$$[\text{CMP}] = \frac{(5{,}800)(0.53) - (2{,}900)(0.675)}{(7{,}650)(5{,}800) - (7{,}550)(2{,}900)} = 4.96 \times 10^{-4}\ \text{M}$$

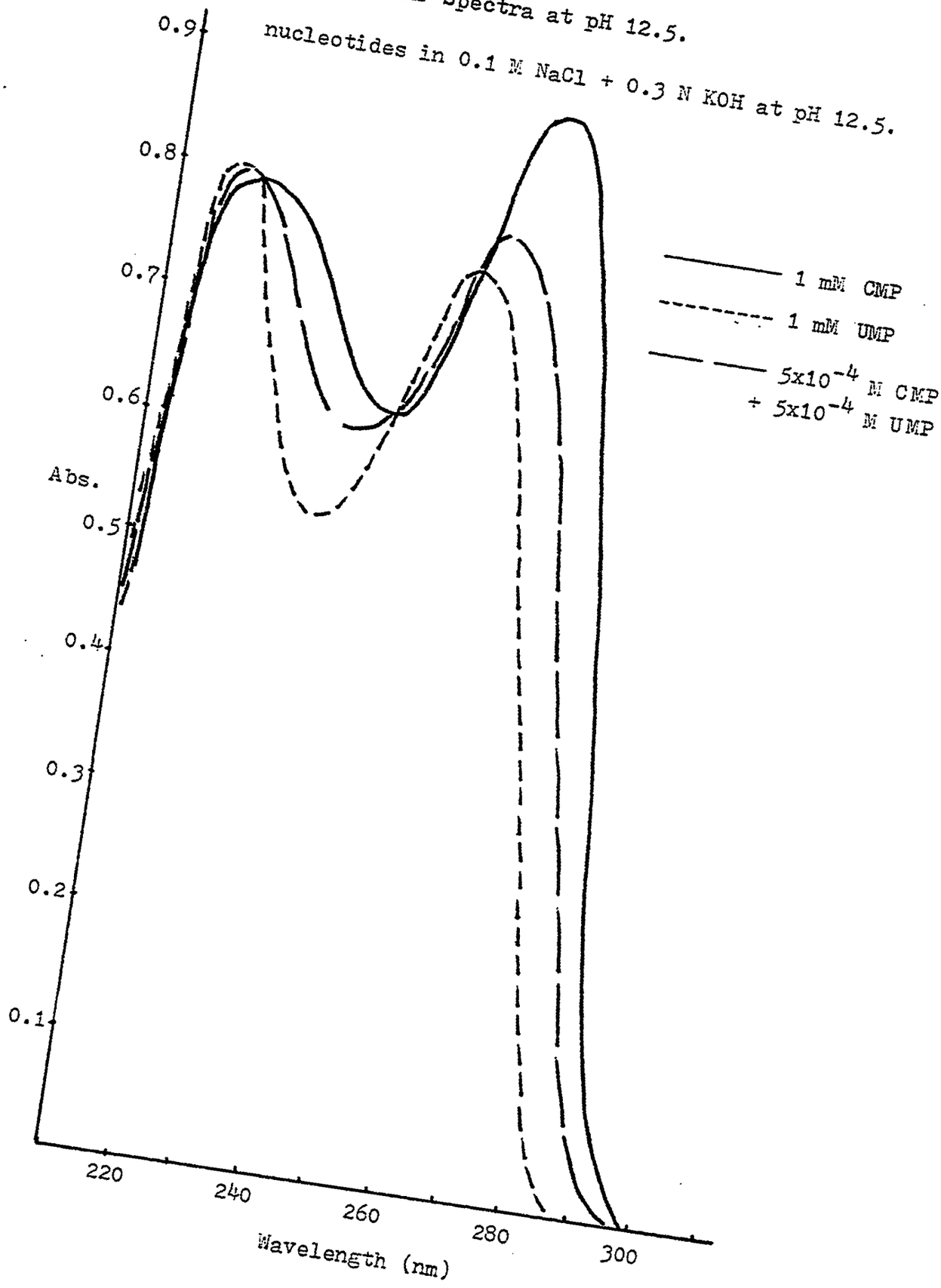
$$[\text{UMP}] = \frac{(7{,}650)(0.675) - (7{,}550)(0.53)}{(7{,}650)(5{,}800) - (7{,}550)(2{,}900)} = 5.17 \times 10^{-4}\ \text{M}$$

General equations for any mixture:

$$[\text{CMP}] = \frac{(5.8)A_{278} - (2.9)A_{234}}{2247}, \quad [\text{UMP}] = \frac{(7.65)A_{234} - (7.55)A_{278}}{2247}$$

Figure 44. CMP and UMP Spectra at pH 12.5.

nucleotides in 0.1 M NaCl + 0.3 N KOH at pH 12.5.



CMP and UMP UV Absorption Spectroscopy Data at pH 6.8.

nucleotides in 0.1 M Na acetate buffer at pH 6.8.

1 mM CMP: λmax = 268nm $A_{268} = 0.89$ Therefore, $\varepsilon_{268} = 8,900$

$A_{258} = 0.745$ $\varepsilon_{258} = 7,450$

$A_{280} = 0.65$ $\varepsilon_{280} = 6,500$

1 mM UMP: λmax = 258nm $A_{258} = 0.995$ Therefore, $\varepsilon_{258} = 9,950$

$A_{280} = 0.28$ $\varepsilon_{280} = 2,800$

λ_1 = 258nm and λ_2 = 280nm

isobestic points at 245 and 266nm $A_{245} = 0.66$ $\varepsilon_{245} = 6,600$

$A_{266} = 0.89$ $\varepsilon_{266} = 8,900$

1:1 Mixture of 1 mM CMP + 1 mM UMP: $A_{258} = 0.88$

$A_{280} = 0.46$

$$[CMP] = \frac{(9,900)(0.46) - (2,800)(0.88)}{(6,500)(9,950) - (7,450)(2,800)} = 4.8 \times 10^{-4} \text{ M}$$

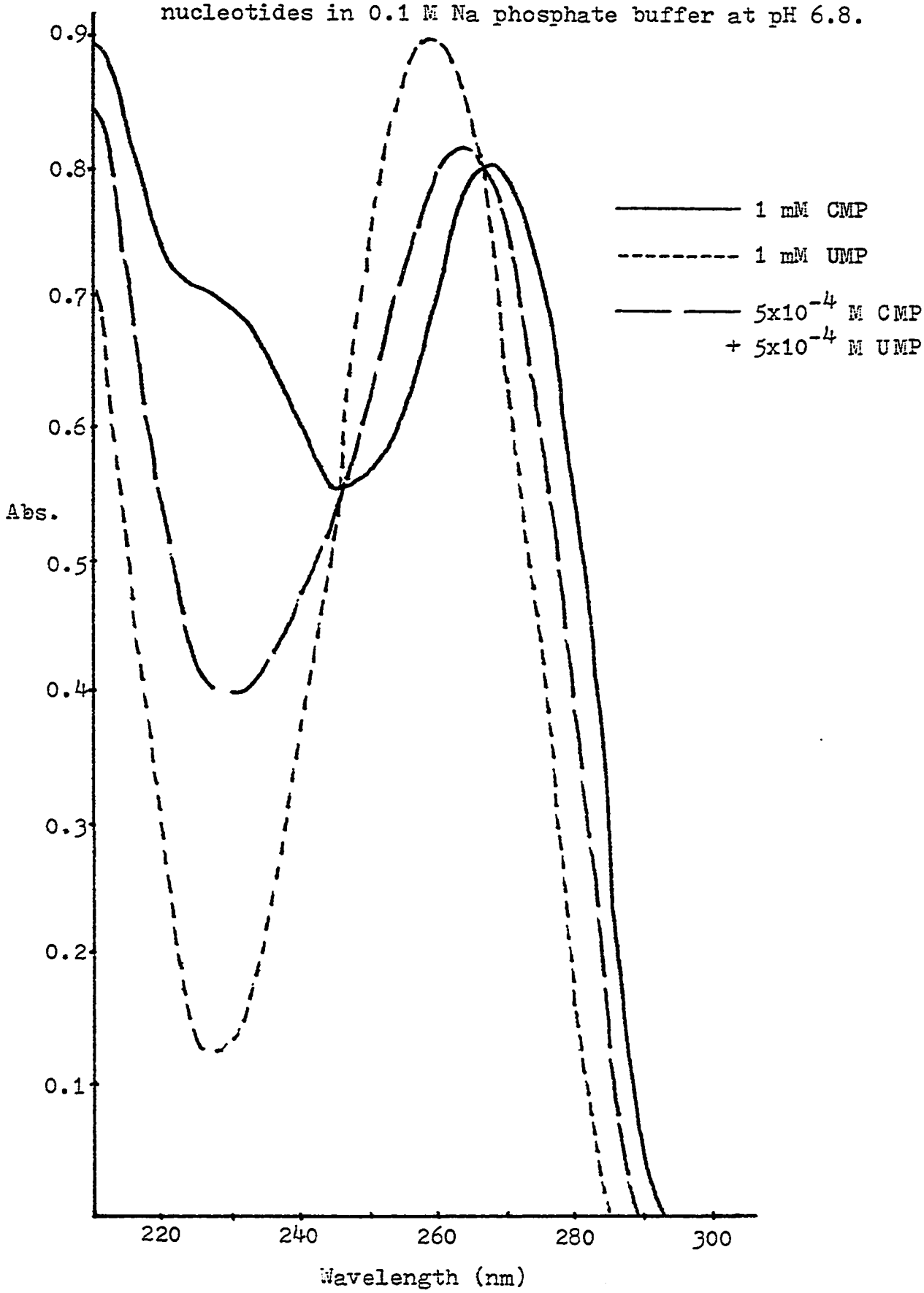
$$[UMP] = \frac{(6,500)(0.88) - (7,450)(0.46)}{(6,500)(9,950) - (7,450)(2,800)} = 5.2 \times 10^{-4} \text{ M}$$

General Equation for any mixture:

$$[CMP] = \frac{(9.95)A_{280} - (2.8)A_{258}}{4381} \qquad [UMP] = \frac{(6.5)A_{258} - (7.45)A_{280}}{4381}$$

Figure 45. CMP and UMP Spectra at pH 6.8.

nucleotides in 0.1 M Na phosphate buffer at pH 6.8.



CMP and UMP UV Absorption Spectroscopy Data at pH 4.1.

nucleotides in 0.1 M Na acetate buffer at pH 4.1.

1 mM CMP: λ max = 273nm $A_{273} = 0.95$ $\varepsilon_{273} = 9,500$

$A_{258} = 0.61$ $\varepsilon_{258} = 6,100$

$A_{278} = 0.91$ $\varepsilon_{278} = 9,100$

1 mM UMP: λ max = 257nm $A_{257} = 0.92$ $\varepsilon_{257} = 9,200$

$A_{258} = 0.91$ $\varepsilon_{258} = 9,100$

$A_{278} = 0.35$ $\varepsilon_{278} = 3,500$

λ_1 = 258nm and λ_2 = 278nm

isobestic points at 240 and 268nm $A_{240} = 0.41$ $\varepsilon_{240} = 4,100$

$A_{268} = 0.825$ $\varepsilon_{268} = 8,250$

1:1 Mixture of 1 mM CMP + 1 mM UMP: $A_{258} = 0.76$

$A_{278} = 0.625$

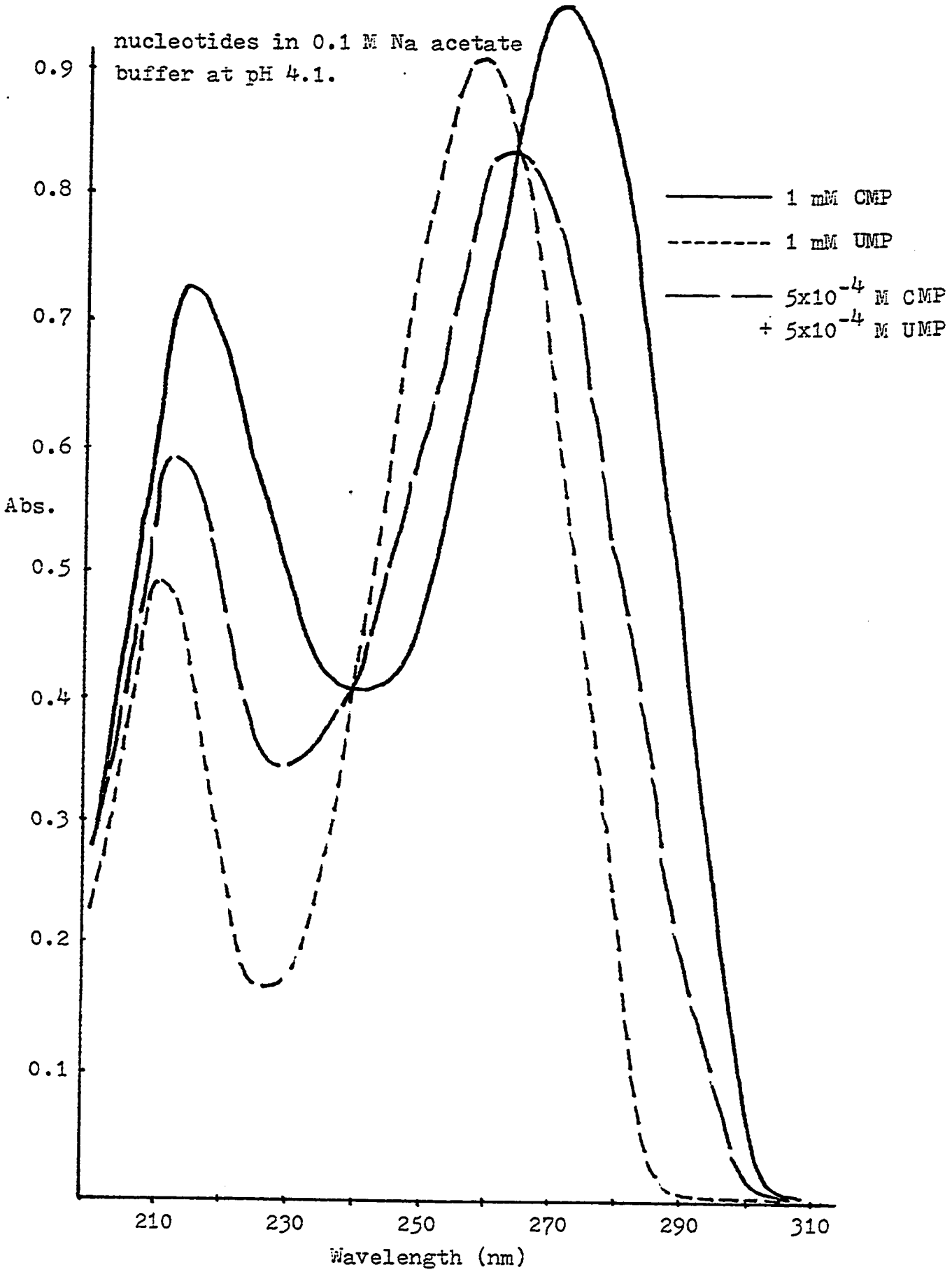
$$[CMP] = \frac{(9,100)(0.625) - (3,500)(0.76)}{(9,100)(9,100) - (6,100)(3,500)} = 4.92 \times 10^{-4} \text{ M}$$

$$[UMP] = \frac{(9,100)(0.76) - (6,100)(0.625)}{(9,100)(9,100) - (6,100)(3,500)} = 5.04 \times 10^{-4} \text{ M}$$

General equations for any mixture:

$$[CMP] = \frac{(9.1)A_{278} - (3.5)A_{258}}{6146} \qquad [UMP] = \frac{(9.1)A_{258} - (6.1)A_{278}}{6146}$$

Figure 46. CMP and UMP Spectra at pH 4.1.



CMP and UMP UV Absorption Spectroscopy Data at pH 2.0.

nucleotides in 0.1 M NaCl + 0.01 N HCl at pH 2.0.

1 mM CMP: λ max = 276nm $A_{276} = 1.30$ $\varepsilon_{276} = 13,000$

$A_{278} = 1.30$ $\varepsilon_{278} = 13,000$

$A_{256} = 0.54$ $\varepsilon_{256} = 5,400$

1 mM UMP: λmax = 258nm $A_{258} = 0.99$ $\varepsilon_{258} = 9,900$

$A_{278} = 0.40$ $\varepsilon_{278} = 4,000$

$A_{256} = 0.97$ $\varepsilon_{256} = 9,700$

λ_1 = 256nm and λ_2 = 278nm

isobestic points at 264 and 232nm $A_{264} = 0.89$ $\varepsilon_{264} = 8,900$

$A_{232} = 0.28$ $\varepsilon_{232} = 2,800$

1:1 Mixture of 1 mM CMP + 1 mM UMP: $A_{256} = 0.76$

$A_{278} = 0.865$

$$[CMP] = \frac{(9,700)(0.865) - (4,000)(0.76)}{(13,000)(9,700) - (5,400)(4,000)} = 5.12 \times 10^{-4} \text{ M}$$

$$[UMP] = \frac{(13,000)(0.76) - (5,400)(0.865)}{(13,000)(9,700) - (5,400)(4,000)} = 4.98 \times 10^{-4} \text{ M}$$

General equations for any mixture:

$$[CMP] = \frac{(9.7)A_{278} - (4.0)A_{256}}{10450} \qquad [UMP] = \frac{(13.0)A_{256} - (5.4)A_{278}}{10450}$$

Figure 47. CMP and UMP Spectra at pH 2.0.

nucleotides in 0.1 M NaCl & 0.01 N HCl at pH 2.0

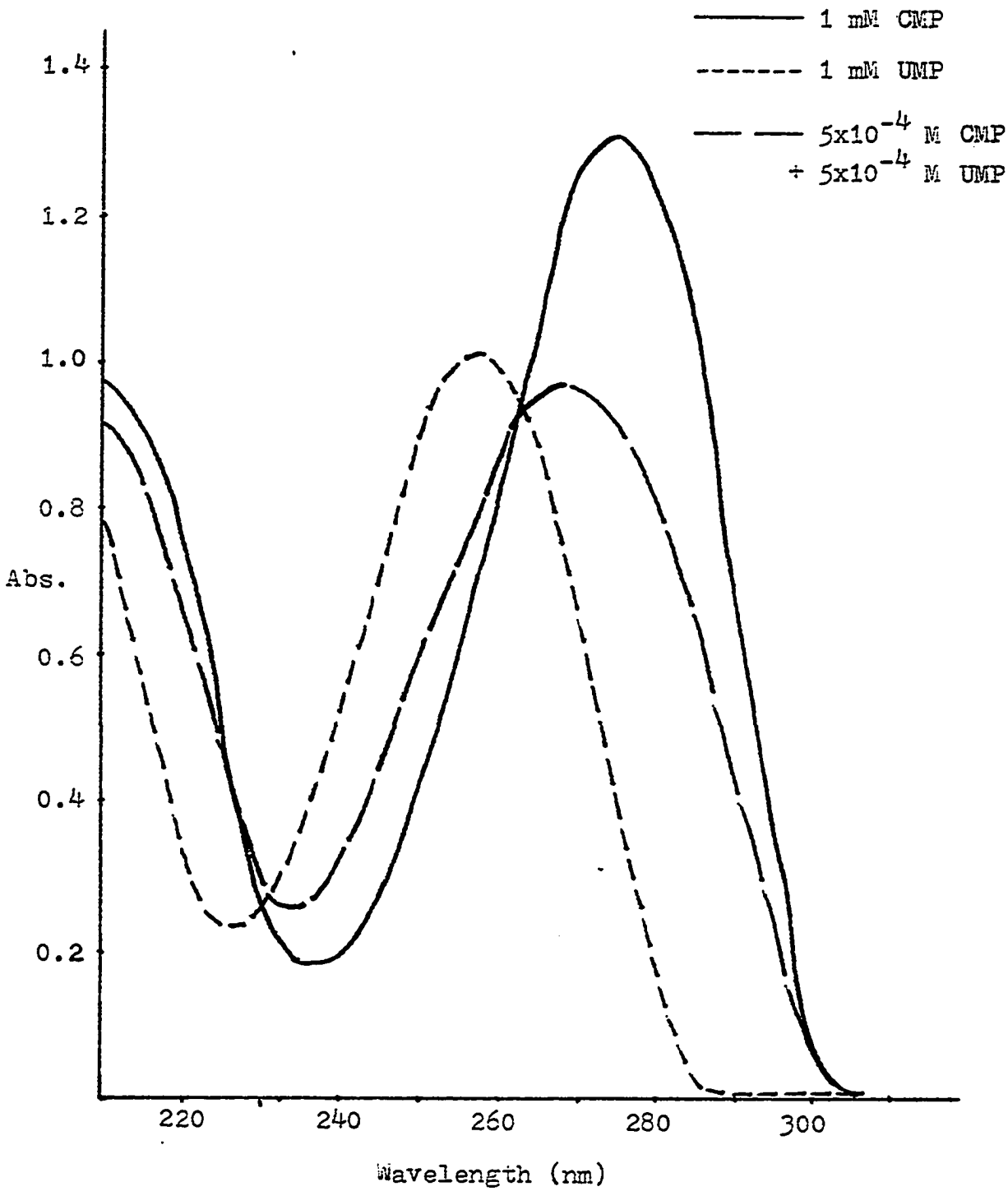


Table 26. Binding of Deoxyguanosine to Chemically Modified Poly C

Equilibrium Dialysis Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 1 hour.

initial [Poly C] = 1.35×10^{-3} M

after reaction: 85% C or 1.15×10^{-3} M CMP

15% U or 0.20×10^{-3} M UMP

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.27	2.07	0.14	1.07	0.00
0.41	3.15	0.22	1.69	0.00
0.68	5.23	0.34	2.61	0.00
0.82	6.30	0.39	3.00	0.30
0.93	7.15	0.42	3.23	0.69
1.07	8.23	0.48	3.69	0.85
1.20	9.23	0.51	3.92	1.39
2.64	20.30	1.04	8.00	4.30
3.44	26.46	1.43	11.00	4.46

38% binding of dG to C residues

Table 27. Binding of Deoxyguanosine to Chemically Modified Poly C

Computer Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 1 hour.

initial [Poly C] = 1.35×10^{-3} M

after reaction: 85% C or 1.15×10^{-3} M CMP

15% U or 0.20×10^{-3} M UMP

n	S	K_1	RMSD
3	4.79×10^{-4} M	987	1.81×10^{-5}
4	4.39×10^{-4} M	1167	1.37×10^{-5}
** 5	4.38×10^{-4} M	1303	1.19×10^{-5}
6	4.37×10^{-4} M	1408	1.24×10^{-5}
7	4.37×10^{-4} M	1493	1.41×10^{-5}

** best value of n based on lowest RMSD

for n=5

$[dG_b]$ exp x 10^4 M	$[dG_b]$ calc x 10^4 M	dG_b/CMP
0.00	0.00	0.000
0.00	0.01	0.000
0.00	0.16	0.000
0.30	0.34	0.026
0.69	0.50	0.060
0.85	0.99	0.073
1.39	1.31	0.120
4.30	4.38	0.373
4.46	4.38	0.387

Figure 48. Binding Isotherm for Deoxyguanosine:Modified Poly C

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 1 hour.

initial [Poly C] = 1.35×10^{-3} M

after reaction: 85% C or 1.15×10^{-3} M CMP

15% U or 0.20×10^{-3} M UMP

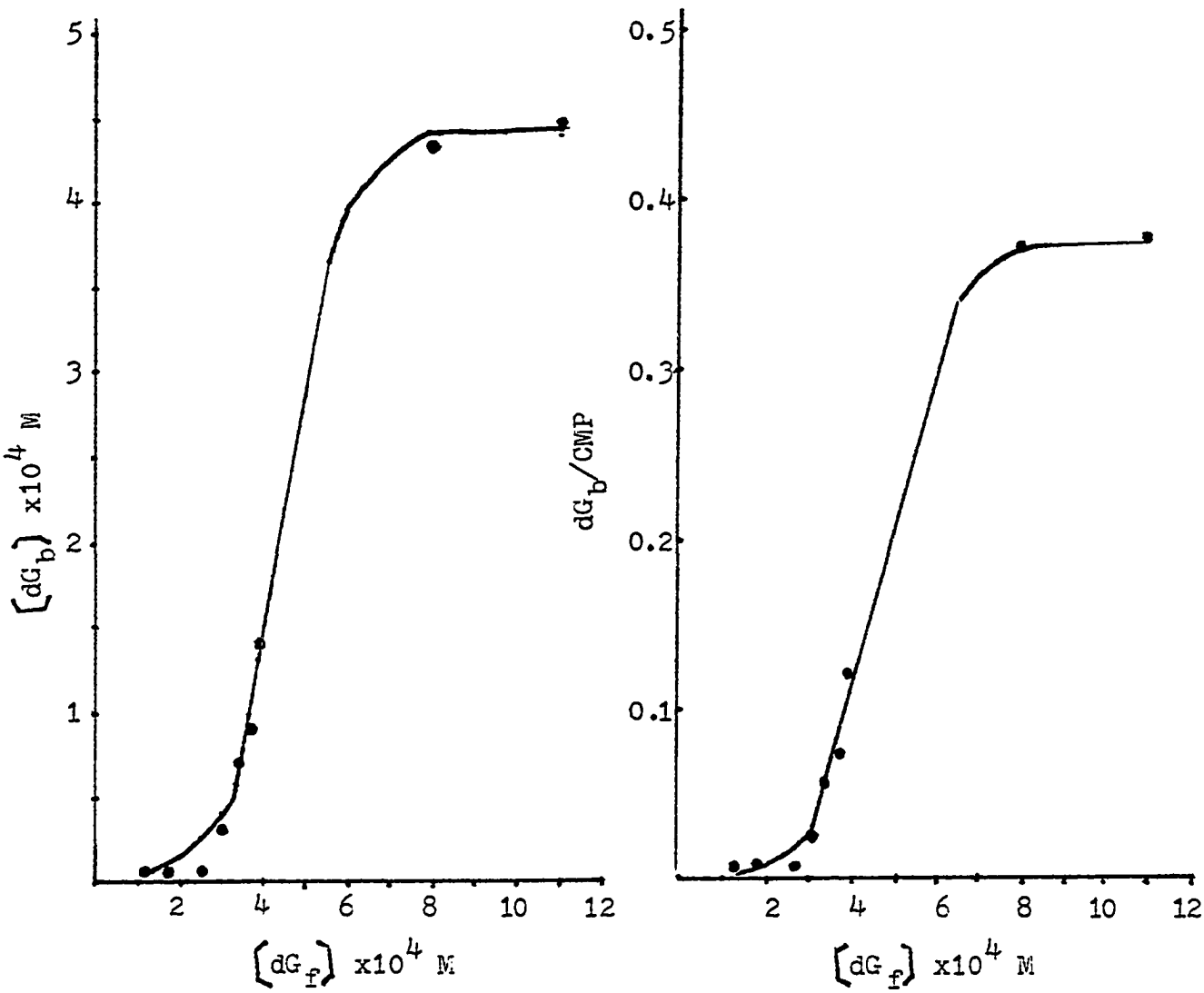


Table 28. Binding of Adenosine to Chemically Modified Poly C.

Equilibrium Dialysis Data

modification with 1M $NaNO_3$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 1 hour.

initial [Poly C] = 1.35×10^{-3} M

after reaction: 85% C or 1.15×10^{-3} M CMP

15% U or 0.20×10^{-3} M UMP

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.46	3.23	0.23	1.61	0.00
0.72	5.07	0.36	2.53	0.00
1.01	7.11	0.50	3.52	0.07
1.16	8.16	0.58	4.08	0.00
1.44	10.14	0.72	5.07	0.00
2.82	19.85	1.40	9.85	0.14
3.92	27.60	1.95	13.73	0.14
5.08	35.77	2.53	17.81	0.14

7% Adenosine bound per UMP

Table 29. Binding of Deoxyguanosine to Chemically Modified Poly C.

Equilibrium Dialysis Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

initial [Poly C] = 1.37×10^{-3} M

after reaction: 75% C or 1.05×10^{-3} M CMP

25% U or 0.32×10^{-3} M UMP

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.38	2.92	0.19	1.46	0.00
0.68	5.23	0.29	2.23	0.77
0.81	6.23	0.34	2.61	1.00
0.92	7.07	0.38	2.92	1.23
1.06	8.15	0.43	3.30	1.55
1.33	10.23	0.56	4.30	1.62
1.83	14.07	0.78	6.00	2.07
2.61	20.07	1.14	8.76	2.54
3.68	28.30	1.66	12.76	2.77

26% binding of dG to C residues

Table 30. Binding of Deoxyguanosine to Chemically Modified Poly C.

Computer Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

initial [Poly C] = 1.37×10^{-3} M

after reaction: 75% C or 1.05×10^{-3} M CMP

25% U or 0.32×10^{-3} M UMP

n	S	K_1	RMSD
1	2.63×10^{-4} M	1088	2.54×10^{-5}
** 2	2.48×10^{-4} M	1413	2.41×10^{-5}
3	2.36×10^{-4} M	1702	2.75×10^{-5}
4	2.29×10^{-4} M	1911	3.10×10^{-5}
5	2.24×10^{-4} M	2075	3.40×10^{-5}

** best value of n based on lowest RMSD

for n=2

$[dG_b]$exp x 10^4 M	$[dG_b]$calc x 10^4 M	dG_b/CMP
0.00	0.11	0.000
0.00	0.26	0.000
0.77	0.65	0.073
1.00	0.88	0.095
1.23	1.09	0.117
1.55	1.33	0.147
1.62	1.91	0.154
2.07	2.41	0.197
2.54	2.48	0.241
2.77	2.48	0.263

Figure 49. Binding Isotherm for Deoxyguanosine:Modified Poly C

modification with 1 M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

initial [Poly C] = 1.37×10^{-3} M

after reaction: 75% C or 1.05×10^{-3} M CMP

25% U or 0.32×10^{-3} M UMP

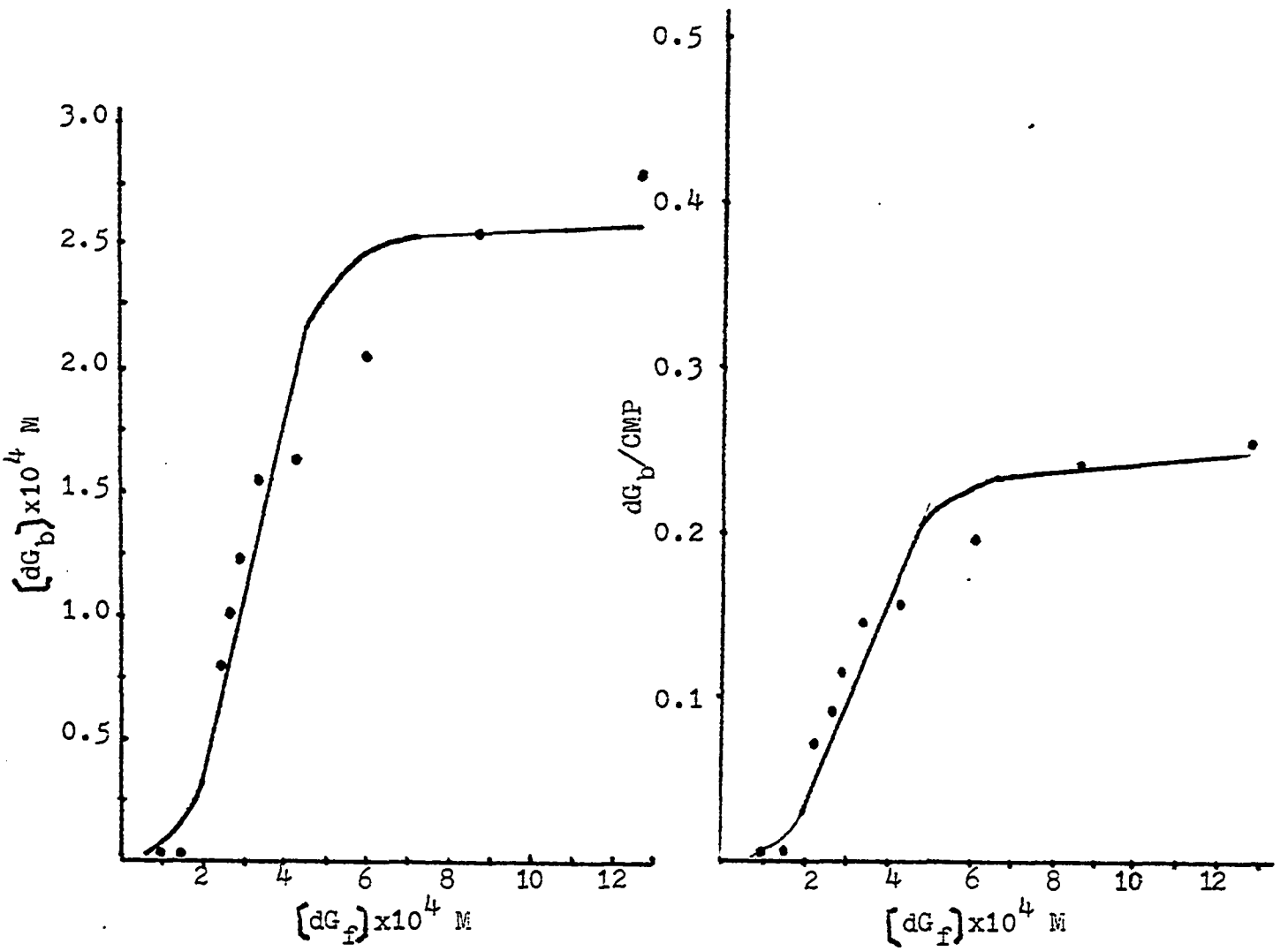


Table 31. Binding of Adenosine to Chemically Modified Poly C.

Equilibrium Dialysis Data

modification with 1 M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

initial [Poly C] = 1.37×10^{-3} M

after reaction: 75% C or 1.05×10^{-3} M CMP

25% U or 0.32×10^{-3} M UMP

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.44	3.09	0.22	1.54	0.00
0.72	5.07	0.36	2.53	0.00
1.03	7.25	0.51	3.59	0.07
1.15	8.09	0.57	4.01	0.07
1.42	10.00	0.71	5.00	0.00
2.56	18.02	1.26	8.90	0.21
3.72	26.19	1.84	12.95	0.28

9% binding of Adenosine per UMP

Table 32. Binding of Deoxyguanosine to Chemically Modified Poly C

Equilibrium Dialysis Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 6 hours.

initial [Poly C] = 2.0×10^{-3} M

after reaction: 45% C or 0.9×10^{-3} M CMP

55% U or 1.1×10^{-3} M UMP

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.36	2.76	0.18	1.38	0.00
0.64	4.92	0.30	2.30	0.32
0.75	5.76	0.32	2.46	0.84
0.86	6.61	0.36	2.76	1.08
0.98	7.53	0.41	3.15	1.23
1.23	9.46	0.53	4.07	1.32
1.82	14.00	0.82	6.30	1.40
2.44	18.76	1.11	8.53	1.69
3.52	27.07	1.64	12.61	1.84
4.67	35.92	2.21	17.00	1.92

21% binding of dG to C residues

Table 33. Binding of Deoxyguanosine to Chemically Modified Poly C

Computer Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 6 hours.

initial [Poly C] = 2.0×10^{-3} M

after reaction: 45% C or 0.9×10^{-3} M CMP

55% U or 1.1×10^{-3} M UMP

n	S	K_1	RMSD
3	1.68×10^{-4} M	1876	1.98×10^{-5}
** 4	1.65×10^{-4} M	2071	1.96×10^{-5}
5	1.63×10^{-4} M	2230	1.98×10^{-5}

** best value of n based on lowest RMSD

for n=4

$[dG_b]$exp x 10^4 M	$[dG_b]$calc x 10^4 M	dG_b/CMP
0.00	0.01	0.000
0.00	0.06	0.000
0.32	0.54	0.035
0.84	0.67	0.093
1.08	0.94	0.120
1.23	1.25	0.136
1.32	1.60	0.146
1.40	1.65	0.155
1.69	1.65	0.187
1.84	1.65	0.204
1.92	1.65	0.213

Figure 50. Binding Isotherm for Deoxyguanosine:Modified Poly C

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 6 hours.

initial [Poly C] = 2.0×10^{-3} M

after reaction: 45% C or 0.9×10^{-3} M CMP

55% C or 1.1×10^{-3} M UMP

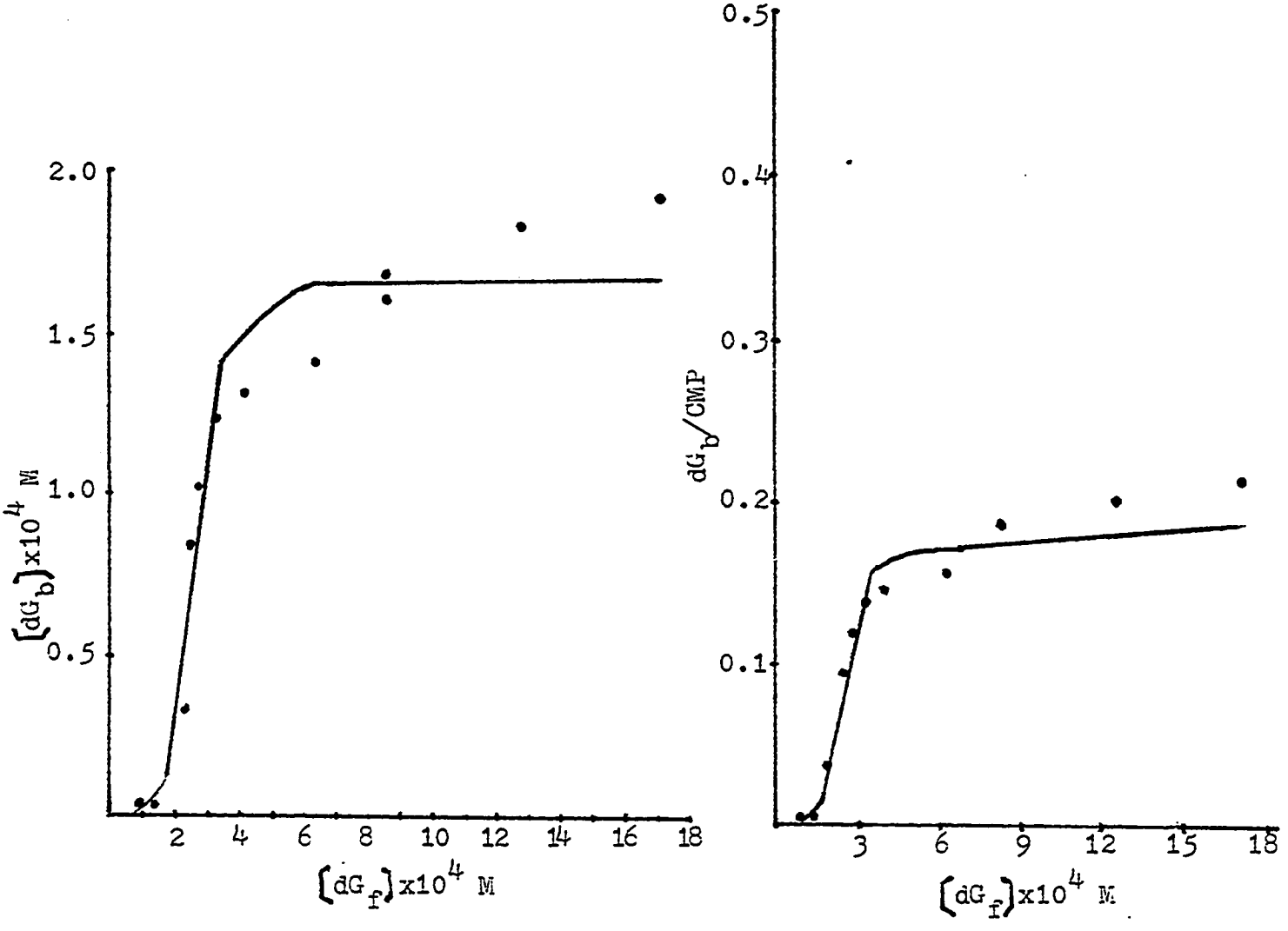


Table 34. Binding of Adenosine to Chemically Modified Poly C

Equilibrium Dialysis Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 6 hours.

initial [Poly C] = 2.0×10^{-3} M

after reaction: 45% C or 0.9×10^{-3} M CMP

55% U or 1.1×10^{-3} M UMP

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.42	2.95	0.21	1.47	0.00
0.73	5.14	0.37	2.60	0.00
0.90	6.33	0.45	3.16	0.00
1.20	8.45	0.60	4.22	0.00
1.31	9.22	0.65	4.57	0.07
2.66	18.73	1.29	9.08	0.57
3.70	26.05	1.79	12.60	0.84
5.35	37.67	2.60	18.30	1.06
6.80	47.88	3.30	23.23	1.41
7.80	54.92	3.79	26.69	1.54
13.20	92.95	6.49	45.70	1.55

14% binding of Adenosine to U residues

Table 35. Binding of Adenosine to Chemically Modified Poly C

Computer Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 6 hours.

initial [Poly C] = 2.0×10^{-3} M

after reaction: 45% C or 0.9×10^{-3} M CMP

55% U or 1.1×10^{-3} M UMP

n	S	K_1	RMSD
1	1.58×10^{-4} M	287	1.25×10^{-5} M
** 2	1.49×10^{-4} M	354	9.03×10^{-6} M
3	1.43×10^{-4} M	427	1.27×10^{-5} M

** best value of n based on lowest RMSD value

for n=2

$[A_b]$exp x 10^4 M	$[A_b]$calc x 10^4 M	A_b/UMP
0.00	0.00	0.000
0.00	0.02	0.000
0.00	0.04	0.000
0.00	0.08	0.000
0.07	0.09	0.00
0.57	0.41	0.051
0.84	0.75	0.076
1.06	1.23	0.096
1.41	1.44	0.128
1.54	1.49	0.140
1.55	1.49	0.140

Figure 51. Binding Isotherm for Adenosine:Modified Poly C

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 6 hours.

initial [Poly C] = 2.0×10^{-3} M

after reaction: 45% C or 0.9×10^{-3} M CMP

55% U or 1.1×10^{-3} M UMP

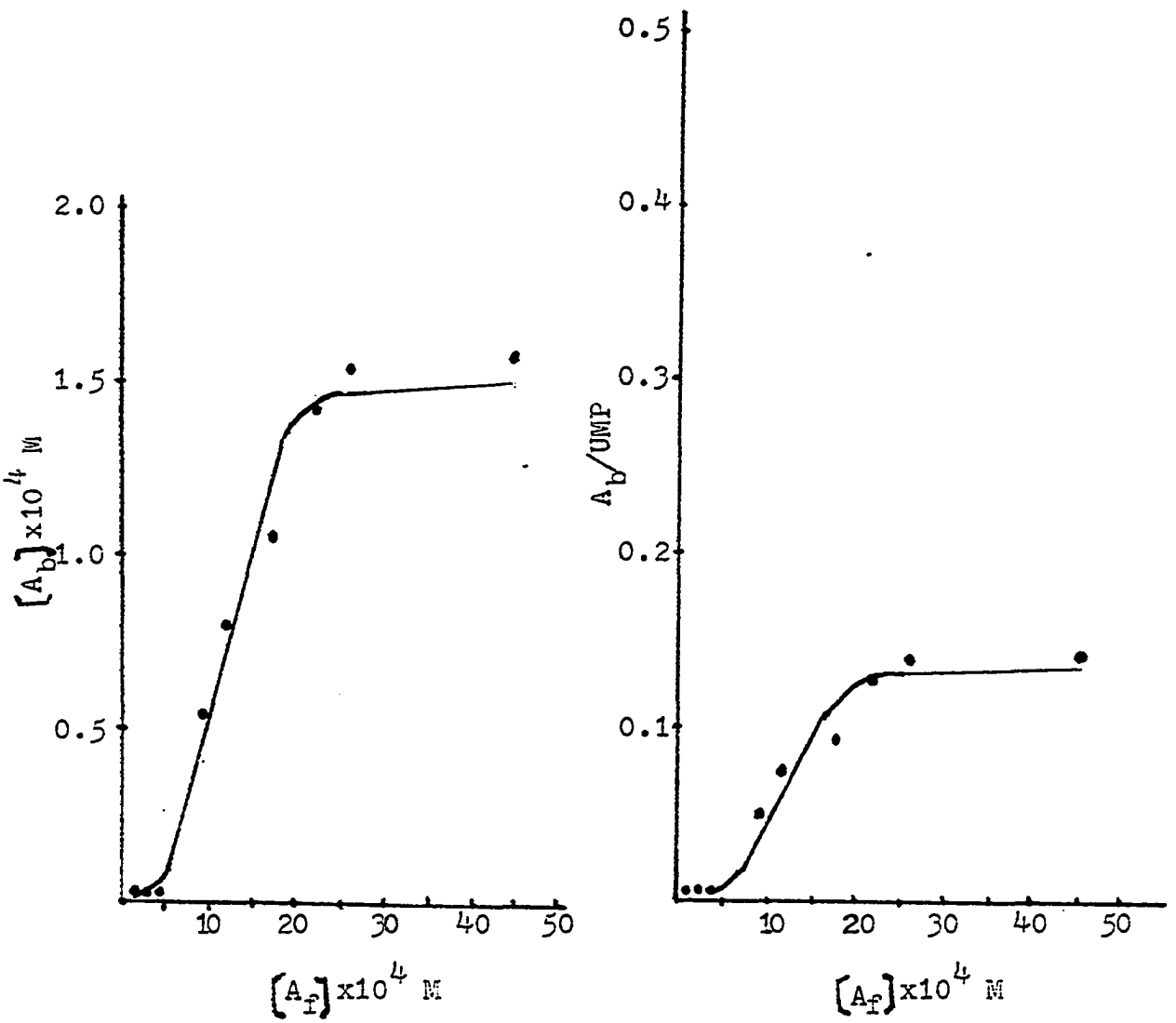


Table 36. Binding of Deoxyguanosine to Chemically Modified Poly C

Equilibrium Dialysis Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

initial [Poly C] = 1.59×10^{-3} M

after reaction: 7% C or 0.12×10^{-3} M CMP

93% U or 1.47×10^{-3} M UMP

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.43	3.30	0.21	1.65	0.00
0.64	4.92	0.32	2.46	0.00
0.90	6.92	0.45	3.46	0.00
1.05	8.07	0.53	4.07	0.00
1.30	10.00	0.65	5.00	0.00
1.92	14.76	0.95	7.30	0.16
2.56	19.69	1.27	9.76	0.16
3.78	29.07	1.88	14.46	0.15
5.12	39.38	2.55	19.61	0.15

12% binding of dG to C residues

Table 37. Binding of Adenosine to Chemically Modified Poly C

Equilibrium Dialysis Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

initial [Poly C] = 1.59×10^{-3} M

after reaction: 7% C or 0.12×10^{-3} M CMP

93% U or 1.47×10^{-3} M UMP

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.71	5.00	0.36	2.53	0.00
0.96	6.76	0.48	3.38	0.00
1.38	9.71	0.68	4.78	0.14
2.76	19.43	1.32	9.29	0.84
3.98	28.02	1.88	13.23	1.56
5.88	41.40	2.79	19.64	2.12
7.35	51.76	3.48	24.50	2.76
8.46	59.57	3.81	26.83	5.91
14.30	100.70	6.67	46.97	6.76

45% binding of Adenosine to U residues

Table 38. Binding of Adenosine to Chemically Modified Poly C

Computer Data

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

initial [Poly C] = 1.59×10^{-3} M

after reaction: 7% C or 0.12×10^{-3} M CMP

93% U or 1.47×10^{-3} M UMP

n	S	K_1	RMSD
1	7.59×10^{-4} M	152	8.66×10^{-5}
** 2	6.93×10^{-4} M	196	7.58×10^{-5}
3	6.86×10^{-4} M	219	7.81×10^{-5}
4	6.86×10^{-4} M	235	8.22×10^{-5}

** best value of n based on lowest RMSD

for n=2

$[A_b]$ exp x 10^4 M	$[A_b]$ calc x 10^4 M	A_b/UMP
0.00	0.03	0.000
0.00	0.06	0.000
0.14	0.13	0.008
0.84	0.57	0.057
1.56	1.22	0.106
2.12	2.69	0.144
2.76	3.92	0.187
5.91	4.48	0.402
6.76	6.88	0.459

Figure 52. Binding Isotherm for Adenosine:Modified Poly C

modification with 1M $NaNO_2$ at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

initial [Poly C] = 1.59×10^{-3} M

after reaction: 7% C or 0.12×10^{-3} M CMP

93% U or 1.47×10^{-3} M UMP

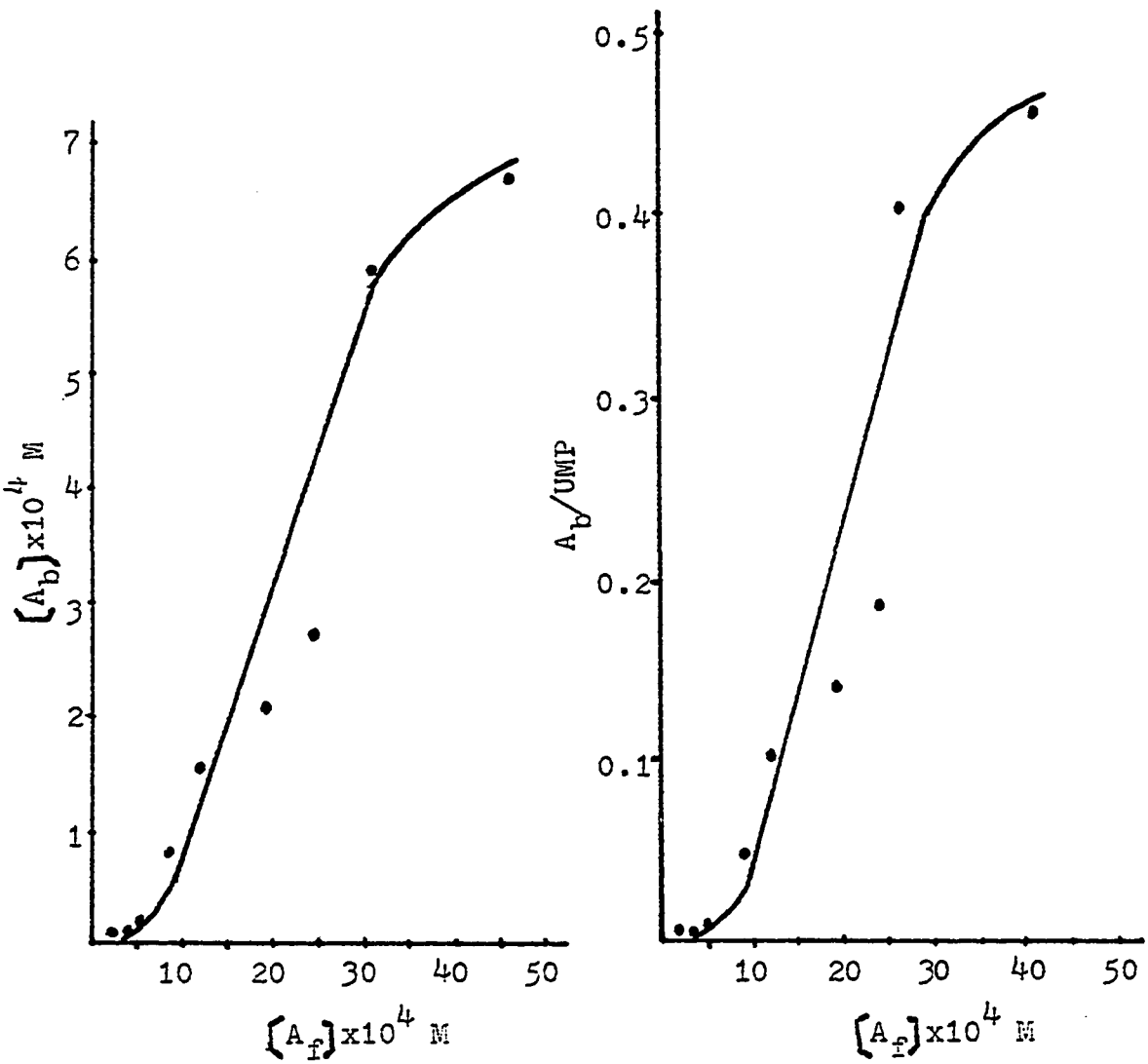


Table 39. Binding of Deoxyguanosine to Poly C,U Copolymer

Copolymer from Miles Labs.

Reported C:U base ratio: 1.0 : 1.3

Experimentally determined base ratio: 45% C : 55% U

$[CMP] = 0.6 \times 10^{-3}$ M $[UMP] = 0.7 \times 10^{-3}$ M

Equilibrium Dialysis Data

copolymer in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.39	3.00	0.19	1.46	0.08
0.64	4.92	0.30	2.30	0.32
0.92	7.07	0.40	3.07	0.92
1.05	8.07	0.45	3.46	1.15
1.32	10.15	0.57	4.38	1.39
1.91	14.69	0.86	6.61	1.46
2.50	19.23	1.16	8.92	1.39
3.72	28.61	1.77	13.61	1.38

23% binding of deoxyguanosine to C residues

Table 40. Binding of Deoxyguanosine to Poly C,U Copolymer.

Copolymer from Miles Labs.

Reported C:U base ratio: 1.0 : 1.3.

Experimentally determined base ratio: 45% C : 55% U

$[CMP] = 0.6 \times 10^{-3}$ M $[UMP] = 0.7 \times 10^{-3}$ M

Computer Data

copolymer in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$.

n	S	K_1	RMSD
3	1.43×10^{-4} M	1812	6.72×10^{-6}
4	1.41×10^{-4} M	1952	3.40×10^{-6}
** 5	1.40×10^{-4} M	2067	3.24×10^{-5}
6	1.39×10^{-4} M	2159	5.06×10^{-5}

** best value of n based on lowest RMSD

for n=5

$[dG_b]$ exp x 10^4 M	$[dG_b]$ calc x 10^4 M	dG_b/CMP
0.00	0.00	0.000
0.08	0.02	0.013
0.32	0.30	0.053
0.92	0.92	0.153
1.15	1.16	0.191
1.39	1.39	0.231
1.46	1.40	0.243
1.39	1.40	0.231
1.38	1.40	0.230

Figure 53. Binding Isotherm for Deoxyguanosine:Poly C,U.

Copolymer from Miles Labs

Reported C:U base ratio: 1.0 : 1.3

Experimentally determined base ratio: 45% C : 55% U

$[CMP] = 0.6 \times 10^{-3}$ M $[UMP] = 0.7 \times 10^{-3}$ M

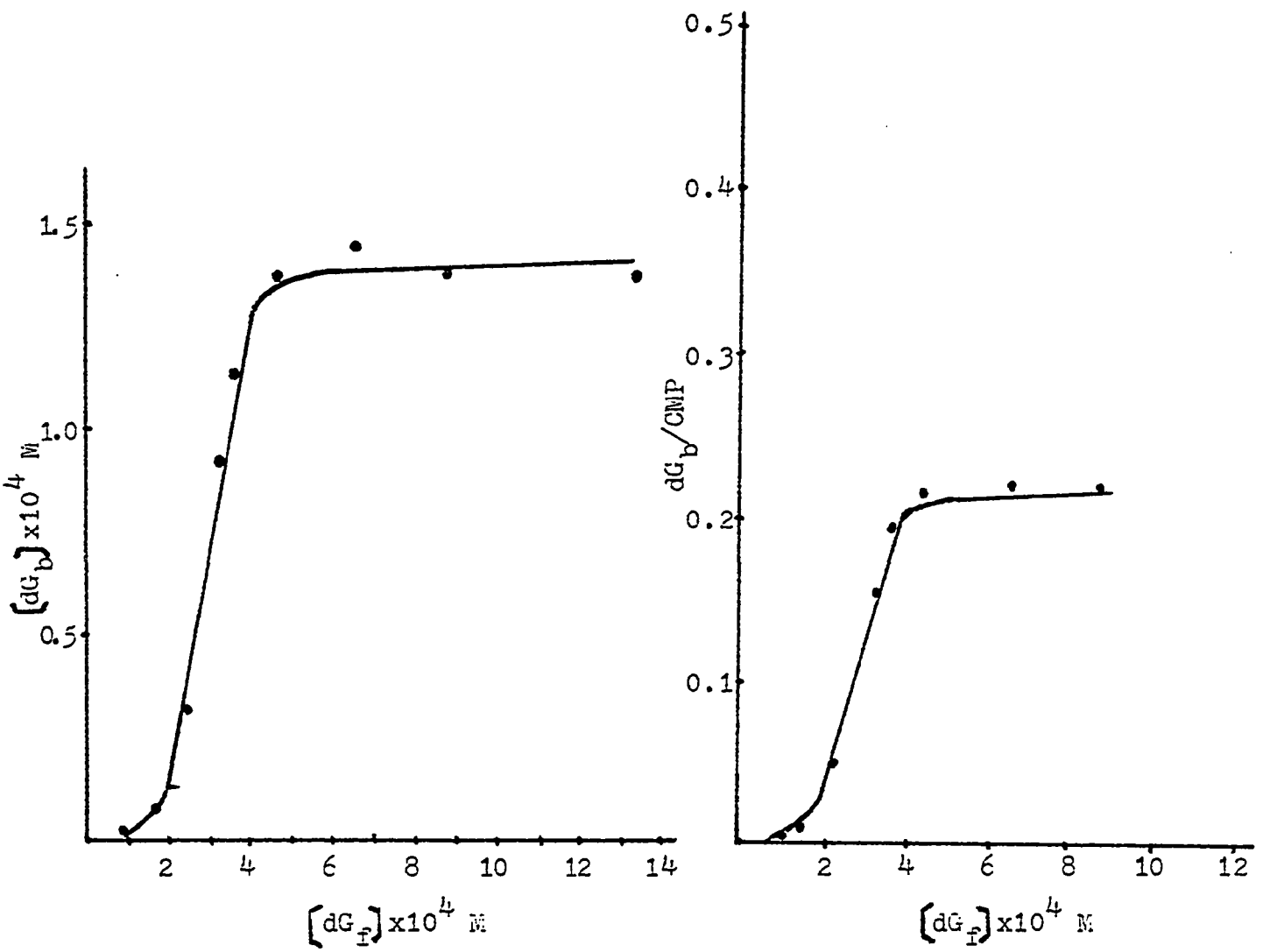


Table 41. Binding of Adenosine to Poly C,U Copolymer

Copolymer from Miles Labs

Reported C:U base ratio: 1.0 : 1.3.

Experimentally determined base ratio: 45% C : 55% U

$[CMP] = 0.6 \times 10^{-3}$ M $[UMP] = 0.7 \times 10^{-3}$ M

Equilibrium Dialysis Data

copolymer in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.44	3.09	0.22	1.54	0.00
0.90	6.33	0.45	3.16	0.00
1.28	9.01	0.64	4.50	0.00
1.40	9.85	0.70	4.92	0.00
2.80	19.71	1.36	9.57	0.56
4.28	30.14	2.09	14.71	0.71
5.94	41.83	2.92	20.56	0.71
7.23	50.91	3.55	25.00	0.91
8.40	59.15	4.14	29.15	0.85

12% binding of Adenosine to U residues

Table 42. Binding of Adenosine to Poly C,U Copolymer

Copolymer from Miles Labs

Reported C:U base ratio: 1.0 : 1.3

Experimentally determined base ratio: 45% C : 55% U

$[CMP] = 0.6 \times 10^{-3}$ M $[UMP] = 0.7 \times 10^{-3}$ M

Computer Data

n	S	K_1	RMSD
4	7.98×10^{-5} M	642	0.69×10^{-5}
5	7.95×10^{-5} M	680	0.67×10^{-5}
** 6	7.95×10^{-5} M	705	0.66×10^{-5}
7	7.95×10^{-5} M	726	0.67×10^{-5}

** best value of n based on lowest RMSD

for n=6

$[A_b]$exp x 10^4 M	$[A_b]$calc x 10^4 M	A_b/UMP
0.00	0.00	0.000
0.00	0.00	0.000
0.00	0.07	0.000
0.00	0.10	0.000
0.56	0.55	0.080
0.71	0.79	0.101
0.71	0.79	0.101
0.91	0.79	0.130
0.85	0.79	0.121

Figure 54. Binding Isotherm for Adenosine:Poly C,U.

Copolymer from Miles Labs

Reported C:U base ratio: 1.0 : 1.3

Experimentally determined base ratio: 45% C : 55% U

$[CMP] = 0.6 \times 10^{-3}$ M $\qquad$ $[UMP] = 0.7 \times 10^{-3}$ M

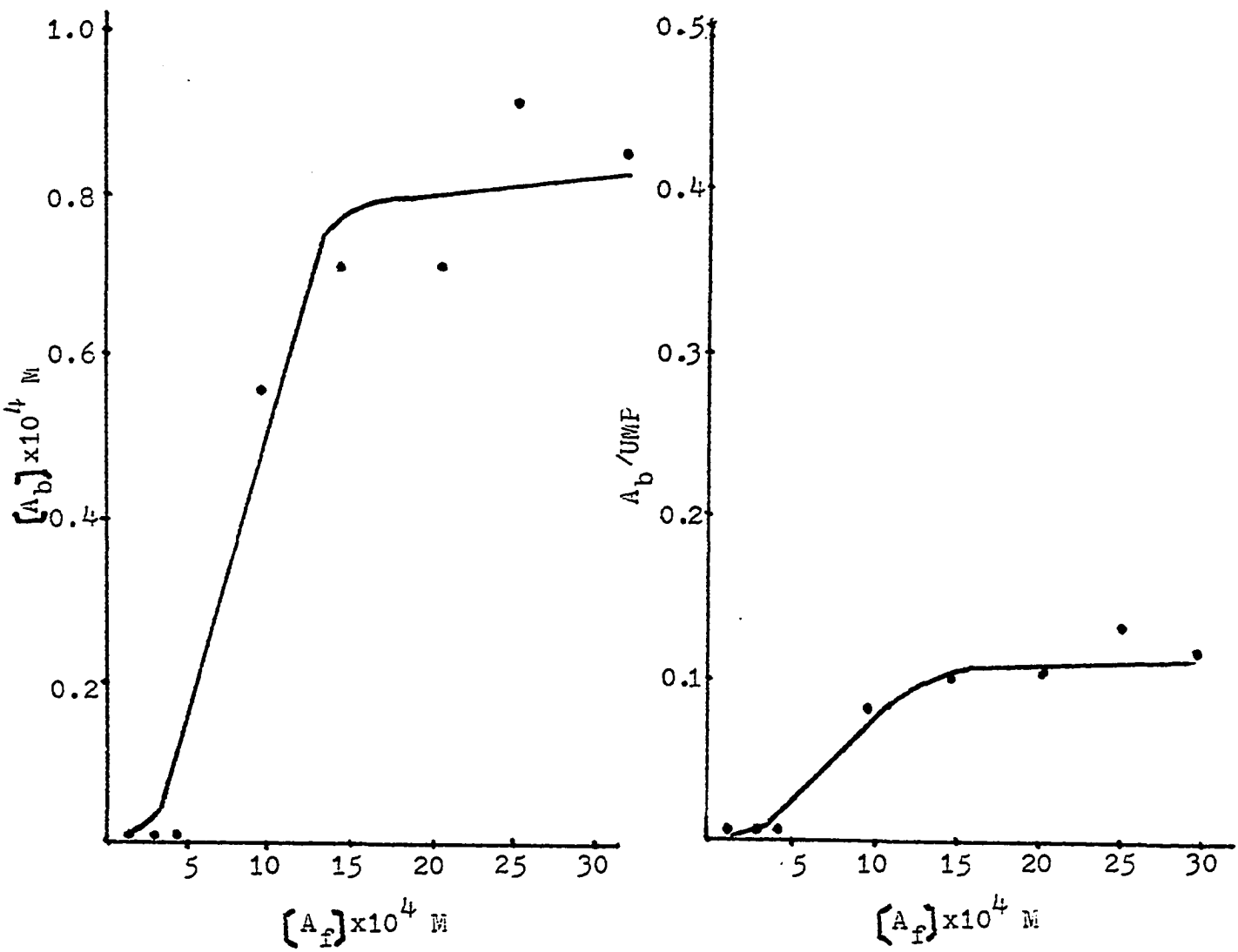


Figure 55. Comparison Binding Isotherms: Binding of Deoxyguanosine To Chemically Modified Poly C.

Equilibrium dialysis at 2°C for 48 hours in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$.

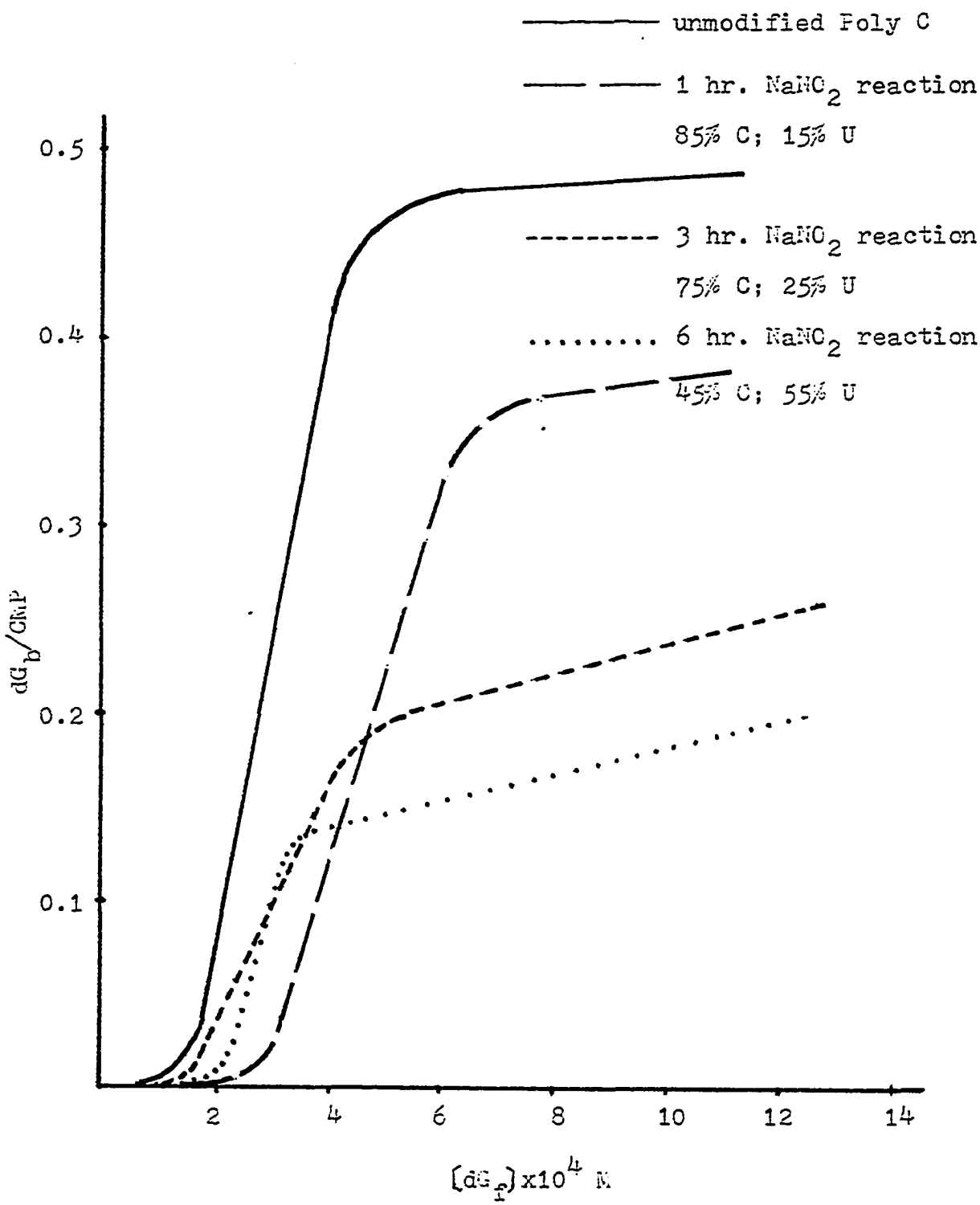


Figure 56. Comparison Binding Isotherms: Binding of Adenosine To Chemically Modified Poly C.

Equilibrium Dialysis at 2°C for 48 hours in 0.1 M Na acetate buffer, pH 4.1 + 1.0 mM $MgCl_2$.

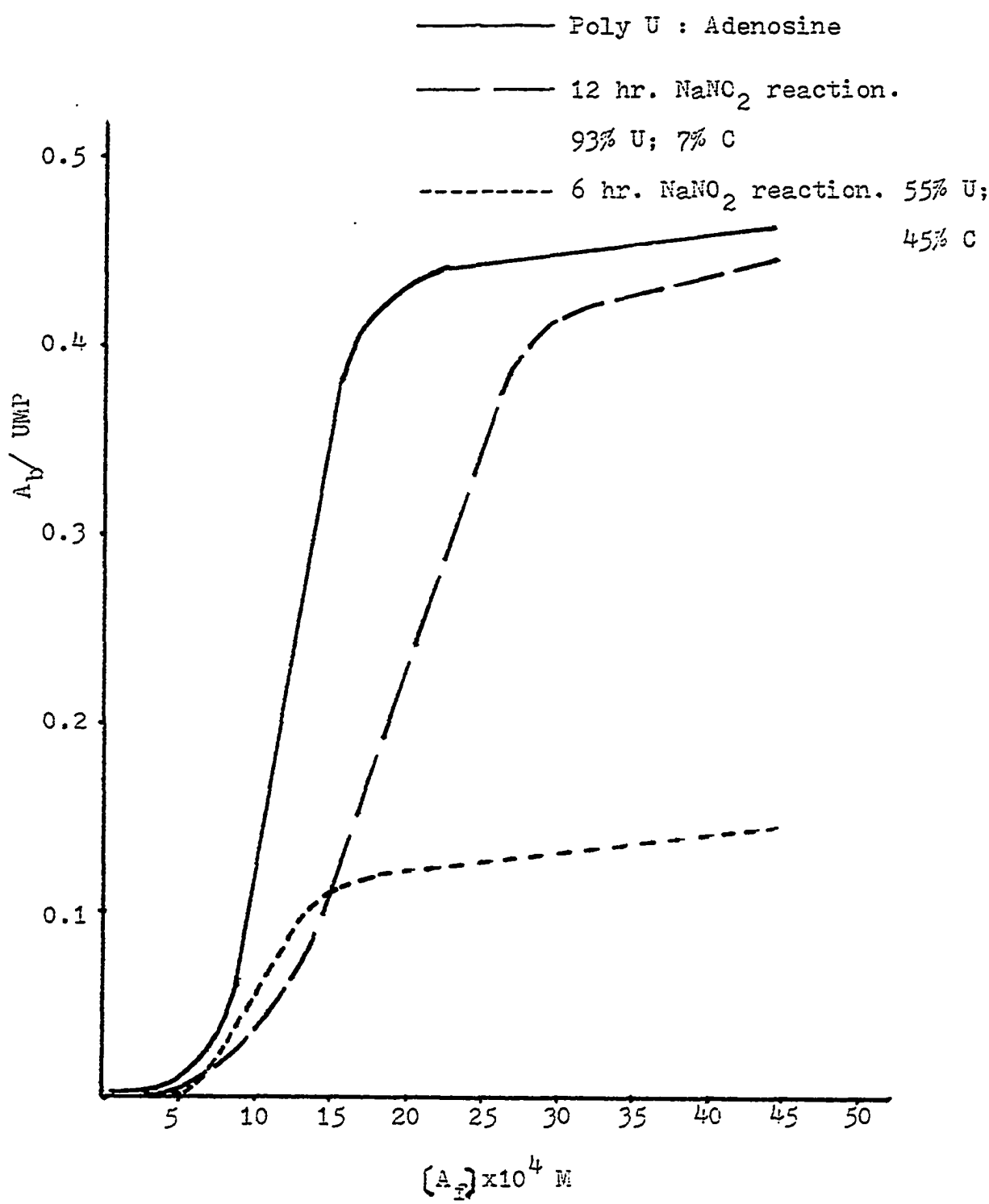


Table 43. Comparison of Binding Data From $NaNO_2$-Modified Poly C Systems

Unmodified Poly C:dG (in 0.1 M Na acetate buffer, 1.0 mM $MgCl_2$, pH 4.1)

n	K_1	$\Delta G(-RT\ln K_1)$
4	1819	-4.08 kcal/mole

1 hour $NaNO_2$ reaction with Poly C (same buffer as above, pH 4.1)

5	1303	-3.90 kcal/mole

3 hour $NaNO_2$ reaction with Poly C (same buffer as above, pH 4.1)

2	1413	-3.94 kcal/mole

6 hour $NaNO_2$ reaction with Poly C (same buffer as above, pH 4.1)

4	2017	-4.14 kcal/mole

Unmodified Poly U:A (in 0.1 M Na acetate buffer, 1.0 mM $MgCl_2$, pH 4.1)

n	K_1	$\Delta G(-RT\ln K_1)$
5	451	-3.32 kcal/mole

6 hour $NaNO_2$ reaction with Poly C (same buffer as above, pH 4.1)

2	354	-3.19 kcal/mole

12 hour $NaNO_2$ reaction with Poly C (same buffer as above, pH 4.1)

2	196	-2.87 kcal/mole

Chromatographic Separation of Reaction Products

-Nitrous Acid-

$NaNO_2$ action on cytosine residues in Poly C changes some of the cytosine residues to uracil residues.

(I) Solvent: 76% Ethanol

Stationary Phase: Cellulose on plastic

Chromatography method: Thin Layer Ascending

Means of Identification: Short wave UV light

Product R_f values: CMP: R_f = 0.46; UMP: R_f = 0.60

(II) Solvent: n-butanol:ethanol:water (80:10:25)

Stationary Phase: Cellulose on plastic

Chromatographic method: Thin Layer Ascending

Product R_f values: CMP: R_f = 0.34; UMP: R_f = 0.50

(III) Solvent: n-butanol:ethanol:water (80:10:25)

Stationary Phase: Whatman 3 MM paper, ascending paper chrom.

Product R_f values: CMP: R_f = 0.28; UMP: R_f = 0.40

(IV) Solvent: 76% Ethanol

Stationary Phase: Whatman # 1 paper dipped in 10% $(NH_4)_2SO_4$

Product R_f values: CMP: R_f = 0.57; UMP: R_f = 0.72

-Hydroxylamine-

(V) Solvent: n-butanol:ethanol:water (80:10:25)

Stationary Phase: Silica Gel Plates for TLC

Product R_f values: CMP: R_f = 0.47; 4-OH-aminocytidine-P R_f = 0.80; 6-hydroxyamino-5,6-dihydro-4-OH-amino cytidine-P R_f = 0.61.

Figure 57. ^{3}H-Poly C Elution on Sephadex G-25 Column.

5-^{3}H polycytidylate from Miles Labs:

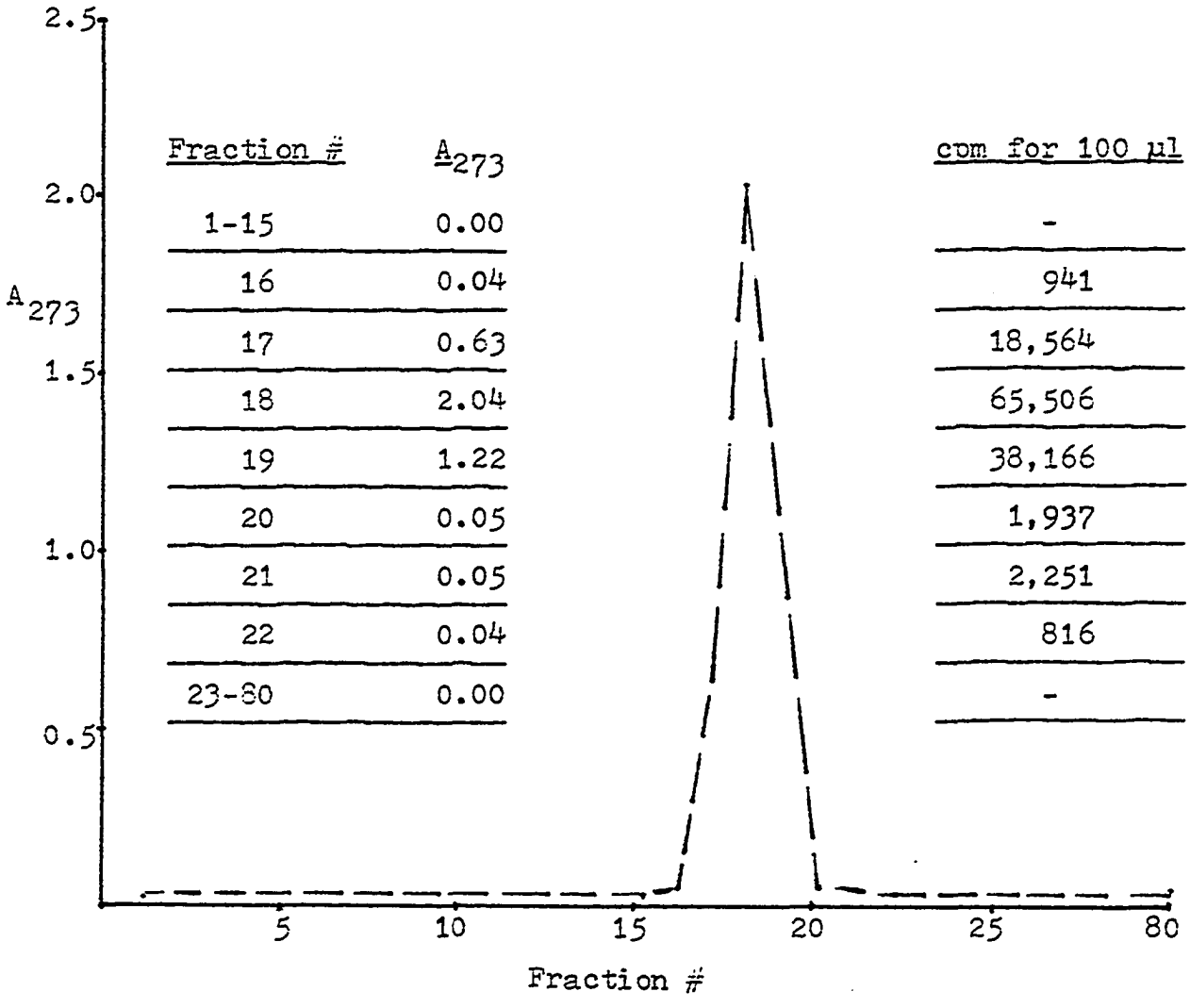
-specific activity: 29.2 μCi/μ mole P

-concentration: 10 uCi in 0.56 ml, in 50% EtOH

-calculated quantity: 11.8 μg Poly C/μCi

Initial Poly C solution: 14 ml Poly C in 0.1 M Na acetate buffer (pH 4.1) + 1.0 mM $MgCl_2$ and 0.5 ml ^{3}H-Poly C. 100 μl of this Poly C solution shows 87,028 cpm. Loaded 8 ml of this solution on Sephadex G-25 column and collected 80, 9 ml fractions using 0.1 M Na acetate buffer (pH 4.1) + 1.0 mM $MgCl_2$ as the eluent.

Fraction #	A_{273}	cpm for 100 μl
1-15	0.00	-
16	0.04	941
17	0.63	18,564
18	2.04	65,506
19	1.22	38,166
20	0.05	1,937
21	0.05	2,251
22	0.04	816
23-80	0.00	-



Modification of Poly C With Hydroxylamine at pH 6.0.

Initial ^{3}H-Poly C in 0.1 M Na acetate buffer at pH 6.0.

λmax = 267nm A_{267} = 0.97 Poly C = 1.44×10^{-3} M

100 ul of Poly C solution shows 33,403 cpm

Modification with 1M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

Sephadex G-25 Gel Filtration

Fraction # (8 ml)	A_{269}	cpm for 100 µl
1-18	0.00	-
19	0.16	5,794
20	0.34	12,440
21	0.11	4,253
22-80	0.00	-

Fractions 19-21 combined and concentrated as described in text.

Modified Poly C solution: λmax = 267nm, A_{267} = 0.65.

100 µl of Modified Poly C solution shows 23,890 cpm.

7 ml of modified Poly C dialyzed against 0.1 M Na acetate buffer at pH 4.1 + 1.0 mM $MgCl_2$. After dialysis: λmax = 273nm, A_{273} = 0.75

100 µl of Modified Poly C solution at pH 4.1 shows 20,217 cpm.

$$\frac{1.44\times10^{-3}\text{M}}{33{,}403\text{cpm}} = \frac{0.89\times10^{-3}\text{M}}{20{,}217\text{cpm}}$$

Total [cytidine] + [modified cytidine] residues in polymer = $\underline{8.9 \times 10^{-4}\ \text{M}}$

Alkaline hydrolysis to nucleotide units: 33% hypochromicity.

Silica Gel TLC reveals: 93% unmodified cytidine; 1% 4-OH-amino cytidine; and 6% 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine.

Table 44. Binding of Deoxyguanosine to Chemically Modified Poly C.

modification with 1 M NH_2OH in 0.1 M Na acetate buffer, pH 6.0 + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

Equilibrium Dialysis Data

conditions: at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$

Total [cytidine] + [modified cytidine] residues in polymer = 8.9×10^{-4} M.

93% are cytidine or 8.27×10^{-4} M

6% are 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 0.53×10^{-4} M

1% are 4-OH-aminocytidine or 0.089×10^{-4} M

$dG_i - A_{273}$	$[dG_i] \times 10^4$ M	$dG_f - A_{273}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.39	3.00	0.19	1.50	0.00
0.66	5.11	0.28	2.19	0.73
0.83	6.38	0.36	2.76	0.85
0.89	6.84	0.37	2.84	1.16
1.05	8.07	0.44	3.38	1.31
1.27	9.76	0.52	4.00	1.76
1.98	15.23	0.79	6.07	3.08
2.42	18.61	1.00	7.69	3.23
3.44	26.46	1.50	11.53	3.40

Migration Check: Modified Poly C:buffer A_{273} = 0.70:0.00

cpm = 18,839 : 211

Table 45. Binding of Deoxyguanosine to Chemically Modified Poly C.

Computer Data

modification with 1 M NH_2OH in 0.1 M Na acetate buffer, pH 6.0 + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

Equilibrium dialysis carried out at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$. Modification of the polymer yielded:

6% 6-hydroxyamino-5,6-dihydro-4-hydroxyamino cytidine or 0.53×10^{-4} M

1% 4-hydroxyaminocytidine or 0.089×10^{-4} M

93% unmodified cytidine or 8.27×10^{-4} M

n	S	K_1	RMSD
1	3.48×10^{-4} M	964	3.09×10^{-5}
** 2	3.35×10^{-4} M	1180	1.50×10^{-5}
3	3.25×10^{-4} M	1374	2.09×10^{-5}
4	3.21×10^{-4} M	1516	2.93×10^{-5}
5	3.18×10^{-4} M	1620	3.63×10^{-5}

** best value of n based on lowest RMSD.

for n=2

$[dG_b]$ exp x 10^4 M	$[dG_b]$ calc x 10^{-4} M	dG_b/CMP
0.00	0.10	0.000
0.00	0.26	0.000
0.73	0.58	0.088
1.16	0.99	0.140
1.31	1.38	0.158
1.76	1.84	0.212
3.08	2.99	0.372
3.23	3.32	0.390
3.40	3.35	0.410

Figure 58. Binding Isotherm for Deoxyguanosine:Modified Poly C

modification with 1M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

total [cytosine] in modified Poly C = 8.9×10^{-4} M

93% unmodified cytosine residues or 8.27×10^{-4} M

6% 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 0.53×10^{-4} M

1% 4-hydroxyaminocytidine or 0.09×10^{-4} M

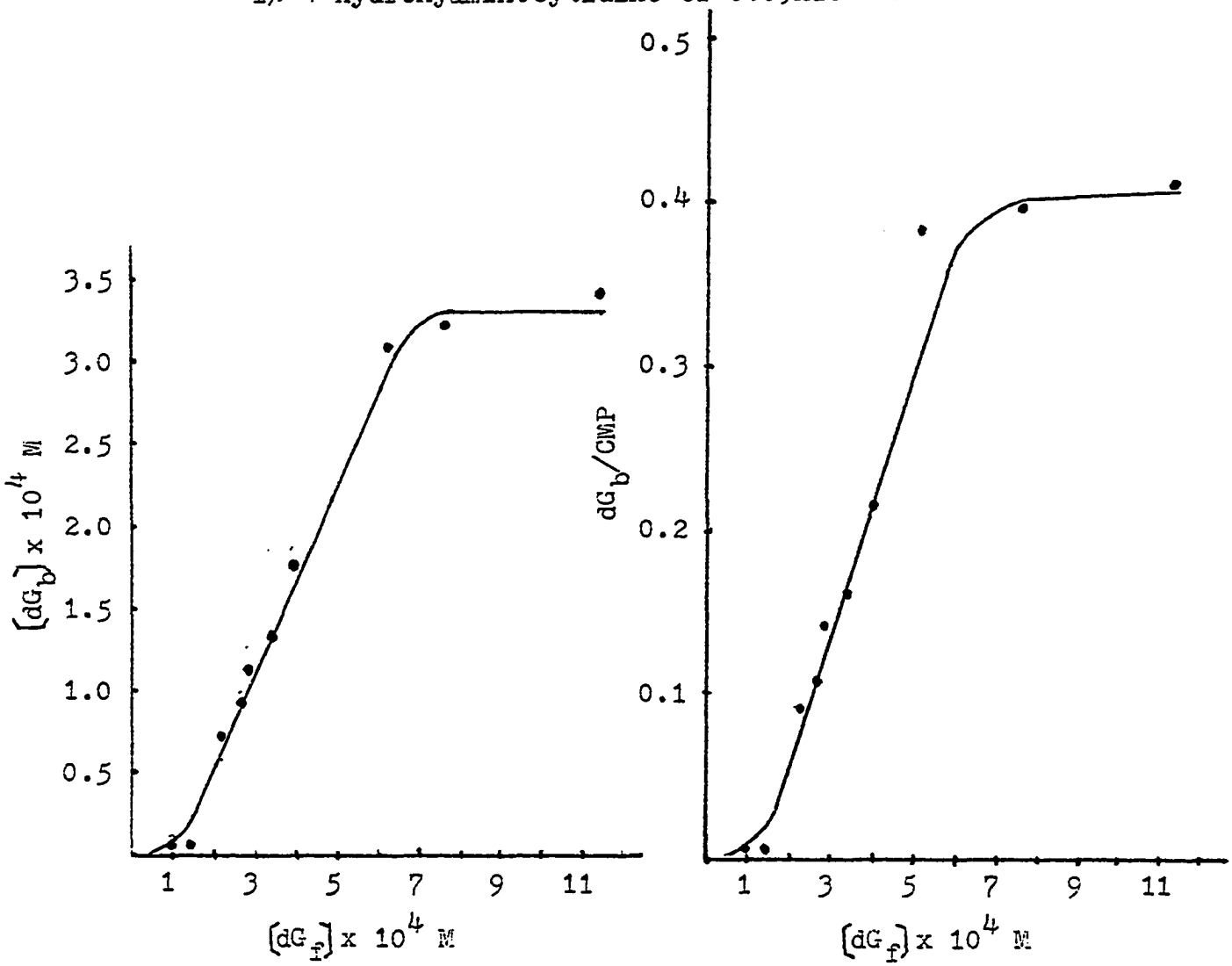


Table 46 Binding of Adenosine to Chemically Modified Poly C.

modification with 1 M NH_2OH in 0.1 M Na acetate buffer, pH 6.0 + 1.0 mM $MgCl_2$ at 37°C for 3 hours.

Equilibrium Dialysis Data

conditions: at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$

Total [cytidine] + [modified cytidine] residues in polymer = 8.9×10^{-4} M

93% are cytidine or 8.27×10^{-4} M

6% are 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 0.53×10^{-4} M

1% are 4-OH-aminocytidine or 0.09×10^{-4} M.

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.43	3.02	0.22	1.54	0.00
0.72	5.07	0.36	2.53	0.00
0.96	6.76	0.48	3.38	0.00
1.40	9.85	0.70	4.92	0.00
2.73	19.22	1.36	9.57	0.07
3.97	27.95	1.98	13.94	0.07
5.72	40.28	2.85	20.07	0.14
7.30	51.40	3.65	25.70	0.00
8.52	60.00	4.25	29.92	0.14

Modification of Poly C With Hydroxylamine at pH 6.0.

Initial ^{3}H-Poly C in 0.1 M Na acetate buffer at pH 6.0.

λmax = 267nm A_{267} = 0.97 [Poly C] = 1.44×10^{-3} M

100 µl of Poly C solution shows 34,578 cpm

Modification with 1 M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

Sephadex G-25 Gel Filtration

Fraction # (9 ml)	A_{269}	cpm for 100 µl
1-15	0.00	-
16	0.06	2,144
17	0.32	11,238
18	0.12	4,551
19	0.01	390
20-80	0.00	-

Fractions 16-18 combined and concentrated as described in text.

Modified Poly C solution: λmax = 267nm A_{267} =0.68

100 µl of modified Poly C solution shows 24,140 cpm.

7 ml of modified Poly C dialyzed against 0.1 M Na acetate buffer at pH 4.1 + 1.0 mM $MgCl_2$. After dialysis: λmax = 272nm, A_{272} = 0.75

100 µl of modified Poly C solution at pH 4.1 shows 22,095 cpm.

$$\frac{1.44\times10^{-3}\ \text{M}}{34{,}578\text{cpm}} = \frac{0.91\times10^{-3}\ \text{M}}{22{,}095\text{cpm}}$$

Total [cytidine] + [modified cytidine] residues in polymer = 9.1×10^{-4} M.

Alkaline hydrolysis to nucleotide units: 24% hypochromicity.

Silica Gel TLC reveals: 86% unmodified cytidine; 2% 4-OH-amino-cytidine; and 12% 6-hydroxyamino-5,6-dihydro-4-OH-amino cytidine.

Table 47. Binding of Deoxyguanosine to Chemically Modified Poly C

modification with 1 M NH_2OH in 0.1 M Na acetate buffer, pH 6.0 + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

Equilibrium Dialysis Data

conditions: at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$

Total [cytidine] + [modified cytidine] residues in polymer = 9.1×10^{-4} M.

86% are cytidine or 7.82×10^{-4} M

2% are 4-OH-aminocytidine or 0.18×10^{-4} M

12% are 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 1.09×10^{-4} M.

dG_i-A_{250}	$[dG_i] \times 10^4$ M	dG_f-A_{250}	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.37	2.84	0.18	1.42	0.00
0.60	4.61	0.26	1.96	0.69
0.79	6.07	0.33	2.53	1.00
0.87	6.69	0.35	2.69	1.31
1.05	8.07	0.43	3.30	1.47
1.28	9.84	0.49	3.80	2.23
3.45	26.53	1.56	12.00	2.53

Migration Check: Modified Poly C:buffer A_{272} = 0.75 : 0.00

cpm = 21,965 : 330

Table 48. Binding of Deoxyguanosine to Chemically Modified Poly C.

Computer Data

modification with 1 M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

Equilibrium dialysis carried out at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$. Modification of the polymer yielded:

12% 6-hydroxyamino-5,6-dihydro-4hydroxyaminocytidine, 7.82×10^{-4} M

2% 4-hydroxyaminocytidine, 0.18×10^{-4} M

86% unmodified cytidine, 7.82×10^{-4} M

n	S	K_1	RMSD
1	2.57×10^{-4} M	1347	3.58×10^{-5}
2	2.59×10^{-4} M	1578	2.08×10^{-5}
** 3	2.51×10^{-4} M	1803	1.77×10^{-5}
4	2.40×10^{-4} M	2002	2.22×10^{-5}
5	2.29×10^{-4} M	2181	2.72×10^{-5}

** best value of n based on lowest RMSD.

for n=3

$[dG_b]$ exp x 10^4 M	$[dG_b]$ calc x 10^4 M	dG_b/CMP
0.00	0.05	0.000
0.00	0.17	0.000
0.69	0.48	0.088
1.00	1.00	0.127
1.31	1.17	0.167
1.47	1.75	0.187
2.23	2.11	0.285
2.53	2.51	0.323

Figure 59. Binding Isotherm for Deoxyguanosine:Modified Poly C

modification with 1M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

total [cytosine] in modified Poly C = 9.1×10^{-4} M

86% unmodified cytosine residues or 7.82×10^{-4} M

12% 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine, 1.09×10^{-4} M

2% 4-OH-aminocytidine or 0.18×10^{-4} M.

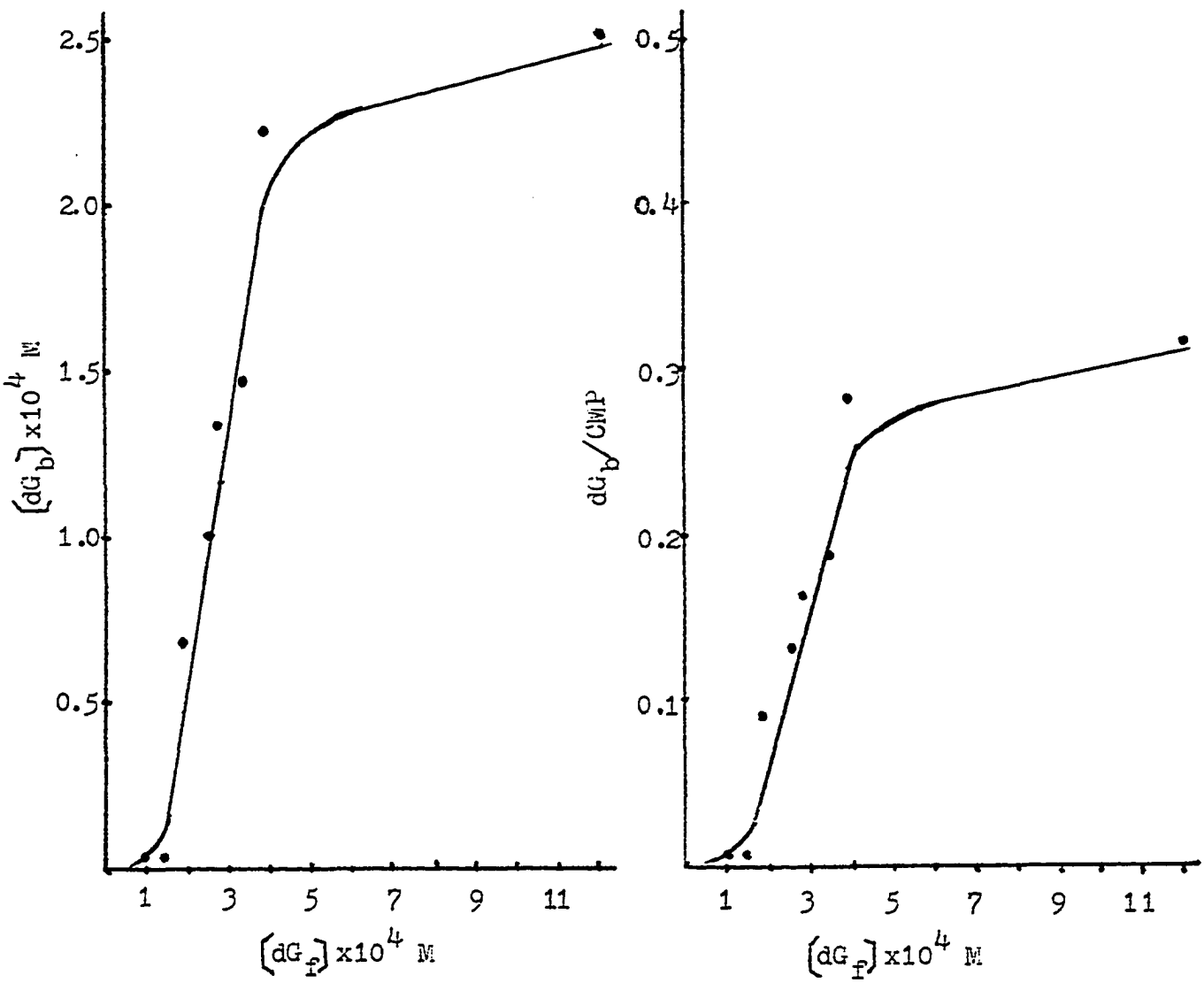


Table 49. Binding of Adenosine to Chemically Modified Poly C.

modification with 1 M NH_2OH in 0.1 M Na acetate buffer, pH 6.0 + 1.0 mM $MgCl_2$ at 37°C for 12 hours.

Equilibrium Dialysis Data

conditions: at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$

Total [cytidine] + [modified cytidine] residues in polymer = 9.1×10^{-4} M

86% are cytidine or 7.82×10^{-4} M

2% are 4-OH-aminocytidine or 0.18×10^{-4} M

12% are 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 1.09×10^{-4} M.

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.42	2.95	0.21	1.47	0.00
0.67	4.71	0.33	2.32	0.06
0.86	6.05	0.43	3.02	0.00
1.34	9.43	0.67	4.71	0.00
2.58	18.16	1.29	9.08	0.00
3.80	26.76	1.90	13.38	0.00
5.60	39.43	2.79	19.64	0.15
6.95	48.97	3.48	24.50	0.00
8.22	57.88	4.12	29.01	0.00

Modification of Poly C With Hydroxylamine at pH 6.0.

Initial ^{3}H-Poly C in 0.1 M Na acetate buffer at pH 6.0.

λmax = 267nm A_{267} = 0.97 [Poly C] = 1.44×10^{-3} M

100 µl of Poly C solution shows 35,837cpm

Modification with 1 M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 24 hours.

Sephadex G-25 Gel Filtration

Fraction # (9 ml)	A_{267}	cpm for 100 µl
1-16	0.00	-
17	0.05	3241
18	0.15	9731
19	0.05	3335
20-80	0.00	-

Fractions 17-19 combined and concentrated as described in text.

Modified Poly C solution: λmax = 267nm

6 ml of modified Poly C dialyzed against 0.1 M Na acetate buffer at pH 4.1 + 1.0 mM $MgCl_2$. After dialysis: λmax = 272nm, A_{272} = 0.43

100 µl of modified Poly C solution at pH 4.1 shows 20,298cpm.

$$\frac{1.44\times10^{-3}\ \text{M}}{35{,}837\text{cpm}} = \frac{8.15\times10^{-4}\ \text{M}}{20{,}298\text{cpm}}$$

Total [cytidine] + [modified cytidine] residues in polymer = 8.15×10^{-4} M

Alkaline hydrolysis to nucleotide units.

Silica Gel TLC reveals: 51% unmodified cytidine; 13% 4-OH-amino cytidine; and 35% 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine.

Table 50. Binding of Deoxyguanosine to Chemically Modified Poly C.

modification with 1 M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 24 hours.

Equilibrium Dialysis Data

conditions: at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$.

Total [cytidine] + [modified cytidine] residues in polymer = 8.15×10^{-4} M

51% are cytidine or 4.15×10^{-4} M

13% are 4-OH-aminocytidine or 1.05×10^{-4} M

35% are 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 2.85×10^{-4} M

$dG_i - A_{250}$	$[dG_i] \times 10^4$ M	$dG_f - A_{250}$	$[dG_f] \times 10^4$ M	$[dG_b] \times 10^4$ M
0.26	2.00	0.13	1.00	0.00
0.36	2.76	0.18	1.38	0.00
0.60	4.61	0.30	2.30	0.00
0.69	5.30	0.33	2.57	0.16
0.85	6.53	0.42	3.26	0.00
0.97	7.46	0.47	3.61	0.24
1.20	9.23	0.59	4.57	0.08
2.36	18.15	1.18	9.07	0.00
3.40	26.15	1.70	13.07	0.00

Table 51. Binding of Adenosine to Chemically Modified Poly C.

modification with 1 M NH_2OH at pH 6.0 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$ at 37°C for 24 hours.

Equilibrium Dialysis Data

conditions: at pH 4.1 in 0.1 M Na acetate buffer + 1.0 mM $MgCl_2$.

Total [cytidine] + [modified cytidine] residues in polymer = 8.15×10^{-4} M.

51% are cytidine or 4.15×10^{-4} M

13% are 4-OH-aminocytidine or 1.05×10^{-4} M

35% are 6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine or 2.85×10^{-4} M

$A_i - A_{257}$	$[A_i] \times 10^4$ M	$A_f - A_{257}$	$[A_f] \times 10^4$ M	$[A_b] \times 10^4$ M
0.43	3.02	0.21	1.51	0.00
0.71	5.00	0.35	2.50	0.00
0.92	6.47	0.46	3.23	0.00
1.34	9.43	0.67	4.71	0.00
2.68	18.87	1.33	9.36	0.06
3.84	27.04	1.89	13.30	0.44
5.31	37.39	2.63	18.52	0.35
6.85	48.23	3.40	23.94	0.35
8.02	56.47	3.97	27.95	0.57

CHAPTER IV

DISCUSSION AND CONCLUSIONS

A discussion of the chemistry of polynucleotide modification with known mutagenic agents involves not only the reactivity of the individual bases, but also the role of secondary structure and conformation of the polymer. The reactivity of particular chemical agents with the polynucleotide depends upon the extent of hydrogen bonding interactions as well as base-stacking interactions.

The reagents we have used for chemical modification of Poly C, nitrous acid and hydroxylamine, are known to be mutagenic in a number of biological systems as they react with the cytosine nucleus in polynucleotides to produce C → U or pseudo U (Ψ) transitions. As a model system we have used this homopolynucleotide (Poly C) since it has been shown to exist in both single-stranded and double-stranded conformations. The extension of experiments from homopolymer:monomer complexes to chemically modified homopolymer:monomer complexes were performed primarily to explore the effects of modification on the binding properties of deoxyguanosine and adenosine with modified Poly C.

Reaction of Nitrous Acid With Poly C

The reaction of Poly C with $NaNO_2$ was carried out at pH 4.1. At this pH Poly C exists primarily in a duplex conformation stabilized both by hydrogen bonding and base-stacking interactions. Although the results of the binding of deoxyguanosine with unmodified Poly C, and of adenosine with Poly U showed a preference for a 2:1 polymer:monomer stoichiometry, we thought that modification of cytosine units in Poly C with nitrous acid might encourage the formation of 1:1 complexes since the addition of the third strand to give 2:1 complexes would be statistically low. However, the data shows that this is probably not the case.

In control experiments (Part II), the interaction of adenosine with Poly C and of deoxyguanosine with Poly U showed no significant binding. These results convincingly demonstrate the hydrogen bonding specificity of the polymer:monomer interactions.

The modification of Poly C with nitrous acid essentially acts to convert the Poly C from a homopolymer to a Poly C,U copolymer. In the course of this reaction the secondary structure of the polynucleotide is altered since hydrogen bonding between cytosine residues in the double helix are disturbed as uracil residues emerge. In addition, base-stacking interactions are reduced since uracil-uracil and uracil-cytosine stacking energies are lower than cytosine-cytosine base-stacking energies. The general breakdown in the secondary structure of the polynucleotide is evidenced by the decreased hypochromicity upon HNO_2 modification.

The interaction of deoxyguanosine with nitrous acid-modified Poly C, as evaluated by equilibrium dialysis dialysis experiments, show that binding of deoxyguanosine was significant with 15% modification of Poly C (showing 0.41 molecules of dG_b/CMP at saturation). With 25% modification, the binding of the nucleoside to the modified polymer decreased to 0.21 molecules of dG_b/CMP. With 93% modification, no significant binding of dG to the polymer was observed.

The cooperative nature of the binding of dG to the modified polynucleotide remained quite apparent in each system, although a higher concentration of free monomer was necessary for initiation of stable complex formation, as compared to the unmodified Poly C: dG system. Upon observation of the concentration of dG_b at binding saturation (all being less than 0.41 m dG_b/CMP), we feel that the C+dG+C triplex is probably the predominant complex species present. Due to the necessity (in the unmodified Poly C: dG system) for a higher free monomer concentration for 1:1 complex formation, the probability of these complexes would probably be evident in a two-phase sigmoidal curve. This was not observed.

The binding of adenosine to chemically modified Poly C was not as appreciable as that found in deoxyguanosine binding. No significant binding of adenosine was observed upon 15% modification and very little binding upon 25% modification. With 55% modification, 0.13 molecules of adenosine were found to bind per UMP and with 93% modification, the binding data showed 0.41 A_b/UMP at saturation. A greater free monomer concentration was required

to initiate stable complex formation, similar to the findings in the modified Poly C:dG system. The cooperative-type sigmoidal binding curves were again apparent in these systems.

The results of these experiments demonstrate that stable complexes can be formed between Poly C,U copolymers (as produced through $NaNO_2$ modification of Poly C) and their complementary monomer nucleosides (deoxyguanosine and adenosine) under suitable conditions. It is clear that the complexes do not approach a 1:1 polymer:monomer stoichiometry, although sections of 1:1 complexes may be present. Instead, the individual nucleosides appear to complex from 0.1 to 0.4 of a molecule of its complementary base, suggesting the probability of 2:1 polymer: monomer complexes as the principal complex species. Assuming random sequences of C and U in the modified polymer, a possible structure for these complexes might involve regions of 2:1 C+dG+C or U+A+U complexes, and regions of non-complementarity where "looped-out" regions might contain 1:1 complexes.

Reaction of Poly C With Hydroxylamine

The reaction of Poly C with hydroxylamine was initially carried out at pH 4.1. It was found that after 24 hours at 37°C, using 1 M NH_2OH, the reaction yielded only 3% modification of the cytosine residues. The 3% modification products were all 4-OH-aminocytidine. Due to this extremely slow reaction it was decided to perform the reaction at pH 6.0, since at this pH Poly C exists primarily in a single-stranded conformation (stabil

ized primarily by cytosine base-stacking interactions). The polynucleotide in this single-stranded conformation was thought to be more susceptible to chemical attack by NH_2OH, as was found to be the case.

At pH 6.0, hydroxylamine action on Poly C was found to result in the formation of the bis-product (6-hydroxyamino-5,6-dihydro-4-OH-aminocytidine) as the predominant reaction product. The accessibility of chemical attack of NH_2OH on cytosine at the 5,6 double bond is apparently a characteristic of the cytosine residues in Poly C at pH 6.0 which is not common to cytosine residues in Poly C at pH 4.1. The addition reaction is rapidly followed by the displacement reaction at C4 to give the bis-product. The mono-substitution product, 4-OH-aminocytidine, was also found to be a product of the reaction, but it was formed at a substantially lower frequency.

Treatment of Poly C with NH_2OH at pH 6.0 for 3-24 hours resulted in 7-49% modification, 6-35% being accounted for by the formation of the bis-product. Thus, under these conditions, the formation of the bis-product dominated over the formation of the mono-product by a factor of approximately 5.

The formation of the bis-product destroys the aromaticity of the cytosine residues and thereby decreases or eliminates cytosine base-stacking in localized regions in the polymer. This acts to significantly break-down the secondary structure as base-stacking interactions are reduced and hydrogen bonding interactions are interrupted. The decrease in functional activity of the homopolymer (Poly C) is apparently the secondary result, as increased

modification proved to eliminate the binding of deoxyguanosine to the modified polymer.

4-OH-aminocytidine is suggested to be the mutational species responsible for C → pseudo U (Ψ) transition mutations in living systems, since this product displays the binding properties of uridine. The formation of this product probably does not alter base stacking interactions significantly, but does cause hydrogen bonding interaction alterations.

Equilibrium dialysis measurements of NH_2OH-modified Poly C with deoxyguanosine and adenosine were performed at pH 4.1. The results of these experiments are not easily explained due to the complexity of events affecting the secondary and tertiary structure of the polynucleotide. The results generally show a decrease and eventual failure of deoxyguanosine binding to Poly C as modification with NH_2OH increased. With 7% modification (6% bis-product) 0.4 molecules of dG were found to bind per CMP, while with 49% modification (35% bis-product) no significant binding of deoxyguanosine to the modified polymer was observed.

Adenosine was found to bind to a small degree with 49% modification (13% 4-OH-aminocytidine). This finding suggests that the presence of the mono-product may contribute to or be responsible for the binding of the nucleoside.

These experiments seem to support the contention that bis-product formation leads to inactivation of the genetic material by destroying secondary structure and thereby interrupting base stacking and hydrogen bond formation. In addition, the data suggests that the presence of 4-OH-aminocytidine encourages the

binding of adenosine residues. The complexity of this system warrents further investigation so that additional information can be obtained for a better explanation of the complexes formed.

BIBLIOGRAPHY

1. Langridge, R., and Rich, A., Nature 198, 725 (1963).

2. Nirenberg, H. W., and Matthaei, J. H., PNAS 47, 1588 (1961).

3. Speyer, J. F., and Lengyel, P., Proc. Nat. Acad. Sci. 48, 63 (1962).

4. Akinrimisi, E. O., Sander, C., and Ts'o, P., Biochemistry 2, 340 (1963).

5. Brahms, J., Maurizot, J. C., and Michelson, A. M., J. Mol. Biol. 25, 465 (1967).

6. Gray, Donald M., Biopolymers 13, 2087 (1974).

7. Gulik, A., Inoue, H., and Luzzati, V., J. Mol. Biol. 53, 221 (1970).

8. Fasman, G. D., Lindblow, C., and Grossman, L., Biochemistry 3, 1015 (1964).

9. Wrobel, A., Rabczenko, A., and Shugar, D., Acta Biochimica Polonica 17, 339 (1970).

10. Aylward, N. N., and Koenig, J. L., Macromolecules 3, 583 (1970).

11. Hartman, Karl A., and Rich, Alexander, Journal of the American Chemical Society 87, 2033 (1965).

12. Ts'o, P., Rapaport, S. A., and Bollum, F. J., Biochemistry 5, 4153 (1966).

13. Voet, D., Gratzer, W. B., Cox, R. A., and Doty, P., Biopolymers 1, 193 (1963).

14. Arnott, S., Chandrasekaran, R., and Leslie, A., J. Mol. Biol. 106, 735 (1976).

15. Steiner, R. F., and Beers, R. F., "Polynucleotides," Amsterdam; Elsevier (1961).

16. Michelson, A. M., "Progress in Nucleic Acid Research," Vol. 6, pp 84, (1967).

17. Cochran, W., Crick, F., and Vand, V., Acta Cryst. 5, 581 (1952).

18. Sarkar, P. K., and Yang, J. T., Biochemistry 4, 1238 (1965).

19. Green, G., and Mahler, H. R., Biochemistry 9, 368 (1970).

20. Marsh, R. E., Bierstedt, R., and Eichhorn, E. L., Acta Cryst. 15, 310 (1962).

21. Inman, R. B., J. Mol. Biol. 2, 624 (1964).

22. Sarocchi, M. T., Courtois, Y., and Guschlbauer, W., J. Biochem. 14, 411 (1970).

23. Guschlbauer, W., PNAS 54, 1441 (1967).

24. Kuhn, W., Ann. Rev. Phys. Chem. 9, 417 (1958).

25. Simmons, N. S., J. Am. Chem. Soc. 83, 4766 (1961).

26. Blout, E. R., J. Am. Chem. Soc., 84, 3193 (1962).

27. Goodman, M., Biopolymers 1, 371 (1963).

28. Gray, D. M., Biopolymers 13, 2087 (1974).

29. Tso, P., Helmkamp, G. K., and Sander, C., Biochim. Biophys. Acta 55, 584 (1962).

30. Helmkamp, G. K., and Tso, P., Biochim. Biophys. Acta 55, 601 (1962).

31. Tinoco, Ignacio, JACS 82, 6409 (1960).

32. Mahler, H. R., Kline, B., and Mehrotra, B. D., J. Mol. Biol. 9, 801 (1964).

33. DeVoe, H., and Tinoco, I., J. Mol. Biol. 4, 518 (1962).

34. Shugar, D., In "The Nucleic Acids," Vol. 3, pp. 54, ed. by Chargaff, E., and Davidson, J. N., (1960) New York, Academic Press.

35. Thomas, R., Soc. Chim. Biol. 82, 6409-(1953).

36. Laland, S. G., Lee, W. A., Overend, W. G., and Peacocke, A. R., Biochim. Biophys. Acta 14, 356 (1954).

37. Beaven, G. H., Holiday, E. R., and Johnson, E. A., In "The Nucleic Acids," ed. by E. Chargaff and J. N. Davidson, Vol. 1, pp. 493, New York, Academic Press (1955).

38. Porschke, D., and Eggers, F., Eur. J. Biochem. 26, 490 (1972).

39. Lawley, P. D., Biochim. Biophys. Acta 21, 481 (1956).

40. Rhodes, William, JACS 83, 3609 (1961).

41. Felsenfeld, G., and Hirchman, Z., J. Mol. Biol. 13, 407 (1965).

42. Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R., and Litt, M., PNAS 45, 482 (1959).

43. Katz, L., and Penman, S., J. Mol. Biol. 15, 220 (1966).

44. Rosenberg, J. M., Seeman, N. C., Roberta, O., and Rich, A., J. Mol. Biol. 104, 145 (1976).

45. Watson, J. D., and Crick, F. H. C., Nature 171, 964 (1953).

46. Podder, W. K., Biopolymers 11, 1395 (1972).

47. Hoogsteen, K., "Molecular Associations in Biology" edited by B. Pullman, 1968 Academic Press.

48. Haselkorn, R., and Fox, F., J. Mol. Biol. 13, 780 (1965).

49. Courtois, Y., Fromageot, P., and Guschlbauer, W., Eur. J. Biochem. 6, 493 (1968).

50. Inman, R. B., and Baldwin, R. L., J. Mol. Biol. 8, 452 (1964).

51. Kyogoku, Y., Lord, R. C., and Rich, A., Science 154, 518 (1966).

52. Shoup, R. R., Miles, H. T., and Becker, E. D., Biochem. Biophys. Res. Commun. 23, 194 (1966).

53. Howard, F. B., Frazier, J., Lipsett, M. N., and Miles, T., Biochem. Biophys. Res. Commun 17, 93 (1964).

54. Howard, F. B., Frazier, J., and Miles, T., PNAS 64, 451 (1969).

55. Sarocchi, M. T., and Guschlbauer, W., Eur. J. Biochem. 34, 232 (1973).

56. Pochon, F., and Michelson, A. M., PNAS 53, 1425 (1965).

57. Lipsett, M. N., Biochem. Biophys. Res. Commun. 11, 224 (1963).

58. Sarocchi, M. T., and Darlix, J. L., Eur. J. Biochem. 46, 481 (1974).

59. Tso, P., and Huang, W., Biochem. 7, 2954 (1968).

60. Lipsett, M. N., J. Biol. Chem. 239, 1256 (1965).

61. Howard, F. B., Frazier, J., Singer, M. E., and Miles, T., J. Mol. Biol. 16, 415 (1966).

62. Marck, C., Thiele, D., Schneider, C., and Guschlbauer, W., Nucleic Acid Res. 5, 1979 (1978).

63. Sarocchi, M. T., Courtois, Y., and Guschlbauer, W., J. Biochem. 14, 411 (1970).

64. Davies, R. J. H., and Davidson, N., Biopolymers 10, 1455 (1971).

65. Huang, W., and Tso, P., J. Mol. Biol. 16, 523 (1966).

66. Burr, J. G., McDowell, T. L., and Christian, S. D., Biochem. Biophys. Res. Commun. 56, 21 (1974).

67. Gellert, M., Lipsett, M., and Davies, D., Proc. Nat. Acad. Sci. 48, 2013 (1962).

68. Chantot, J., Haertle, T., and Guschlbauer, W., Biochemie 56, 501 (1974).

69. Guschlbauer, W., and Courtois, Y., FEBS Letters 1, 183 (1968).

70. Miles, H. T., Proc. Nat. Acad. Sci. 47, 791 (1961).

71. Thiele, D., and Guschlbauer, W., Biopolymers 10 (1971).

72. Ulbricht, T., Swan, R., and Michelson, A., Chem. Commun. 63 (1966).

73. Morgan, A. R., and Wells, R. D., J. Mol. Biol. 37, 63 (1968).

74. Zamenhof, Stephen, "Progress in Nucleic Acid Research," Volume 6, 1 (1967).

75. Davidson, N., "Introduction to Nucleic Acids," (1968).

76. Crick, F. H. C., "Progress in Nucleic Acid Research," Volume 1, 187 (1963).

77. Humphreys, G. O., Willshaw, G. A., Smith, H. R., and Anderson, E. S., Molecular gen. Genet. 145, 101 (1976).

78. Shugar, D., Huber, C. P., and Birnbaum, G. I., Biochim. Biophys. Acta 447, 274 (1976).

79. Cohn, W. E., "Progress in Nucleic Acid Research and Molecular Biology," Volume 16 (1976).

80. Shapiro, R., and Pohl, H., Biochemistry 7, 448 (1968).

81. Budowsky, E. I., Mutation Research 27, 1 (1975).

82. Vanderbilt, A. S., and Tessman, I., Genetics 66, 1 (1970).

83. Phillips, J. H., and Brown, D. M., "Progress in Nucleic Acid Research," Volume 7 (1967).

84. Singer, B., and Fraenkel-Conrat, H., "Progress in Nucleic Acid Research," Volume 9 (1969).

85. Fraenkel-Conrat, H., and Singer, B., Biochim. Biophys. Acta 262, 264 (1972).

86. Singer, B., and Fraenkel-Conrat, H., "Progress in Nucleic Acid Research," Volume 9 (1969).

87. Schuster, H., and Schramm, G., Naturforsch. 13, 697 (1958).

88. Mundry, K. W., and Gierer, A., Vererbungsl. 89, page 614 (1958).

89. Gierer, A., and Mundry, K. W., Nature 182, 1,457 (1958).

90. Zimmerman, F. K., Mutation Research 39, 127 (1977).

91. Wittmann, H. G., Vererbungsl. 93, 491 (1962).

92. Veldhuisen, G., Poelman, M. C., and Coehn, J. A., Biochim. Biophys. Acta 161, 115 (1968).

93. Vielmetter, W., and Wieder, C. M., Naturforsch. 14, 312 (1959).

94. Howard, B. D., and Tessman, I., J. Mol. Biol. 9, 372 (1964).

95. Konicek, J., and Malek, I., Folia Microbiol 15, 48 (1970).

96. Nilson, L. R., Ljung, A., and Uhnoo, B., Hereditas 67, 293 (1971).

97. Litman, R., and Taylor, E., Compt. Rend. 249, 838 (1959).

98. Alper, Mark, and Ames, Bruce, J. of Bacteriology 121, 259 (1975).

99. Johnson, W. B., Mutation Research 27, 17 (1975).

100. Shapiro, Robert, "Progress in Nucleic Acid Chemistry," Volume 8, (1968).

101. Tessman, I., Poddar, R. K., and Kumar, S., J. Mol. Biol. 9, 352 (1964).

102. Singer, B., and Fraenkel-Conrat, H., Virology 60, 485 (1974).

103. May, M. S., Dissertation Abstracts 30, 3,021 (1969).

104. Bellemare, G., Jordan, B. R., Serra, J. R., and Monier, R., Biochemie 54, 1,453 (1972).

105. Mishra, A. K., Roy P., and Das, S. K., Current Science 44, 267 (1975).

106. Postel, E. H., and Goodgal, S. H., J. of Bacteriology 94, 1,802 (1967).

107. Robinson, D. J., J. Gen. Virol. 18, 215 (1973).

108. Blumauerova, M., Callieri, D. A., Stajner, K., and Vanek, K., Folia Microbiol. 19, 133 (1974).

109. Kodama, M., Umeda, M., and Tsutsui, T., Mutation Research 40, 119 (1976).

110. Rustia, Mario, Cancer Research 34, 3,232 (1974).

111. Takahashi, M., Ikegami, M., and Osaki, T., Bull. Univ. Osaka Pref. 2, 1 (1971).

112. Bose, S., and Naskar, S. K., Bull. Botan. Soc. Bengal 29, 49 (1975).

113. Reddy, T. P., Reddy, C. S., and Reddy, G. M., Ind. J. of Exp. Biol. 11, 222 (1973).

114. Hutchinson, F., and Stein, J., Mole. gen. Genet. 152, 29 (1977).

115. Kiang, M. C., and Halloran, G. M., Mutation Research 33, 373 (1975).

116. Dhillon, K. S., and Dhillon, T. S., Mutation Research 22, 223 (1974).

117. Herrington, M. B., and Takahashi, I., Mutation Research 20, 275 (1973).

118. Augustine, V. J., Palanichamy, K., and Siddiq, E. A., Radiation Botany 15, 267 (1975).

119. Brogger, Anton, Hereditas 69, 19 (1971).

120. Germanov, A., B., Parasiuk, N. A., and Sokolov, M. I., Acta Virol. 17, 377 (1973).

121. Malling, H. V., Hereditas 68, 219 (1971).

122. Salganik, R. I., Vasjunina, E. A., Poslovina, A. S., and Andreeva, I. S., Mutation Research 20, 1 (1973).

123. Tiraby, J. G., and Fox, M. S., Genetics 77, 449 (1974).

124. EPA Report: "Potential Industrial Carcinogens and Mutagens" (May 1977).

125. Fresse, E., Bautz, E., and Fresse, E. B., Proc. Natl. Acad Sci. 47, 845 (1961).

126. Verwoerd, D. W., Kohlhage, H., and Zillig, W., Nature 192, 1,038 (1961).

127. Budowsky, E. I., Sverdlov, E. D., Shibaeva, R. P., Monastyrskaya, G. S., and Kochetkov, N. K., Biochim. Biophys. Acta 246, 300 (1971).

128. Schalke, P. M., and Hall, C. D., J. Chem. Soc. Commun., 391 (1976).

129. Blackburn, G. M., Jarvis, S., Ryder, M. C., and Solan, V., J. Chem. Soc., 370 (1975).

130. Budowsky, E. I., Sverdlov, E. D., and Spasokukotskaya, T. N., Biochim. Biophys. Acta 287, 195 (1972).

131. Phillips, J. H., and Brown, D. M., "Progress in Nucleic Acid Research," Volume 7, 349 (1967).

132. Singer, B., and Fraenkel-Conrat, H., "Biological Effects of Polynucleotides," edited by R. F. Beers and W. Braun (1971), Springer-Verlag.

133. Kochetkov, N. K., and Budowsky, E. I., "Progress in Nucleic Acid Research," Volume 9 (1969).

134. Bresler, S. E., Kalinin, V. L., and Perumov, D. A., Mutation Research 5, 1 (1968).

135. Singer, B., and Fraenkel-Conrat, H., Biochemistry 9, 3,694 (1970).

136. Krieg, David R., "Progress in Nucleic Acid Research," Volume 2 (1963).

137. Wilson, R. G., and Caicuts, M. J., J. Biol. Chem. 241, 1,725 (1966).

138. Brown, D. M., and Phillips, J. H., J. Mol. Biol. 11, 663 (1965).

139. Brown, D. M., and Schell, P., Nucleotides (1964).

140. Brown, D. M., Hewlins, J. E., and Schell, P., J. Chem. Soc., 1,925 (1968).

141. Phillips, J. H., and Brown, D. M., J. Mol. Biol. 21, 405 (1966).

142. Phillips, J. H., and Brown, D. M., J. Mol. Biol. 12, 816 (1965).

143. Lawley, P. D. J. Mol. Biol. 24, 75 (1967).

144. Small, G. D., and Gordon, M. P. J. Mol. Biol. 34, 281 (1968).