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OPTICAL PROPERTIES OF ADENYLIC ACID AND ITS OLIGOMERS

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Special Thesis

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# OPTICAL PROPERTIES OF ADENYLIC ACID AND ITS OLIGOMERS

Stephen L. Davis

Ph. D. Thesis

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## Table of Contents

ABSTRACT..... v

INTRODUCTION..... 1

MERCURIC BINDING TO NUCLEOTIDES..... 5

A Summary of Previous Work..... 5

Preliminary Experiment..... 9

Interaction of Adenylic Acid Oligomers With Mercuric Chloride: Experimental..... 20

Reaction of Mercuric Chloride With Adenylic Acid.... 31

Chloride Release..... 39

Nature of the Spectral Shift..... 50

Summary of Mercuric Binding to AMP..... 51

Mercuric Chloride Binding to ApAp..... 52

The Determination of Stoichiometry in the ApAp-$HgCl_2$ Complex Formation..... 65

Mercuric Chloride Binding by Heterodimers..... 74

The Binding of Ap Trimer by Mercuric Chloride..... 85

The Binding of Polyadenylic Acid by $HgCl_2$..... 91

Summary of Mercuric Chloride Ap Oligomer Binding.... 93

DENATURATION OF ADENYLIC ACID DIMER..... 94

Denaturation by Ethanol..... 94

Methods..... 97

Results..... 97

Discussion..... 100

Summary..... 115

Denaturation of the Dimer by Trimethyl Phosphate.... 116

Table of Contents (Continued)

General Properties of Nucleosides in Trimethyl Phosphate ..... 124

LOW TEMPERATURE EFFECTS ..... 139

Theory ..... 139

Spectra From Ice Samples ..... 153

Methods ..... 153

ApAp and AMP ..... 156

Thymine Dimer ..... 163

Summary ..... 168

Light Absorption from Ethanol Solutions at Low Temperatures ..... 173

Methods ..... 173

ApA and AMP ..... 175

Spectra of Other Purines ..... 177

Spectra of Some Pyrimidines ..... 193

Summary ..... 202

CONCLUSIONS ..... 203

APPENDIX A ..... 205

Chromatography Preparation of Adenylic Acid Oligomers ..... 205

APPENDIX B ..... 214

Characterization of Some Adenylic Acid Oligomers ..... 214

Extinction Coefficients of Oligomers ..... 214

References ..... 223

# OPTICAL PROPERTIES OF ADENYLIC ACID AND ITS OLIGOMERS

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## ABSTRACT

Each of the nucleotide molecules possesses characteristic physical properties which are altered when the molecule is linked into a polymeric chain. Among the properties so altered are the absorption and rotation of light, the degree of complex formation with certain metal ions, and the degree of conformational change which occurs upon going from an aqueous to a non-aqueous solvent environment.

One could, theoretically, prepare a linear array of nucleotides of length n, composed of some arbitrarily chosen sequence of molecules. It was more practical to restrict our experiments to the monomer units, several dimer units, and longer polymers, the latter composed only of adenylic acid residues. Our experiments emphasized some of the differences in properties between the monomer and dimer of adenylic acid.

The dimer of adenylic acid forms complexes with mercuric chloride more readily than does the monomer, and longer polymers of adenylic acid, more readily still.

As one changes the solvent environment of adenylic acid dimer from water to binary solutions composed of ethanol/water or trimethyl phosphate/water, the spectral characteristics of

the dimer come to resemble those of the monomer. We investigated this phenomenon in terms of conformational changes likely involved.

Theoretically, one ought to be able to observe splittings in the optical absorption bands of a molecule composed of two like, light absorbing units. One cannot observe such a splitting at room temperature in the absorption spectrum of the adenylic acid dimer. We utilized several methods of low temperature spectroscopy in an endeavor to resolve the predicted splitting.

It proved feasible with some of our experimental procedures to look at properties of other pertinent molecules (all constituents of nucleic acids, or analogs of such constituents.) We did not hesitate to do so.

# INTRODUCTION

Nucleic acids are biological polymers of the greatest importance. They subserve the functions of cellular reproduction and of protein synthesis.

The nucleic acids are built from nucleotide molecules which are linked together by phosphodiester bonds.[1] There are a number of kinds of nucleotide molecules. The physical properties, and biological function of the polymer depend upon the sequence into which the nucleotide units are ordered. Given a sequence of nucleotide units, and some defined solvent environment (a virus, the nucleus or cytoplasm of a living cell, a test tube) a nucleic acid necessarily assumes a particular physical conformation. This conformation is the result of the interactions between the nucleic acid and the molecules of its environment, as well as intramolecular interactions among the nucleotide residues of which the polymer is composed.[2]

When in its native conformation, the nucleic acid can carry out some particular biological process (or processes) for which its presence is vital. One is thus led to study, in artificial systems, the sorts of interactions which can occur between nucleotides and molecules of their solvent environment. Likewise by looking at properties of small polymers of nucleotides (dimers, trimers, etc.) one may begin to get an idea of the intramolecular interactions which occur in nucleic acids.

The simplest system from which to glean knowledge of intramolecular interactions is one in which all the monomer building blocks are the same. A large portion of our experimental work involves the study of differences in behavior between the monomer, adenylic acid and its dimer.

Katz was the first to study the interactions of nucleic acids with mercuric ions.[4] Yamane and Davidson showed the great differences in the reactivity of mercuric ion with nucleic acids and with their separated monomer residues.[5] We thought it would be interesting to look at the reactivity of mercuric ion with polymers of adenylic acids of varying chain lengths, viz. the monomer, dimer and trimer.

In the course of work on the mercuric interactions with adenylic acid oligomers, we also looked at the mercuric interactions with several heterodimer molecules in an attempt to clarify some points about the reaction mechanism.

The conformations of biological polymers (proteins and nucleic acids) give rise to characteristic optical absorption and rotation spectra. The optical characteristics are greatly altered when the solvent environment of the molecules is changed.[6,7] The effect of solvent changes upon the spectra of the nucleotide monomers, e.g., adenylic acid, on the other hand, are real but hardly spectacular. The dimer of adenylic acid exhibits spectral characteristics vastly different from those of the monomer. How would solvent changes effect the conformation of the dimer and hence its spectral characteristics? Is it necessary to have a long polymer before solvent changes

will have large effects, or will such changes equally effect a short polymer like the dimer or trimer?

We employed two main solvent systems for studying the adenylic acid dimer in non-aqueous environments, ethanol/water, and trimethyl phosphate/water. We studied, in addition, the general effects of a trimethyl phosphate solvent upon the spectra of several nucleoside molecules.

In observing the optical absorption spectrum of the adenylic acid dimer, the most readily apparent difference between its spectrum and that of the monomer is the diminished integrated intensity of the 260 mu electronic absorption band in the dimer (per unit residue of monomer). Theoretically, there should be yet another observable effect of putting two similar light absorbing molecules together in a dimer. One should be able to observe a splitting in the electronic absorption band due to a coulombic interaction between the light absorbing portions of the nucleotides.[8] At room temperature this splitting is not observable. Perhaps by going to low temperatures, we could resolve such a splitting. Such a splitting, if found could give us a better idea of the strength of the base-base interactions between the adenine groups of the two adenylic acid residues in the adenylic acid dimer.

Our low temperature work was of two sorts. One involved spectral changes at liquid nitrogen temperatures occurring in molecules when the latter had as their solvent environment, thin blocks of ice. In addition to looking at spectral changes

occurring with adenylic and its dimer under these circumstances, we also looked at the absorption spectra of a number of constituents of nucleic acids. The most interesting of this latter work involved the spectral changes occurring with thymine molecules under the experimental conditions. These changes correlated very nicely with the work done by Wang on thymine dimer formation.[3] Our other low temperature solvent system was ethanol near -100°C in which we took the spectra of a number of nucleic acid constituents and analogs.

## MERCURIC BINDING TO POLYNUCLEOTIDES

### A Summary of Previous Work

Katz performed the first experiments involving the complexing of nucleic acids with mercury (II) ions.[4] He found that the viscosity of solutions of DNA was decreased at relatively low concentrations of Hg(II), and that the electrophoretic and light-scattering properties of such solutions were likewise changed. It appeared from his measurements that the DNA molecules aggregated to form dimers, but that there was a great decrease in the size of each individual DNA molecule. The size of the dimer aggregate turned out to be no larger than that of the uncomplexed molecule. Furthermore, he found that all the physical properties of his original solutions were restored upon adding sufficient KCN to bind with the mercuric ions.

When Katz used oxyacid salts of mercuric as a complexing agent (mercuric nitrate, or mercuric acetate) he obtained, upon their addition to DNA solutions, an appreciable amount of precipitation.[4]

With an added amount of $HgCl_2$ such that the total mercuric/nucleotide residues was equal to 1.15, Katz found that .47 moles of this mercuric was bound per nucleotide. His first thoughts upon the matter were that the binding was at the phosphate groups. Other metal ions, even at concentrations, 10x that of the mercury chloride did not lead to any such drastic changes in physical properties of DNA solutions.

Thomas demonstrated that the ability of $HgCl_2$ to so markedly alter the physical properties of DNA solutions was due to the ability of this compound to bind, not to the phosphate groups, but rather to the nucleotide bases.[11] He demonstrated this very simply by showing the marked change in the UV spectra of the solutions upon the addition of $HgCl_2$. Thomas demonstrated that when the $HgCl_2$/nucleotide ratio was less than .6, an isosbestic point occurred amongst a series of DNA solutions, titrated with mercuric chloride.[11] With a ratio between .6 and 10, a second and different isosbestic point appeared. This situation, Thomas attributed to the occurrence of two types of reaction sites upon the DNA molecule.[11] Yamane and Davidson subsequently found similar behavior for a variety of DNA molecules.[5] Thomas speculated that the $HgCl_2$ reacted either with the amino groups of the nucleotides, or possibly with their pi-electron system.[11]

Just as Katz showed the reversibility of the mercuric induced changes in solutions of DNA, so Thomas found that the optical changes, caused by the addition of mercuric could be completely reversed by decomplexing the mercuric with KCN.[4,11]

Perhaps the most convincing evidence shown by Dove and Yamane of the reversibility of the mercuric complexing is that transforming activity of E. coli DNA can be regained by removing added mercuric from this DNA with appropriate agents.[24] Katz and Santilli showed, similarly, that RNA from tobacco mosaic virus reacts reversibly with $HgCl_2$, both in its physical

characteristics as well as in its biological property of infectivity.[25] RNA shows similar optical changes when it is complexed with $HgCl_2$. These include red shifts in the spectra, and at low concentrations of mercuric chloride, isosbestic points.

As they added $HgCl_2$ [or $Hg(ClO_4)_2$] to solutions of DNA Yamane and Davidson observed the simultaneous release of protons.[5] At the initial addition of the metal, a maximum of two protons were released per $HgCl_2$ [or $Hg(ClO_4)_2$] added. This ratio declined as increasing mercuric was added to the solutions, but it must be kept in mind that metal addition to the solutions, not to the nucleotides, was the parameter being measured. Since the structure of the DNA double helix is completely restored upon removing the mercuric, one must postulate that the two strands of the double helix were never completely disrupted. One possibility which the authors suggested could account for protonation data, as well as for the reversibility of the reaction was a cross strand linkage of the mercuric between the $N_3$ of thymidine residues, and the amino nitrogen of adenosine groups, displacing in this fashion two protons. They likewise postulated some similar linking between C and G groups. Katz did an extensive analysis of the data of Yamane and Davidson which led him to believe that the initial mercuric caused a shift in the two strands of the double helix.[5,26] This shift occurs in such a fashion that two thymidine molecules come into register, and are cross-linked by a mercuric ion.

When Yamane and Davidson added chloride to remove mercuric from DNA, they found that solutions became less acidic as the DNA took up its lost protons.[5]

The release of protons in binding mercuric ion to thymidine or guanidine clearly seems to be from the $N_3$ of the former and from $N_1$ of the latter.[17] But the site of binding in adenosine and in cytidine is much less clear.

Unlike mercuric dihalides, or the mercuric cation, the methylmercuric cation, $CH_3Hg^+$, seem able to coordinate only one ligand.[29] Gruenwedel and Davidson used this cation to complex solutions of DNA. Unlike the mercuric dihalides and the mercuric cations, it ought not to be able to cross-link the two strands of a DNA double helix. And, indeed, although the methyl-mercuric can be removed from the DNA, the double helix structure is permanently destroyed.[30]

## Preliminary Experiment

Yamane and Davidson studied the reversible mercuric ion binding to polynucleotides as well as to the adenosine molecule.[5] The difference in the behavior of the mercuric ion towards the monomer and polymer forms of the nucleotides was very pronounced. The complex formation of mercuric ion with short oligomers of adenylic acid should display characteristics somewhere between what was seen in the polymeric and monomeric nucleotide forms. A preliminary set of experiments to test this hypothesis showed that it was correct.

We used a commercial preparation of AM(5')P from Pabst Laboratories.[9] The polyadenylic acid used was likewise a commercial preparation, from Miles Laboratories.[10] The dimer, trimer, and tetramers of adenylic acid (ApA, ApApA, ApApApA) were a gift from Myron Warshaw of our lab, who had previously prepared them by chromatographic procedures. All the samples used in this preliminary study were in solutions containing .1M $NaClO_4$, and buffered with acetate/acetic acid, .01M/.001M, to a pH of 5.7. Each sample contained roughly $5x10^{-5}$M adenosine residue, to which, for complex formation study, we added $5x10^{-4}$ M $HgCl_2$. Spectral changes occurring as a result of complex formation were recorded with a Cary 15 spectrophotometer and a Cary 60 spectropolarimeter. The reference cell of the spectrophotometer contained a like amount of mercuric chloride as in the sample cell as well as the buffer and the sodium perchlorate.

The results of these experiments are illustrated in Figs. 1 through 9. Each figure contains two spectra, one with and the other without mercuric chloride present. The absorption spectra, especially, give a rough idea of the relative binding abilities these oligomers have with mercuric chloride. The estimate may be improved by observing the ratio of the absorption peaks at 285 and 260 mu. At the former wavelength, the absorption is due primarily to the complex species which has formed with the mercuric chloride. The degree of mercuric binding is easily seen to increase as the oligomer is lengthened.

The conformations which the oligomers of adenylic acid, and especially which the polymer of this molecule possess lead to a large amount of optical rotation in the near uv. Binding of mercuric chloride is seen to disrupt that conformation, and greatly reduce the optical rotation.

Previous workers have shown that agents which complex mercuric ion strongly, will reverse the binding of the metal to polynucleotides.[4,5,11] Likewise, our observed spectral changes were reversible by .1N NaCl. Furthermore, .2N perchloric acid reverses the binding of the mercuric chloride.

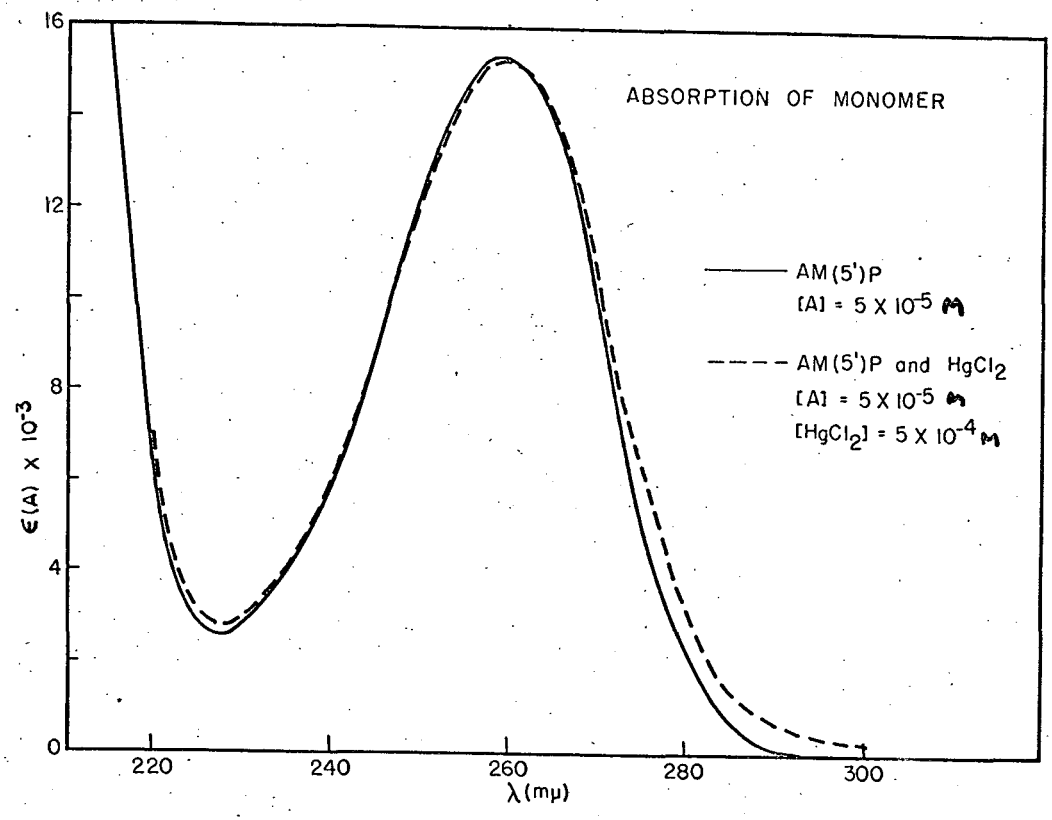



Figure 1. Optical absorption of adenylic acid, with mercuric as a complexing ag-
agent, and alone.

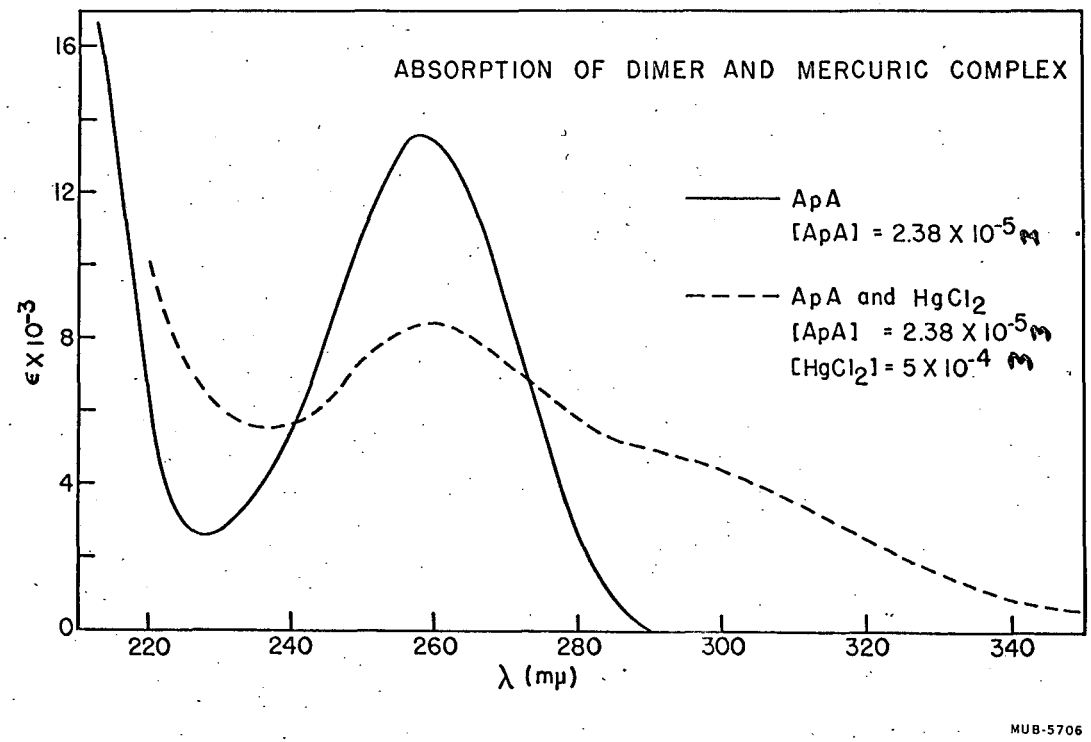



Figure 2. Optical absorption of ApA, with and without mercuric chloride as a complexing agent.

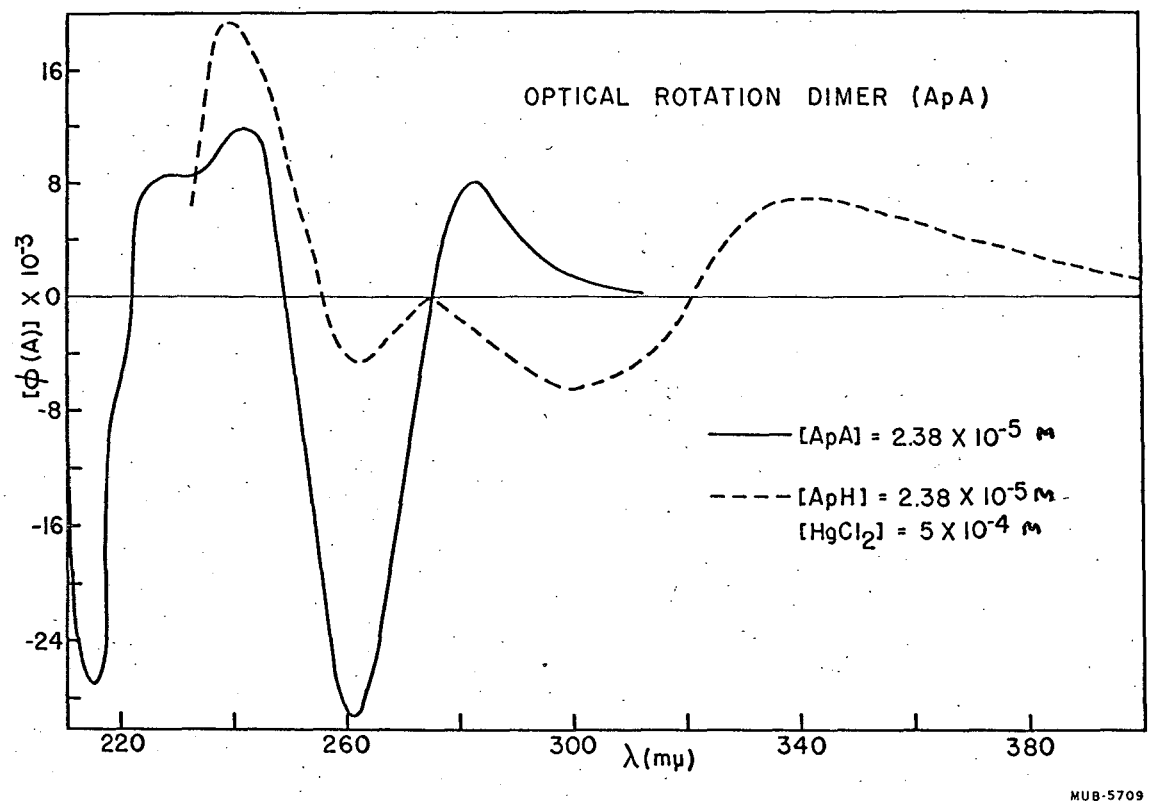



Figure 3. Optical rotatory dispersion of ApA, with and without mercuric chloride as a complexing agent.

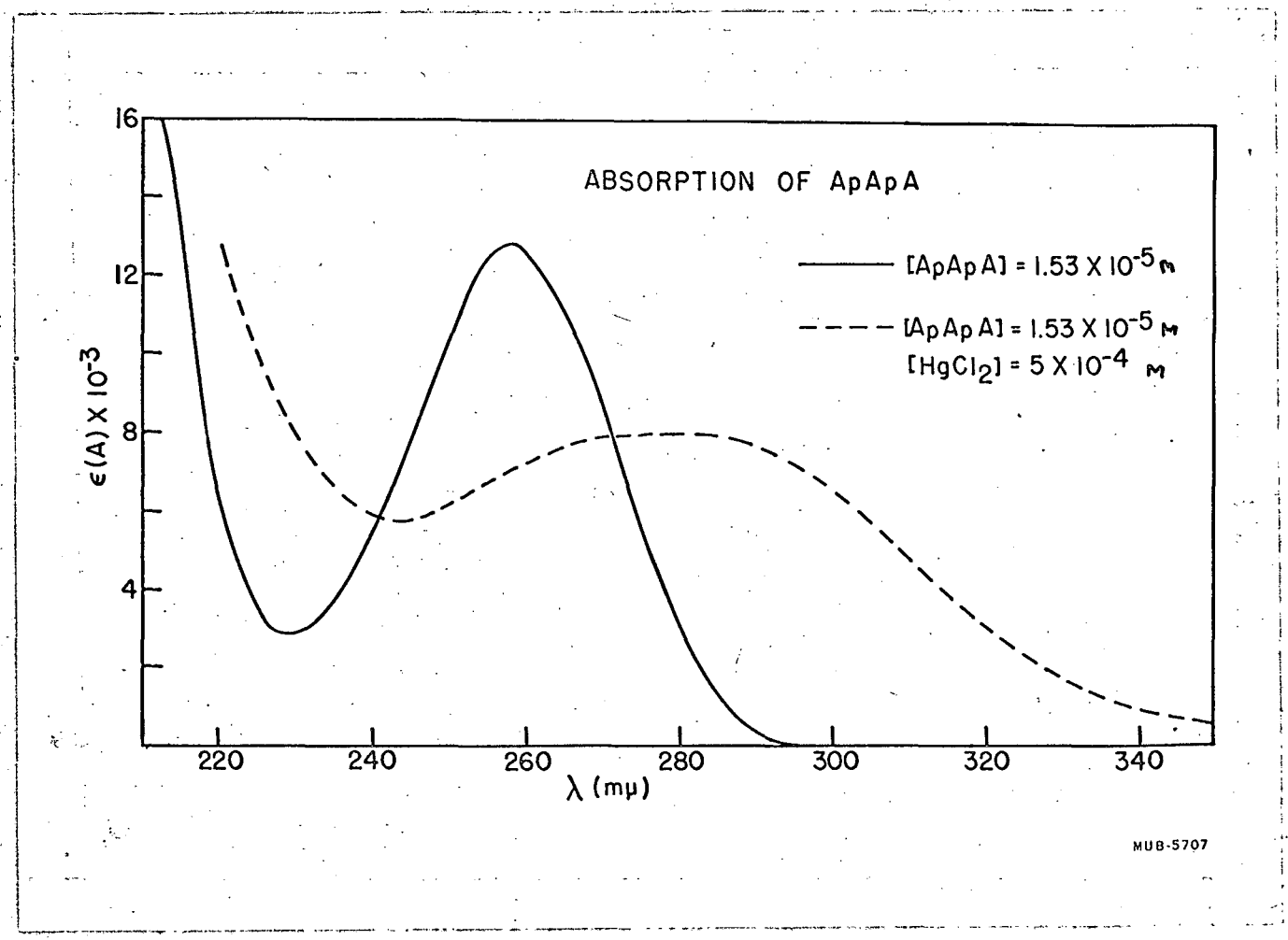



Figure 4. Optical absorption of ApApA, with and without mercuric chloride as a complexing agent.

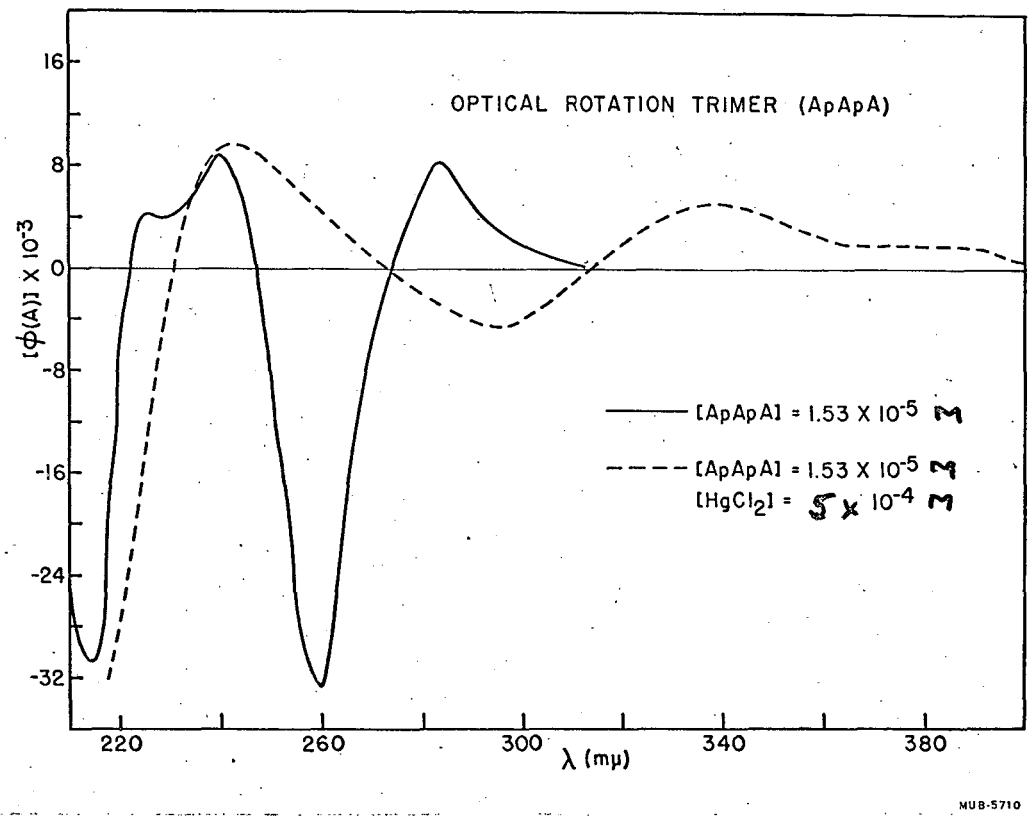



Figure 5. Optical rotatory dispersion of ApApA, with and without mercuric chloride as a complexing agent.

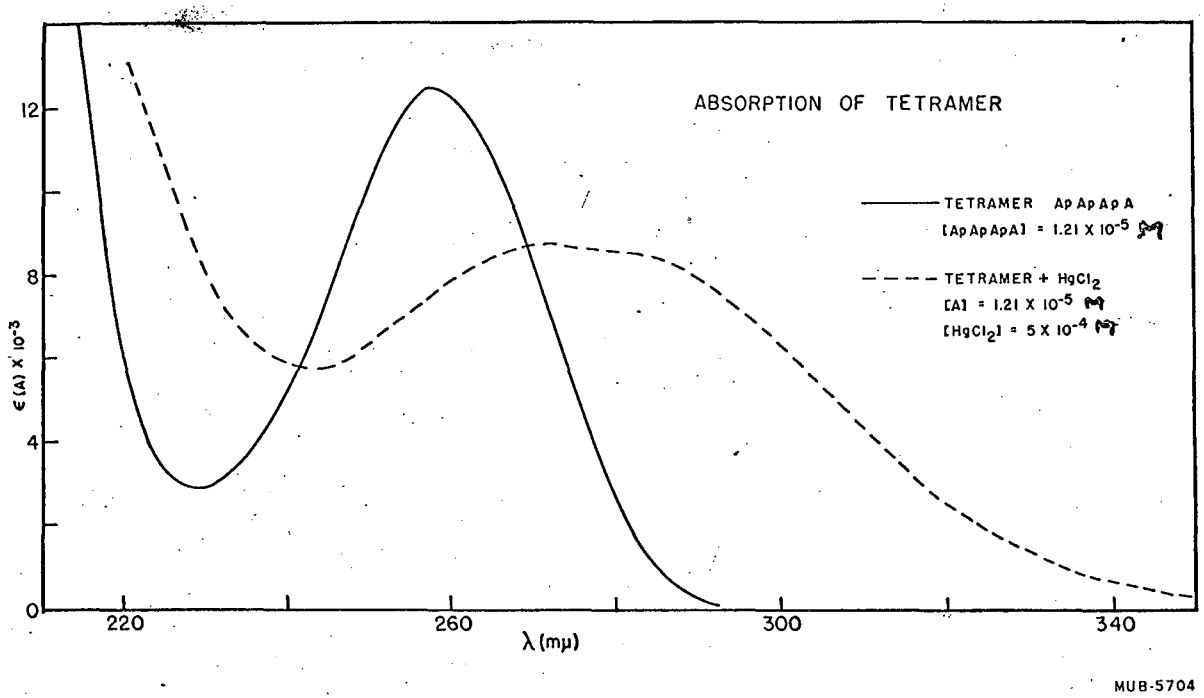



Figure 6. Optical absorption of $(Ap)_3A$, with and without mercuric chloride as a complexing agent.

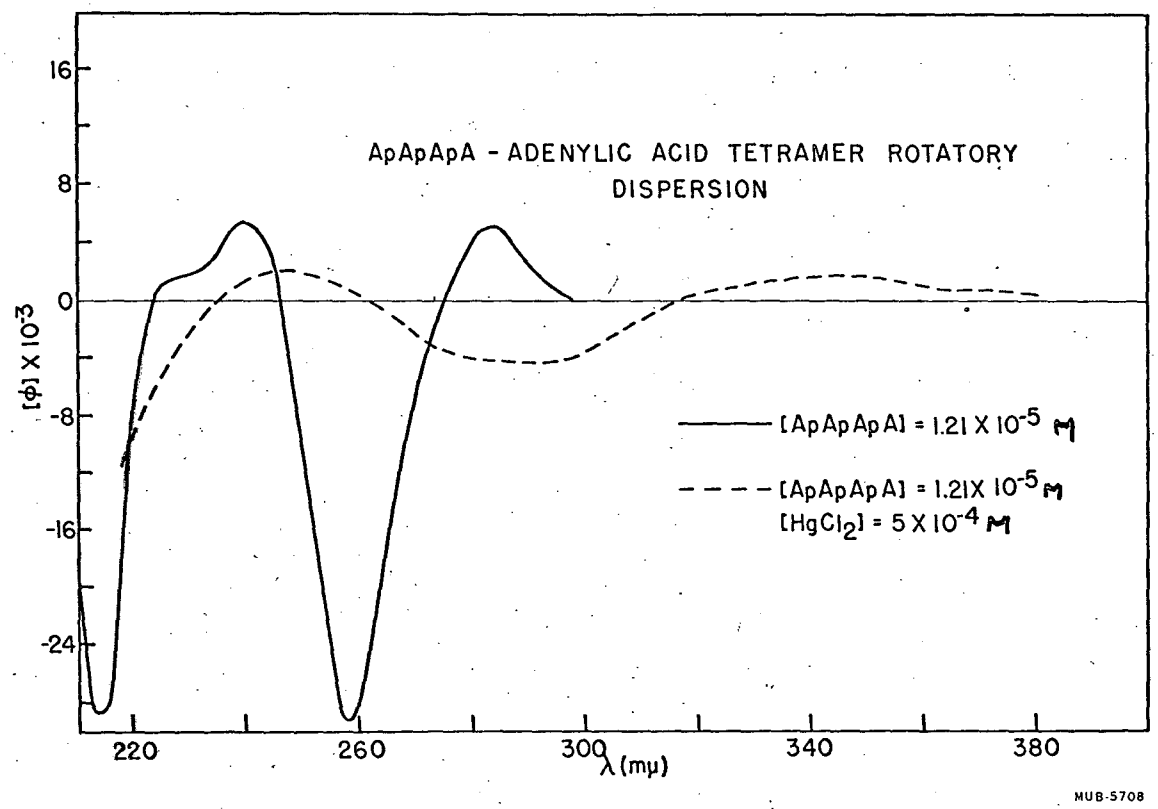



Figure 7. Optical rotatory dispersion of $(Ap)_3A$, with and without mercuric chlorchloride as a complexing agent.

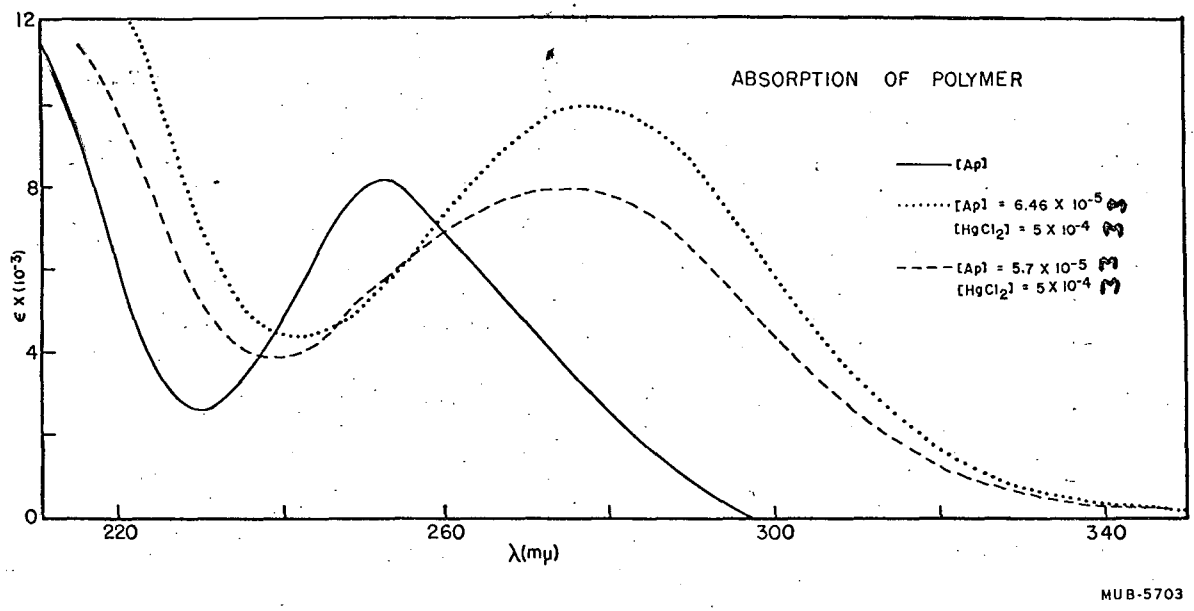



Figure 8. Optical absorption of polyadenylic acid, with and without mercuric chloride as a complexing agent.

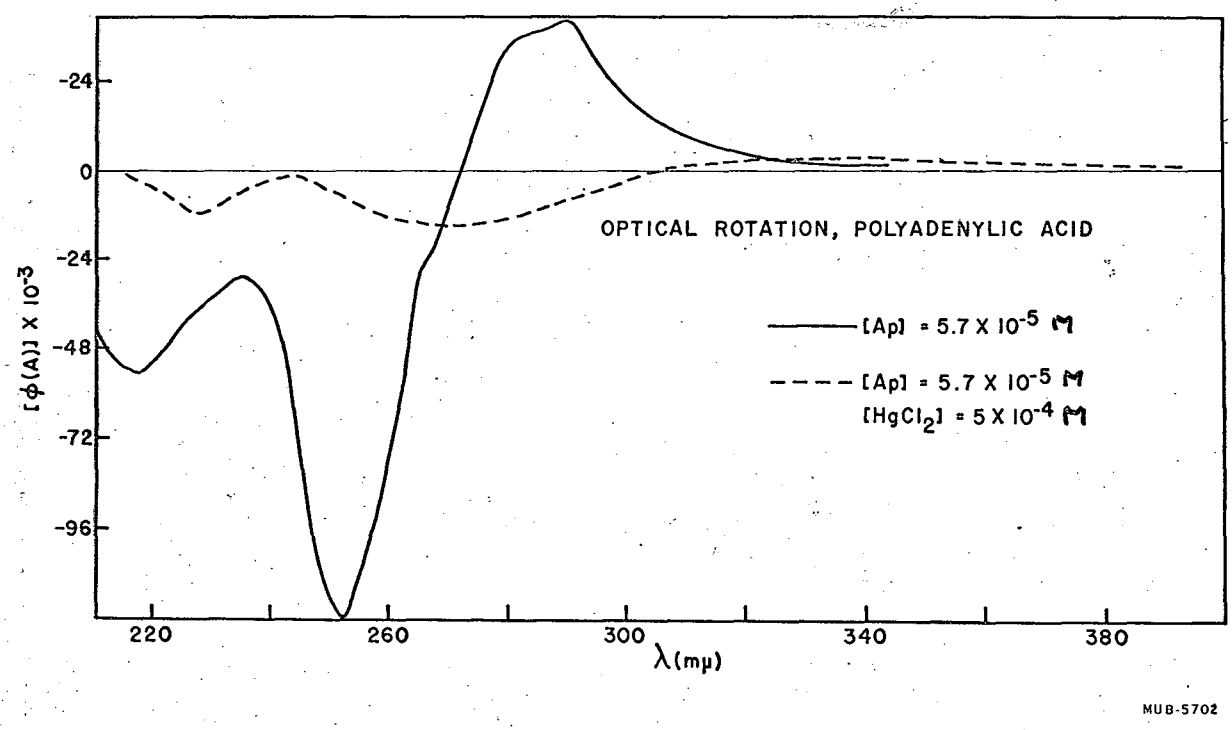



Figure 9. Optical rotatory dispersion of polyadenylic acid, with and without mercuric chloride as complexing agent.

## Interaction of Adenylic Acid Oligomers With Mercuric Chloride: Experimental

Following the preliminary experiments, we attempted to obtain some quantitatively more useful data on the binding of mercuric chloride to the monomer, dimer and the trimer of adenylic acid.

The conditions of these experiments, unless otherwise noted were the same as for our preliminary experiments. Nucleotide solutions were prepared containing $HgCl_2$ in increments of $5x10^{-5}$ M between $5x10^{-5}$ and $10^{-3}$ M. In these series of absorption measurements, mercuric chloride was not present in the reference cell. Instead, for reference, we recorded absorption spectra of solutions of mercuric of the above concentrations. These are shown in Fig. 10. The monomer, dimer, and trimer we used in this experiment possessed mixed terminal (2', 3') phosphate groups. These oligomers were prepared by column chromatography as detailed in an appendix to this paper.

Figures 11 through 18 show the changes in absorptive and rotational properties of the oligomers with increasing concentrations of mercuric chloride.

What stands out most clearly in these sets of spectra is the much greater spectral changes shown by the oligomers, than by the monomer in reacting with mercuric chloride. It behooves us to ascertain the nature of the interaction of $HgCl_2$ with the monomer, and to see in what fashion the reaction with the oligomers differ from this.

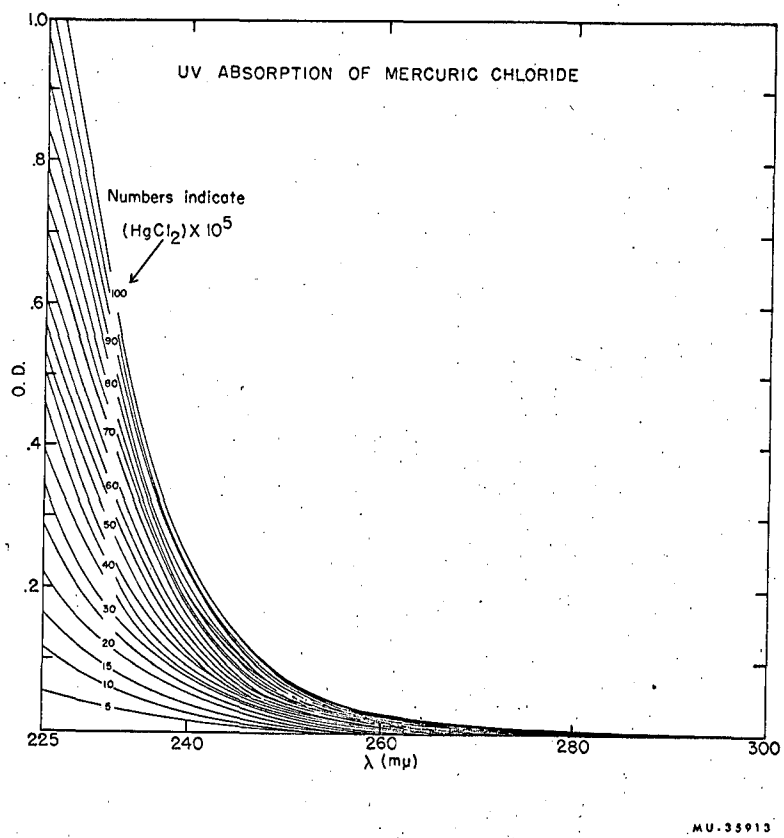



Figure 10. Optical absorption of mercuric chloride.

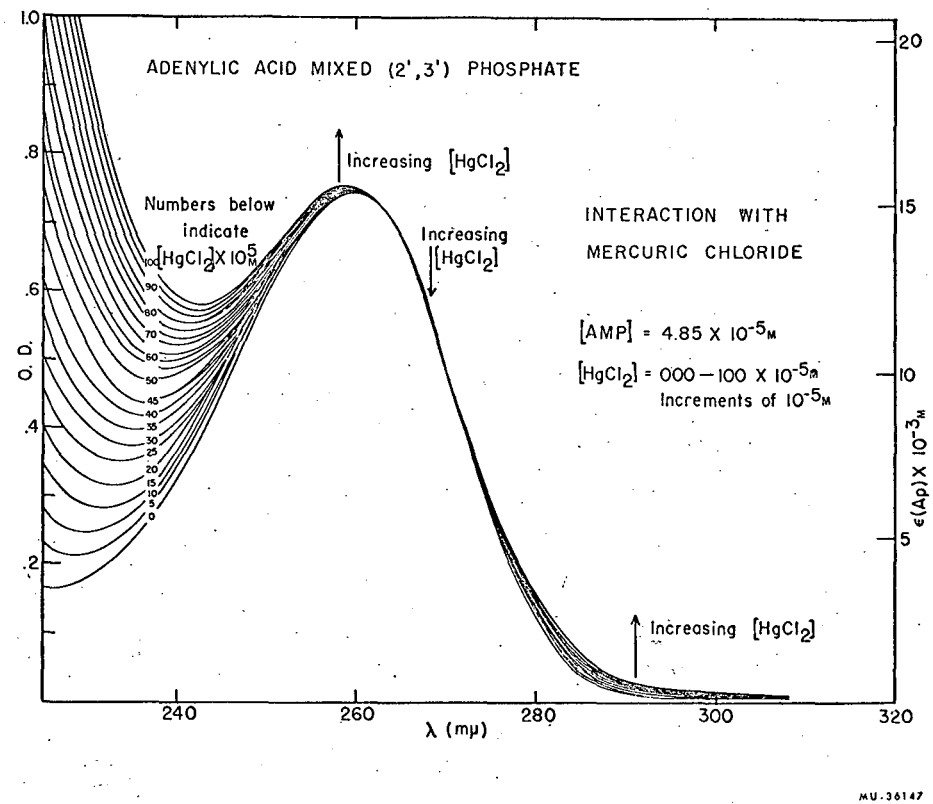



Figure 11. Optical absorption of adenylic acid with increasing concentrations of mercuric chloride.

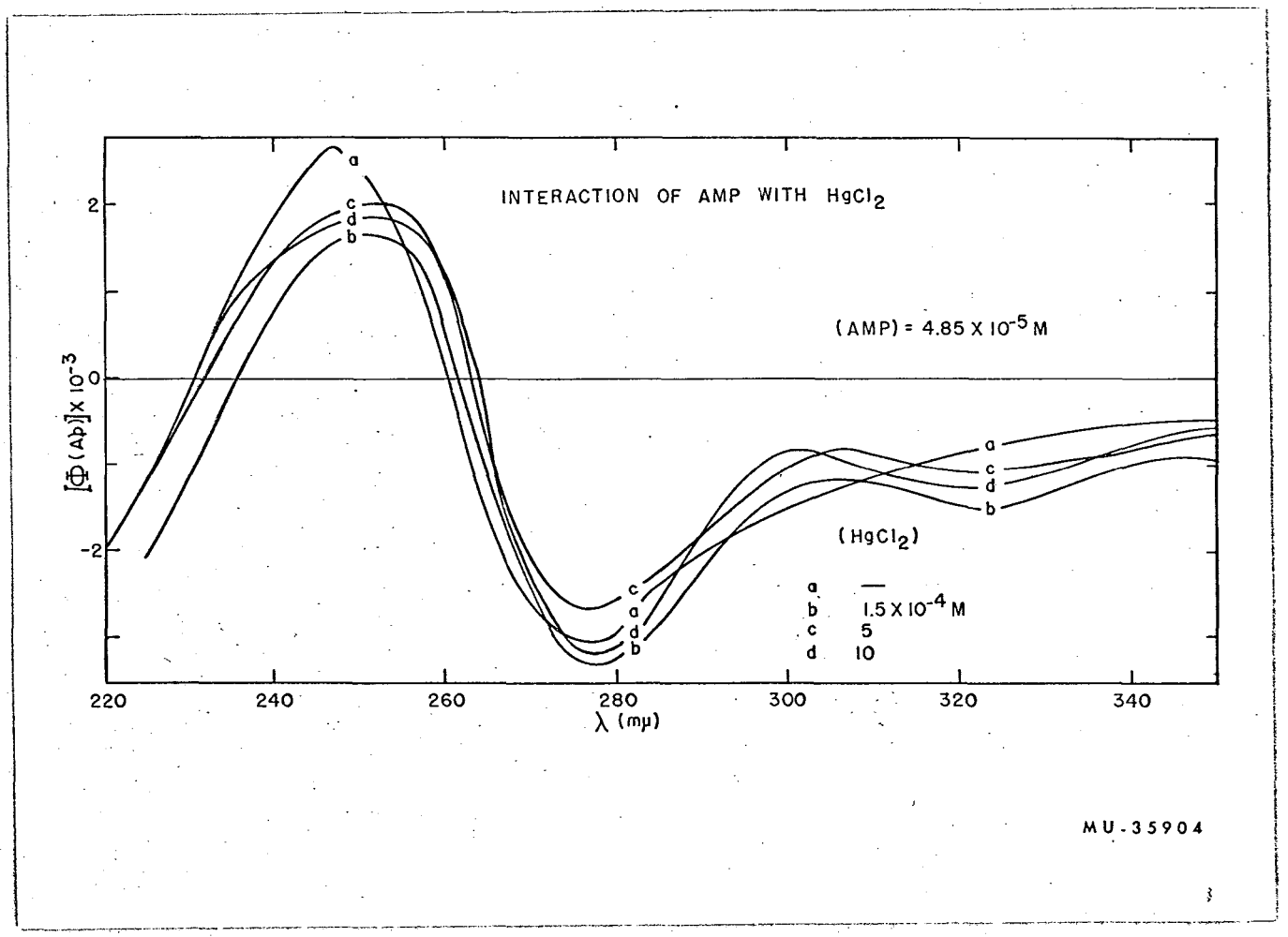



Figure 12. Optical rotatory dispersion of adenylic acid with increasing concentrations of mercuric chloride.

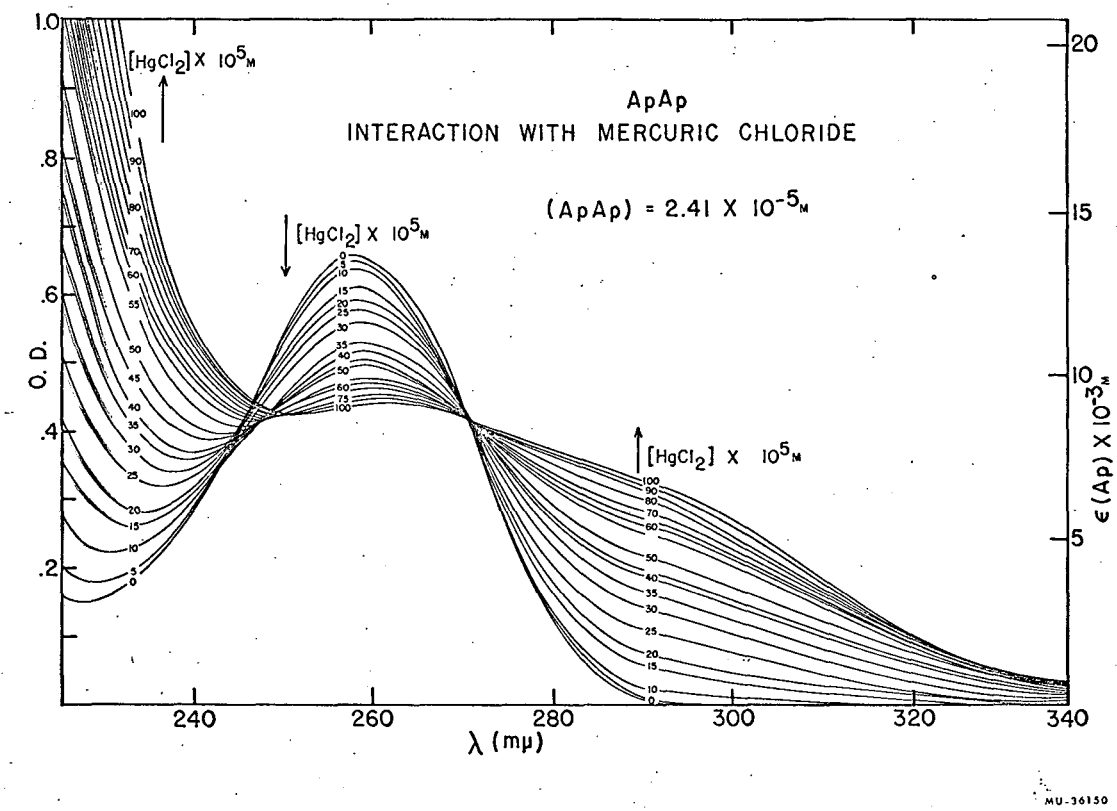



Figure 13. Optical absorption of adenylic acid dimer with increasing concentrations of mercuric chloride.

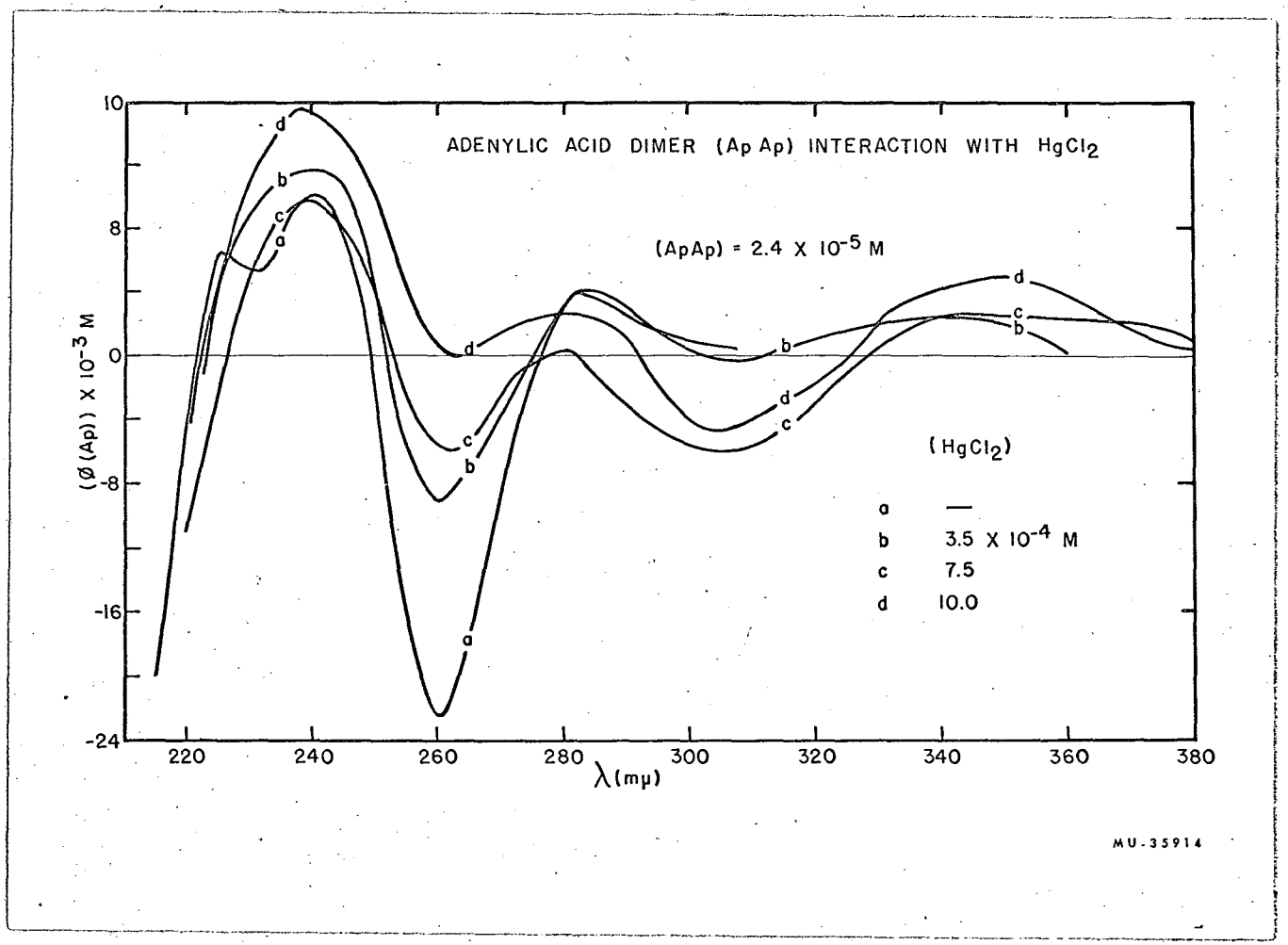



Figure 14. Optical rotatory dispersion of adenylic acid dimer with increasing concentrations of mercuric chloride.

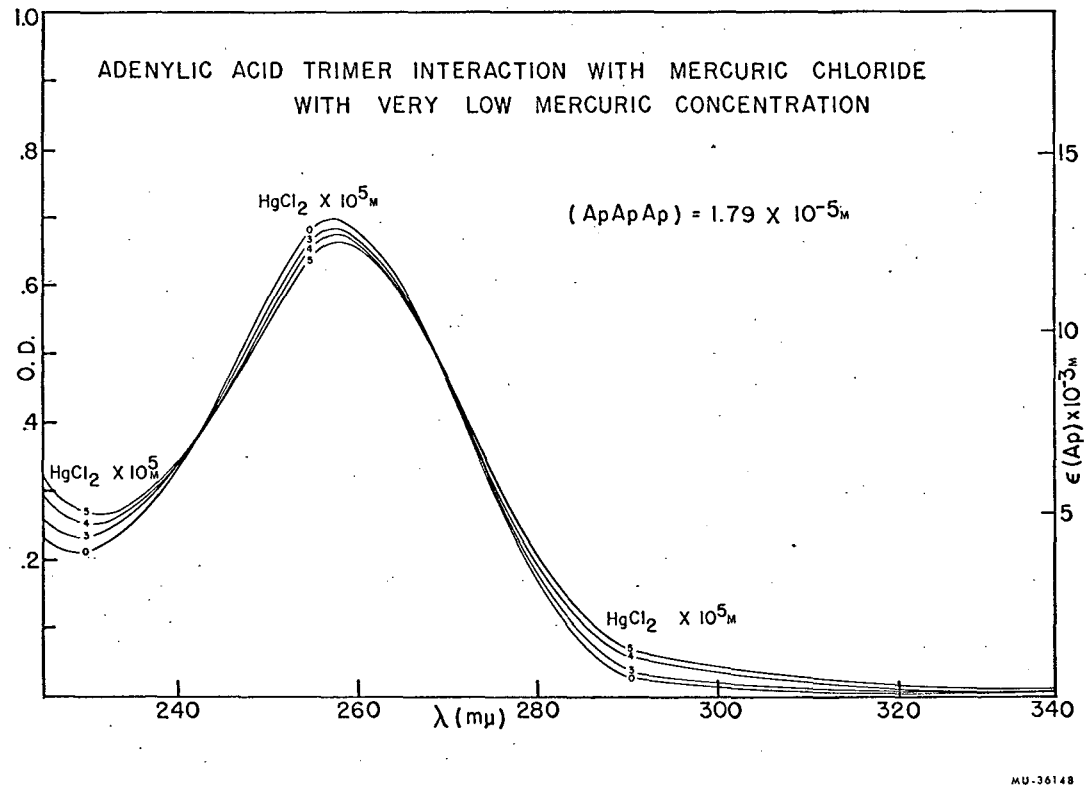



Figure 15. Optical absorption of $(Ap)_3$ with increasing concentrations of mercuric chloride, very low concentrations of mercuric.

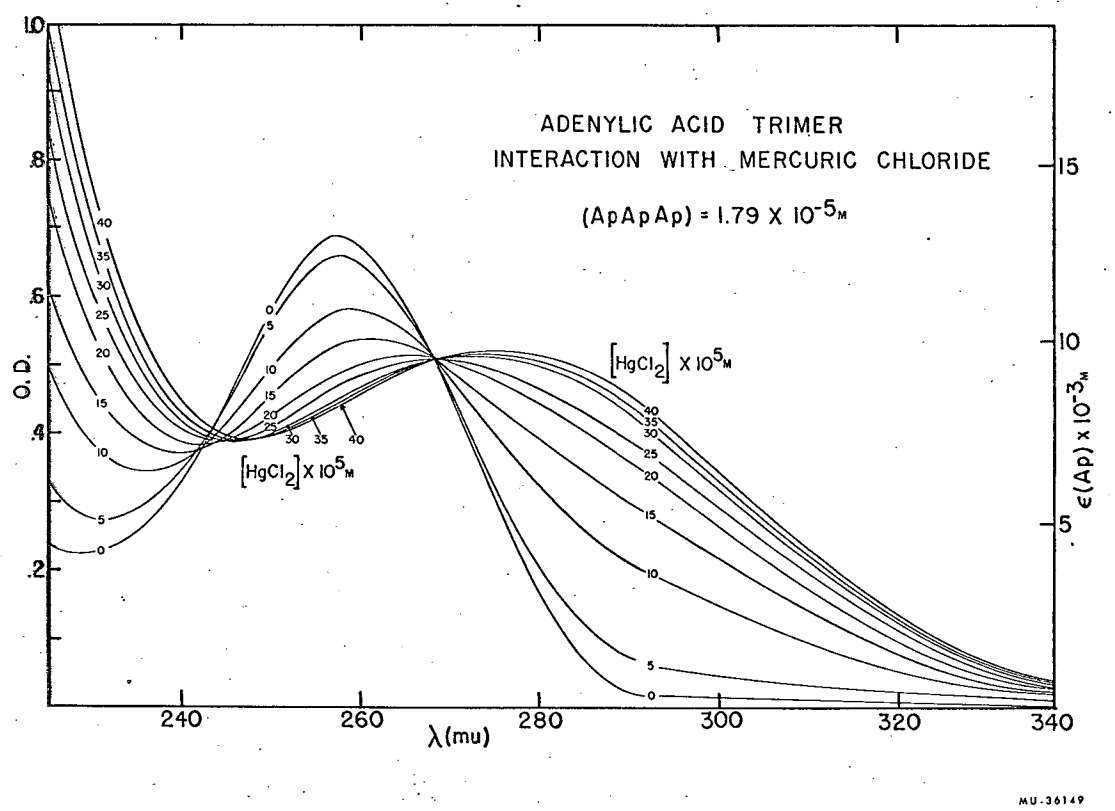



Figure 16. Optical absorption of $(Ap)_3$ with increasing concentrations of mercuric chloride, with intermediate amounts of mercuric.

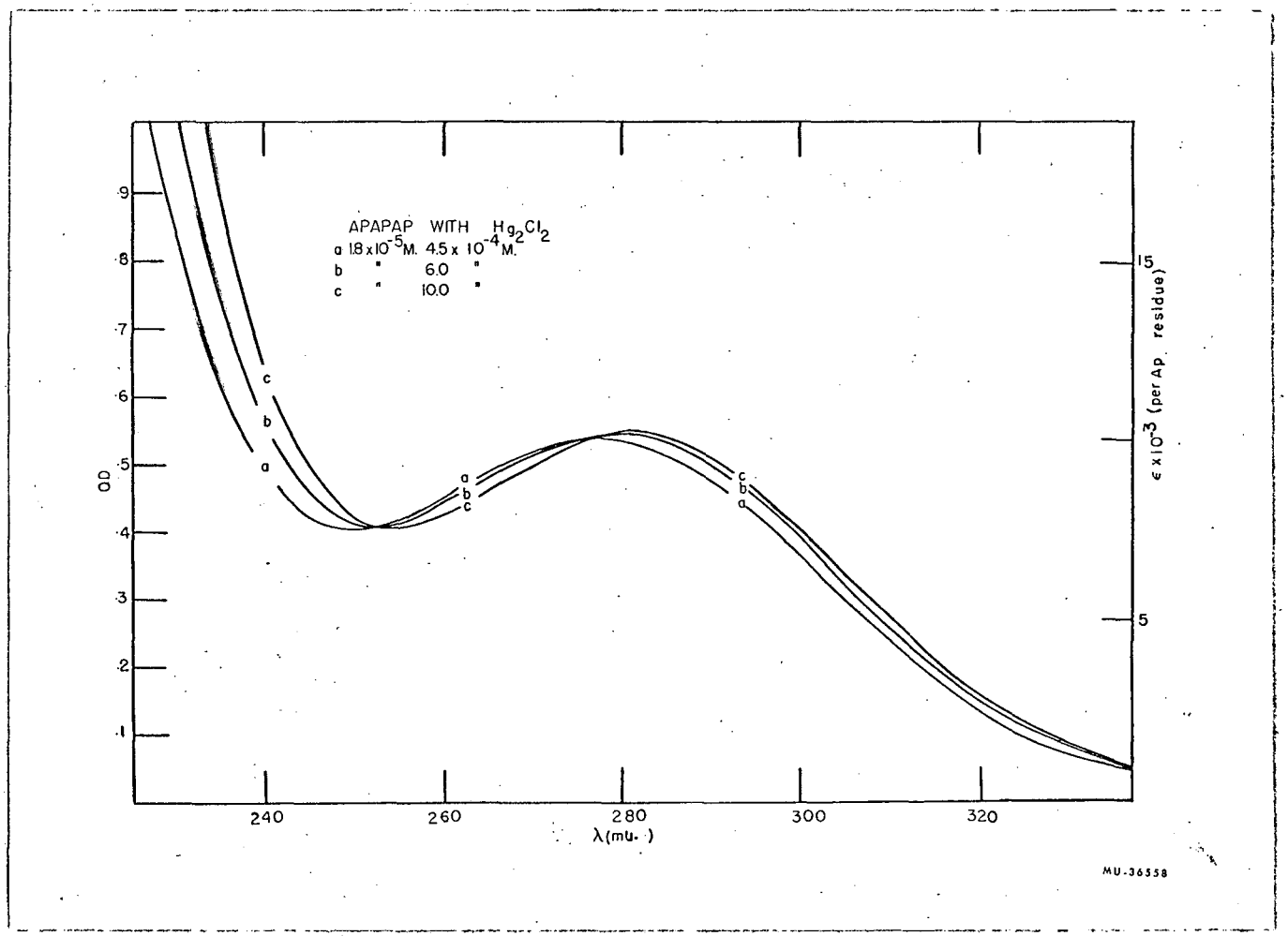



Figure 17. Optical absorption of $(Ap)_3$ with increasing concentrations of mercuric chloride with increasing concentrations of mercuric chloride, at high concentrations of mercuric.

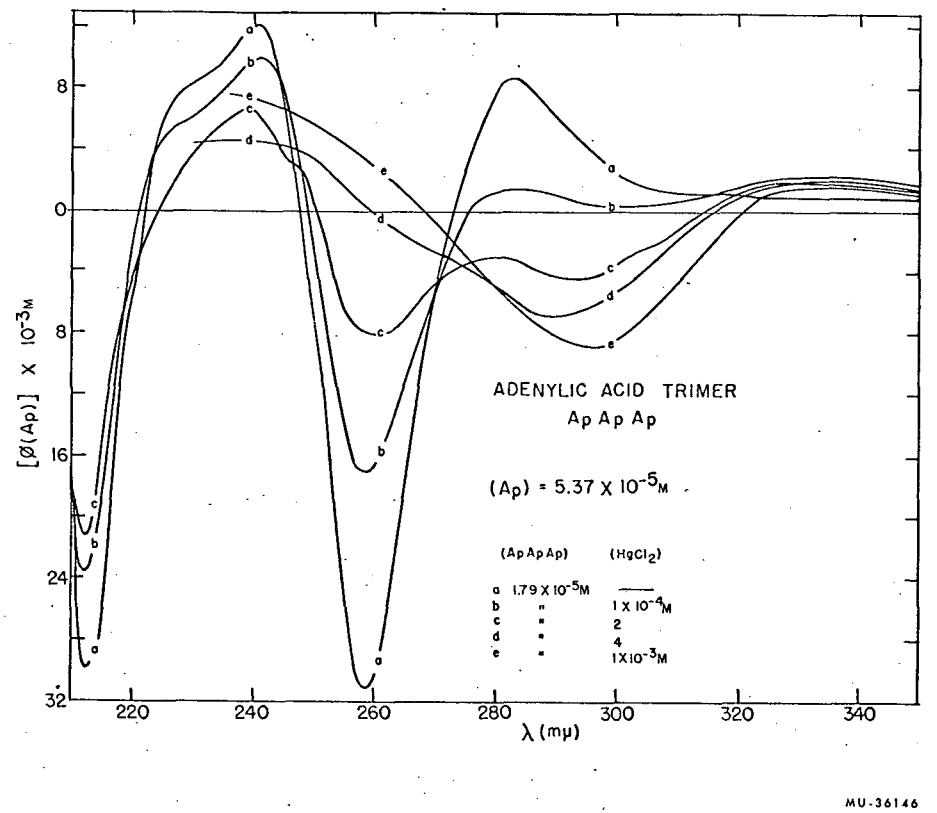



Figure 18. Optical rotatory dispersion of $(Ap)_3$ with increasing concentrations of mercuric chloride.

Throughout the range of increasing mercuric chloride concentrations, the monomer and the dimer of adenylic acid contained isosbestic points in their absorption spectra (Figs. 11 and 13). This indicates that in these ranges, at least, only a single mercuric complexed species is formed. Likewise the spectra of the trimer possess an isosbestic point when the concentration of $HgCl_2$ is less than $4 \times 10^{-4}$ M. (Figs. 15 and 16). Above this concentration of mercuric chloride, the isosbestic point is lost, indicating the formation of yet another species of complex. Our discussion will be concerned chiefly with complex formation in ranges of mercuric chloride concentrations where the absorption spectra of the oligomers possess isosbestic points.

The mercuration reaction for any oligomer of adenylic acid may be written crudely as

$$a_n(A_p)_n + b_n\ HgCl_2 \rightleftharpoons [(Ap)_n]_{a_n}(HgCl_2)_{b_n}$$

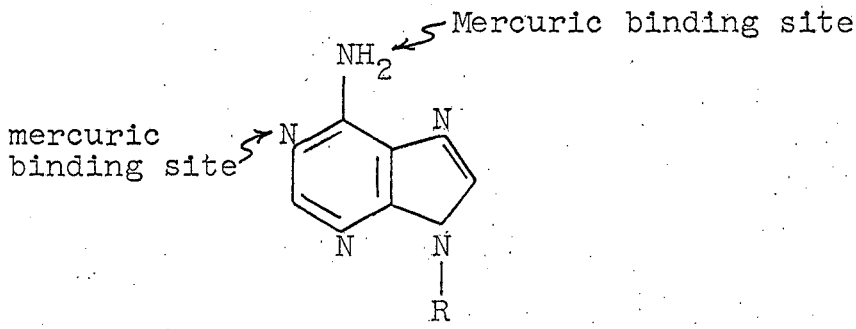
where n represents the length of the adenylic acid (Ap) oligomer. What are the stoichiometries for the reactions, and what are the equilibrium constants? Probing more deeply, what is the reaction site, and could there be more than one such site? Are protons or chloride ions, or both released in the reaction? How does the reaction differ in the monomer and in the oligomers? Finally, what are the changes in electronic configuration which give rise to the spectral changes observed with complex formation?

## Reaction of Mercuric Chloride With Adenylic Acid

Yamane and Davidson have shown that with an excess of mercuric perchlorate, a 1:1 mercuric:adenosine complex is formed, and with this formation, a single proton is released.[5] Simpson believes that the proton is displaced from the amino group of the adenosine molecule by the mercuric ion.[12]

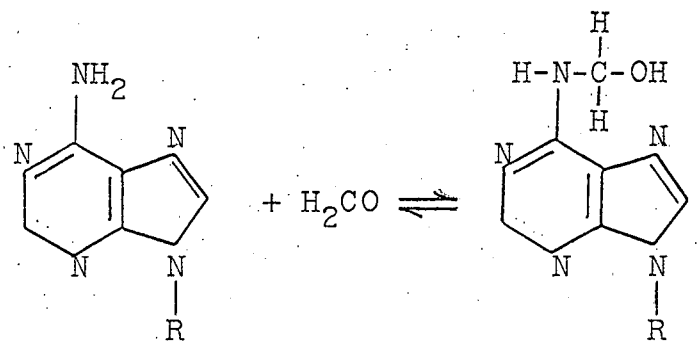
When silver ion reacts with 9-methyladenine or 5'-deoxy-adenylic acid, one mole of protons is released per mole of silver bound, and only one such mole of silver is bound.[13] In binding with adenine, silver appears to react at both the 9 N site, as well as at the amino group. At any rate two moles of silver are bound by adenine, and two moles of protons are released into the solution. The reaction of silver ion with N,N-dimethyl 9-methyladenine is much weaker than with the above molecules, and there is no measurable proton release.

According to Simpson there are two major binding sites for mercuric ion upon the adenosine molecule.[12] One is on the amino group, and the other upon the $N_1$ ring nitrogen.




Simpson believes that the reaction at the amino group occurs preferentially in neutral or basic media. That on $N_1$ tends

to occur most strongly in weakly acidic media. Eichorn and Clark believe they have shown unequivocally, binding by mercuric chloride to the amino position at pH 9.[14] There is a distinctive spectral shift in the UV absorption of adenosine with the addition of mercuric chloride to the solution. When these authors added formaldehyde to a solution of adenosine, there was also a distinctive spectral shift, presumably due to the reaction




When in addition to the formaldehyde, the authors added mercuric chloride to the solution of adenosine they found no additional change in spectrum beyond what already existed with the formylation. In general, the pK for mercuration of a nitrogenous group is rather similar to the pK for its protonation.[15]

Adenosine is protonated at pH 3.7 upon the $N_1$ site as shown by infrared spectroscopy.[16] One might expect mercuration to occur upon that site rather than upon the amino group.

The interaction of mercuric with aniline is the simplest example of the mercuration of an aromatic amino group. With $HgCl_2$ $10^{-3}$ M, and aniline about $10^{-4}$ M. The complex formation is rather slight. With an oxyacid salt of mercuric (the nitrate)

the complex formation is more pronounced (Fig. 19). When a like concentration of pyridine is complexed by mercuric nitrate, there is also an absorption excited in the region of 300 mu as shown in Fig. 20. Quantitatively, the aniline reacts more strongly than the neutral pyridine molecule towards mercuric, but the nature of the spectral changes are qualitatively the same. In adenosine, then, one cannot on the basis of the foregoing experiment, choose the ring nitrogen or the amino nitrogen as the binding site of the mercuric chloride at pH 5.7, under our reaction conditions. Perhaps I.R. spectra might help to clarify things. If the peak of the I.R. spectrum due to the amino group's vibration is greatly altered upon the molecules mercuration, then the binding site would very likely be that amino group.

At a pH of 3.77, an excess of mercuric chloride does not cause the release of protons from AM(2',3')P. There is no evidence of spectral changes upon the addition of mercuric chloride to AMP at this pH. Raising the pH of the mercuric chloride, AMP solution to 5.7, does result in complex formation. At the relatively high concentrations we used ($10^{-3}$ $HgCl_2$, $5x10^{-4}$ AMP) we got some precipitate formation. From the drop in the absorption at 260 mu at least 12% of the AMP had been complexed by the mercuric chloride. This would have an easily measurable $6x10^{-5}$ M proton release had the mercuric chloride displaced hydrogen ions from the adenylic acid. It did not.

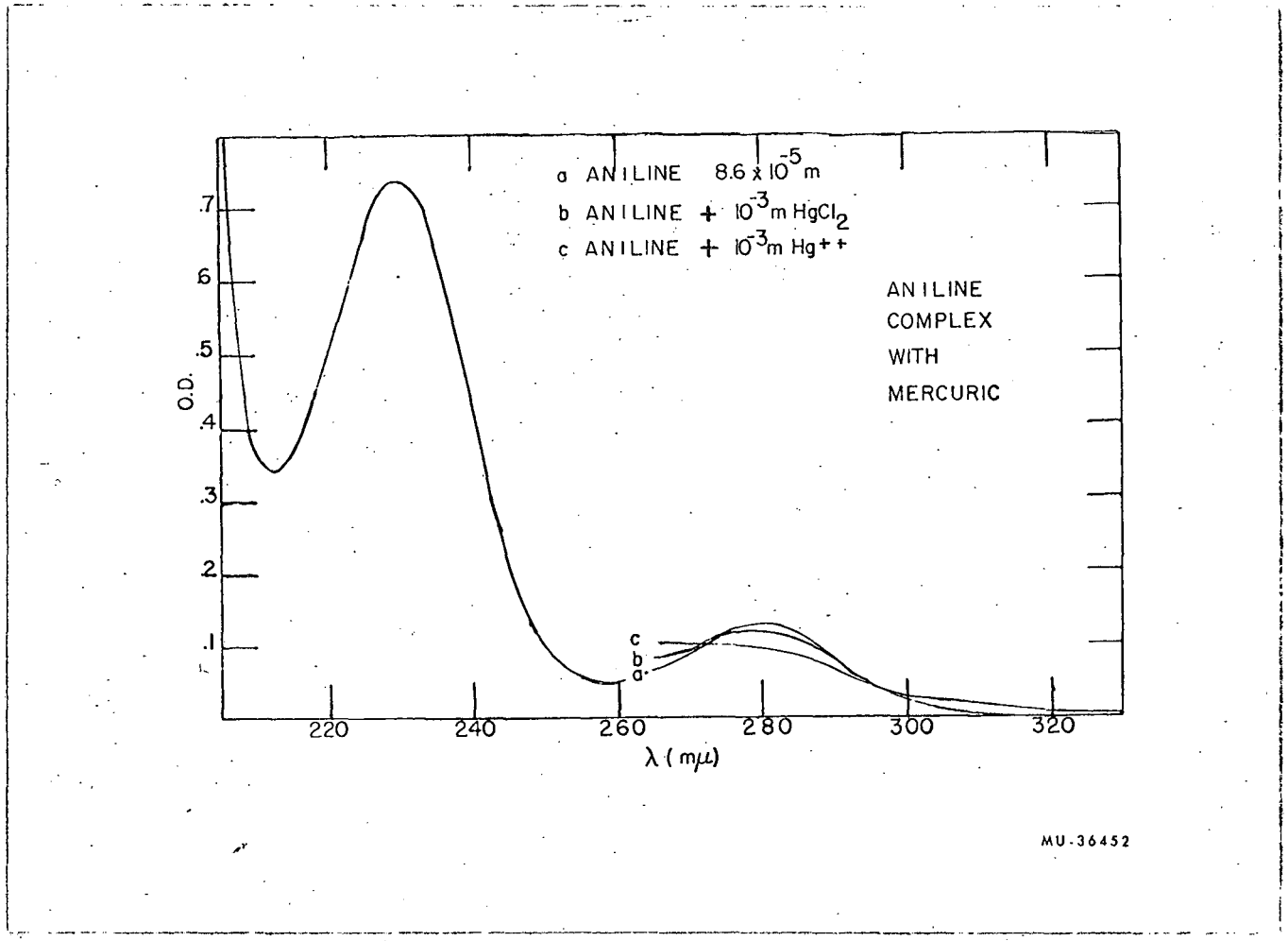



Figure 19. The interaction of mercuric with aniline.

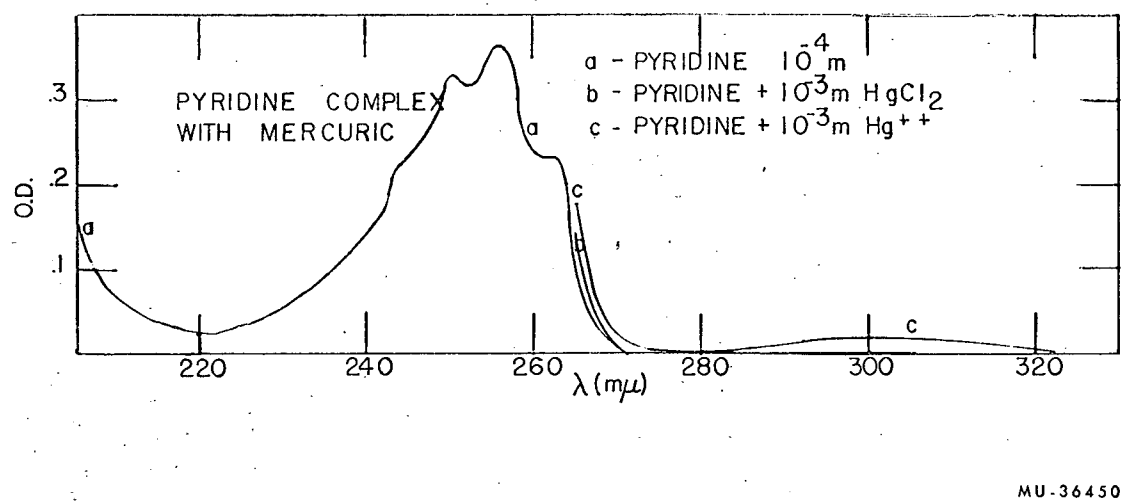
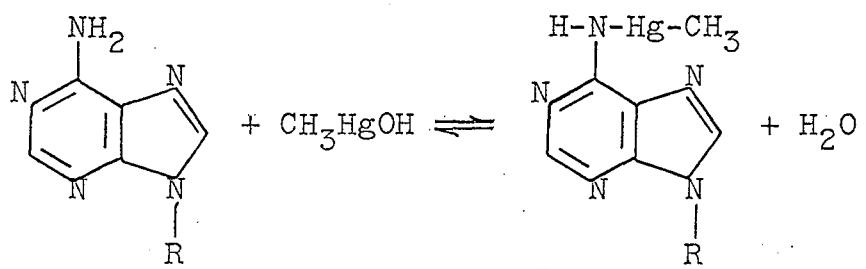



Figure 20. The interaction of mercuric with pyridine.

Likewise, addition of mercuric chloride to adenosine, under conditions where a complex will form does not appear to result in the release of hydrogen ions.

The mechanism which Simpson proposed for the mercuration of adenosine by methyl mercuric hydroxide, $CH_3HgOH$, is shown below.



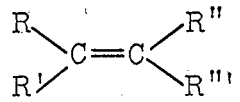

His proposed mechanism possesses, he believes, an equilibrium constant of 12.5. He arrives at this figure through the spectral changes occurring with the addition of methylmercuric hydroxide to solutions of adenosine. With very high concentrations of methylmercuric hydroxide, he obtains a species which has an extinction at 285 mu of 20,000, which he believes is the extinction coefficient at that wavelength for the species drawn above. From his data, Simpson further believes that in the pH range of 4-6, mercuration upon the ring nitrogen, $N_1$, enhances the binding of mercuric to the amino group.[12]

Our own data, Fig. 11, on the binding of $HgCl_2$ to AM(2',3')P possess an easily distinguishable isobestic point, implying, as earlier stated, that the reaction in our system is at a single site, with the production of a single species of complexed molecule. Whether this site is the ring nitrogen or the amino group remains an enigma.

In DNA, the amino and $N_1$ nitrogens of adenosine are involved in hydrogen bonding. These should, then, be considered as the likely sites of mercuration. If one prepares crystals from 1/1 mixtures of 9-ethyladenine and 1-methyluracil, the unit cells of the crystals formed contain one each of these heterocycles. The $O_4$ of the uracil is hydrogen bonded to the amino group of the adenosine. The $N_3$ of the uracil is hydrogen bonded to the $N_7$ of the adenine.[34] Likewise in forming, from poly A and poly U a 2/1 U/A triple helix polymer the $N_7$ of the adenines is involved in this triple stranded formation and is involved in hydrogen bond formation. Thus the $N_7$ of adenosine might well be involved in some manner in the mercuration reaction.

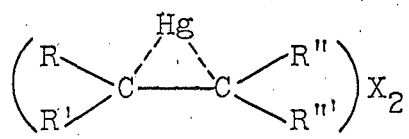
The reaction of mercuric (II) with nitrogenous groups is common enough but the reaction with double bond systems to form reversible complexes has not been so widely investigated.[15] Chatt investigated the complexing of mercuric (II) by olefins.[23] If one reacts an olefin

(a)



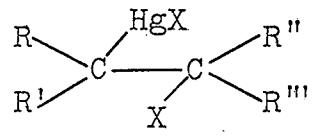

with $HgX_2$ one initially gets an addition product

(b)




In a variety of organic solvents, this reaction probably progresses to a compound

(c)




Indeed, one may isolate stereoisomers of the above type of compound. In aqueous solution, the reactions with mercuric are often reversible, possibly not proceeding beyond the weak addition product illustrated in (b). Especially liable to this type of addition reaction are alpha-beta unsaturated ketones such as are believed to exist in uridine and guanosine.

While most subsequent literature on the addition of Hg(II) to nucleotides implicate either aromatic amino groups or ring nitrogens as the reaction sites, the possibility of addition across double bonds cannot be completely eliminated.

## Chloride Release

The next problem is whether chloride is released when mercuric chloride binds AMP. Is the reaction best written as

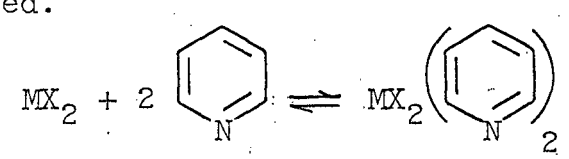
$$HgCl_2 + AMP \rightleftharpoons HgClAMP^+ + Cl^- \qquad (I)$$

or as

$$HgCl_2 + AMP \rightleftharpoons HgCl_2AMP. \qquad (II)$$

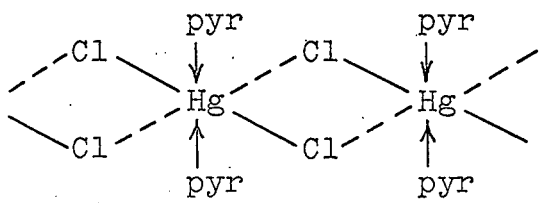
Our evidence for this is quite indirect. From calculations we performed on the binding of $HgCl_2$ to dimer or trimer adenylic acid molecules, it would appear that (II) is the likely reaction mechanism. Details of why we believe this to be so appears later. Mercuric, further, has the ability to bind six or even eight ligand groups under the appropriate conditions.[27,28] A number of studies indicate that the halides remain with mercuric, when mercuric dihalides complex additional nitrogenous groups as ligands.[32,33]

Clark and Williams showed reactions between metal halides, including mercuric chloride, with the pyridine molecule as illustrated.[32]

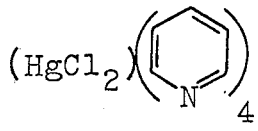



They studied the IR spectra of the adducts formed. They were able to analyse the pyridine ring vibrations and their change upon metal complexing. They could observe the metal halide stretch vibrations, and they could look at the metal pyridine vibrations. They performed the IR measurements in a Nujol mull.

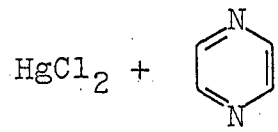
They believe (on the basis of x-ray crystallographic measurements) that the mercuric chloride-pyridine is probably in the form of a polymer.



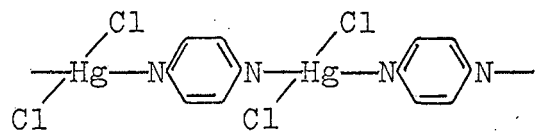

Each mercuric of this polymer has two strong mercuric-chloride bonds, two weaker chloride bonds, and each mercuric is further coordinated to two pyridine molecules. The vibration of the Hg-Cl bond in $HgCl_2$ itself occurs at a frequency of 375 $cm^{-1}$. The ring vibrations of pyridine occur at 405 and 604 $cm^{-1}$. On the addition of mercuric chloride to pyridine, the ring vibrations appear with frequencies of 420 and 641 $cm^{-1}$. The stronger of the two types of mercuric-chloride bonds appears at a frequency of 292 $cm^{-1}$. The crystallographically determined weak mercuric-chloride and the mercuric-nitrogen bonds are likely at a frequency below 200 $cm^{-1}$, and could not be measured with their IR absorption spectrophotometer. When the mercuric chloride-pyridine octahedral complex is dissolved in pyridine, the polymer appears to disassociate with the formation of a tetra pyridine complex,




Stidham and Chandler report the formation of a similar complex of mercuric chloride with pyrazine.[33]

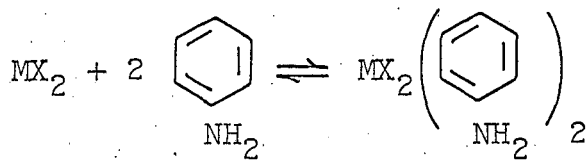


From an aqueous solution they obtain a precipitate whose structure corresponds to 1 $HgCl_2$/1 pyrazine. The infrared spectra indicates that the precipitate is composed of mercuric chloride tetrahedrally bound as a polymer.



The authors were unable to observe, in their infrared spectra, either the metal pyrazine or the metal halide vibrations. The lack of the former they attributed to the dependence of this frequency upon the chain length of the polymer formed. Since this polymer length would likely be highly random, the band corresponding to the metal pyrazine stretch should be quite broadened. That they were unable to identify the metal halide bond, they attributed to the formation of a pi bond between the mercuric and the chloride. They postulated that the electrons in this bond would be delocalized into the pyrazine ring with a weakening of the Hg-Cl bond strength below the minimum resolvable frequency of their spectrophotometer.

Jungbauer and Curran using IR techniques have shown that a vast array of divalent metal halides react with aniline as shown below, without the release of halide acid.[31]



Let us begin to construct a model of the reaction of $HgCl_2$ with AMP. On the basis of our own titrations, we do not find any proton release in the mercuration of AMP at pH 5.7, using $HgCl_2$ as a complexing agent.

On the basis of indirect evidence, we assume that no $Cl^-$ is released when $HgCl_2$ binds adenylic acid.

Since with concentrations of $HgCl_2$ between $5x10^{-5}$ and $10^{-3}$ M our $4.8x10^{-5}$ M solution of adenylic acid contain isosbestic points in their absorption spectra, we feel safe in concluding that mercuration is at a single site, and that a single species of mercuric complexed molecule is formed. We will assume that there is only 1 $HgCl_2$/1 AMP. In general, the reaction constant for the formation of a fourth ligand bond with mercuric, $k_4$, is significantly less than that for the formation of the third ligand bond, $k_3$. Without looking at the IR spectra as we mentioned earlier, to state whether the reaction site were the $N_1$, amino nitrogen, or maybe even $N_7$ would be mere guesswork. We prefer to remain noncommital to this question.

Having assumed a reaction model,

$$HgCl_2 + AMP \rightleftharpoons HgCl_2AMP$$

we should like to get an idea of the equilibrium constant for this reaction.

Consider Figs. 10 and 11 at 260 mμ. The absorption at 260 mμ in Fig. 11 is due to (a) uncomplexed AMP, (b) $HgCl_2$ complexed AMP, and (c) $HgCl_2$. We can subtract the contribution

of the latter from the absorption curves in Fig. 10. Consider in both Figs. 10 and 11, the case when the concentration of $HgCl_2$ is $10^{-3}$ M. If we subtract the contribution of the $HgCl_2$ we find that there is a net decrease in the 260 mμ absorption of .01 OD units. If we assume that the extinction coefficient of the complexed species at 260 mμ is negligible compared to the 15,300 of the AMP we may obtain a minimum value for the fraction of the AMP complexed. It is merely the fractional decrease of the 260 mμ absorption. With the AMP in solution along with $10^{-3}$ M $HgCl_2$ this fractional decrease is of the order .015. The calculated equilibrium constant would then be given by

$$K = (HgCl_2AMP)/(HgCl_2)/(AMP) \cong (HgCl_2AMP)/(HgCl_2)_0(AMP)_0$$

where the subscript 0, simply indicates the total amount of the constituent in the reaction mixture whether complexed or not. Since the fractional amount of $AMP_0$ complexed is .015 (f = .015) we have

$$K \cong f(AMP)_0/(AMP)_0(HgCl_2)_0 = f/HgCl_{2_0} = .015/.001 = 15$$

This is a minimum value for the equilibrium constant of complex formation, and if account were taken of the value of the absorption of the species $HgCl_2AMP$ at 260 mμ a higher value for the equilibrium constant would be found.

There is another method to estimate the equilibrium constant for the formation of the $HgCl_2AMP$ complex. Assume that the AMP-$HgCl_2$ ligand bond, which results in the absorption increase

at 285 mμ, is the same in the monomer, dimer, polymer, etc. Assume that in the monomer, for whatever reasons, less of these bonds are formed, giving rise to a less dramatic spectral change. In the trimer and the polymer, with relatively high concentrations of $HgCl_2$ we find that the extinction at 285 mμ, per adenylic acid residue, is near 10,000.

The optical density at 285 mμ in our AMP $HgCl_2$ mixtures may be written as:

$$OD(285\ m\mu) = OD(AMP,\ 285\ m\mu) + OD(HgCl_2AMP\ complex,\ 285\ m\mu)$$

At this wavelength, there is no correction necessary for absorption of the mercuric chloride. Calling the fractional amount of AMP complexed, f, and the total amount of AMP, complexed and free, $AMP_0$, we have

$$OD(285\ m\mu) = (1-f)\ \epsilon(AMP\ free,\ 285\ m\mu)\ AMP_0 + f\ \epsilon(HgCl_2AMP,\ 285\ m\mu)\ AMP_0$$

The extinction of AMP itself at 285 mμ, ε(AMP free, 285 mμ), we know to be 870. We have estimated the extinction of the complexed species to be 10,000 at this wavelength. From our data in Fig. 11, and using our proposed reaction mechanism, we calculate values of the equilibrium constant, K, for several values of mercuric chloride concentrations. See Table I.

There could be a number of reasons for lack of agreement between the values of K, calculated at different $HgCl_2$ concentrations. The mercuric chloride might be binding to some other sites(s) in such a way as not to effect the absorption spectra.

Table I. Binding of Adenylic Acid (2',3') Phosphate by Mercuric Chloride

| $HgCl_2$ Total | $HgCl_2$ Free | AMP Total | AMP Complexed | AMP Free | f | K(lit/mole) |
|---|---|---|---|---|---|---|
| - | - | $4.85 \times 10^{-5}$ | - | $4.85 \times 10^{-5}$ | - | - |
| $15 \times 10^{-5}$ | $14.8 \times 10^{-5}$ | " | $.16 \times 10^{-5}$ | $4.69 \times 10^{-5}$ | .033 | 230 |
| $50 \times 10^{-5}$ | $49.7 \times 10^{-5}$ | " | $.27 \times 10^{-5}$ | $4.58 \times 10^{-5}$ | .056 | 120 |
| $100 \times 10^{-5}$ | $99.6 \times 10^{-5}$ | " | $.38 \times 10^{-5}$ | $4.47 \times 10^{-5}$ | .078 | 90 |

The assumption that the extinction of $HgCl_2$ at 285 mμ is 10,000 might be much in error. The mercurated species of the monomer might really possess totally different spectral characteristics than those of the oligomers, i.e., the ligand bond between the monomer and $HgCl_2$ might be of quite a different nature than those in higher polymers. The spectral change of the monomercurated species under these conditions might really be very small. This is a possibility that we cannot prove or disprove, for at much higher concentrations of $HgCl_2$, or of AMP, we tend to get precipitate formation.

More dramatic spectral changes occur when adenylic acid or adenosine is complexed with oxyacid salts of mercuric, e.g., the perchlorate or the nitrate, or with methylmercuric hydroxide.[12] Figure 21 shows the effect upon the absorption spectrum of AMP when mercuric perchlorate is used as a complexing agent. Over the range of concentrations of mercuric perchlorate used these spectra do not possess an isosbestic point, indicating that the reaction involves the formation of more than one species of complex.

The changes in the optical rotatory dispersion spectra of AMP as $HgCl_2$ is added is seen in Fig. 12. The very low signal level, and the high noise level in the recording leads to a difficulty in resolving such true spectral changes as might occur in the rotatory dispersion of AMP as small fractions of the molecules are complexed. The only consistency in the ORD spectra of the monomer, oligomers and polymer of mercuric complexed adenylic acid is the appearance of a red shifted

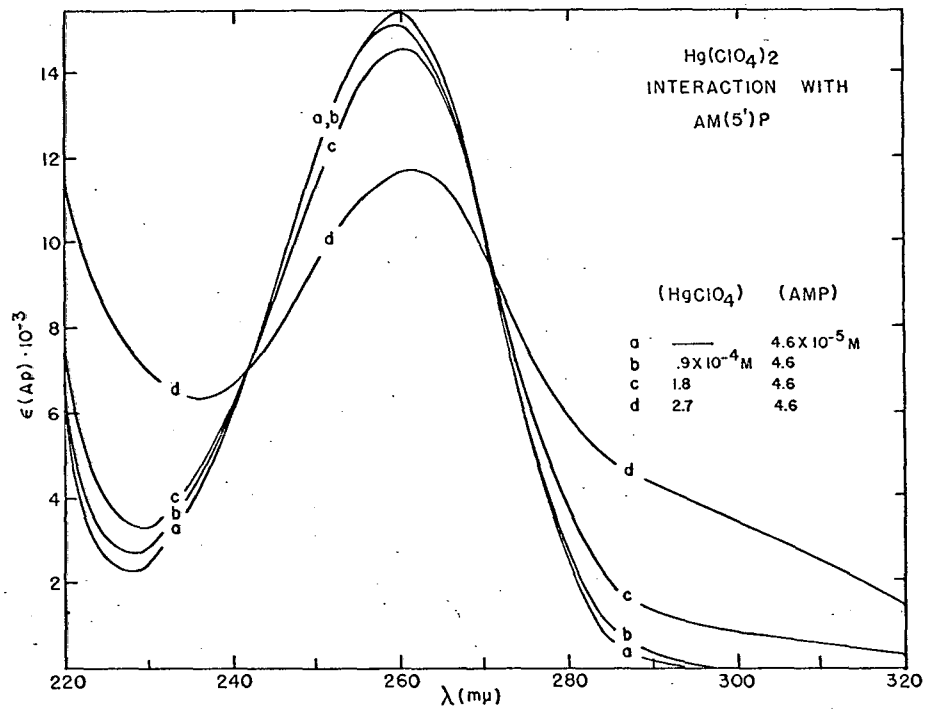



Figure 21. Interaction of mercuric perchlorate with adenylic acid: change in absorption spectrum.

cotton effect with a crossing point in the range of 310-330 mμ. A hint of such an effect is seen in the AMP with $10^{-3}$ M $HgCl_2$. This high wavelength cotton effect is seen much more clearly when AMP is complexed with mercuric perchlorate (Fig. 22).

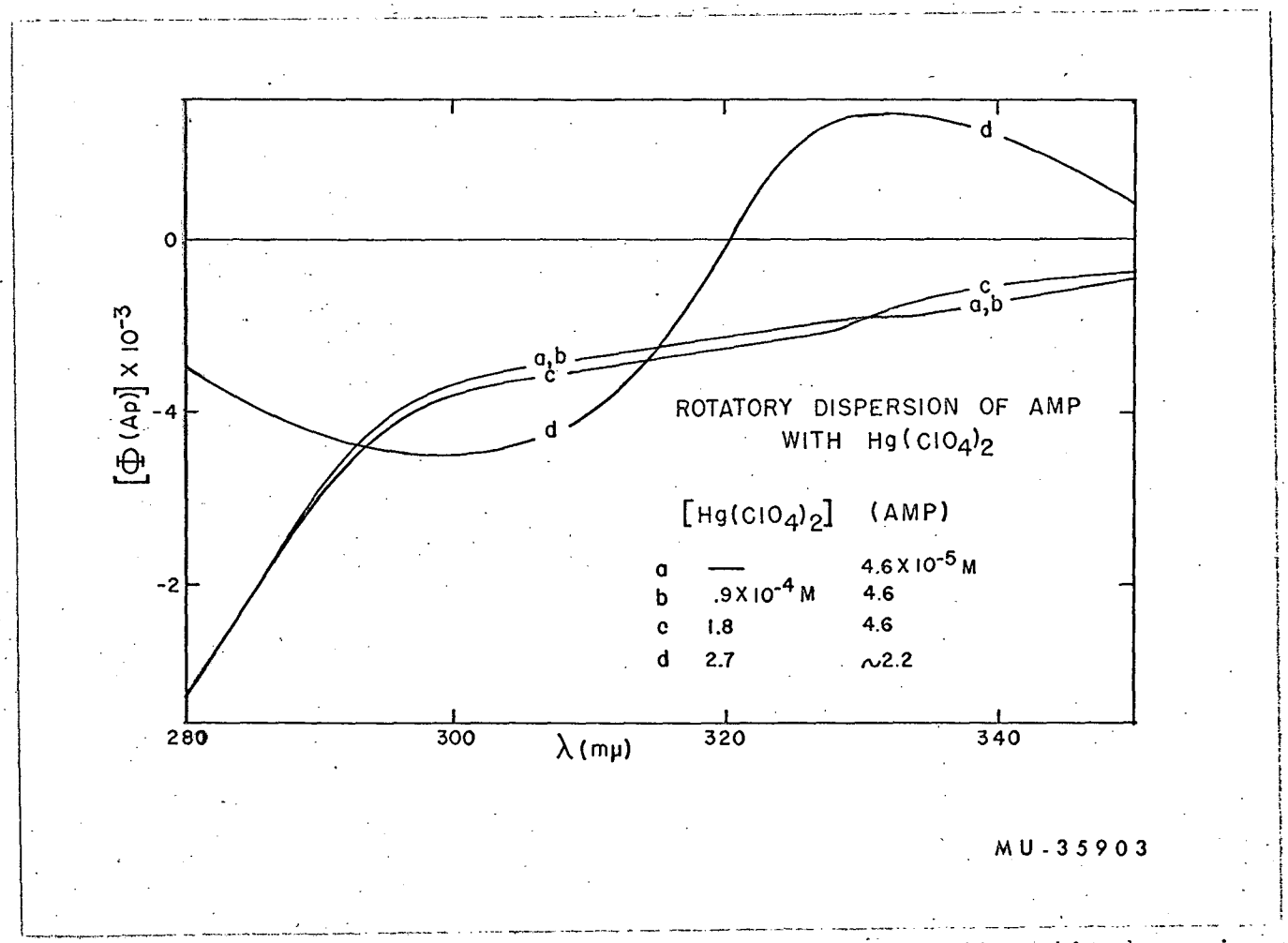



Figure 22. Interaction of mercuric perchlorate with adenylic acid: change in rotatory dispersion.

## Nature of the Spectral Shift

The nature of the absorption spectral changes in mercuric complexes of the adenylic acids (and other nucleotides and polynucleotides) appear to be due to charge transfer phenomena.

The absorption spectrum of mercuric chloride, near 200 mμ seems due to a charge transfer phenomenon from the chloride (or maybe the pi bond between the chloride and the mercuric) to the mercuric ion. Similar phenomena occur in all the other mercuric halide complexes.[35]

Orgel suggests that metal amine complexes exhibiting strong electronic absorptions do so because of charge transfer phenomena from the ligand to the metal.[36]

Bonds between mercuric and halide ions are largely covalent in character, and likely, the mercuric amine, or mercuric-ring nitrogen bonds are also of this type. From this and the foregoing discussion it is possible that there is some delocalization of the ring pi electron system into the mercuric nitrogen bond of the nucleotides. It would then be possible that the charge transfer phenomenon involves the transfer of an electron from this entire pi system to the mercuric ion.

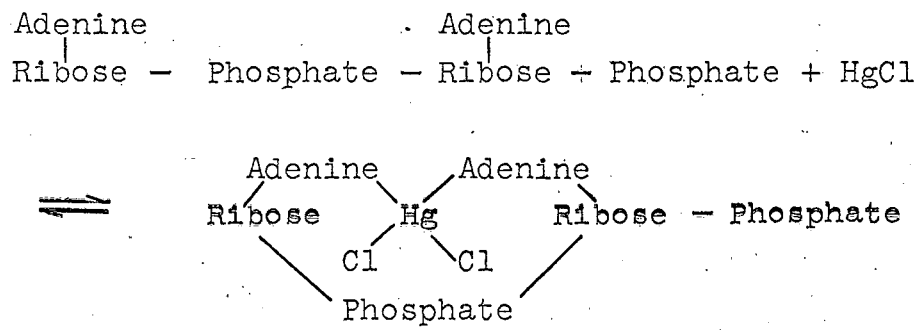
## Summary of Mercuric Binding to AMP

We may say that under our conditions of reaction, $HgCl_2$ reacts 1/1 with AMP to form a complex. The reaction site is probably the amino nitrogen or the ring, $N_1$, nitrogen. No proton is released in this reaction, at least not at pH 5.7. The chloride ions likely remain complexed to the mercuric, and the addition of an AMP molecule represents the formation of a third ligand bond. If the ligand bond is of the same nature as occurs in the much more highly reactive oligomers of adenylic acid, then the equilibrium constant for the mercuration is of the order of 100 lit/mole.

## Mercuric Chloride Binding to ApAp

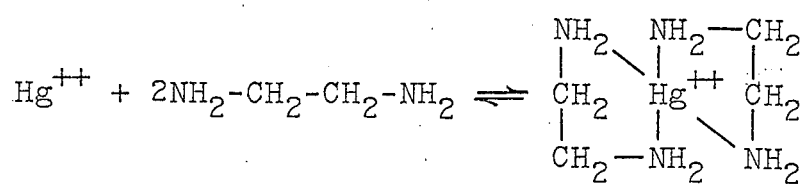
The reaction of the ApAp dimer with $HgCl_2$, we see from Fig. 13, results in much greater spectral changes than its interaction with the monomer. Either the nature of the interaction (the specific reaction site and/or the strength of the ligand bonds) are different than in the monomer, or simply more Ap residues are bound by the mercuric chloride. We proceed with the discussion of the dimer (as well as trimer and polymer) complex assuming a similar sort of bond is formed at the same sites in all the oligomers including the monomer.

Because the absorption spectra of the dimer with mercuric chloride contain an isosbestic point, we are justified in again believing that over the range of $HgCl_2$ used for a complexing agent, that a single metal bound species is formed. A possible reason for the greater strength of reaction between the dimer, than between the monomer and $HgCl_2$ could be the formation of a chelated species as diagrammed below.




In assuming similarities between the reaction of $HgCl_2$ with the monomer and the dimer, we assume that neither $H^+$ nor $Cl^-$ is liberated in the reaction.

According to Bjerrum the reaction of mercuric ion with diaminoethane is described as



with the amino ligands of the two bidentate chelates tetrahedrally bound.[15] In its interactions with ethyl amine, mercuric ion reacts strongly with only two of the ligands. The point here is that the first two ligand bonds in mercuric complexes are relatively easy to form, e.g., the above complex with the two amino ethane groups, the dihalides of mercuric, or even the complex between AMP and mercuric perchlorate Figs. 21 and 22. But to form the third and fourth coordinate bonds requires either a strongly reacting molecule such as one capable of forming a chelate, e.g., diaminoethane or ApAp, the dimer of adenylic acid, or a large excess of reactant such as $Cl^-$ in forming $HgCl_4^=$.

From the fractional change in absorption at 260 mμ we may, as with the monomer, estimate a minimum value for the amount of ApAp complexed by the mercuric chloride. (By assuming no absorption at 260 mμ for the complexed species, and correcting from Fig. 10 for the absorption at 260 mμ of the $HgCl_2$.) Since the chelated species likely has a non-zero extinction at 260 mμ, the actual fraction chelated is greater than this calculated minimum. Thus from Fig. 13, the minimum fraction of chelate

formed when $10^{-3}$ M $HgCl_2$ is added to a solution $2.4 \times 10^{-5}$ M in ApAp is .37.

The relative absorption at 285 mμ is also indicative of the fraction of ApAp chelated bu mercuric chloride. What is not known is the extinction coefficient of this chelated species at that wavelength. As with the monomer, we assume an extinction coefficient of 10,000 for the $HgCl_2$AMP complex which would amount to 20,000 for the $HgCl_2$ApAp complex, if both mercuric adenylic acid ligand bonds were the same (and the same as that in the monomer complex). The extinction of uncomplexed ApAp at 285 mμ is 1870 (935 per Ap residue).

Designating f as the fraction of the ApAp molecules chelated, and $ApAp_0$ as the total (chelated + free) ApAp we have:

$$OD_{285} = OD_{285}(\text{free ApAp}) + OD_{285}(\text{chelated ApAp})$$

$$OD_{285} = \epsilon(\text{free ApAp, } 285\ m\mu)(1-f)ApAp_0 + \epsilon(\text{chelated ApAp, } 285\ m\mu)fApAp_0$$

From the optical densities of Fig. 13 we may calculate the values of f at different concentrations of mercuric chloride, and from these may calculate values for the equilibrium constant of the reaction.

Table II gives the calculated values of f and of K for different concentrations of $HgCl_2$, under the assumption that ε(chelated ApAp, 285 mμ) is equal to 20,000. While all the values of the equilibrium constant are of the same order of magnitude the agreement between them could be better. One

Table II. The Equilibrium Constant for the Formation of the Chelated Complex $HgCl_2ApAp$: Calculated Values as a Function of $(HgCl_2)$ and of Assumed $\epsilon(HgCl_2ApAp, 285\ m\mu)$

| $(HgCl_2)_0$ Total concentration of mercuric chloride | $\epsilon$ = 15,000 | | $\epsilon$ = 20,000 | | $\epsilon$ = 25,000 | | $\epsilon$ = 30,000 | | $\epsilon$ = 35,000 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | f | K | f | K | f | K | f | K | f | K |
| $10 \times 10^{-5}$ | .047 | 500 | .034 | 360 | .027 | 280 | .022 | 230 | .019 | 192 |
| $25 \times 10^{-5}$ | .316 | 1910 | .229 | 1210 | .179 | 890 | .148 | 702 | .125 | 580 |
| $50 \times 10^{-5}$ | .641 | 3690 | .465 | 1770 | .364 | 1170 | .299 | 867 | .254 | 690 |
| $75 \times 10^{-5}$ | .837 | 7050 | .606 | 2100 | .475 | 1230 | .391 | 867 | .332 | 670 |
| $100 \times 10^{-5}$ | .932 | 14040 | .675 | 2120 | .529 | 1140 | .435 | 780 | .370 | 590 |

possibility was to see if the agreement could be improved by using different values for $\epsilon$(chelated ApAp, 285 m$\mu$).

We prepared a computer program which varied the value of this extinction coefficient, and from each such value calculated sets of equilibrium constants and sets of values of f, the fraction of ApAp calculated at different concentrations of $HgCl_2$. We ran the program on an IBM 7090.

As discussed, for the greatest concentration of $HgCl_2$ used in these experiments, $10^{-3}$ M, the extinction coefficient cannot be allowed to give a value for the fraction of ApAp chelated which is less than .37. This sets an upper limit to $\epsilon$(chelated ApAp, 285 m$\mu$) of 35,000.

Now assume that at $10^{-3}$ M $HgCl_2$ 100% of the ApAp is chelated, an obvious upper limit to f. The $OD_{285}$ under these conditions is .35, and the concentration of total dimer, $ApAp_o$ now all assumed to be complexed, is $2.4 \times 10^{-5}$ M. This gives a lower, physically allowed, limit to the value of the extinction of $.35/2.4 \times 10^{-4} \cong 14500$.

In Table II sets of values for f and K appear, each set based upon some value of $\epsilon$(chelated ApAp, 285 m$\mu$) chosen within the above physical limitations. The best agreement among a set of such values of K occurs by assuming a value for the extinction near its upper allowed limit.

That we do not get better agreement among a set of equilibrium constants may be due (as pointed out in the discussion on $HgCl_2$ binding to the monomer) to some binding of

$HgCl_2$ at an additional site which does not much effect the light absorption properties of the aromatic ring portion of adenylic acid. Thomas points out that some of the mercuric dihalide is dissociated in water.[11] This charged species might very well react with the negatively charged phosphate groups of the nucleotides. While the optical absorption spectra show an isosbestic point, Fig. 13, the rotatory dispersion spectra of the dimer upon incremental addition of $HgCl_2$ do not (Fig. 14). This might indicate that some additional mercuration is occurring which alters the conformation of the molecule, while only minimally effecting the optical absorption.

From the same data of Fig. 13, there is some further indication that $Cl^-$ is not released in the reaction of $HgCl_2$ with ApAp. Let us assume our same model for chelation, but also assume that upon forming the complex with the adenine rings $HgCl_2$ does lose its $Cl^-$ ions.

$$HgCl_2 + ApAp \rightleftharpoons HgApAp^{++} + 2Cl^-$$

$$K_A = (HgApAp^{++})(Cl^-)^2/(HgCl_2)(ApAp)$$

Assume that the $\epsilon(HgApAp^{++}, 285\ m\mu) = 20{,}000$. Using this value while it did not give perfect agreement between calculated equilibrium constants in Table II, at least the values obtained for different concentrations of $HgCl_2$ are within the same order of magnitude. Using this, for the extinction we compare, in Table III, the calculated values for the equilibrium constants (a) with the above model for the chelation reation, and (b) with

Table III. Comparison of Chelation Models Assuming.

$\epsilon$(chelated ApAp, 285 m$\mu$) = 20,000

| $(HgCl_2)_0$ Total mercuric chloride | f fraction of ApAp chelated | K(lit/mole) | $K_A$(mole/lit) |
|---|---|---|---|
| $10 \times 10^{-5}$ M | .034 | 360 | $32 \times 10^{-10}$ |
| $25 \times 10^{-5}$ M | .229 | 1210 | $1500 \times 10^{-10}$ |
| $50 \times 10^{-5}$ M | .465 | 1770 | $8300 \times 10^{-10}$ |
| $75 \times 10^{-5}$ M | .606 | 2100 | $18400 \times 10^{-10}$ |
| $100 \times 10^{-5}$ M | .675 | 2120 | $23000 \times 10^{-10}$ |

our previously assumed model, $HgCl_2 + ApAp \rightleftharpoons HgCl_2ApAp$. Clearly, assuming that no chloride is released leads to a significantly better agreement among calculated equilibrium constants as the concentration of mercuric chloride is varied.

There is yet a third way to get some information on the equilibrium constant of the dimer-mercuric chloride complex formation. At relatively high concentrations of $HgCl_2$, the concentration of uncomplexed mercuric chloride is nearly equal to that of the total mercuric chloride concentration, since only a small fraction is actually complexed with the dimer. $[HgCl_2] \cong [HgCl_2]_0$. At 285 mμ, when even a small percentage (say greater than 25%) of the dimer is complexed, almost all the absorption is due to the complex alone.

$$OD_{285} \cong \epsilon(ApApHgCl_2,\ 285\ m\mu)[ApApHgCl_2].$$

The equilibrium constant for the complex formation, K, is equal to

$$[ApApHgCl_2]/[HgCl_2][ApAp]$$
$$= [ApApHgCl_2]/[HgCl_2][(ApAp)_0 - ApApHgCl_2]$$

Abbreviating the above extinction as merely $\epsilon$, and $OD_{285}$ as simply OD, and utilizing the two above approximations concerning mercuric concentration and light absorption at 285 mμ we have

$$K = \frac{OD/\epsilon}{[HgCl_2]_0[(ApAp)_0 - OD/\epsilon]}$$

This can be put into the form

$$1/\epsilon + (1/\epsilon K)(1/[HgCl_2]_0) = [ApAp]_0/OD$$

which is of the form $y = mx + b$. By plotting $[ApAp]_0/OD$ vs. $1/[HgCl_2]_0$, and extrapolating the line found to essentially infinite mercuric chloride concentrations, one finds the intercept on the y axis, b, which gives the value of $1/\epsilon$. From this, and the slope of the line, m, which is equal to $1/K\epsilon$, one may find the equilibrium constant for the reaction.

Figure 22a is a plot of $[ApAp]_0/OD_{285}$ vs. $1/[HgCl_2]_0$. The data for this plot is taken from Fig. 13. $[ApAp]_0$ is $2.41 \times 10^{-5}$ M. We have drawn a somewhat arbitrarily chosen best straight line through the points obtained. The points which represent the lowest concentrations of mercuric chloride are neglected in drawing this line, for at low mercuric concentrations, the two approximations just given do not hold.

From the y axis intercept and the slope of our best straight line we find that the value of $\epsilon(ApApHgCl_2, 285\ m\mu) = 26,400$, and the equilibrium constant for the reaction is equal to 1360. If we assume, as heretofore, that the $ApApHgCl_2$ chelate is composed of the two Ap molecules of the dimer similarly complexed to an $HgCl_2$ molecule, then per unit Ap residue, or per mercuric-nitrogen bond, the extinction coefficient at 285 mμ is equal to 13,200.

A more complete reaction with the dimer (judging from OD 285 mμ) occurs when the mercuric is used as its oxyacid salt rather than as the chloride. These larger changes are observable in either the absorption or rotatory dispersion spectra, Figs. 23 and 24. Neither of these sets of spectra possess

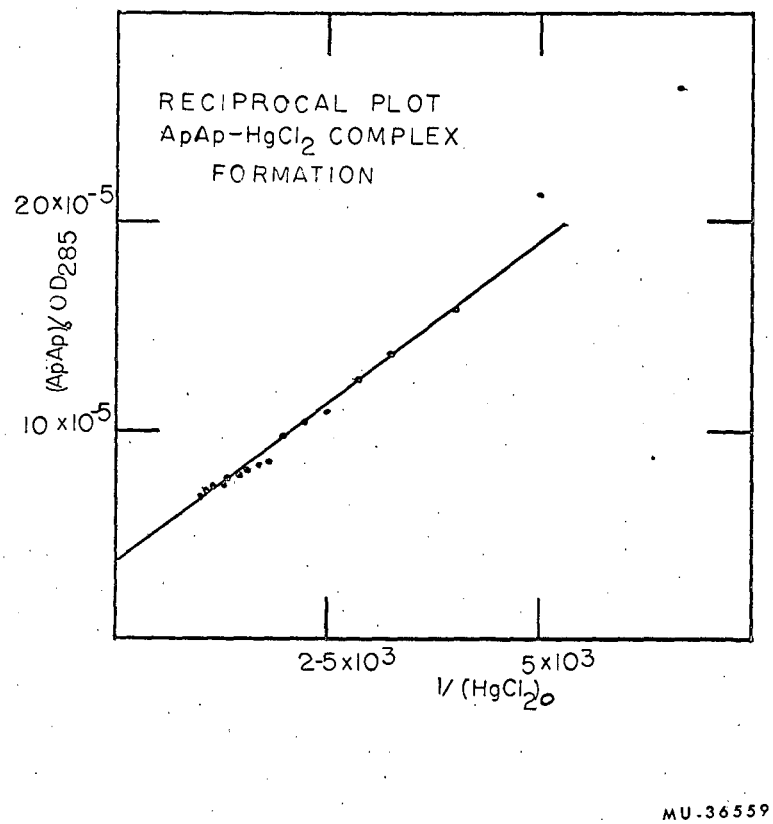



Figure 22a. Reciprocal plot of ApAp-$HgCl_2$ complex formation.

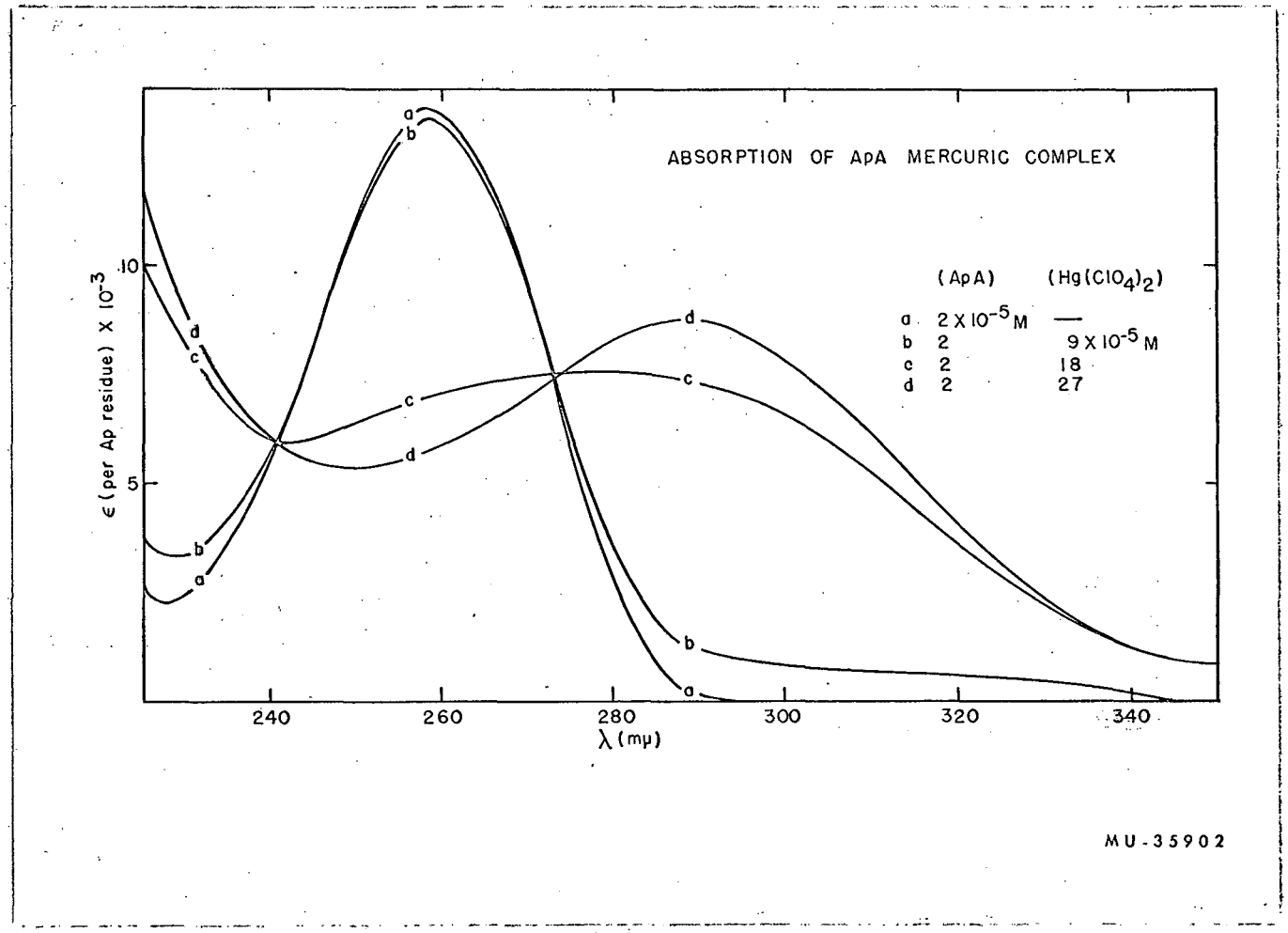



Figure 23. Interaction of mercuric perchlorate with ApA: change in absorption spectrum.

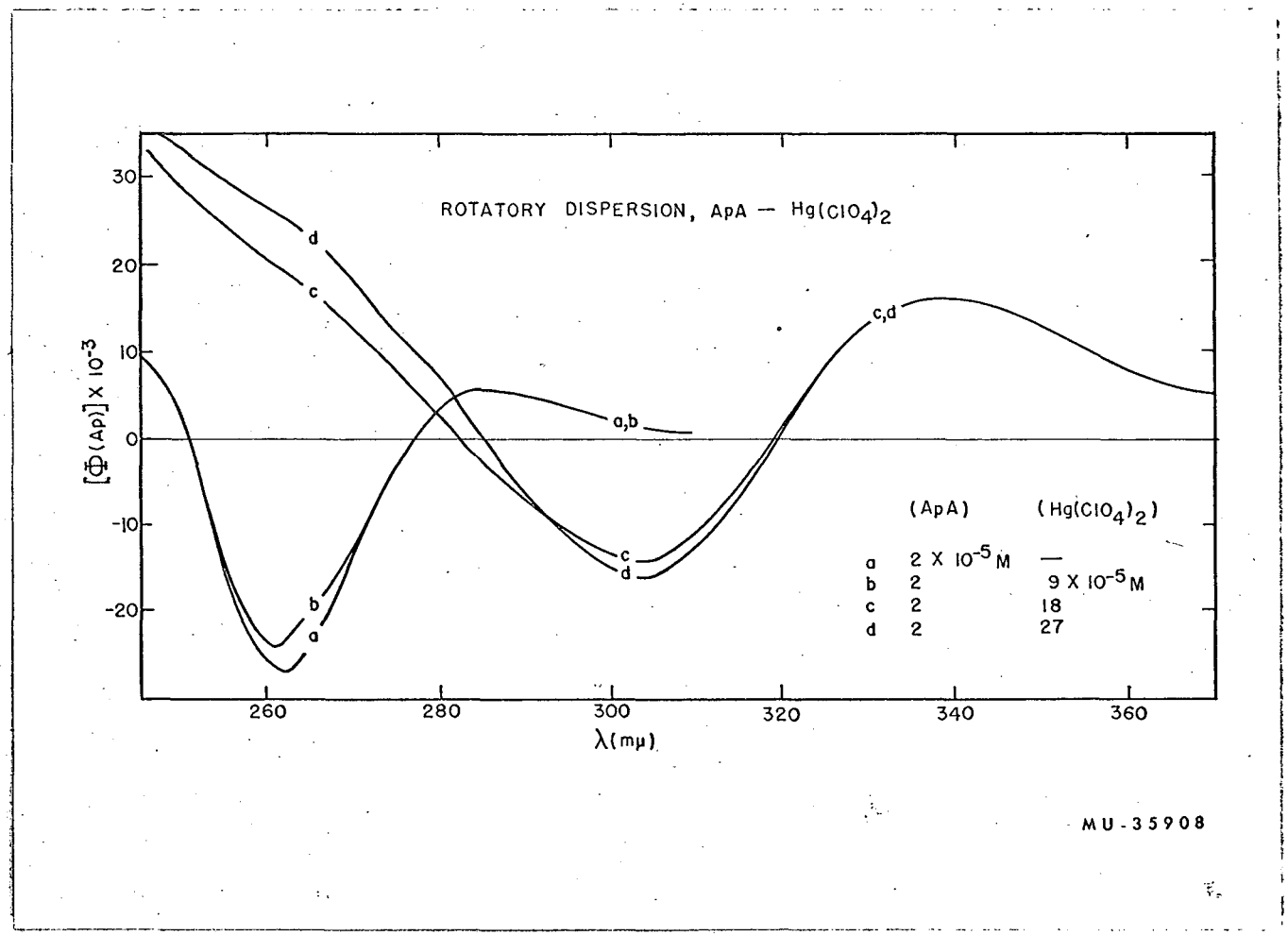



Figure 24. Interaction of mercuric perchlorate with ApA: change in rotatory dispersion.

isosbestic points, indication that more than one species of complex molecule is formed. At still higher concentrations of mercuric perchlorate, there is a tendency to get precipitate formation.

Our model has assumed that there is a 1/1 mercuric chloride/ ApAp chelate formed. It would be nice to verify that the two molecules truly do combine in this ratio.

## The Determination of Stoichiometry in the ApAp-$HgCl_2$ Complex Formation

Suppose there is a chemical reaction in which

$$nX + Y \rightleftharpoons X_nY$$

and suppose that one wishes to find the value of n. If $X_nY$ possesses a physical characteristic not possessed by either X or Y, e.g., optical absorption, optical rotation, etc., then it is possible to find the ratio n, characteristic of the product $X_nY$.[58]

Let

$$X = [HgCl_2];\ Y = [ApAp];\ X_nY = [ApAp(HgCl_2)_n]$$

The absorbancies of the two reacting molecules at 300 mμ are very small, that of the complex which is formed from them is quite large.

Let

$$X_0 = [HgCl_2]_{total} = [HgCl_2] + n[ApAp(HgCl_2)_n]$$

$$Y_0 = [ApAp]_{total} = [ApAp] + [ApAp(HgCl_2)_n]$$

If we set the sum of the two reactants equal to some constant, and vary the ratio of the reactants $X_0 + Y_0 = A$ at what ratio, $X_0/Y_0$ do we achieve a maximum of complex formation? Put in another way, at what ratio of reactants is there a maximum OD at 300 mμ (where only the complex significantly absorbs light)?

$$X_n + Y \rightleftharpoons X_nY$$

$$K = X_nY/X^nY$$

$$K = Z/(X_o-nZ)^n(Y_o-Z)$$

Differentiating to find the conditions for maximal complex formation.

$$dZ/dX_0 = 0 \quad dZ/dY_0 = 0$$

under the restraint of both reactants, $X_0 + Y_0 = A$

$$nZ/(X_0-nZ)^{n+1}(Y_0-Z) = Z/(X_0-nZ)^n(Y_0-Z)$$

$$n(Y_0-Z) = (X_0-nZ)$$

$$X_0/Y_0 = n$$

Whether protons, or chloride ions are released in this reaction or not, carrying through the mathematics will show that the maximum complex formation still occurs when the ratio of the reactants, $X_0/Y_0$, is equal to the ratio of their composition in the complex, n.

We prepared solutions, whose compositions are shown in the table below. The sum of the $ApAp_0$ and the $HgCl_{2_0}$ was, in all cases, equal to $5x10^{-4}$ M. All solutions contained a pH 5.7 acetate buffer (.01/.001 acetate/acetic acid) and all were .1M in $NaClO_4$.

Composition of Solutions

| $ApAp_0$ | $HgCl_{2_0}$ |
|---|---|
| $5.00 \times 10^{-4}$ M | - |
| 4.00 | $1.00 \times 10^{-4}$ M |
| 3.00 | 2.00 |
| 2.50 | 2.50 |
| 2.25 | 2.75 |
| 2.00 | 3.00 |
| 1.67 | 3.33 |
| 1.00 | 4.00 |

Figures 25 through 28 show both absorption and rotatory dispersion measurements of the mixtures of solutions above. Figures 25 and 27 are the results of spectra taken through a 1 mm path length of solution. Figures 26 and 28 result from the utilization of a 1 cm path length. The former two figures give an overall picture of the spectra, the later two magnify the region of especial interest. This is the region where both rotation and absorption are primarily due to the presence of the mercuric complex.

We see that both the 290 mμ absorption, and the 320 mμ cotton effect are maximal with an $HgCl_{2_0}/ApAp_0$ ratio of 3/2, Fig. 28A. According to the theory, then, this should be the ratio in which the two constituents of the complex combine. It is hard to reconcile this with the seeming simplicity of the reaction as judged from the existence of isosbestic points in the absorption spectra.

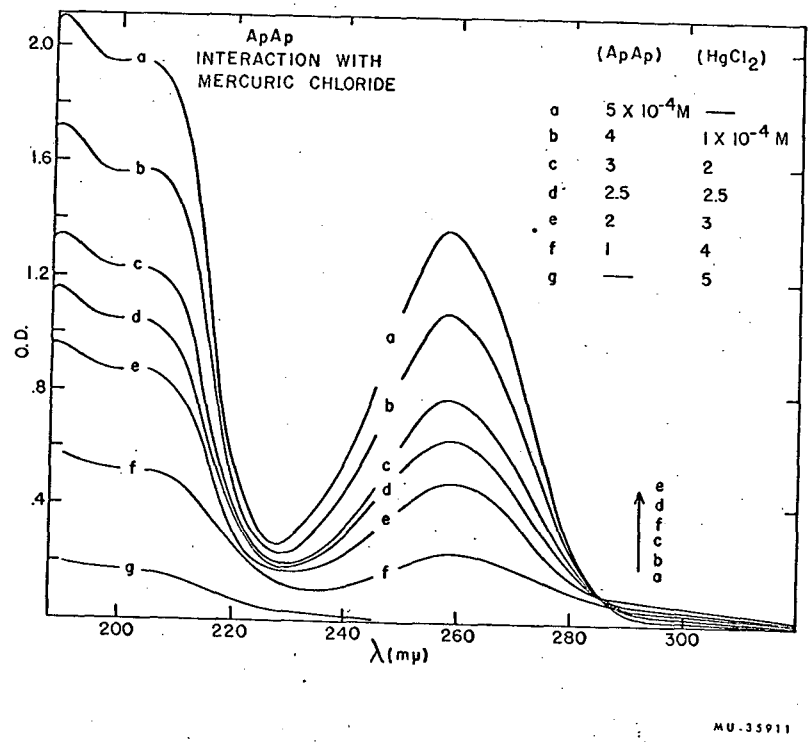



Figure 25. Absorption spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of reactants changes.

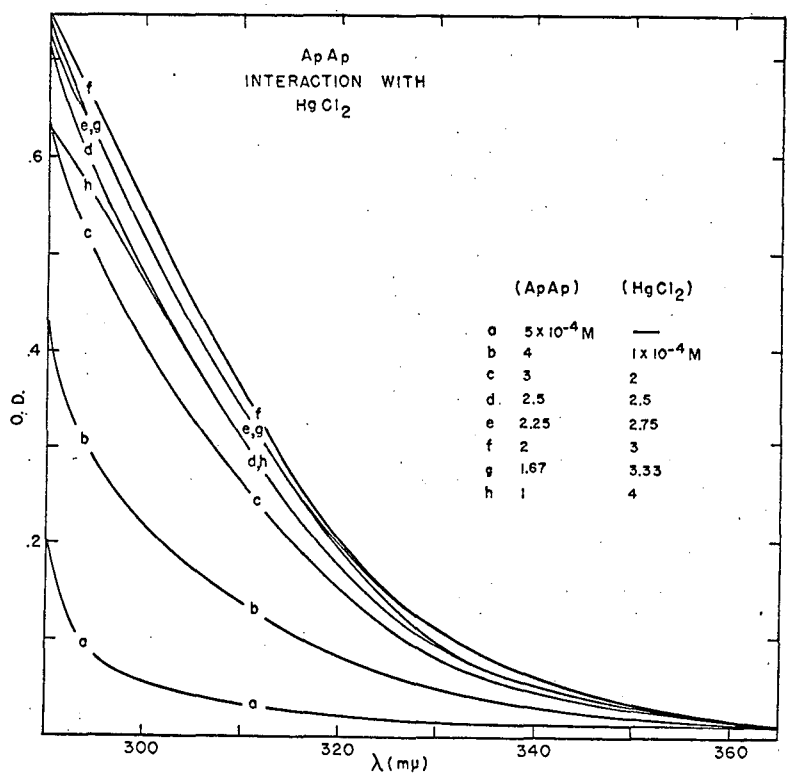



Figure 26. Absorption spectra of ApAp + $HgCl_2$ at same total concentration for both reactants as ratio of reactants changes. (Expanded absorption at red end of absorption band.)

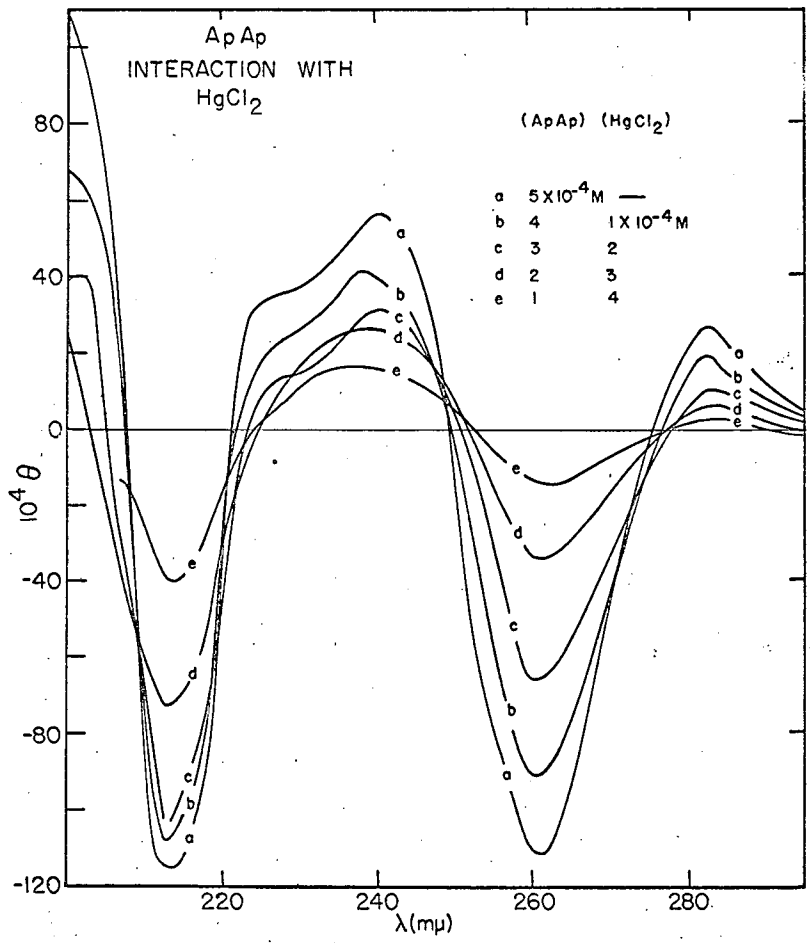



Figure 27. Rotatory dispersion spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of [illegible] reactants changes.

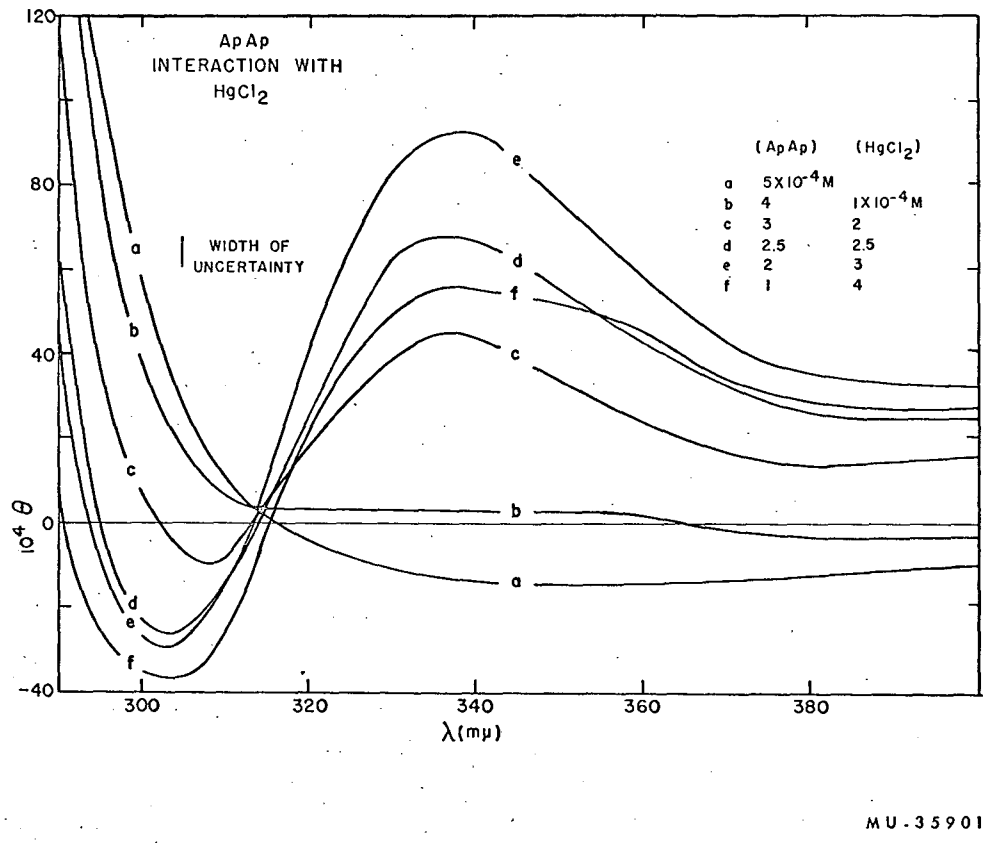



Figure 28. Rotatory dispersion spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of reactants changes. (Expanded scale at red end of rotatory dispersion band.)

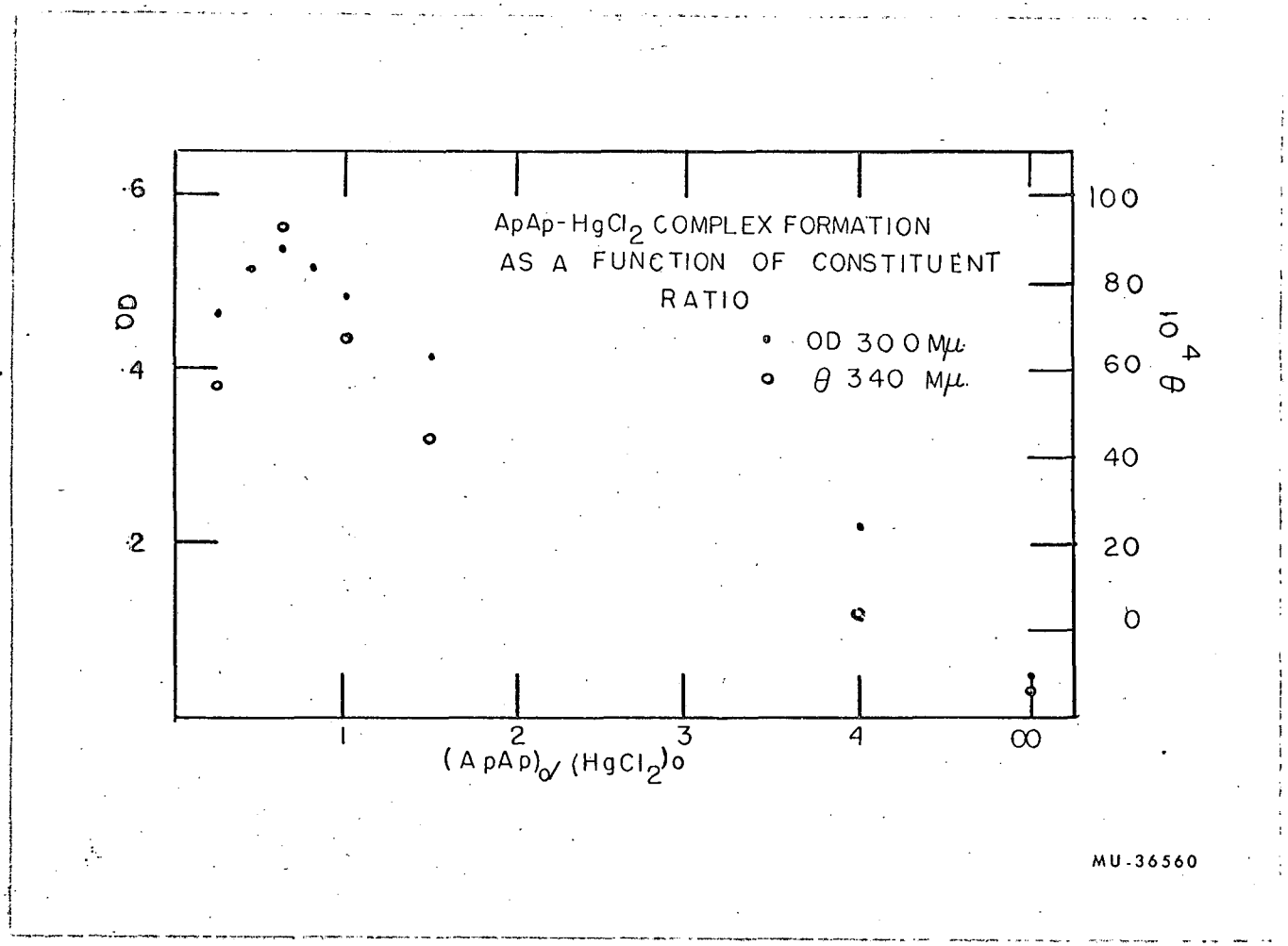



Figure 28a. ApAp-$HgCl_2$ complex formation as a function of constituent ratio.

Suppose that there also exists another site upon the ApAp which competes for the mercuric chloride. The reaction at that site must have, as its distinguishing characteristic, no involvement with the aromatic portions of the molecule, and hence no direct effect upon its 260 mμ electronic absorption band. We may write this reaction as

$$ApAp + HgCl_2 \rightleftharpoons (HgCl_2)^{*}ApAp$$

where the * indicates a mercuric chloride bound at the second reaction site of the ApAp molecule. Leaving the definitions of other quantities unchanged, we redefine Z as being the total concentration of ApAp complexed by $HgCl_2$ at the site which gives rise to the 290 mμ absorption and to the 320 mμ cotton effect. This includes ApAp complexed at the second site additionally.

$$Z = [ApApHgCl_2] + [HgCl_2{}^{*}ApApHgCl_2]$$

T, on the other hand is defined as the total ApAp complexed at the second site by $HgCl_2$, whether or not the ApAp also has an $HgCl_2$ otherwise complexed.

$$T = [HgCl_2{}^{*}ApAp] + [HgCl_2{}^{*}ApApHgCl_2]$$

If it is assumed that the reaction responsible for the 290 mμ absorption band and for the 320 mμ cotton effect is a 1/1 $HgCl_2$/ApAp complex formation, how would the formation of the second site complex alter the ratio, $X_o/Y_o$ at which we would see the maximum of these effects? This ratio would no longer be at unity, but rather would occur when

$$([HgCl_2]_0 - T)/(ApAp_0) = 1 \text{ or } (HgCl_2)_0/[ApAp_0]$$
$$= 1 + T/[ApAp_0]$$

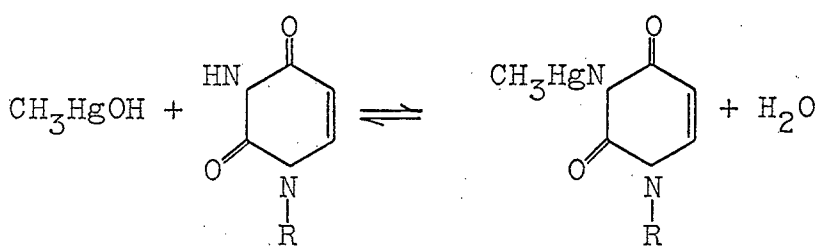
This could well account for the observed 3/2 ratio obtained.

## Mercuric Chloride Binding by Heterodimers

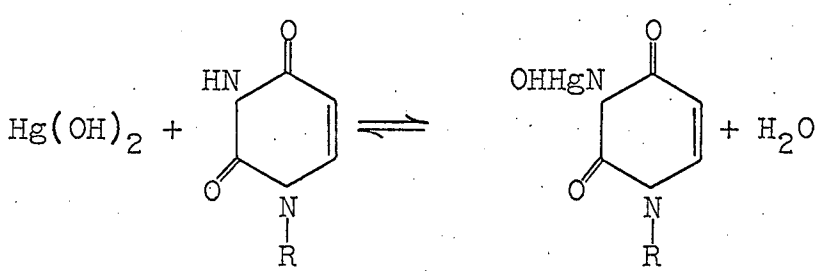
In trying to elucidate further, the nature of the reaction of mercuric chloride with the adenylic acid dimer, we looked at the complexing reactions of some analogous compounds, ApU and ApC.

Consider the reaction of mercuric chloride with uridylic acid. See Fig. 29. The spectral change here is quite the same as Simpson obtained with a many times excess of mercuric or methyl mercuric ion, and probably represents a fairly complete reaction of the uridylic acid with the mercuric chloride.

Simpson found that the reaction




proceeds with an equilibrium constant (log K = 4.3), and that the reaction




proceeds with a constant (log K = 5.1).[12] That the hydrogen of the cyclic imide structure is replaced is to be expected, for such generally occurs upon mercuration of such structures.[17] Furthermore, the small change in spectral properties upon

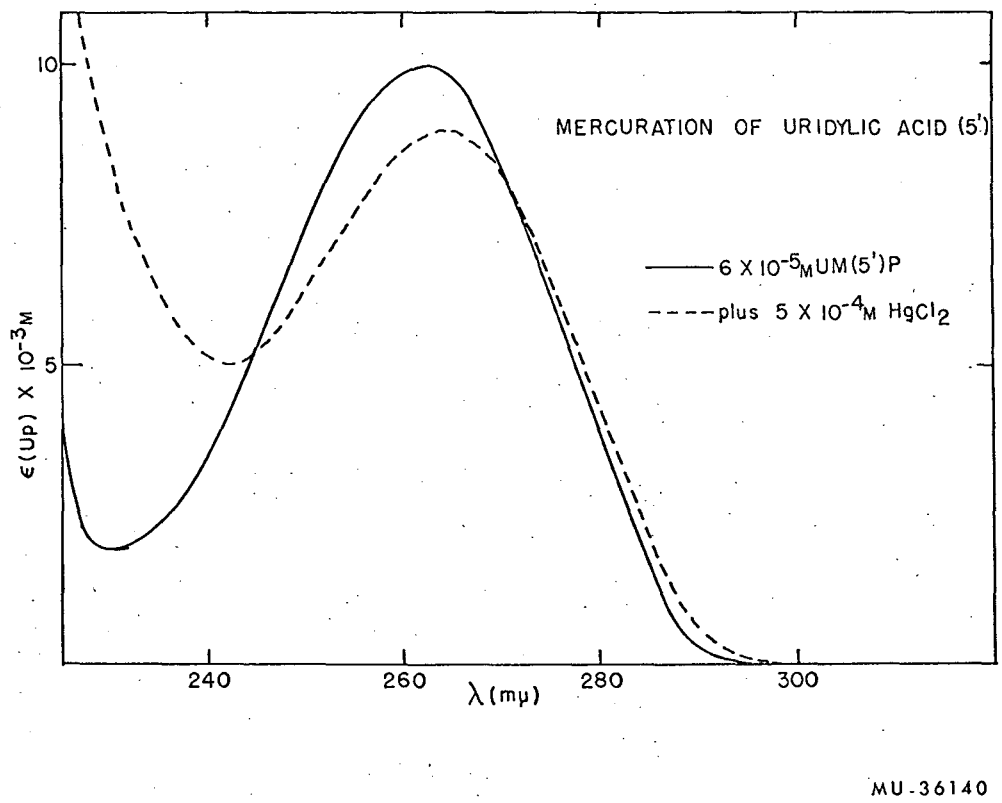
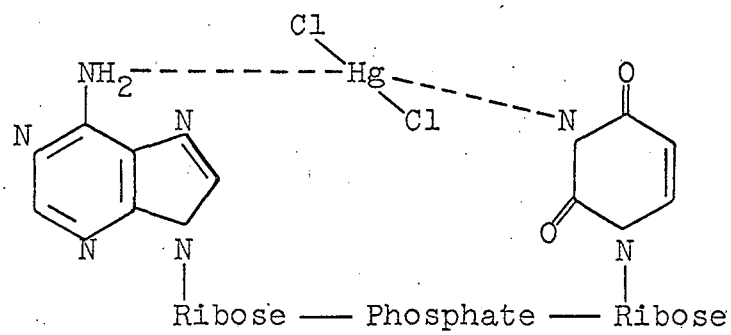



Figure 29. Interaction of mercuric chloride with uridylic acid. Absorption spectra.

mercuration of a ring nitrogen is not inconsistent with the equally small changes found upon mercuration of the pyridine molecule. Figure 20. The reaction of uridylic acid, $6x10^{-5}$ M with $HgCl_2$, $5x10^{-4}$ M, took place in an acetate buffered medium, pH 5.7 with .1N $NaClO_4$ present. There was no $HgCl_2$ in reference chamber of the Cary 15 recording spectrophotometer, see Fig. 10.

If one uses instead of uridylic acid alone, an equimolar combination of adenosine and uridylic acid each $3x10^{-5}$ M, and all other conditions as above, the spectral changes are indicative of a mercuration of uridylic acid alone, Fig. 30. The changes in rotatory dispersion spectra with the mercuration of this mixture of compounds may be seen in Fig. 31. Compare changes in adenylic acid alone which occur upon mercuration, Figs. 11 and 12. If one now leaves all conditions the same, but links the equimolar quantities of adenosine and uridylic acid into the ApU dimer, the reaction with mercuric chloride, based upon spectral changes, is greatly enhanced, Figs. 32 and 33. The spectral changes are of the same nature seen with ApA, and likely the same sort of chelate formation is involved. Our hypothesized structure is illustrated below.



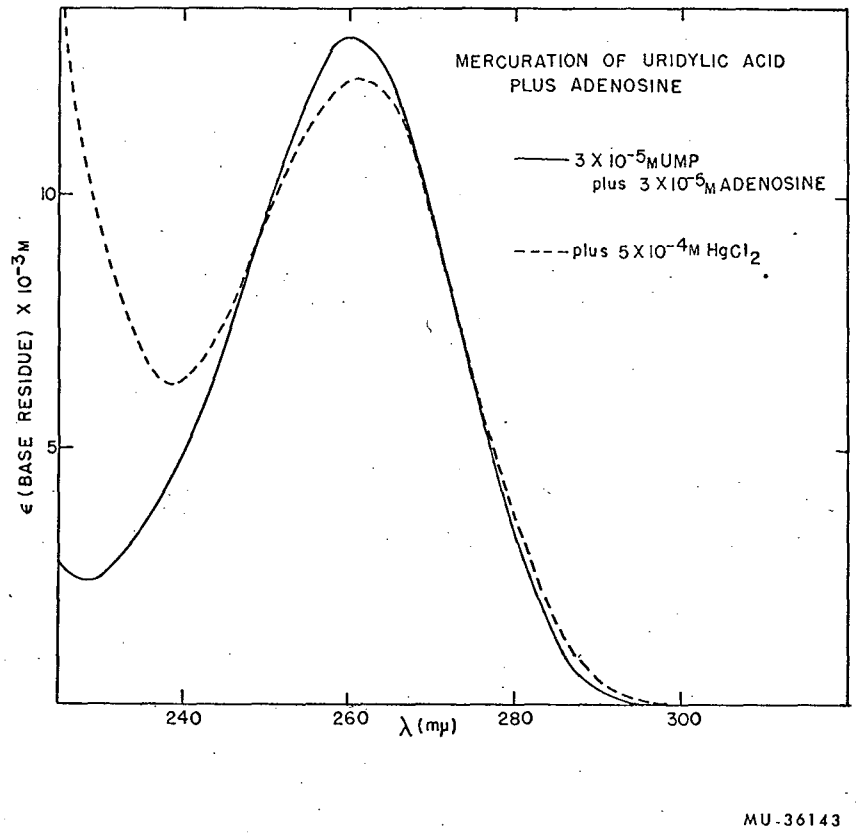



Figure 30. Interaction of mercuric chloride with an equal parts mixture of uridylic acid and adenosine. Absorption spectra.

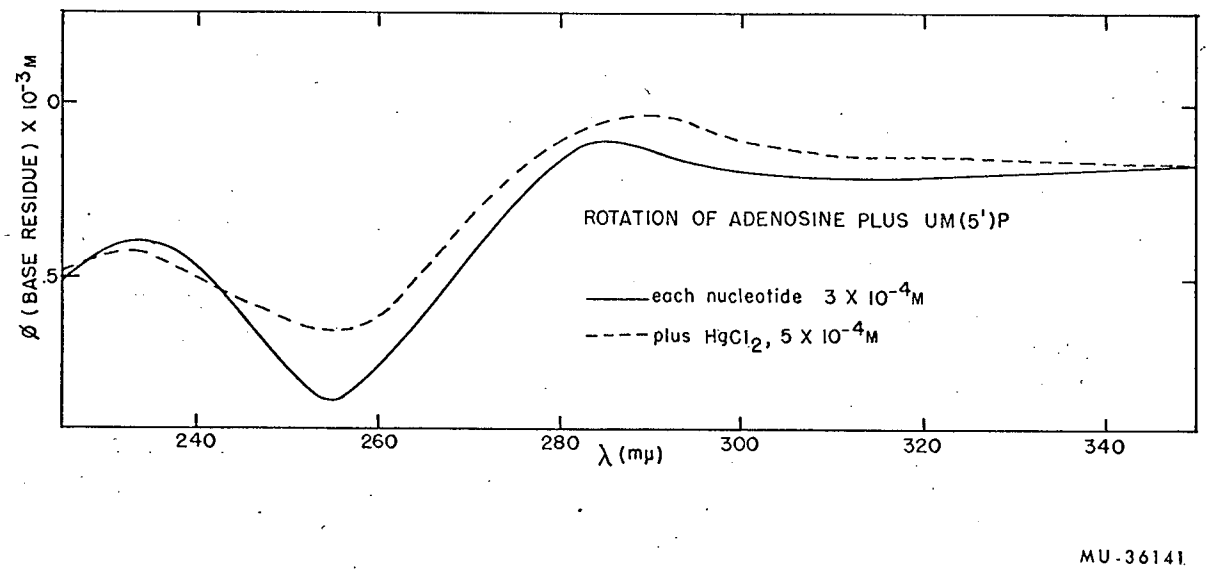



Figure 31. Interaction of mercuric chloride with an equal parts mixture of uridylic acid and adenosine. Rotatory dispersion spectra.

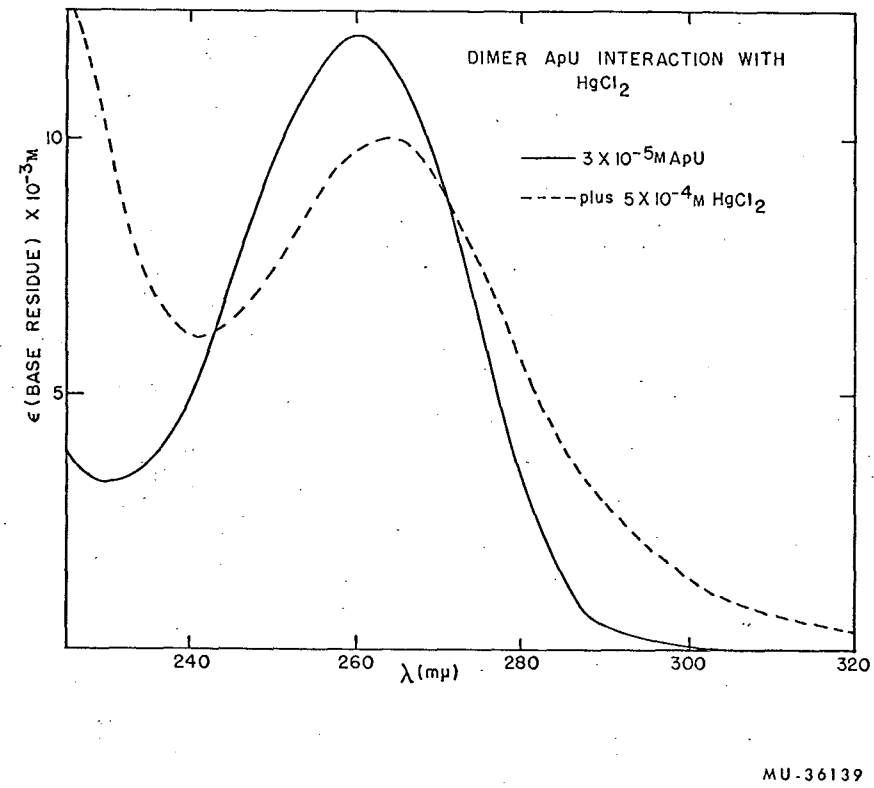



Figure 32. Interaction of mercuric chloride with ApU dimer. Absorption spectra.

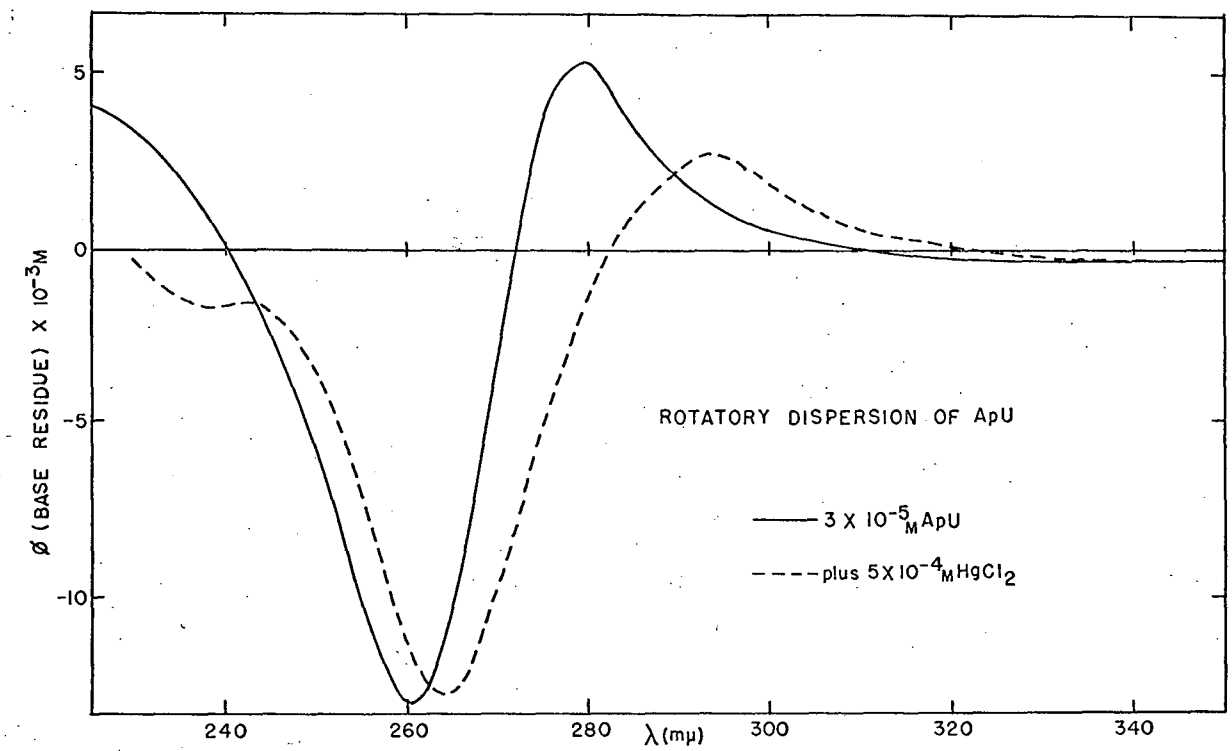
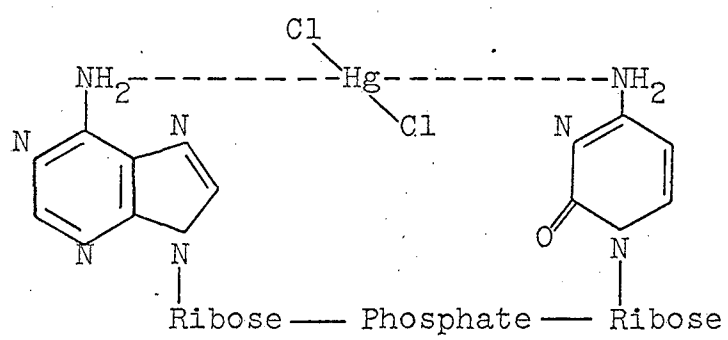



Figure 33. Interaction of mercuric chloride with ApU dimer. Rotatory dispersion spectra.

A similar account may be given for the binding of $HgCl_2$ to mixtures of adenosine and cytidylic acid, as opposed to its binding to the ApC dimeric molecule, Figs. 34, 35, and 36. In the experiments with ApC, there are a few changes in the experimental conditions to be noted. Each base residue was present at a concentration of $2.3 \times 10^{-5}$ M, and in the absorption spectrum, the reference blank was $5 \times 10^{-4}$ M in $HgCl_2$. Data upon the extinction coefficients of the two dimers used was provided by the work of Warshaw.[18]

We illustrate a probable structure for the mercuric complexed ApC below. The reaction site of cytidine poses many of the same problems as does that of adenosine. Is the site really the amino group, or could it be at the ring nitrogen?[12]



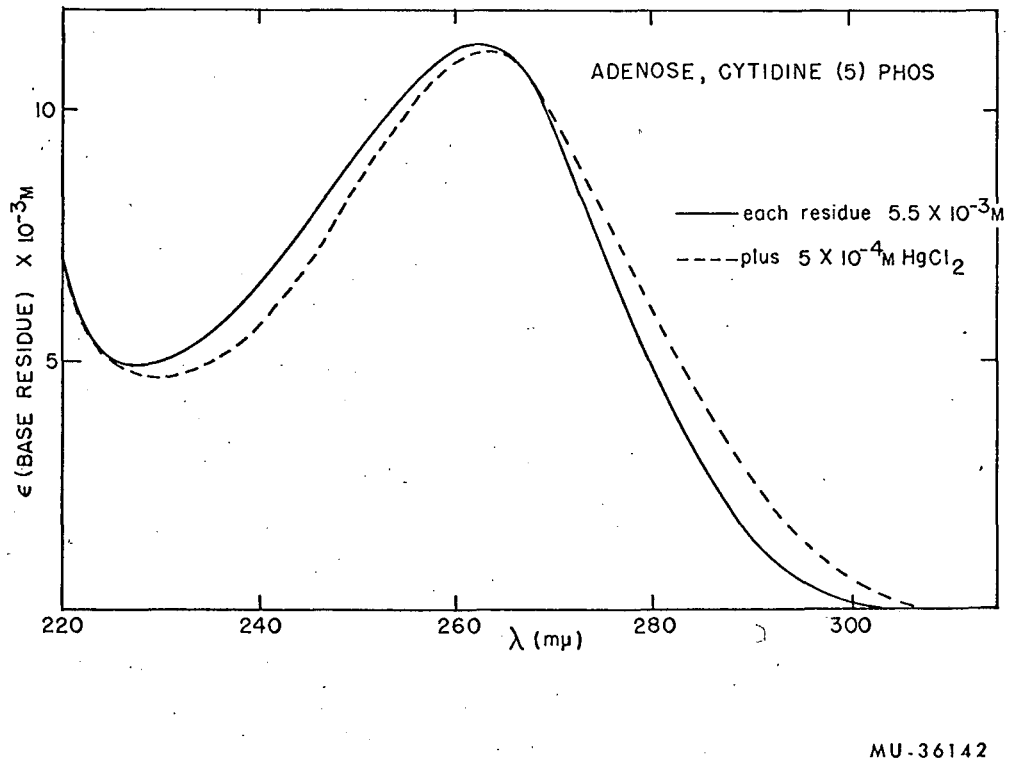



Figure 34. Interaction of mercuric chloride with equal parts of cytitylic acid and adenosine.

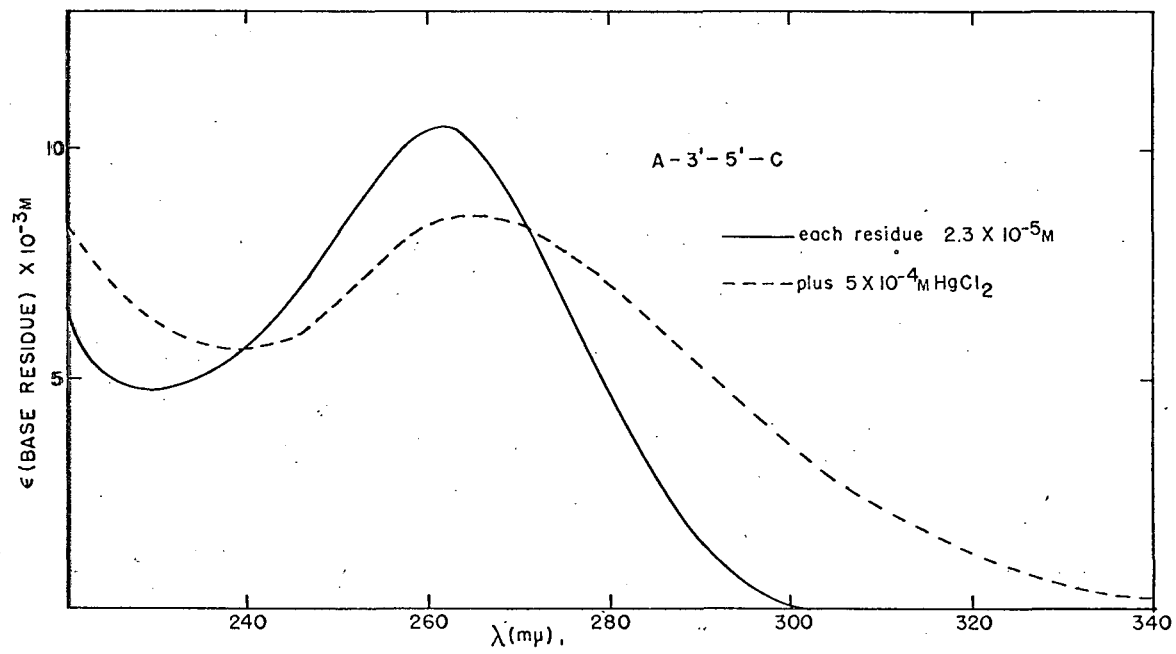



Figure 35. Interaction of mercuric chloride with ApC dimer. Absorption spectra.

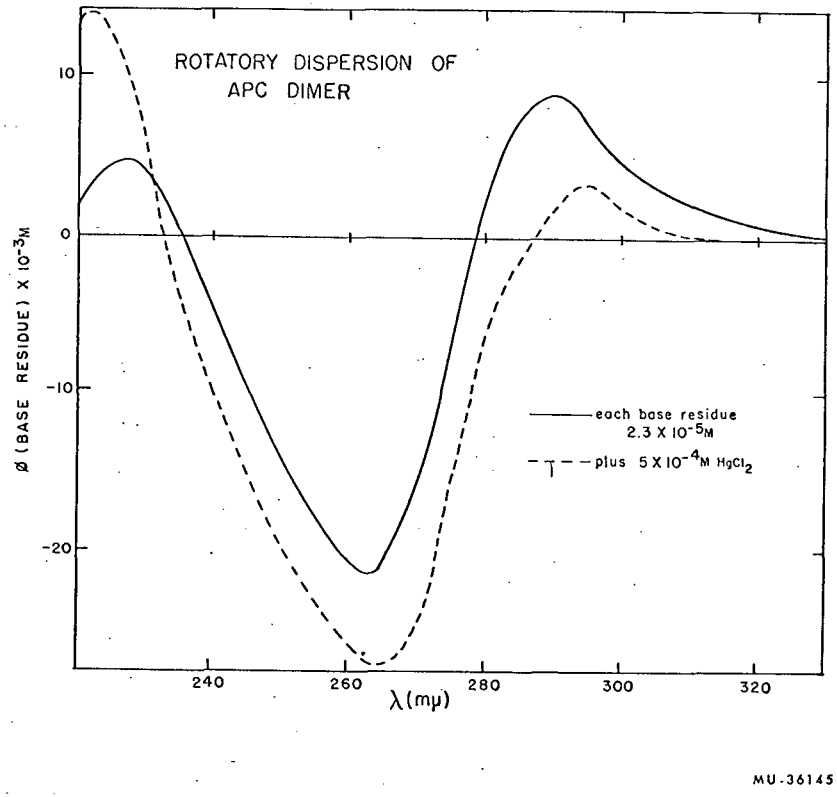



Figure 36. Interaction of mercuric chloride with ApC dimer. Rotatory dispersion spectra.

## The Binding of Ap Trimer by Mercuric Chloride

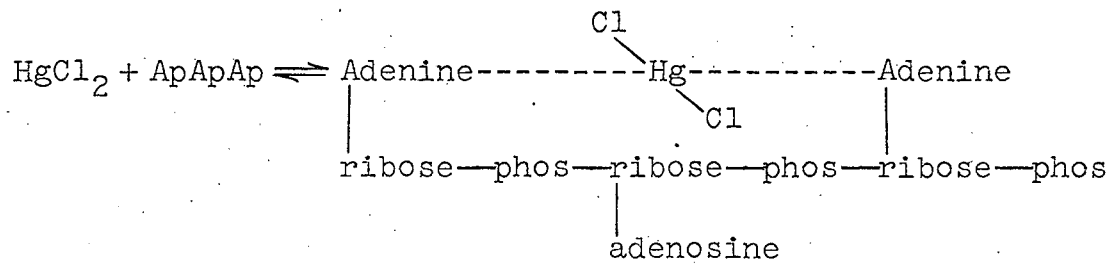
The complex formation of mercuric chloride with the adenylic acid trimer, ApApAp behaves in a fashion similar to that with the dimer, but it is a stronger reaction, Figs. 16, 17 and 18.

We have used a trimer concentration of $1.8 \times 10^{-5}$ M, with other conditions of the reaction as in the complex formation with the monomer and dimer of adenylic acid.

At concentrations of $HgCl_2$ less than $4 \times 10^{-4}$ M, the absorption spectra possess an isosbestic point. This leads one to suspect the formation, under these conditions, of a single species of complexed molecule. If the reaction were the same as that with the dimer, it would involve the chelating of mercuric between two adenine residues. In the dimer (on the basis of Courtauld Models[19]) there appears to be some steric difficulties in so accommodating the mercuric chloride. In the trimer, the $HgCl_2$ could be chelated by (and easily accommodated between) the two end adenosine residues, leaving the middle residue to react separately at sufficiently high concentrations of $HgCl_2$. This ease of accommodation could well be the reason for the greater degree of reaction of the trimer over that of the dimer.

Above $4 \times 10^{-4}$ M $HgCl_2$, the isosbestic point seen at lower concentrations is lost, probably due to the formation of some second site complex, involving the middle adenosine group. Keeping our attention on the situation below an $HgCl_2$

concentration of $4x10^{-4}$ M, one may propose the reaction model shown below




As with the dimer, we can find a maximum and minimum value for the fraction of trimer bound at the first mercuric chloride chelation sites. If we assume that at $4x10^{-4}$ M $HgCl_2$ all trimers are so chelated, we may calculate a minimum value for $\epsilon$(chelated trimer, 285 mμ). From Fig. 16, the absorption at 285 mμ of the trimer in the presence of $4x10^{-4}$ $HgCl_2$ is .495. The concentration of trimer is $1.8x10^{-5}$ M. The minimum value for chelate extinction is thus $.495/1.8x10^{-5} \cong 27,000$, or 9,000 per Ap residue.

The minimum fraction of trimer which can be bound is obtainable, as with the dimer, from the fractional change in the absorption at 260 mμ, appropriately corrected for the absorption of $HgCl_2$. Again from Fig. 16, this fraction comes to .34 (calculated also at $HgCl_2$ concentration of $4x10^{-4}$).

The extinction coefficient for the unchelated trimer at 285 mμ is 3900, or 1300 per Ap residue. By guessing values for the extinction of the chelated trimer at 285 mμ consistent with the above stated physical limitations, we may calculate equilibrium constants for the mercuric chloride binding.

$$OD_{285} = \epsilon(\text{free ApApAp, 285 m}\mu)(1-f)\text{ApApAp}_0$$
$$+ \epsilon(\text{chelated ApApAp, 285 m}\mu)f\text{ApApAp}_0$$

where f is the fraction of the trimer which is bound, and $ApApAp_0$ is the total concentration of trimer, bound and unbound. Table IV gives values for f, and for K according to the selected value of the extinction. As with the dimer, better agreement among a set of equilibrium constants occurs at the higher physically allowed values of the extinction coefficient. What deviation there is among a set of equilibrium constants, as the mercuric chloride concentration is varied, is likely due to the same causes discussed for dimer chelation.

Assuming that chloride ions are released in the mercuration of the trimer leads to a set of equilibrium constants whose values diverge from one another by many orders of magnitude. This is just what we have found in the dimer.

For the dimer, using the absorption at 285 mμ, we plotted $[ApAp]_0/OD$ vs. $1/[HgCl_2]_0$ where the subscript 0, indicates the total concentration of the particular constituent (complexed as well as free).

This allowed us, as discussed, to estimate a value for $\epsilon(ApApHgCl_2, 285\ m\mu)$ as well as for the equilibrium constant for the dimer-mercuric chloride complex formation. In a similar fashion, using Fig. 16, we plot $[ApApAp]_0/OD_{285}$ vs. $1/[HgCl_2]_0$. We draw the best straight line through these points, again, as in the dimer, neglecting points at the lowest few mercuric chloride concentrations. The intercept of this line on the

Table IV. Equilibrium Constant and Chelate Formations as $\epsilon$(chelated ApApAp, 285 m$\mu$) is Varied to Arbitrary Values.

| $(HgCl_2)_0$ Total mercuric chloride in reaction mixture | $\epsilon$ = 30,000 | | $\epsilon$ = 40,000 | | $\epsilon$ = 50,000 | | $\epsilon$ = 60,000 | | $\epsilon$ = 70,000 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | f | K | f | K | f | K | f | K | f | K |
| $5 \times 10^{-5}$ M | .105 | 2460 | .077 | 1710 | .060 | 1300 | .049 | 1060 | .041 | 890 |
| $10 \times 10^{-5}$ M | .395 | 7180 | .290 | 4290 | .226 | 3050 | .186 | 2360 | .158 | 1930 |
| $15 \times 10^{-5}$ M | .575 | 9830 | .418 | 5050 | .328 | 3380 | .269 | 2540 | .228 | 2030 |
| $20 \times 10^{-5}$ M | .715 | 12970 | .512 | 5500 | .401 | 3430 | .330 | 2530 | .280 | 1990 |
| $25 \times 10^{-5}$ M | .774 | 14720 | .561 | 5340 | .440 | 3240 | .361 | 2320 | .307 | 1810 |
| $30 \times 10^{-5}$ M | .840 | 18480 | .608 | 5360 | .476 | 3110 | .391 | 2190 | .332 | 1690 |
| $35 \times 10^{-5}$ M | .875 | 20430 | .630 | 5040 | .494 | 2860 | .406 | 1990 | .344 | 1530 |
| $40 \times 10^{-5}$ M | .890 | 24600 | .653 | 4860 | .512 | 2680 | .421 | 1850 | .359 | 1410 |

y axis, and its slope give us, respectively, $1/\epsilon(ApApApHgCl_2$, 285 mμ) and $1/K\ \epsilon(ApApApHgCl_2$, 285 mμ), from which the extinction coefficient and the equilibrium constant may be found.

This plot is shown in Fig. 36A. We find that the equilibrium constant is 8900, and the extinction coefficient of the chelated species at 285 mμ is 35,700, or about 12,000 per Ap residue.

If we contend that all $HgCl_2$-nitrogen bonds are the same in the dimer and the trimer, we are faced with a problem. In ApAp where we likely have two mercuric nitrogen bonds (one per Ap residue) the extinction per residue at 285 mμ is close to 13,000. If, in ApApAp, we assume that only the two end residues are involved in the chelate formation, we would have an extinction per (mercuric bound) Ap residue of 18,000. If on the other hand we allowed for the possibility that in chelation with the trimer, all three Ap residues could somehow bond to the mercuric chloride, then the extinction per mercuric chloride bound Ap residue would be close to 12,000, a value not inconsistent with the 13,000 found for the dimer.

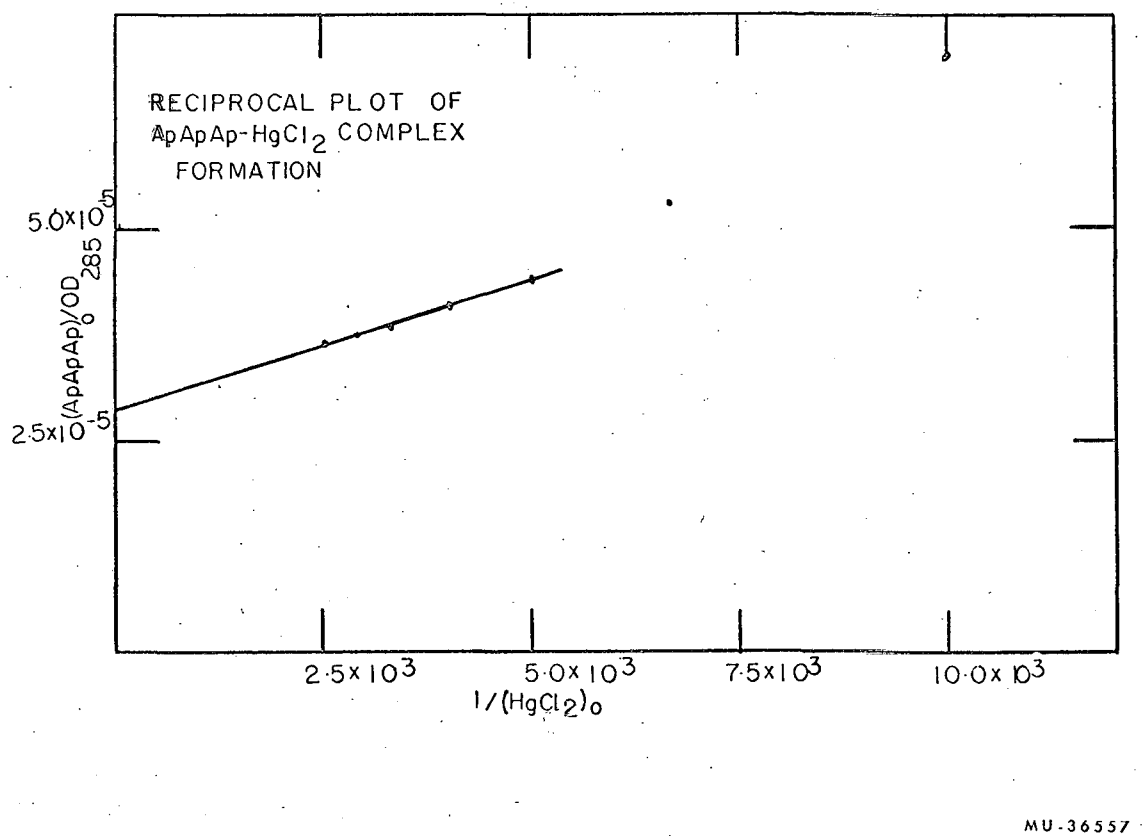



Figure 36a. Reciprocal plot of ApApAp-$HgCl_2$ complex formation.

## The Binding of Polyadenylic Acid by $HgCl_2$

Kawade performed experiments of a similar nature, using $HgCl_2$ to bind polyadenylic acid.[20] His conditions differed a bit from the ones we employed with the oligomer binding experiments. He used a pH 7 phosphate buffer, and a .14M concentration of sodium perchlorate. His conditions imply that the poly A he used was in the form of a single stranded polymer.[21]

The spectra of the solutions of poly A, upon addition of mercuric chloride, do not possess an isosbestic point. We may still obtain some idea of the strength of binding. Take the extinction of the $AMPHgCl_2$ complex to be 10,000 at 285 mμ. The extinction per Ap residue of poly A at 285 mμ is 1600.[22] In his experiments, the solutions of poly A contained $5.65 \times 10^{-5}$ M Ap residue. On the basis of the change in $OD_{285}$ with repeated addition of $HgCl_2$, one may get a rough idea of the fraction of Ap residues complexed. These data, gleaned from Fig. 11 of Kawade's paper are given in the table below.

Binding of $HgCl_2$ to Single Stranded Polyadenylic Acid
(From Kawade)

| $(HgCl_2)_0$ | $HgCl_2$ free | $ApHgCl_2$ | Ap Uncomplexed |
|---|---|---|---|
| $.9 \times 10^{-5}$ | - | $.9 \times 10^{-5}$ | $4.75 \times 10^{-5}$ |
| 1.8 | - | 2.3 | 3.35 |
| 3.0 | - | 3.8 | 1.85 |
| 4.5 | - | 4.4 | 1.25 |
| 6.0 | $1.7 \times 10^{-6}$ | 4.6 | 1.05 |

In light of the foregoing experiments upon the oligomers of adenylic acid, the most plausible reaction mechanism would seem to involve the chelation of the mercuric chloride by two residues of the poly A. The $HgCl_2$ could be bound between residues n and n + 2 of the polymer, as well as between n and n + 3, etc. The polymer chain would thus be twisted and coiled in every conceivable fashion, and one would not expect the appearance of an isosbestic point.

## Summary of Mercuric Chloride Ap Oligomer Binding

From the evidence of both a direct as well as an indirect nature it appears that Ap oligomers react with mercuric chloride according to the following mechanism:

$$HgCl_2 + (Ap)_n \quad HgCl_2(Ap)_n$$

where n is 1, 2, and 3, as we are dealing, respectively, with the monomer, dimer or trimer of adenylic acid. The equilibrium constants for these reactions are an order of magnitude separated from one another, being in the range of 50-200 lit/mole for the monomer, 500-2000 lit/mole for the dimer, and 5000-20,000 for the trimer.

The reaction between the Ap residues of polyadenylic acid and mercuric chloride is even stronger than that between the oligomers and $HgCl_2$, but it is a more complex reaction, with many mercuric chloride molecules likely attached to each polyadenylic acid strand.

# DENATURATION OF ADENYLIC ACID DIMER

## Denaturation by Ethanol

It had been our original intent to study the interactions of the base residues at low temperatures. We tried, and failed to find augmented interactions between the rings of ApA with the compound below -100°C dissolved in ethanol.

The extinction maximum of adenosyl 3'-5' adenylate (ApA) in ethanol (per adenosine residue) was 15600 ± 300. Likewise, adenosine possessed an extinction in this solvent of 15600 ± 300. We could not accurately determine the extinction of adenylic acid in pure ethanol. The solubility of this latter compound appears to vary greatly with the moisture content of both solute and the ethanol. But one may probably assume correctly that its absorption characteristics in the near UV do not differ significantly from those of the nucleoside. In ethanol, then, the hypochromism of ApA vanishes.

The foregoing, leads to the conclusion that ethanol, far from being a solvent in which we could hope to see augmented base-base interactions, is a solvent in which the interactions are reduced. When it became available to us, a spectropolarimeter provided measurements to show, quite spectacularly, that base-base interactions of ApA in ethanol are greatly reduced. The specific rotation of the dimer (per adenosine residue) is reduced to near that of adenosine itself, that is, by nearly an order of magnitude. Some of these phenomena are illustrated in Figs. 38 and 39.

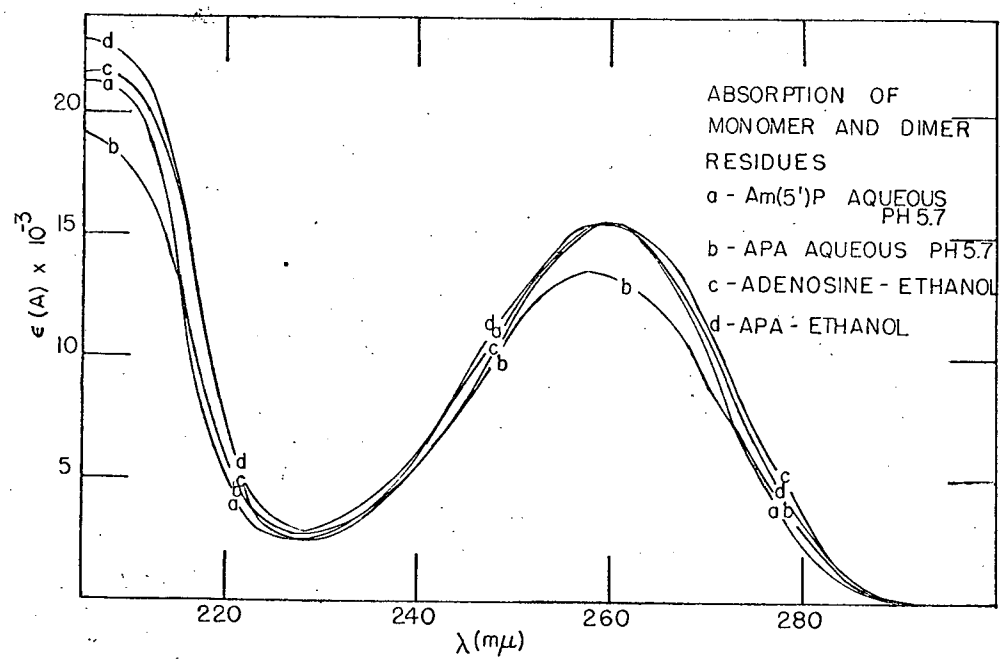



Figure 38. Absorption spectra of Amp and ApA in ethanol and in water.

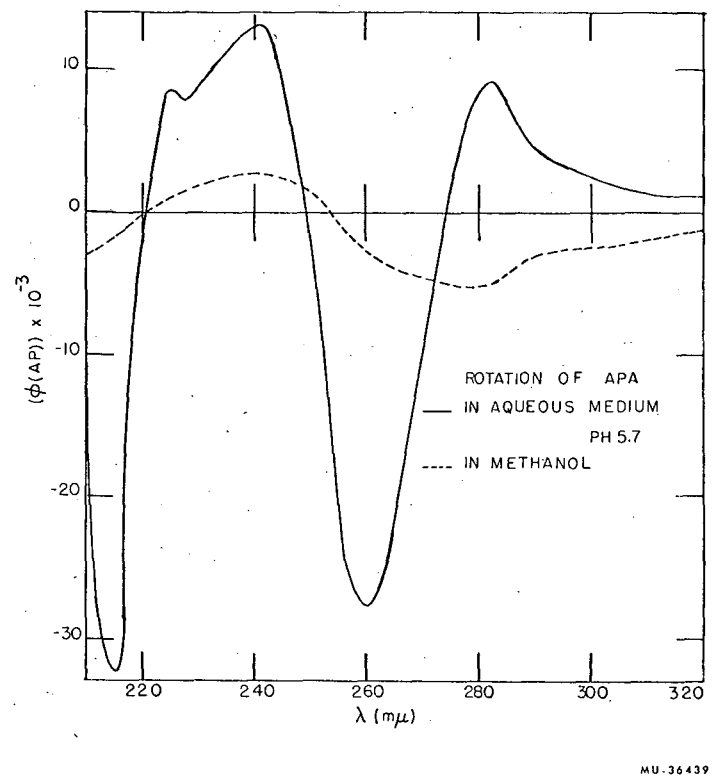



Figure 39. Rotatory dispersion of ApA in methanol and in water.

To obtain a clearer idea of the denaturation effect of ethanol upon ApA, and from this perhaps some information upon the strength of the base-base interaction in that molecule, we investigated spectral changes in the adenylic acid dimer dissolved in ethanol/water mixtures. Because of its availability, we used the dimer possessing a terminal phosphate for the following experiments.

Methods

We prepared a stock solution of ApAp, .1M in $NaClO_4$ and buffered to pH 5.7 with .01/.001 sodium acetate/acetic acid. A similar solution, but lacking the ApAp, served as a standard solvent. We also prepared an ethanol solution containing like amounts of sodium perchlorate, sodium acetate, and acetic acid. The test solutions contained: a fixed volume of stock solution; measured volumes of alcohol solution; and adequate standard solvent to achieve a fixed final volume. From a known volume of ethanol, and a known final volume, we were able to compute, for each test mixture, the weight percentage of ethanol present. We obtained the spectra with a Cary 15 spectrophotometer and a Cary 60 spectropolarimeter.

Results

Figures 40 and 41 illustrate the effect of increasing concentrations of ethanol upon the optical absorption and optical rotatory dispersion spectra of ApAp. From the fact that both sets of spectra contain isosbestic points throughout the concentration range of ethanol we used, we feel confident

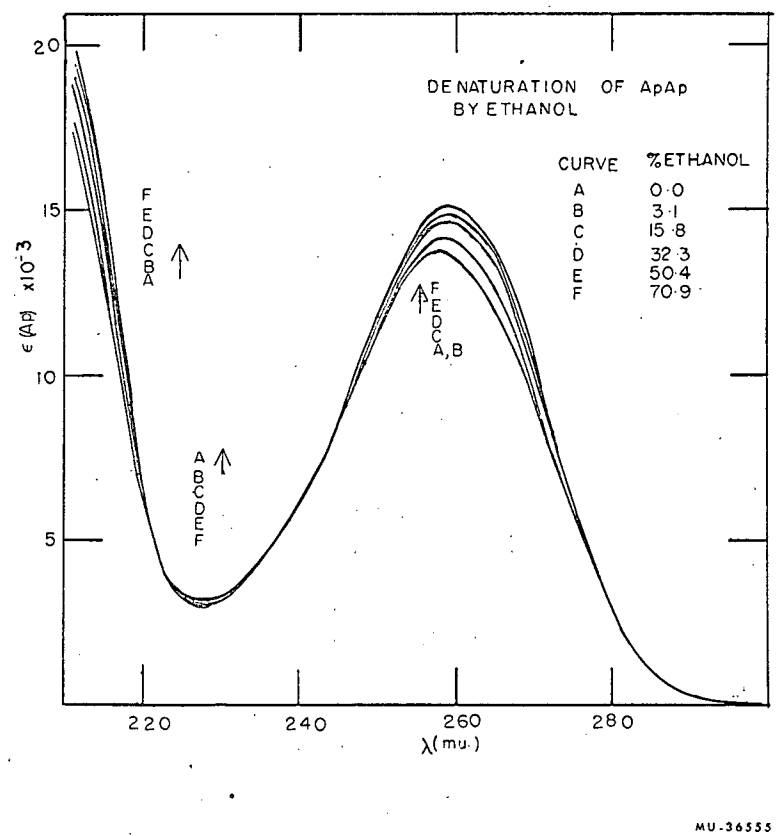



Figure 40. Denaturation of ApAp by ethanol. Optical absorption spectra.

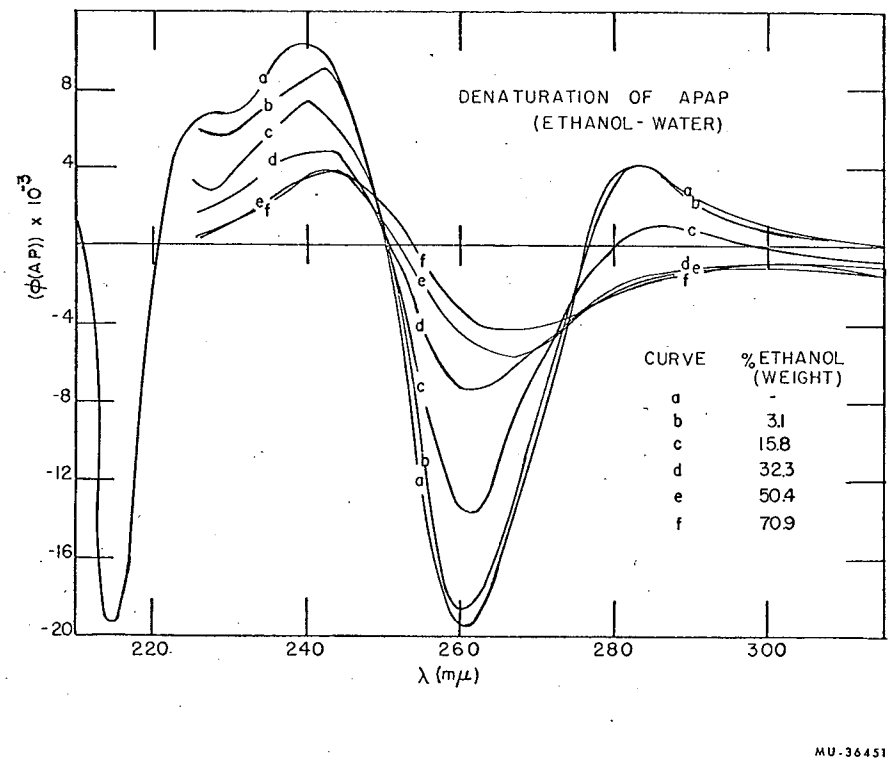



Figure 41. Denaturation of ApAp by ethanol. Optical rotatory dispersion spectra.

that there are only two species of ApAp present. We will refer to these species as being "stacked" and "unstacked".

Discussion

To what physical mechanism may one attribute the denaturation or "unstacking" of the ApAp molecule? One could not rule out protonation of the adenosine rings as a possible factor in the observed spectral changes. Michelson shows that when the adenosine rings are protonated ApA does not exhibit hypochromism.[37] Further, Warshaw demonstrated that the protonated form of ApA exhibits low specific rotation.[18]

Julliard discusses data relating to the effect of methanol upon the alteration of the pK values of organic acids.[38] The pK of benzoic acid, for instance, is changed from 4.21 in water to 5.9 in 55% methanol to 9.35 in absolute methanol. There is as yet no consistent way to predict how the pKs will change. We measured spectra of the absorption of adenosine, in roughly 95% ethanol, with several different concentrations of HCl. Under these conditions, it is found that a protonation occurs between concentrations of HCl of $10^{-3}$ M and $10^{-5}$ M. This indicates that the pK of adenosine (and hence probably of ApA or ApAp) in ethanol is between 3 and 5. Especially from this latter piece of evidence one may conclude that protonation is likely not a factor in the observed denaturation of the dimer by ethanol. Though Hanlon and Klotz discuss the possibility that some of the phenomena occurring in "helix-breaking"

solvents may be due to protonation occurring upon the macromolecules, such does not appear to be the case in our system.[39]

The more usual way of looking at denaturation phenomena is to consider the magnitude of interactions among portions of the macromolecule, e.g., intramolecular hydrogen bonds, van der Waals forces, and the like. One compares the magnitude of these sorts of interactions with those which could exist between solute and solvent. If the former are energetically more favorable, one is likely to have intact, (undenatures) macromolecules.[2] Situations which lead to stronger solvent-solute interactions would also tend to lead to the disruption of the intramolecular structure, and hence to some degree of denaturation. As one changes solvent environment from water to organic solvents, such disruptions are more likely to occur as interactions increase between the organic macromolecules and the solvent. Obviously the strength of such interactions depends upon the particular solvent is employed. If one denatures proteins or polypeptides with an organic solvent, one can, by adding small amounts of a second solvent, even further increase the degree of denaturation, as measured by changes in optical absorption or optical rotatory dispersion measurements. This is the method of solvent perturbation of Herskovits and Herskovits and Mescanti.[6,7]

Since macromolecules tend to be most stable, and possess a maximum of intramolecular ordering in aqueous solutions, the idea of the ice-cage came to prominence.[40,41] The basic idea

of the ice cage is that solvent-solvent interactions (or ordering) contributes a great deal of the stabilization energy to the structure of macromolecules (in water). Nemethy explains the disruption of macromolecules by urea as being due to the disruption of an ice like structure supposedly surrounding such a macromolecule.[42a] In a dissolved macromolecule, surrounding each relatively non-polar portion of the molecule is a supposed sheath of ordered water or ice. For such an arrangement, the entropy is decreased over a situation in which there would not be such an ordering phenomenon. Since

$$\Delta S < 0$$

$$-T\Delta S > 0$$

and

$$\Delta F > 0$$

A more energetically favorable state of the molecule would be one in which the non-polar portions of the molecules were packed together, for this would tend to reduce the total amount of water ordered by the macromolecule. Such a packing together of the non-polar portions of a macromolecule would constitute its ordered or non-denatured form. Jencks does not accept this argument, for he points out that solutes having an ordering or disordering effect upon the structure of water, do not correlate this property with their ability to denature macromolecules.[43]

Klotz describes the ability of methane, under high pressure to form about itself an orderly array of water.[42] This methane

water structure is stable to temperatures greater than 30°C. Klotz refers to this theory of the importance of solvent-solvent stabilization as the hydrotactoid hypothesis, as opposed to the hydrophobic hypothesis. This latter treats the energy of intramolecular reactions among non-polar groups as providing the major amount of energy for stabilization of the macromolecules. Further evidence for the hydrotactoid theory is provided by the fact that as one adds to water, solutes favoring (ostensibly) its ordering, there is a shift in the i.r. spectrum of water closer to that spectrum seen in ice.[42]

Kuwahra and Kaneko discuss the ordering effect of solute molecules upon a number of classes of solvents. Their model compounds were various length polymers of polydimethyl siloxane.[44] Thus the idea of ordering of solvents by solutes is not restricted to the ordering of water molecules.

Sinanoglu and Abdulnur offer a rather concrete theory of the hydrotactoid type.[40,41] They consider the solvent as macroscopic, and the nucleotides as existing in cavities contained in the solvent. When the dimer is stacked, it exists in a cavity, circumscribed about itself. The area of the cavity (which we assume to be spherical) we call $A_1$. When the molecule becomes unstacked, we assume the creation of a new cavity. While each of the cavities surrounding a nucleotide residue are smaller than the one surrounding the dimer, the total surface area of the cavities is bigger. Let us call this area $2A_2$ where $A_2$ is the area of one of the cavities. Reasonable values for the radii of these cavities are

$$r_1 = 7.3 \text{ Å}$$

$$r_2 = 5.6 \text{ Å}$$

We obtained these figures by estimation from Courtald Models.[19] They were based upon the radii of the smallest spheres which could be circumscribed about the van der Waals volume of the monomer and dimer respectively. The surface areas of the cavities would be

$$A_1 = 670 \text{ Å}^2$$

$$2A_2 = 782 \text{ Å}^2$$

The energy in creating these cavities would be expended, according to Sinanoglu and Abdulnur primarily by surface tension forces of the solvent.[40,41] As one increases the concentration of ethanol, the surface tension of the solvent is lowered, and it ought to become easier to create greater cavity areas. Hence the equilibrium for the reaction

$$\text{ApAp}_{\text{stacked}} \rightleftharpoons \text{ApAp}_{\text{unstacked}}$$

ought to become more favorable. We can estimate the equilibrium constant for this reaction

$$K = \frac{[\text{ApAp}]_{\text{unstacked}}}{[\text{ApAp}]_{\text{stacked}}}$$

from the lessening of the hypochromism as the concentration of ethanol is increased. We can obtain the surface tension of ethanol from appropriate tables.[45] One must assume that the

local solvent environment about the ApAp or AMP is the same as the bulk solvent in its ratio of ethanol/water. Only then would the surface tension in a cavity wall equal that, say, at the room air/solvent interface. We can estimate the free energy involved in creating the expanded area of the cavity for the unstacked dimer

$$E = (2A_2 - A_1)\gamma$$

where $\gamma$ is the surface tension of the solution. And we may estimate the standard free energy of the reaction from the equation

$$\Delta F^o = - RT \ln K.$$

For the different concentrations of ethanol which we used, the measured and calculated quantities appear in the table below.

Energetics of Cavity Formation in Dimer Unstacking

| % Ethanol | Surface Tension, $\gamma$ (dynes/cm) | Energy ergs/molecule | Energy kcal/mole |
|---|---|---|---|
| 0.0 | 73 | $8.18 \times 10^{-13}$ | 11.8 |
| 3.1 | 58 | 6.50 | 9.3 |
| 15.8 | 41.5 | 4.65 | 6.7 |
| 32.3 | 37.5 | 3.53 | 5.1 |
| 50.4 | 27 | 3.03 | 4.3 |
| 70.9 | 25 | 2.80 | 4.0 |

If we assume that at room temperature, in aqueous solution, (pH = 5.7, $NaClO_4$ = .1M) nearly all the dimer is stacked

it is easy enough to calculate the value for the equilibrium constants (stacked dimer to unstacked dimer) at different concentrations of ethanol. But there is an uncertainty in this for we lack a true knowledge of the degree of unstacking of the bases under these conditions. Under these circumstances, one can only assume several figures for this property. Assume successively that in room temperature aqueous medium as above, ApAp is totally stacked, 10%, 20%, 30%, 40% and 80% unstacked. Under these conditions it is possible to calculate a family of curves for the values of the equilibrium constants vs. the percent of ethanol in an ethanol water mixture (Fig. 42). From these values of K, we may construct curves for $\Delta F^{o}$ (= -RT ln K) vs. the percent ethanol in the ethanol water mixtures. Again, we get a family of curves depending upon the guess as to the relative amount of unstacking of the dimer ApAp at room temperature in an aqueous medium (Fig. 43).

We arrived at these data by a fairly straight forward process. The extinction of the monomer, either in water or in ethanol is close to 15,400 at 260 mμ. When completely unstacked, or nearly so, as it is in 100% ethanol ApAp has an extinction/Ap residue close to 15,400 (within experimental error). Thus in any alcohol/water mixture, we consider the extinction/Ap residue for an unstacked dimer to be 15,400.

In aqueous solution the extinction of the ApAp dimer/Ap residue is 13,700. Depending upon the percent of room temperature unstacking we choose to hypothesize we find a "true"

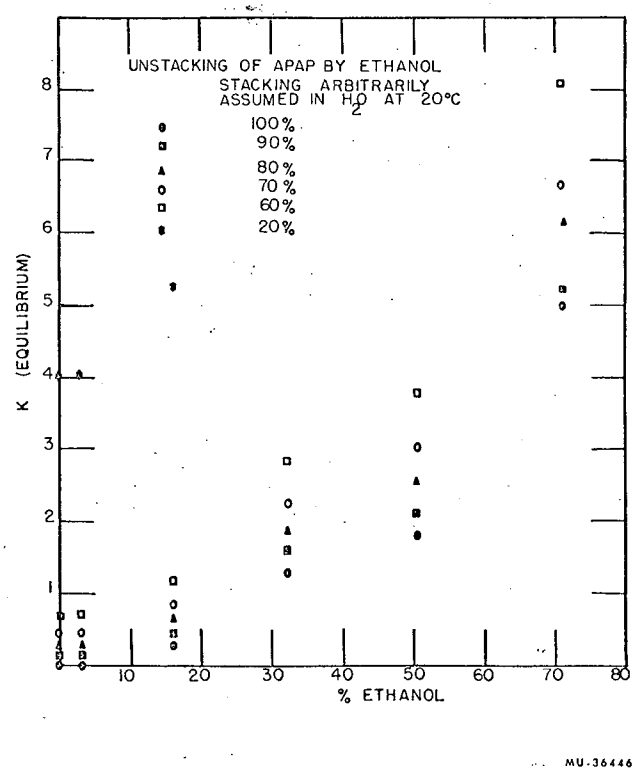



Figure 42. Unstacking of ApAp by ethanol. Equilibrium constants.

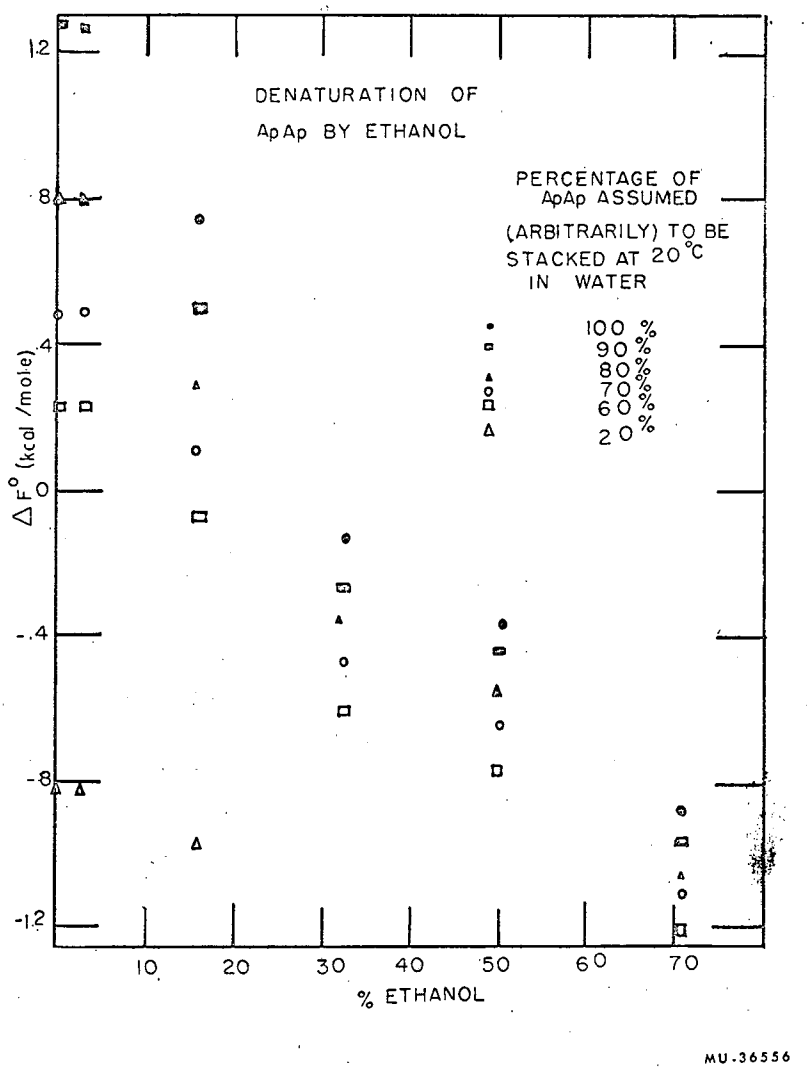



Figure 43. Unstacking of ApAp by ethanol. Energetics.

extinction/Ap residue for the dimer.

$$13{,}700 = 15{,}400\ (2f) + \epsilon(\text{dimer/Ap residue})2(1-f)$$

where f is the fraction of the unstacked dimer.

With increasing concentrations of ethanol, the degree of unstacking increases, and using the dimer extinctions calculated above, and the OD at 260 mμ we can calculate the percent of unstacking at any alcohol concentration. From these values, one may draw the two sets of curves described above (Figs. 42 and 43). Can the decreased surface tension, and with it the increased ease (decreased free energy) of cavity formation account for the greater equilibrium towards unstacking seen in ethanol/water solutions? To be quantitative, we first make an arbitrary assumption that at room temperature, in aqueous solution, the ApAp is 20% unstacked, i.e., f is initially .2. The change of standard free energy in water for unstacking, $\Delta F^{o}_{H_2O}$, is equal to .83 kcal/mole. The free energy necessary for cavity formation in water is 11.8 kcal/mole. Thus there are factors other than cavity formation involving in the unstacking. As we increase the alcohol concentrations to, say, 50.4%, the free energy for unstacking, $\Delta F^{o}_{50\%\ \text{ethanol}}$ decreases to become equal to -5.5 kcal/mole. Since the surface tension of the solvent, $\gamma_{50\%\ \text{ethanol}}$, has been lowered, the free energy involved in cavity formation has decreased to 4.3 kcal/mole. Cavity formation is easier by 11.8-4.3 = 7.5 kcal/mole in a 50.4% ethanol/water mixture. This is reflected in a reaction whose overall ease (its free energy change) has been increased by 5.5 + .83 kcal/mole = +6.3 kcal/mole.

The decrease of free energy for cavity formation in a 50.4% ethanol/water mixture is more than enough of a decrease to provide for the similar decrease for unstacking of ApAp in the same ethanol/water mixture. In the Table VI we list, for each concentration of ethanol, the standard free energy of unstacking of the ApAp, and the free energy for cavity formation. Further, for each percent of alcohol the differences between these free energies, and the corresponding values in pure water appear. One sees that in each instance, the change in free energy for cavity formation is more than adequate to account for a similar change in the ease of unstacking. Ease of unstacking of the dimer increases monotonically with increasing concentrations of ethanol in the ethanol/water binary mixture. The surface tension decreases in a monotonic fashion. While the latter phenomenon provides sufficient free energy for the former, there does not seem to be any simple relationships between them. For this, there could be several reasons. Perhaps the choice of f, the fraction of ApAp unstacked at room temperature was faulty, maybe it is not 20%. More likely, as Sinanoglu and Abdulnur pointed out, there is no guarantee that the surface tension (and its changes with the composition in the solvent) is the same locally, e.g., surrounding a small cavity in which there is a macromolecule) as it is in the bulk of the solution.[40] If one assumes an ice-like cage to surround the organic molecule, the local solvent properties would most definitely not equal the bulk properties of the solvent. Thus Garst et al. show a truly tiny amount of a second solvent may selectively

Table VI. Unstacking of ApAp in Ethanol/Water Mixtures Compared to Ease of Cavity Formation in Such Mixtures (kcal/mole)

| % Ethanol | Standard free energy of unstacking in ethanol/water $\Delta F^o_{ethanol/H_2O}$ | $\Delta F^o_{ethanol/water} - \Delta F^o_{H_2O}$ | Free energy of cavity formation in ethanol/water $E_{ethanol/H_2O}$ | $E_{ethanol/H_2O} - E_{H_2O}$ |
|---|---|---|---|---|
| | .83 | | 11.8 | |
| 3.1 | .81 | - .02 | 9.3 | -2.9 |
| 15.8 | .29 | - .55 | 6.7 | -5.0 |
| 32.3 | - .36 | -1.19 | 5.4 | -6.7 |
| 50.4 | - .55 | -1.38 | 4.3 | -7.4 |
| 70.9 | -1.07 | -1.91 | 4.0 | -7.6 |

solvate a solute molecule while leaving the bulk properties of the solvent quite unchanged.[46]

Though we have chosen 80% as the value for the stacking of ApAp in $H_2O$ at room temperature, we see that even if stacking were considerably higher, the surface tension considerations would still provide for the major share of change in free energy for the unstacking.

This is all a very simplified picture of things, and it seems almost inevitable that other factors must enter.

In the denaturation of ApAp, both the absorption and rotatory dispersion spectra possess isosbestic points, and the first thought to occur is that the denaturation is a simple unstacking phenomenon. Thus the stacked form of the molecule would have some absorption spectrum, $a_1(\lambda)$, and some rotational spectrum, $b_1(\lambda)$. The unstacked molecule would have some absorption spectrum $a_2(\lambda)$, and a rotatory dispersion spectrum, $b_2(\lambda)$. We have so far discussed the denaturation of ApAp in these terms.

An added proof that the unstacking model is the correct one would be if the relative change in rotation and absorption were to parallel one another. We will mean, by total unstacking, the situation in which both the optical absorption and optical rotatory dispersion spectra of the dimer become identical to the spectra of the monomer in alcohol. We normalize the change in either rotatory dispersion or in absorption spectra (at a given wavelength) to the change which would occur in "total unstacking" as defined above. The extinction at 259.5 for the

monomer in ethanol is 15,400 within experimental error, and we define it to be the same for "totally unstacked" dimer. Likewise, within experimental error, the specific optical rotation of the monomer in ethanol at 265 mμ is -1500 and we define it to be the same for "totally unstacked" dimer.

The dimer, ApAp, in water, has an extinction coefficient at 259.5 mμ of 13,500, and a specific rotation at 265 mμ of -16,000. (All these constants are given on a per unit residue Ap basis.)

We may then calculate the percent relative unstacking on the basis of both absorption spectra and rotatory dispersion spectra, as a function of the fraction of ethanol used as solvent. The data for the changes in optical absorption comes from Fig. 40, and for changes in rotation from Fig. 41. The formula for percent relative unstacking based upon absorption changes at 259.5 mμ, and upon rotational changes at 265 mμ are respectively

$$r_{OD} = \frac{(100)[\epsilon(Ap)_{259.5} - 13,500]}{(15,400 - 13,500)}$$

and

$$r_{ORD} = \frac{(100)(16,000 - [\Phi(Ap)]_{265})}{(16,000 - 1500)}$$

where $\epsilon(Ap)_{259.5}$ and $[\Phi(Ap)]_{265}$ are the extinction and specific rotation per Ap residue in the ApAp dimer with some given ethanol/water mixture as solvent; $r_{OD}$ and $r_{ORD}$ are the calculated percent relative unstacking in that ethanol/water mixture.

The results of performing the above computations are shown in the table below.

Percent Relative Unstacking of ApAp in Ethanol/Water Mixtures as Solvents, based upon Changes in Absorption at 259.5 mμ and changes in Rotation at 265 mμ.

| Percentage Ethanol | Extinction 259.5 mμ | % Relative Unstacking $r_{OD}$ | Rotation 265 mμ | % Relative Unstacking $r_{ORD}$ |
|---|---|---|---|---|
| 0 | 13500 | 0 | 16000 | 0 |
| 03.1 | 13500 | 0 | 15200 | 06 |
| 15.8 | 14100 | 32 | 11200 | 33 |
| 32.3 | 14700 | 63 | 6800 | 64 |
| 50.4 | 14900 | 74 | 5500 | 73 |
| 70.9 | 15100 | 84 | 4400 | 80 |

Within experimental error, the changes in absorption and in rotation follow one another very closely. The 6% change at 3% ethanol would be too small a change to readily detect in the absorption spectrum.

From the fair degree of correlation in rotational and absorptive changes, we feel that the denaturation seen, at least within the limits of ethanol concentrations used, is the result of a simple unstacking.

Summary

We see that ethanol denatures the dimer, ApAp, which we regard as "stacked" when in aqueous solution. The "unstacking" which we observe is most likely not due to protonation or deprotonation of the ApAp. One possible way to look at the breakdown of structure in ApAp is to consider it as secondary to the breakdown of an ice-like cage structure surrounding the ApAp molecules, as increasing quantities of ethanol are added. The more macroscopic theory of Sinanoglu and Abdulnur, which gives prime consideration to the surface tension of the solvent in denaturation phenomena, gives results not inconsistent with experimental observation.

## Denaturation of the Dimer by Trimethyl Phosphate

Trimethyl phosphate as a solvent for adenylic acid dimer behaves much like ethanol. Looking at the spectra for the optical rotation and optical absorption of this compound in trimethyl phosphate, we find that both phenomena are greatly changed from what appears in aqueous solutions. The optical rotation is greatly diminished towards the level of the monomer. The hypochromatic effect seen in the dimer disappears (see Figs. 44 and 45).

We carried out some measurements on the denaturation of the ApAp dimer in binary solvents composed of varying ratios of water and trimethyl phosphate. (The terminal phosphate on ApAp precludes its solubility in 100% trimethyl phosphate, but it is soluble in up to 85% trimethyl phosphate.)

We prepared stock solutions of ApAp, .1M in sodium perchlorate, and buffered to pH of 5.7 with sodium acetate, acetic acid, .01/.001. We prepared a similar standard solvent, containing all but the ApAp. To equal volumes of our stock solutions, we added various volumes of trimethyl phosphate (itself made .1M in sodium perchlorate), and diluted all our solutions to an equal final volume with standard solvent. With these solutions we measured the spectra of the optical absorption and of the optical rotatory dispersion of ApAp in differing concentrations of trimethyl phosphate solvent. The measurements were made with the Cary 15 spectrophotometer, and the Cary 60 spectropolarimeter. Figures 46 and 47 show the results of these measurements.

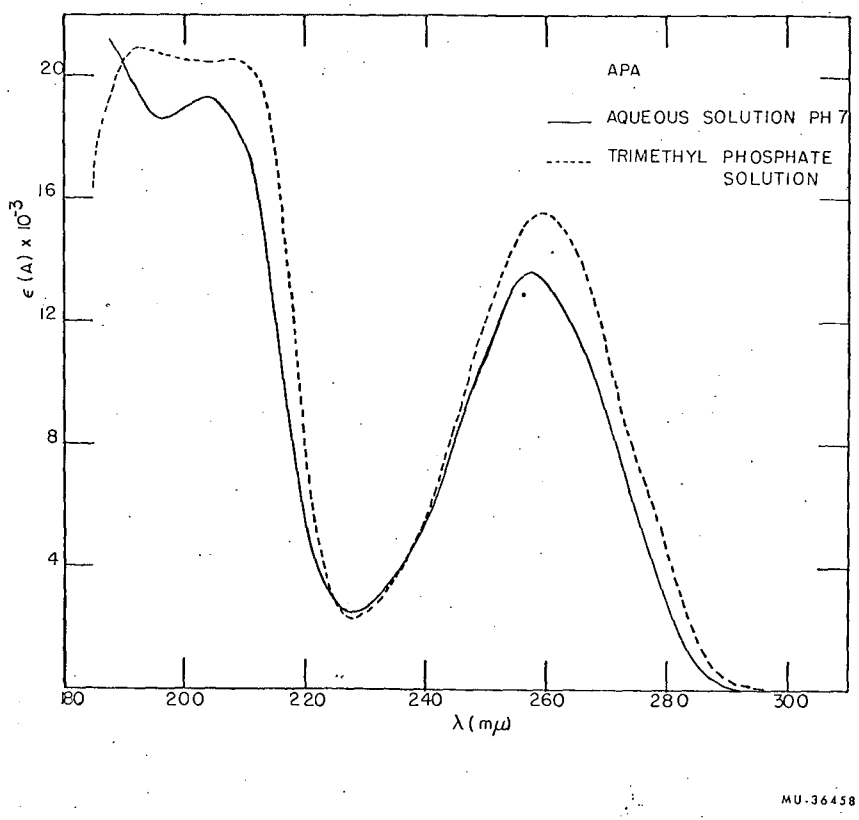



Figure 44. ApA in water and in trimethyl phosphate. Optical absorption spectra.

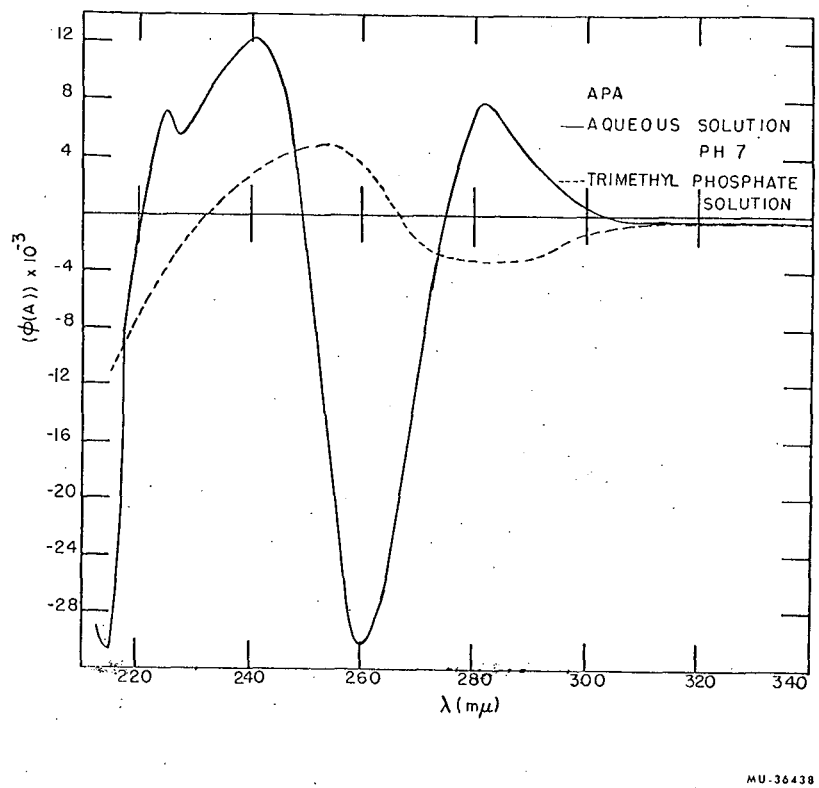



Figure 45. ApA in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

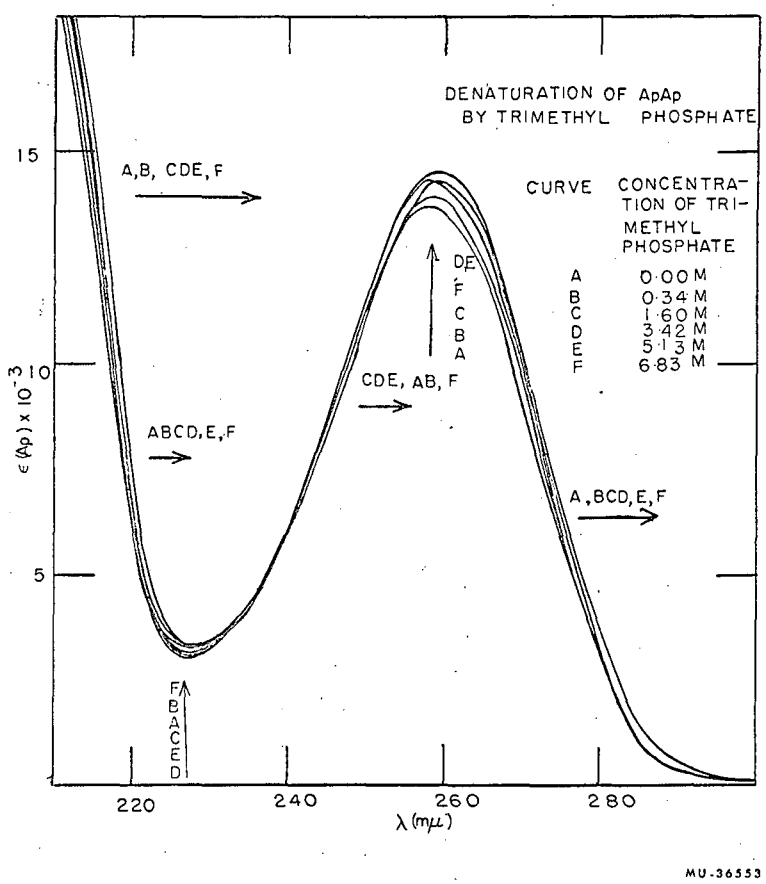



Figure 46. Denaturation of ApAp by trimethyl phosphate. Optical absorption spectra.

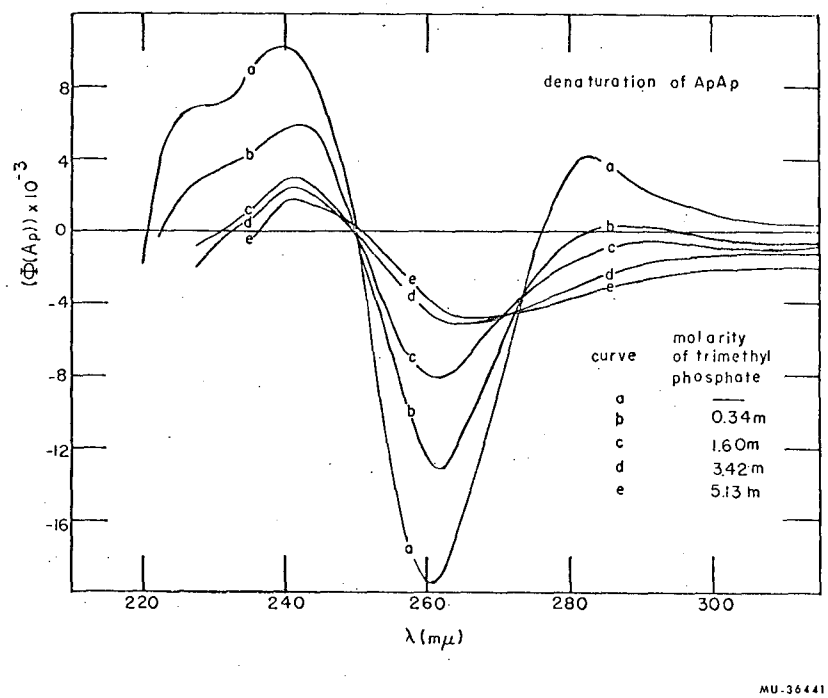



Figure 47. Denaturation of ApAp by trimethyl phosphate. Optical rotatory dispersion spectra.

These denaturation curves do not so clearly define an isosbestic point as did similar curves made in ethanol/water solvents. This is especially the case at high concentrations of trimethyl phosphate.

Friedel, Schaffert, and Sieder measured the surface tensions of trimethyl phosphate water mixtures.[47] From their values we were able to determine the surface tensions of our solutions, which are indicated in the table below. We were interested in the surface tension values (in comparison to those of similar ethanol/water mixtures) for possible use in applying Sinanoglu and Abdulnur's theory of denaturation.[40]

| Concentration of Trimethyl Phosphate | Surface Tension (dyne/cm) |
|---|---|
| - | 73 |
| .34 M | 57.5 |
| 1.60 M | 50.0 |
| 3.42 M | 46.5 |
| 5.13 M | 44.5 |
| 6.83 M | 43.5 |

The free energy required to create two cavities for the unstacked ApAp from the one cavity which ostensibly exists surrounding the stacked molecule is calculated below, for the differing trimethyl phosphate/water solutions. The basis of these calculations appears in the previous section where the same calculations appear for ethanol/water binary solvents.

In the following table we give these energies calculated in terms of kcal/mole expended in cavity formation when ApAp is unstacked.

| Concentration of Trimethyl Phosphate | Free Energy of Cavity Formation in Unstacking ApAp (kcal/mole) | $E_{H_2O}-E_{TMP/H_2O}$ (kcal/mole) |
|---|---|---|
| - | 11.75 | - |
| 0.34 M | 9.26 | 2.49 |
| 1.60 M | 8.03 | 3.72 |
| 3.42 M | 7.50 | 4.25 |
| 5.13 M | 7.19 | 4.56 |
| 6.83 M | 7.07 | 4.65 |

Qualitatively, the results of more elaborate calculations such as we performed with the ethanol/water mixtures, should be much the same. Indeed almost any organic solvent, miscible with water, should lower the surface tension, and shift the equilibrium from one favoring stacked dimers to one favoring unstacked dimers.

What is the possibility that the ApAp adenine rings are protonated in trimethyl phosphate, and for this reason become unstacked? It is unlikely because a 10% water solution of trimethyl phosphate has a pH of near neutral. Furthermore, adding sodium methoxide, a powerful acceptor of protons to the ApAp dissolved in trimethyl phosphate does not in the least alter the spectrum. Indeed, in the succeeding section of this paper we shall show that of all the nucleosides dissolved in

trimethyl phosphate, probably only the pK of cytosine is shifted sufficiently from that occurring in water to allow some degree of protonation of the ring.

At higher concentrations of trimethyl phosphate there is a distinct broadening, especially of the red side of the spectral absorption curve. Likewise in this limb, there is a distinct inflection. This could be due to one of two causes. First, some aggregation might have occurred. This is unlikely, because an exactly similar shift, broadening, and inflection appear in the spectrum of adenosine dissolved in trimethyl phosphate (Fig. 54). Another possibility comes from the work of Clark and Tinoco.[48] It appears likely that the 260 mμ absorption band of adenosine is composed of two separate electronic absorption bands, which fortuitously overlap in water solutions. However in an organic solvent such as trimethyl phosphate, the two bands may become resolvable, one of them becoming considerably red shifted. This provides the broadening seen in the spectrum, as well as the inflection. At any rate, it seems that increasing concentrations of trimethyl phosphate (from 0-85%) in water/trimethyl phosphate solutions of ApAp give rise to two phenomena. The first is an unstacking of the ApAp, with a concomitant lessening of hypochromism and of the specific rotation. The second is a spectral change just as occurs in the monomer, with the appearance of a red broadening and an inflection in this portion of the spectral band.

General Properties of Nucleosides in Trimethyl Phosphate

Charney and Gellert interested in the interactions amongst nucleotides in polynucleotides, looked at the spectra of some nucleosides (and analogous compounds) dissolved in acetonitrile.[49] There is evidence that in polynucleotides, the heterocyclic bases exist in a hydrophobic environment. For this reason, it might prove interesting to ascertain some of the characteristics of the mononucleotides when they are subject to a non-aqueous environment.

Trimethyl phosphate has the advantage over acetonitrile of dissolving all the natural ribonucleosides, and we wished to see the effects of this solvent upon the optical properties of these molecules.

For rotation measurements, we used a Cary 60 spectropolarimeter, and for absorption measurements, a Cary 15 spectrophotometer. We used commercial preparations of ribonucleosides from Cal Biochem.[50] As a standard for extinction values of the ribonucleosides in aqueous solutions, we used data from the Pabst Corporation.[51]

From a measurement of absorption of a given weight concentration of a nucleoside in water, and the knowledge of its extinction coefficient therein, we were able to obtain the extinction of a nucleoside in trimethyl phosphate. It was only necessary to determine the absorption of a known weight concentration of nucleoside in a trimethyl phosphate solution, with the absorption of the weight concentration in water known.

We did all weighings on a Mettler[52] balance accurate to ±.005 mg. All our samples had a total weight of about 1 mg, giving us an overall accuracy of about 1%.

Charney and Gellert found decreased light absorption (with respect to aqueous solutions) for inosine, 9-pyranosyl adenine, and cytidine, dissolved in acetonitrile.[47] The integrated intensities of the near UV spectra were reduced by roughly 20%, 10% and 5%, respectively. There is a slight increase in the integrated absorption (of perhaps 1%), in the 280 mμ electronic absorption band of 5-bromodeoxyuridine in acetonitrile, although its lower wavelength band shows a definite decrease in absorption. They were able to rule out either protonation of the compounds or base aggregation as being responsible for the spectral changes. The changes they observed had to be due to solvent-solute interactions in the acetonitrile different from those in water.

Unlike their 5-bromodeoxyuridine, uridine in trimethyl phosphate shows a definite decrease in absorption intensity in both 260 mμ and 220 mμ bands. There is little likelihood that protonation of the molecule plays a part in the spectral changes observed. The shape of the spectrum is little changed. The rotatory dispersion spectrum is considerably red-shifted (the cross-over moving from 271.5 to 276 mμ), and the lower wavelength portions of the spectrum are shifted even more (Figs. 48 and 49).

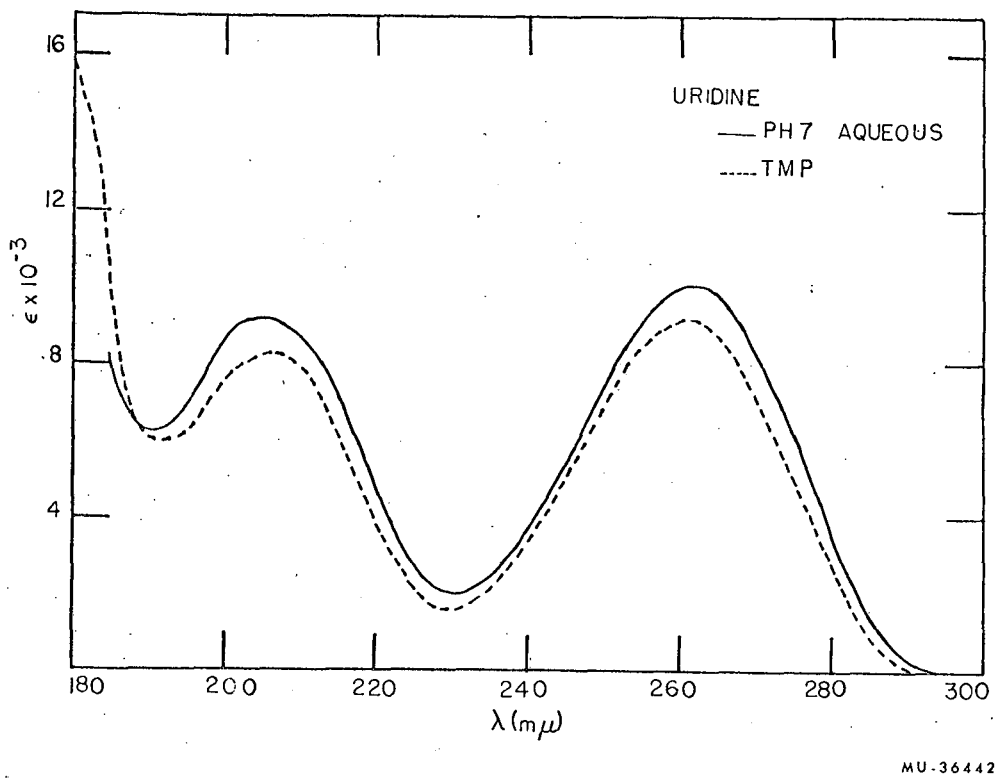



Figure 48. Uridine in water and in trimethyl phosphate. Optical absorption spectra.

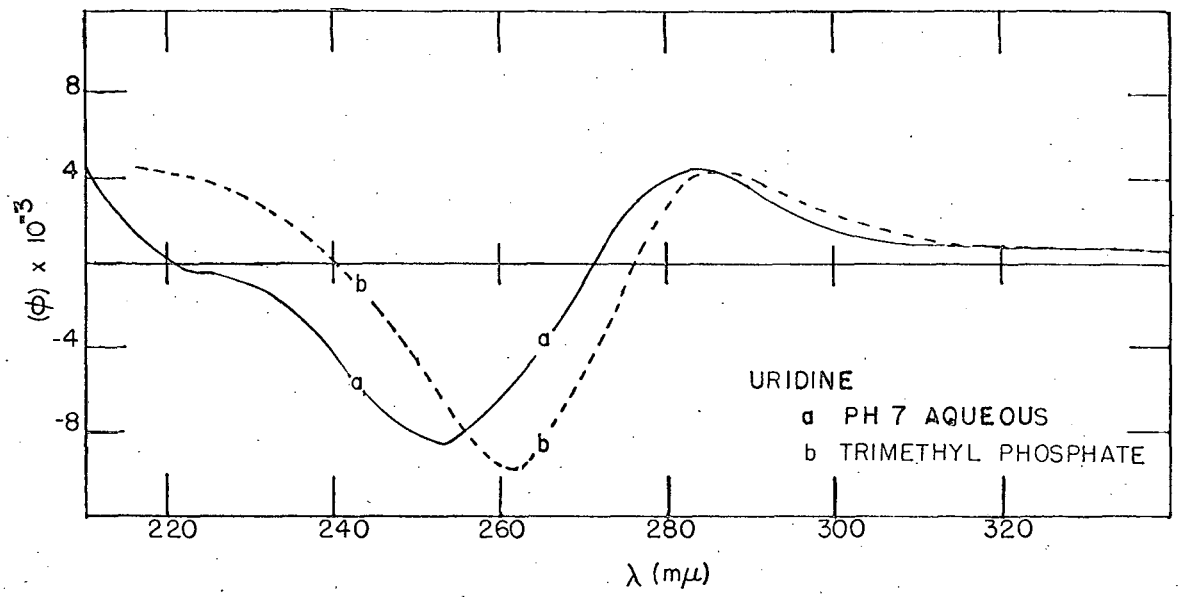



Figure 49. Uridine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

Guanosine exhibits some rise in peak height upon solution in trimethyl phosphate. The absorption spectrum is shifted in ways quite differently from what would be expected with protonation. Further, since the pK for protonation in guanosine is 1.6,[53] such an occurrence is not too likely even with the possibilities of changed pKs in an organic solvent. There is a slight blue shift in the cross over of the very weak and very noisy rotatory dispersion spectrum of this molecule. The data of Yolles on the ORD of guanosine shows a very dramatic change in spectrum upon protonation, which our spectrum lacks[54] (Figs. 50 and 51).

Cytidine alone of the nucleosides exhibits what appears to be a definite protonation when dissolved in trimethyl phosphate. The absorption spectrum has characteristics midway between those of the protonated and unprotonated species in aqueous solution. Furthermore, the cross over of the ORD is red shifted, consistent with what occurs in the protonated species.[54] It is quite possible that the spectra we see represent a mixture of protonated and unprotonated species. Why is cytidine, of all the nucleosides, probably the only protonated species in the organic solvent? Likely because the pK for protonation is the highest of the four nucleosides, being about 4.5 (Figs. 52 and 53).[53]

Adenosine, from its pK of 3.45 would be, next to cytidine, the most likely of the nucleosides to be protonated in trimethyl phosphate,[53] if protonation were due to an upward shift in pKs

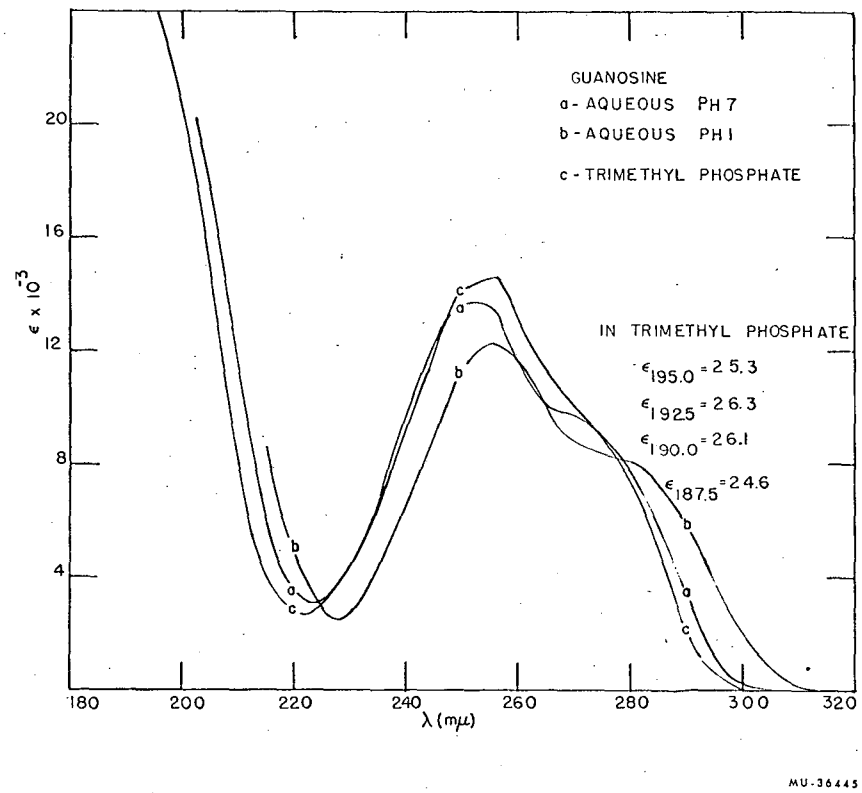



Figure 50. Guanosine in water and in trimethyl phosphate. Optical absorption spectra.

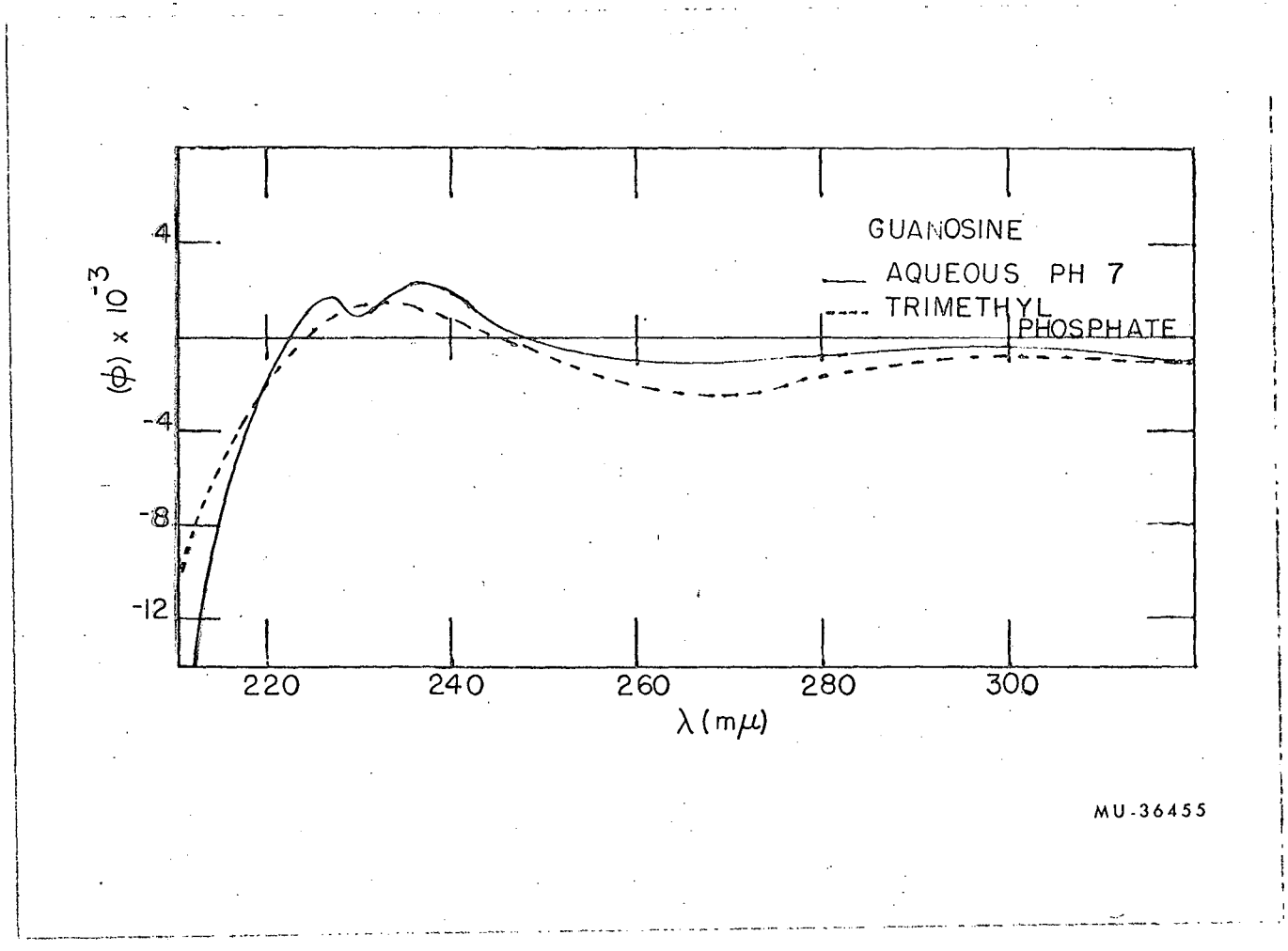



Figure 51. Guanosine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

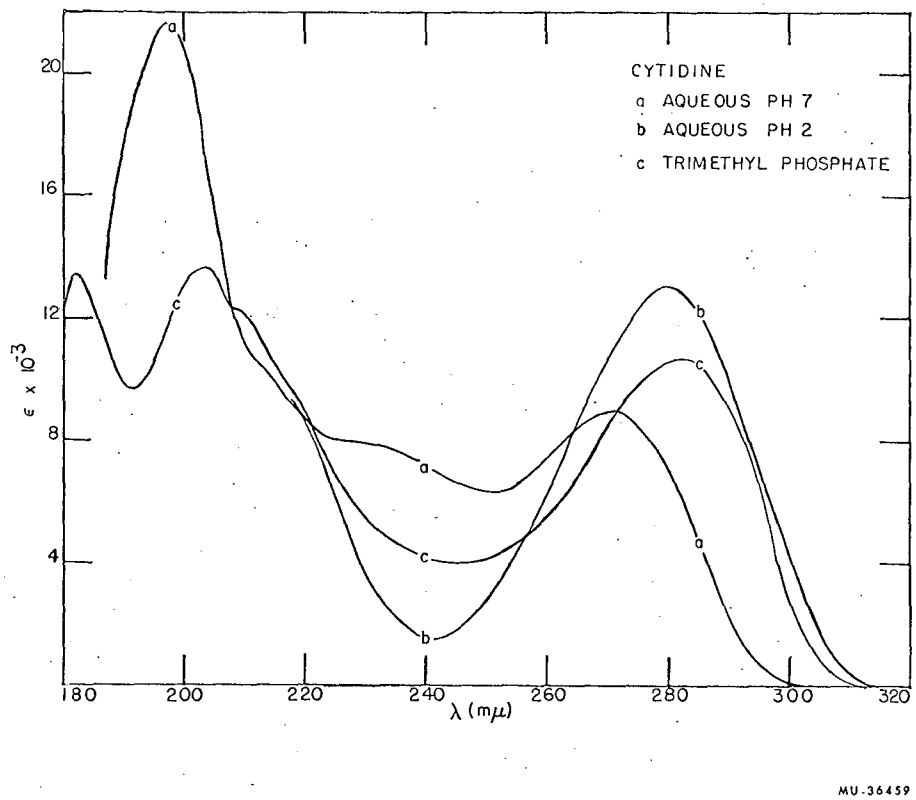



Figure 52. Cytidine in water and in trimethyl phosphate. Optical absorption spectra.

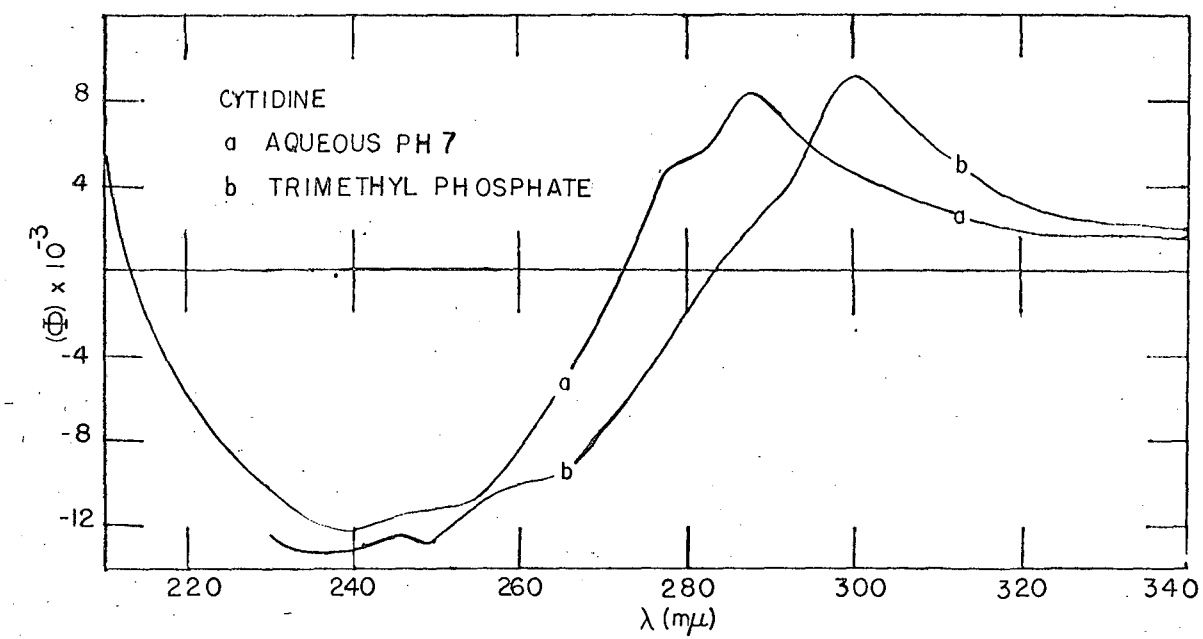



Figure 53. Cytidine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

of the nucleosides in this solvent. Yolles' data on adenosine shows the cross over point of the ORD spectra for protonated adenosine shifted from 260 to 245 mμ. Our ORD spectrum of adenosine in trimethyl phosphate is red shifted to 267.5 mμ. It is likely that some of the changes in the adenosine spectra are due to the composite nature of the 260 mμ absorption band,[48] (Figs. 54 and 55).

Using a computer program designed by Tinoco, and modified by Warshaw we calculated some properties of the electronic absorption bands of the nucleoside residues, dissolved in both neutral aqueous solution as well as in trimethyl phosphate.[55,56]

The <u>oscillator strength</u> of an absorption band is defined as the integrated area under the absorption curve, with the extinction coefficient plotted as a function of frequency ($cm^{-1}$). The integral is multiplied by a constant, such that the oscillator strength is dimensionless. If, as in our spectra, the absorption bands overlap, one must make extrapolations to zero of each overlapping absorption band, of times in an arbitrary fashion.

$$\text{oscillator strength} \equiv f = 4.315\text{x}10^{-9} \int \epsilon d\nu$$

where $\epsilon$ is the extinction coefficient, and $\nu$ is the frequency in wavenumbers.

The <u>dipole strength</u>, D, is defined as

$$.92\text{x}10^{-38} \int \frac{\epsilon d\nu}{\nu}$$

and its square root is the <u>transition moment</u>.

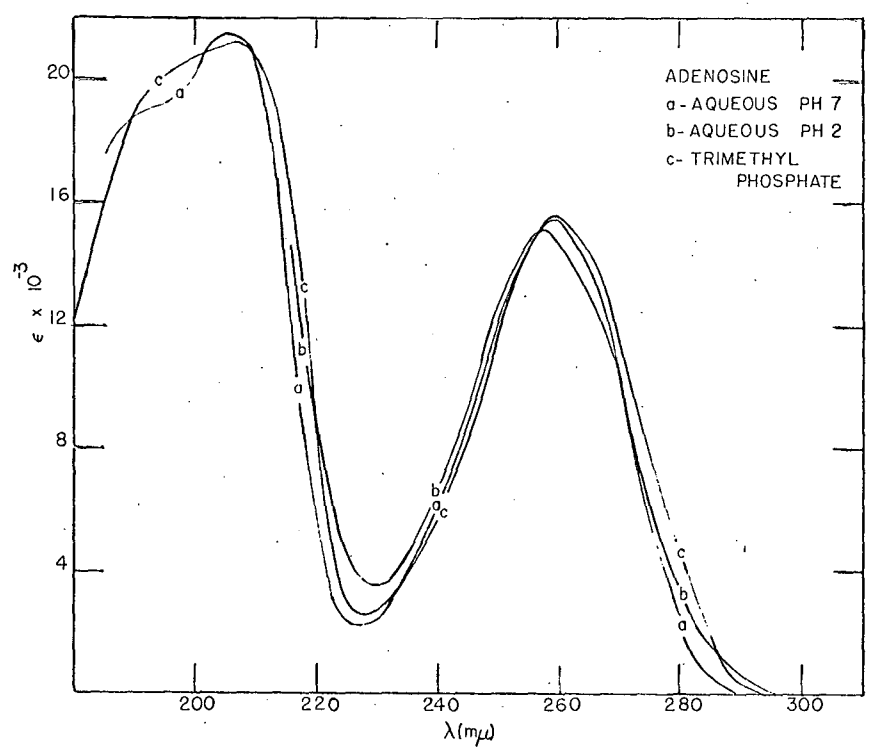



Figure 54. Adenosine in water and in trimethyl phosphate. Optical absorption spectra.

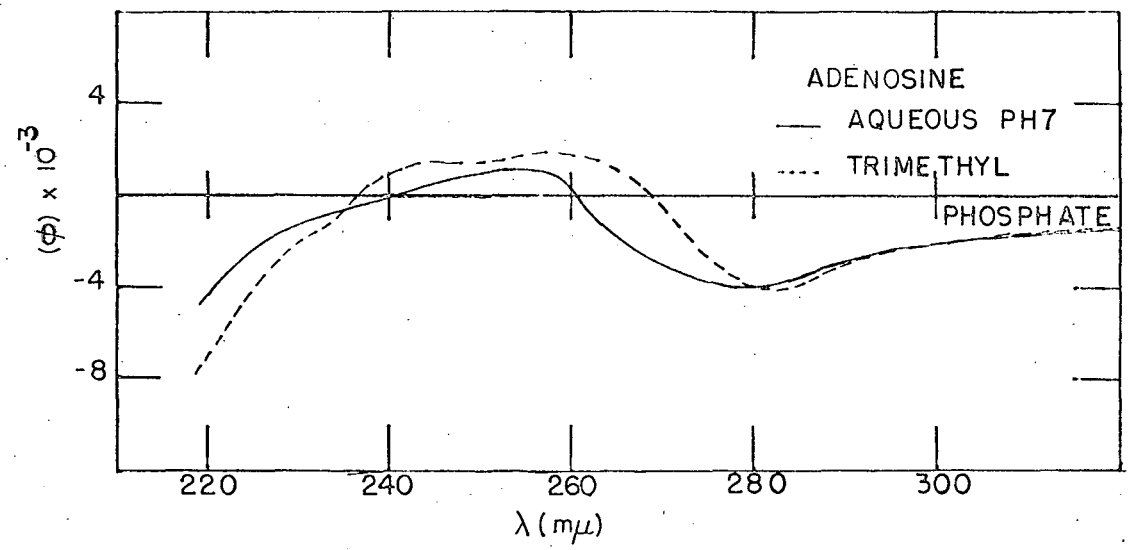



Figure 55. Adenosine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

The units of dipole strength are debyes$^2$, and of transition moment, debyes.

The first moment or average frequency of an optical absorption band is defined as

$$\bar{\nu} = \frac{\int \epsilon d\nu}{\int \frac{\epsilon}{\nu} d\nu}$$

and its reciprocal is the average wavelength.

$$\bar{\lambda} = \frac{1}{\bar{\nu}}$$

The second moment of an optical absorption band is given by the integral

$$\overline{\nu^2} = \frac{\int \nu \epsilon d\nu}{\int \frac{\epsilon}{\nu} d\nu}$$

Its square root defines the second moment average frequency, and the reciprocal of the latter is the second moment average wavelength. In the following two tables (VII and VIII) TMP refers to the solvent, trimethyl phosphate.

A comparison in absorption properties for the spectra of: (a) AM (2',3')P in pH 7 phosphate buffer; (b) AM (2',3')P in trimethyl phosphate; (c) ApA in pH 7 phosphate buffer; and (d) ApA in trimethyl phosphate can similarly be extracted from the raw spectral absorption data with the use of the same computer program. These properties are shown in Table VIII.

Table VII. Properties of Ribonucleosides in Trimethyl Phosphate (TMP) *vs.* Properties in Aqueous Solutions

| | Guanosine | | Cytosine | | Adenosine | | Uridine | |
|---|---|---|---|---|---|---|---|---|
| | Water | TMP | Water | TMP | Water | TMP | Water | TMP |
| Oscillator Strength | .410 | .410 | .379 | .305 | .322 | .333 | .222 | .201 |
| Transition Moment (debyes) | 4.71 | 4.71 | 4.46 | 4.15 | 4.18 | 4.36 | 3.49 | 3.32 |
| Dipole Strength $(\text{debyes})^2$ | 22.21 | 22.18 | 19.95 | 17.25 | 17.44 | 18.18 | 12.15 | 10.99 |
| Average Frequency $(\text{cm}^{-1})$ | 39203 | 39237 | 40315 | 37494 | 39203 | 38877 | 38728 | 38889 |
| Average Wavelength (mμ) | 255 | 255 | 248 | 267 | 255 | 257 | 258 | 257 |
| Second Moment $\times 10^{-10}$ $(\text{cm})^{-2}$ | .154 | .155 | .164 | .142 | .154 | .152 | .150 | .152 |

Table VIII. Properties of Adenylic Acid and the ApA Dimer in Water and in Trimethyl Phosphate

| | Adenylic Acid (2',3')Phosphate | | ApA | |
|---|---|---|---|---|
| | Water | TMP | Water | TMP |
| Oscillator Strength | .329 | .353 | .297 | .340 |
| Transition Moment (debyes) | 4.21 | 4.38 | 4.01 | 4.31 |
| Dipole Strength $(\text{debyes})^2$ | 17.75 | 19.16 | 16.05 | 18.54 |
| Average Frequency $(\text{cm}^{-1})$ | 39297 | 39095 | 39281 | 38965 |
| Average Wavelength (mμ) | 255 | 256 | 255 | 257 |
| Second Moment $\times 10^{-10}$ $(\text{cm})^{-2}$ | .155 | .153 | .154 | .153 |

# LOW TEMPERATURE EFFECTS

## Theory

The room temperature optical absorption spectral curve of the adenylic acid dimer is hopochromic with respect to adenylic acid. The absorption spectra are shown in Fig. 56, and the molecular structures are diagrammed in Fig. 57. The hypochromism is defined as

$$H = 1 - F_{Oa}/f_{Oa}$$

where $F_{Oa}$ is the oscillator strength for a given electronic transition Oa, per residue of chromophore (per adenylic acid residue in the ApA dimer), and $f_{Oa}$ is the oscillator strength for the same transition in the monomer (adenylic acid).[57] If, as in the ApA dimer, we have two linked chromophores, each with the same absorption band, and if coulombic interactions can occur between them, the absorption band should be split.[8] In ApA, no such splitting is observable at room temperature. If one could lower the temperature at which the spectrum were recorded, it might be possible to observe such a splitting in the electronic absorption band of ApA.

Such a phenomenon is a common enough thing in many physical processes. Suppose one has two vibrating structures, and suppose their vibrations are of a similar frequency. If one couples the two structures, e.g., with a spring, but couples them loosely, there will exist two frequencies at which the objects will vibrate together. These latter are the normal modes of

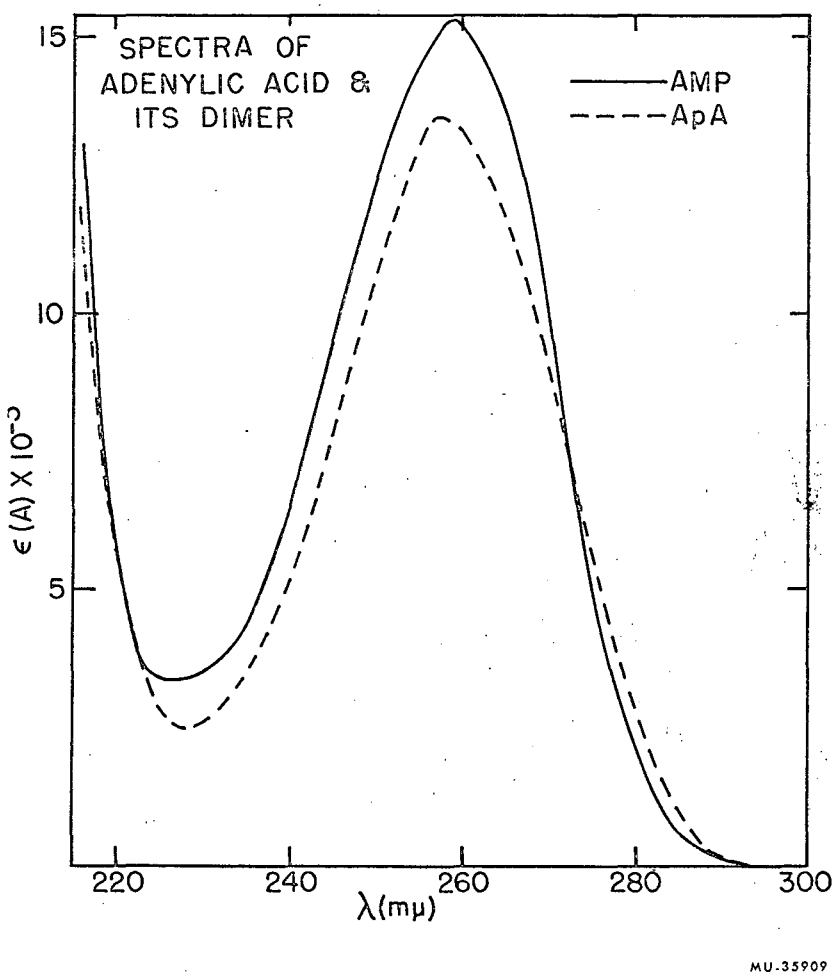
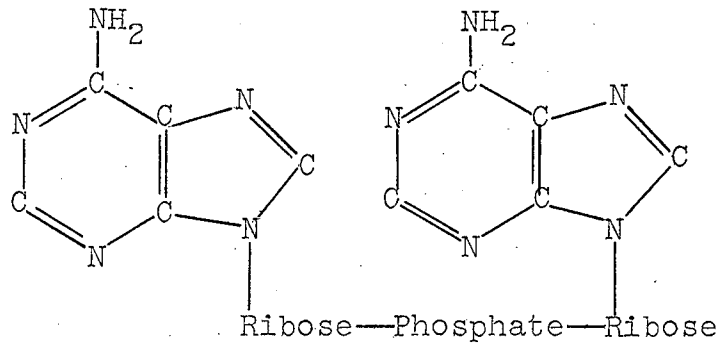
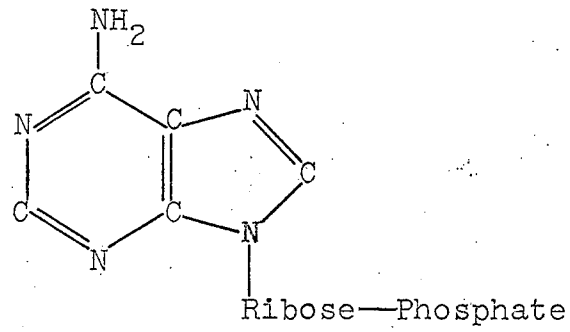



Figure 56. Spectra of adenylic acid and its dimer.




Adenylic Acid Dimer



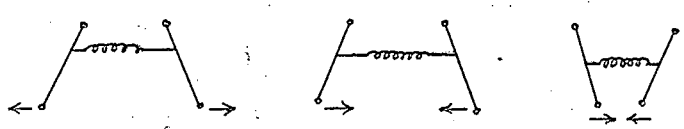

Adenylic Acid

Figure 57

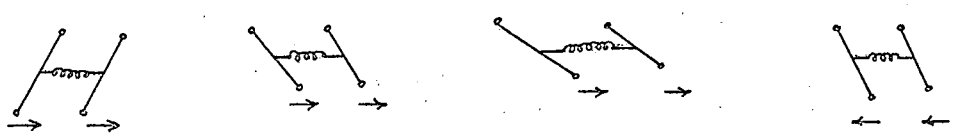
oscillation. If the coupling is adequately loose, the frequencies of the normal modes will be close to the original frequency of vibration of the separated objects. In quantum mechanical realms, similar situations can arise. If we loosely couple two like objects each capable of absorbing light, in a given band, the ensemble should possess two light absorption bands, and each should only be a small amount removed from that possessed by a single unit. In the case of ApA, the adenylic acid dimer, the chromophores are the two adenine rings, and the coupling is a coulombic interaction between them.

We are interested only in the electrons of the adenine rings, for only these are involved in light absorption in the near UV range. We follow the notation of Tinoco.[57] In the unexcited state, we call the wave function of an adenosine molecule $\phi_{01}$. When excited by the absorption of light, the wave function of the aromatic electrons of adenosine will become $\phi_{a1}$ which is the $a^{th}$ excited state of the molecule, and where $\phi_{01}$ and $\phi_{a1}$ are appropriately orthonormalized wave functions. If we have a dimer, ApA, and there were no interactions between the adenine rings, we could call the ground state wave function of the entire dimer, $\psi_0^0 = \phi_{01}\phi_{02}$. If the conformation of the two rings were such that they would not interact under any conditions except when the one or the other of the rings absorbed a photon, the wave function of the dimer could be written as either $\psi_{a1}^0 = \phi_{a1}\phi_{02}$ or $\psi_{a2}^0 = \phi_{01}\phi_{a2}$ depending upon whether adenosine 1, or adenosine 2 were the excited ring. If there is some sort of

coulombic interaction between the two aromatic rings when either has been excited one may consider the situation as analogous to having moved from rest, one of two pendula coupled by a spring.[59] In the case of the pendula, energy would pass back and forth from one to the other of the pendula, until it was dissipated by friction. This is a non-stationary state for the coupled pendula. There are also, as mentioned two stationary states, or normal modes for the pendula, in which the energy is shared by the system as a whole. In one of these normal modes, the pendula move in unison, in the other, in anti-unison with one another, and they continue to move in this fashion until all energy is lost by friction.



anti-unison motion of pendula



unison motion of pendula

If there is a coulombic interaction between the adenosine molecules, there exists, as with the pendula, two stationary electronic states for the molecule as a whole, the normal modes of the molecule. These wave functions for the excited molecule are designated $\psi^{0}_{A1}$ and $\psi^{0}_{A2}$. In order that the problem be tractable, we must consider that there is no interaction between

the rings in the ground state in which case, the wave function for the unexcited molecule as a whole may be written as before, $\psi_0^0 = \phi_{01}\phi_{02}$.[57,8] The two excited stationary states may be expressed as $\psi_{A1}^0 = \frac{1}{\sqrt{2}}(\psi_{a1}^0 + \psi_{a2}^0)$ and as $\psi_{A2}^0 = \frac{1}{\sqrt{2}}(\psi_{a1}^0 - \psi_{a2}^0)$. $\psi_{a1}^0$ and $\psi_{a2}^0$ describe wave functions in which only one of the chromophores has been excited. The normal modes may further be expressed as $\psi_{A1}^0 = \frac{1}{\sqrt{2}}(\phi_{a1}\phi_{02} + \phi_{01}\phi_{a2})$ and $\frac{1}{\sqrt{2}}(\phi_{a1}\phi_{02} - \phi_{01}\phi_{a2})$ where the $\phi_{ij}$ are wave functions referring only to a single adenosine residue. The factor $1/\sqrt{2}$ is necessary to normalize the wave function. If the coulombic interaction between the chromophores in the dimer is expressed by $U_{1,2}$ the energies of the two stationary excited modes of the molecule ought, in the first approximation to equal $E_0 \pm U_{1,2}$ where $E_0$ is the energy of an excited monomer relative to the ground state, i.e., is the difference in energies between the wave function $\phi_{01}$ and $\phi_{a1}$ or between $\phi_{02}$ and $\phi_{a2}$.[59]

Physically, $U_{1,2}$ is the result of a coulombic interaction which can be approximated by a dipole-dipole interaction between the adenine rings in the dimer. Each of the adenine rings possesses a transition electric dipole moment of about 2.8 debye.[2] The perpendicular distance between the two dipoles ought to about equal the van der Waal thickness of the adenine. (Assuming that in the dimer, the two adenine groups are lined up parallel, and stacked the one above the other.) The perpendicular distance between the centers of the two rings is about 3.5 Å and $U_{1,2} = p_1p_2/kr^3$ where $p_1 = p_2 = 2.8\text{x}10^{-18}$, the transition dipole moment for each adenine ring; $r = 3.5\text{x}10^{-8}$ cm which

is the distance between the centers of the two bases, and k is the dielectric constant of the solvent which is about equal to the square of the index of refraction at optical frequencies. For water, then, k is about equal to 1.7. $U_{1,2} = 2.29 \times 10^{-15}$ ergs. In terms of separation in wave numbers this would be 560 $cm^{-1}$ and a separation of this nature of two bands should be extremely hard to resolve. To each point of the monomer spectrum, of energy $E_0$, there corresponds points of the two dimer spectra with energies $E_0 \pm U_{1,2}$. Likewise, the peak of the absorption band should be split into two peaks, separated by like amounts of energy. At 260 mμ the difference in wavelength corresponding to 560 $cm^{-1}$ is about 4 mμ. If the peaks were smooth and bilaterally symmetrical there would be no chance of resolving them. If two peaks are gaussian shaped with width at half peak maximum of 30 mμ. Jaffee and Orchin show that the resolution of two peaks is possible only if the separation is some 25 mμ.[60]

Figure 58a shows the spectral curve of adenosine between 250 and 270 mμ. If we were to split this band and separate the two bands so split by ±5 mμ, we would have the situation shown in Fig. 58b. The resultant of these two bands is seen as Fig. 58c. No splitting is resolvable here.

If we introduce some arbitrary jaggedness into the adenosine spectrum (presumably the sort of vibrational structure we would see at low enough temperatures) we would have a spectrum shown as Fig. 58d. If we split this electronic band and separate

the two bands thereby created, we have the situation of Fig. 58e. The resultant of these clearly shows the splitting of the band, Fig. 58f.

Thus if we bring solutions of adenosine to adequately low temperatures, we should see a spectrum like Fig. 58d, and if we were to bring the ApA dimer to like temperatures, we should see two vibrational peaks for each one seen in the monomer. But the vibrational peaks must be large enough, and sharp enough for the splitting to be resolvable.

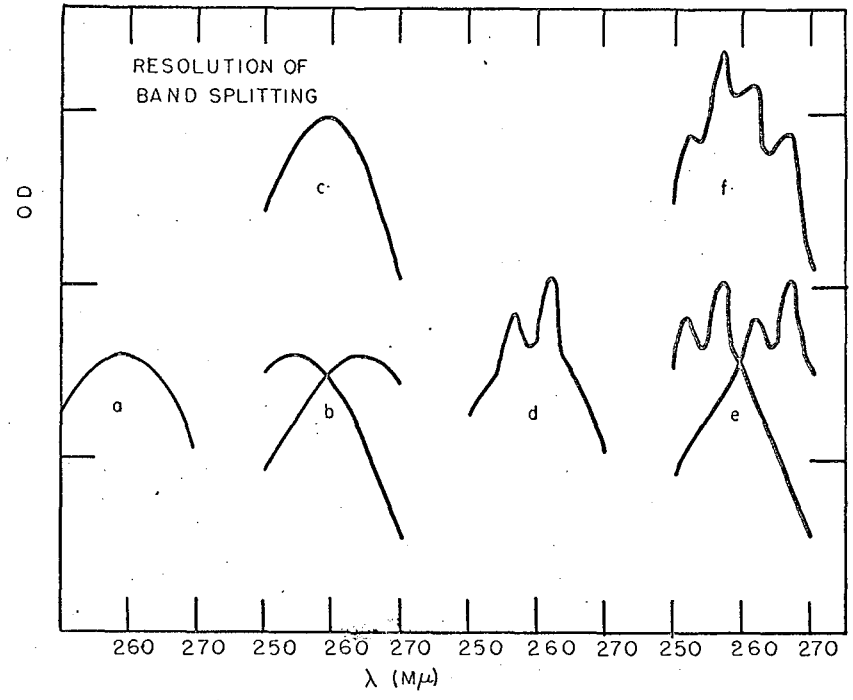



Figure 58. Resolution of band splitting in optical absorption spectra.

-148-
-149-
-150-
-151-
-152-

Omitted

Our sample holder consisted of a single quartz plate, spring loaded between two recessed copper blocks (Fig. 59). The assembly, with its shallow layer of solution, could be placed upon a block of dry ice to freeze the sample. We then placed the assembly, with its frozen sample into a quartz-windowed dewar flask, and covered it with liquid nitrogen. The whole unit fit into the 1462 Scatter Transmission Assembly.

We used plastic electric tape as a gasket between the quartz plate and its restraining copper block. So that the sample would spread evenly over the quartz plate, we periodically shined it with Shamva Metallographic Polish.[61]

We encountered a number of experimental difficulties. While we could easily pipette a specified volume (and hence thickness) of a solution onto the quartz plate, the sample often froze unevenly. This resulted in uncertainty of sample thickness, and prevented us from measuring the absolute absorptions of our samples. It further resulted in some irreproducibility of scattering characteristics from sample to sample. Such irreproducibility was especially troublesome below 245 mμ. The worst problem we encountered was the fierce bubbling of the liquid nitrogen in our dewar surrounding our copper sample holder. This bubbling gave us a great deal of noise in our recorded spectra. To get around this problem, we sometimes tried to cool the copper sample holder assembly to liquid nitrogen temperature, by immersion in $N_2$, but delayed taking the spectrum until the level of the nitrogen had gone just

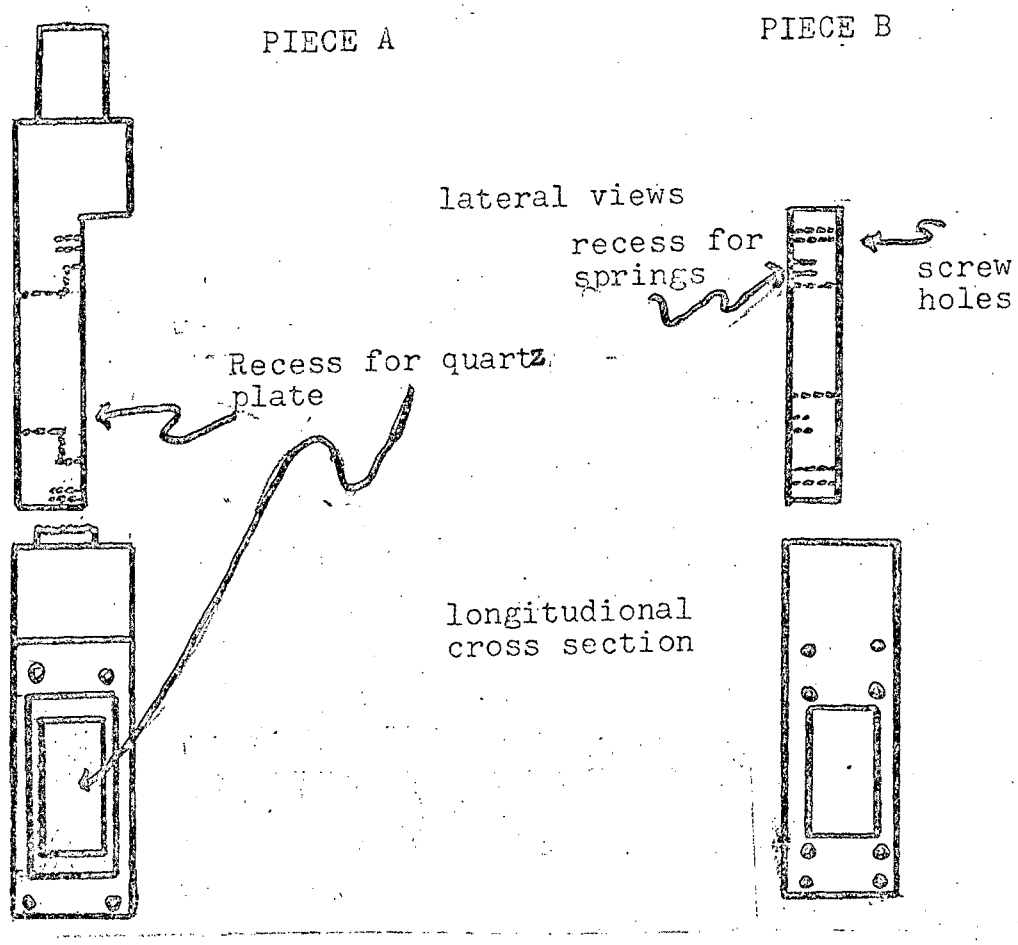


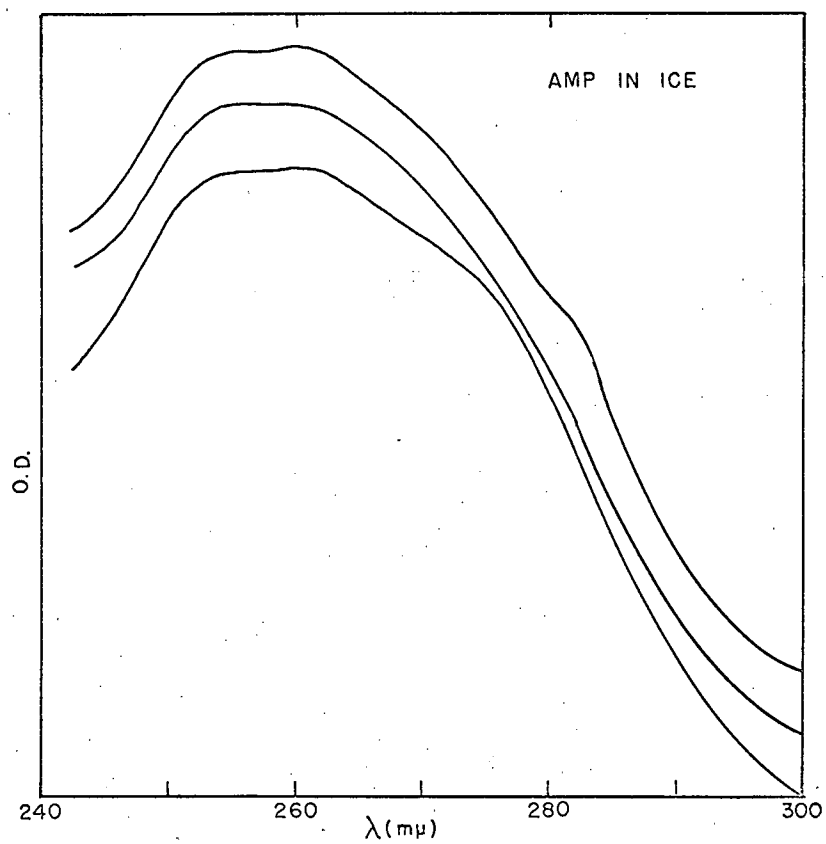

Figure 59. Ice film sample holder.
(Drawn to full scale.)

below the ice sample. Because of rapid temperature changes with this method, as well as problems with moisture fogging the sample, we were still left with a certain amount of irreproducibility. All in all, our method would allow us to see only very prominent spectral changes or splittings.

ApAp and AMP

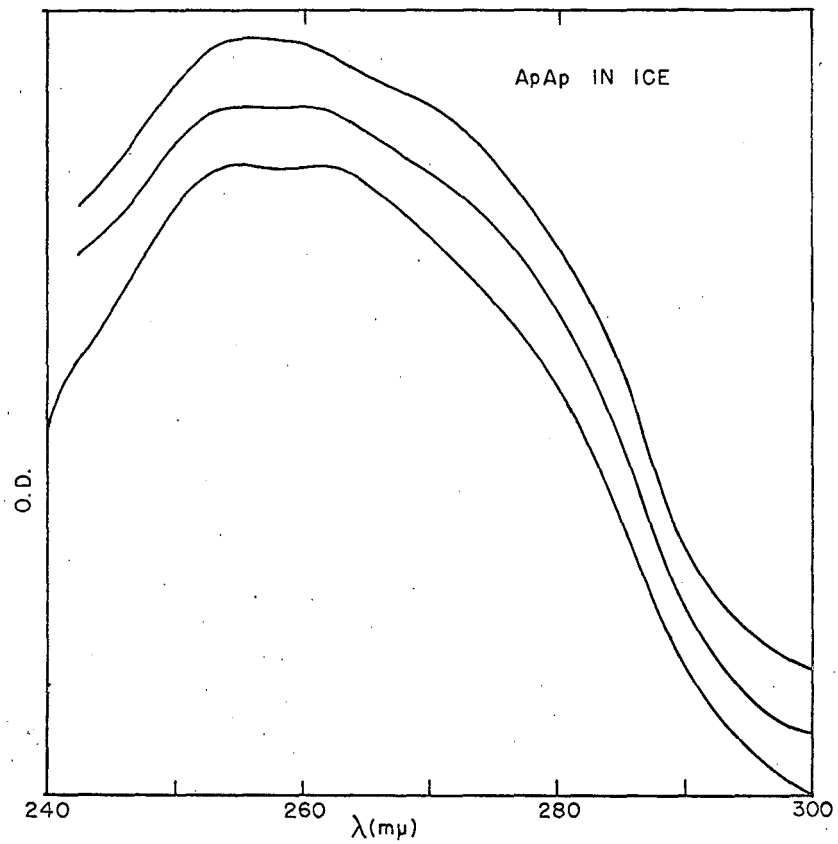
Both the monomer and the dimer of adenylic acid possess spectra which differ considerably from those in neutral aqueous solution. There are inflections in the spectral curves, both above and below the peak maximum. These are reminiscent of the vibration structure which we found in the low temperature ethanol solutions of these molecules (Fig. 71). There are no prominent differences in the ice samples between the monomer and the dimer. Both are considerably broadened over the spectra seen in aqueous solution. The spectra of the monomer and of ApAp were taken with the ice covered by liquid nitrogen (Figs. 60A and 60B). The recordings were very noisy, and we have included in this figure several spectra taken with each sample. From these, one may get an idea of the degree of reproducibility of our method.

We took the spectra of the four ribonucleosides (Figs. 61 through 64) after the nitrogen had evaporated to below the level of the samples. Like the spectra of the AMP and ApAp, the spectra of the nucleosides are considerably broadened over those in aqueous solutions.




MU-35906

Figure 60A. Several spectra of adenylic acid, taken in an ice sample.




MU-35907

Figure 60B. Several spectra of ApAp taken in an ice sample.

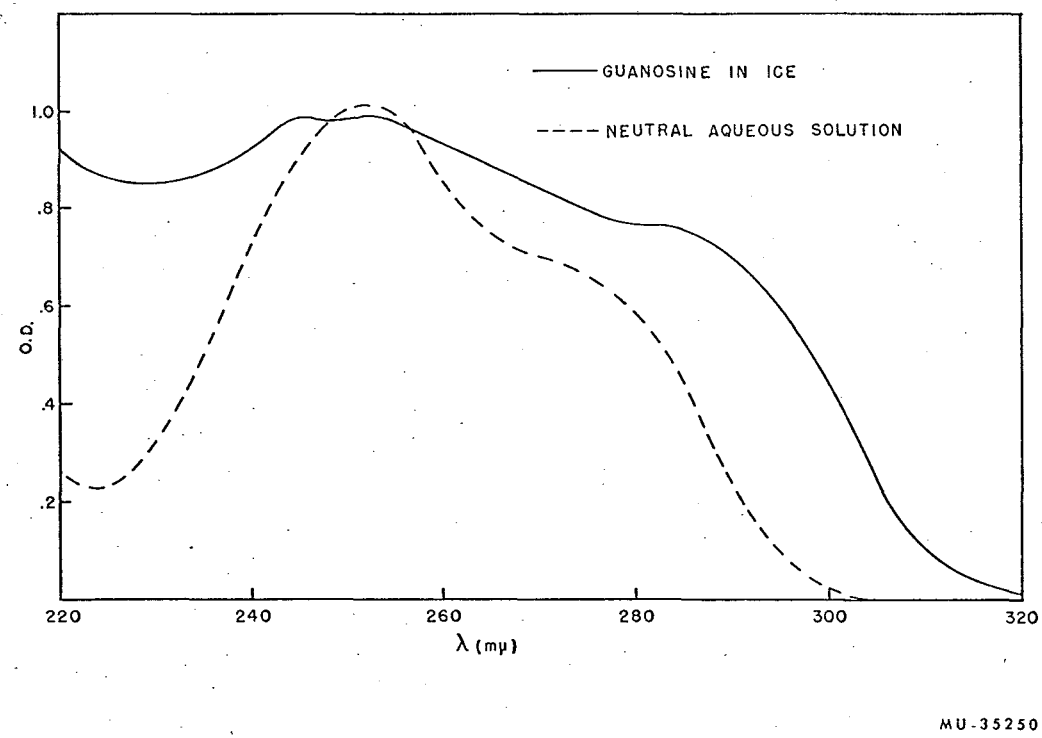



Figure 61. Optical absorption of guanosine, recorded form ice sample.

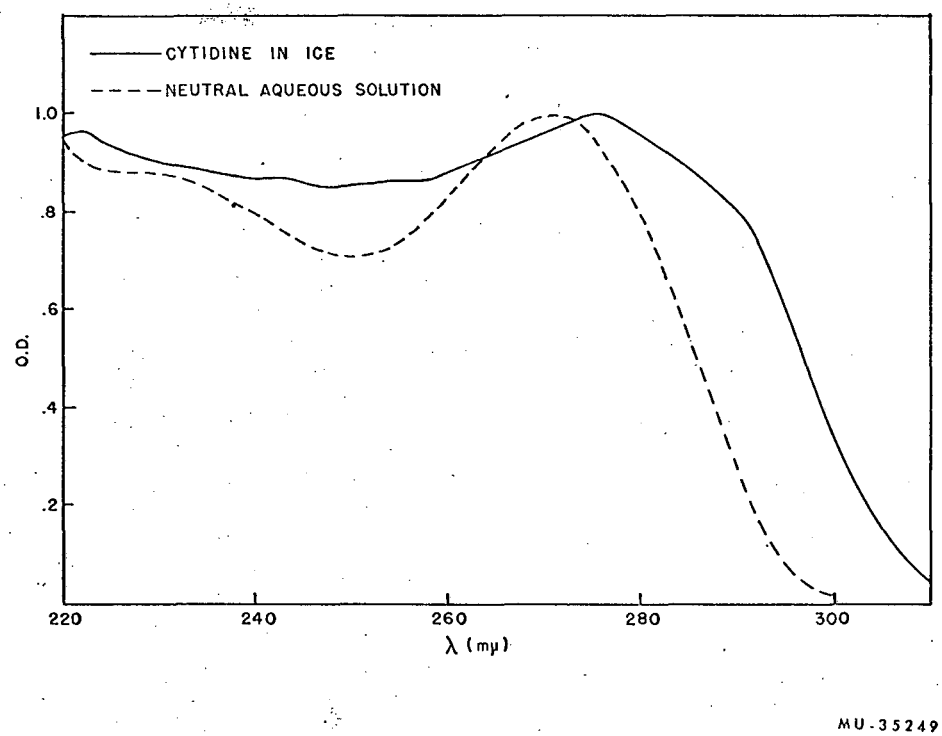



Figure 62. Optical absorption of cytidine, recorded from ice sample.

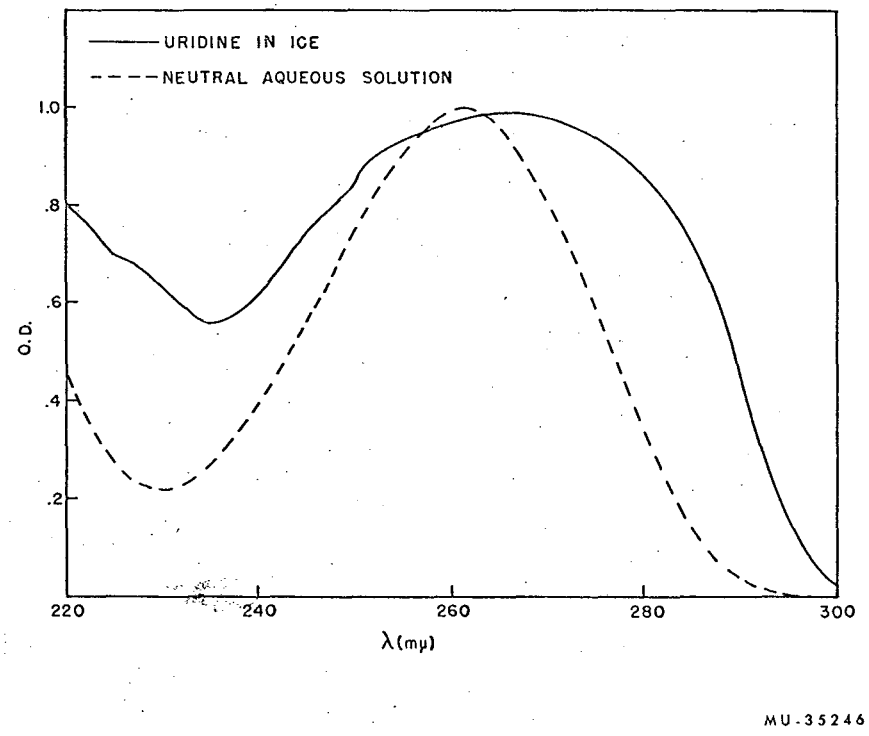



Figure 63. Optical absorption of uridine, recorded from ice sample.

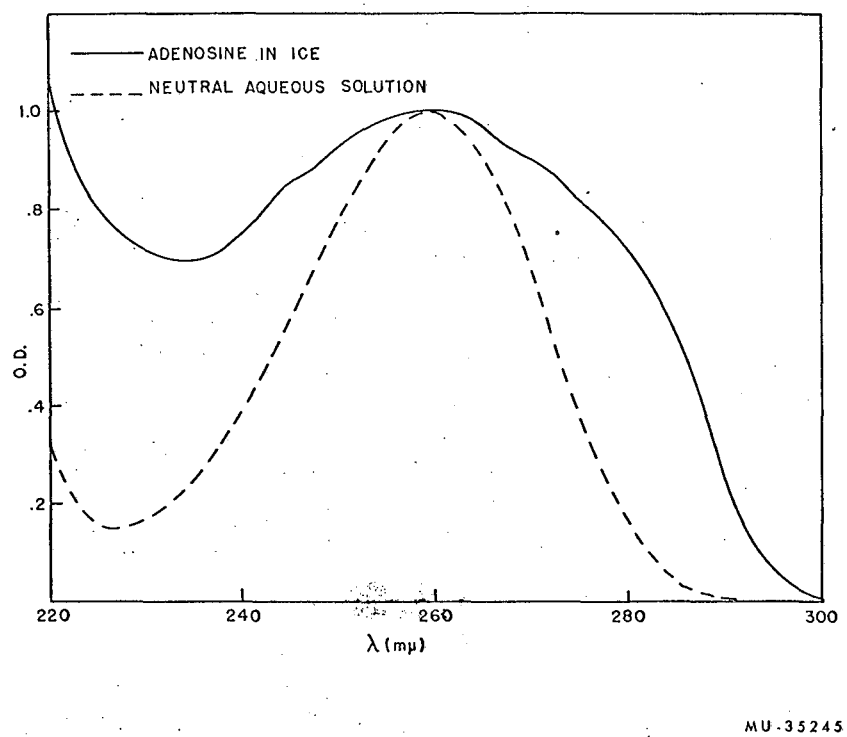



Figure 64. Optical absorption of adenosine, recorded from ice sample.

Thymine Dimer

If one shines intense ultraviolet light on a dish containing a frozen thymine solution, the molecules will dimerize at a rate far surpassing the rate of the same phenomenon in aqueous solution.[3,62] Likewise the optical absorption spectrum of thymine solution is greatly changed when it is frozen into an ice (Fig. 65). This spectrum is very similar to one obtained by Sinsheimer, Scott, and Loofbourow from thin films of sublimed thymine, at low temperatures.[63] A highly aggregated state of the thymine molecules should tend to favor the photoreaction which gives rise to the dimer formation. Such an aggregated system would also give rise to spectral changes due to interactions amongst the chromophoric thymine molecules. In Sinsheimer's sublimed films, there probably exists a more or less crystalline state of the thymine molecules, giving rise to his observed spectral changes. Likely, in our frozen samples, we also possess a highly aggregated system of thymine molecules. Wang believes that when water solutions of thymine, or solutions of a similar nature are frozen, the solute collects in very concentrated "puddles" of liquid solvent, scattered throughout the ice phase.[3,62] Such a phenomenon would explain the aggregation effects. Harrison shows evidence for the existence of water filled "puddles" or pores in ice.[64]

The formation of the thymine dimer is greatest on irradiation at a wavelength of from 275-280 mμ, the wavelength range of the most dramatic spectral changes we observe. A number of products may form in this dimerization reaction, one of which

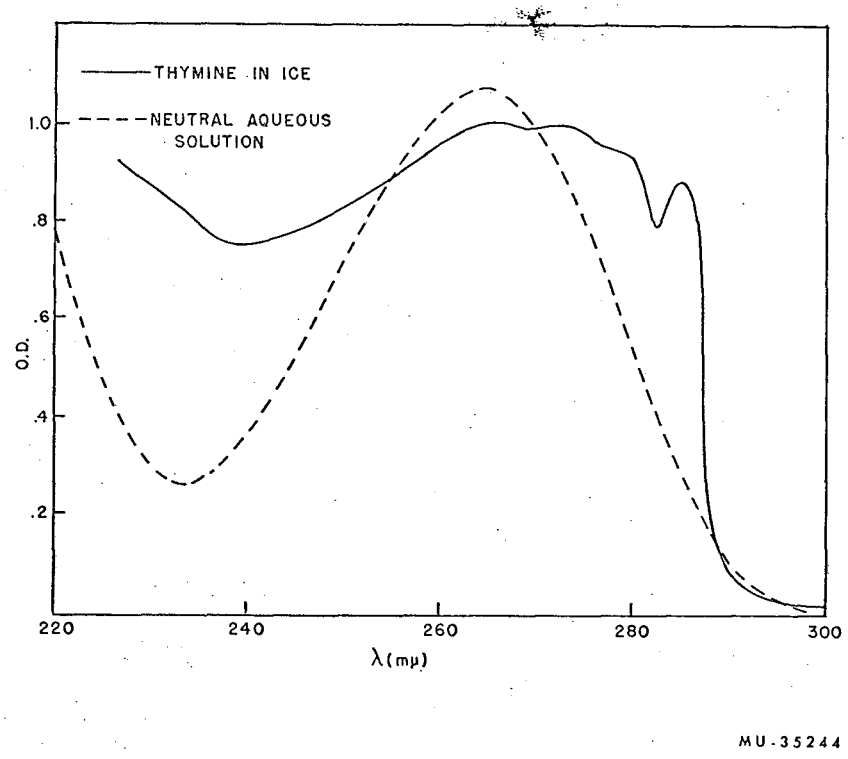
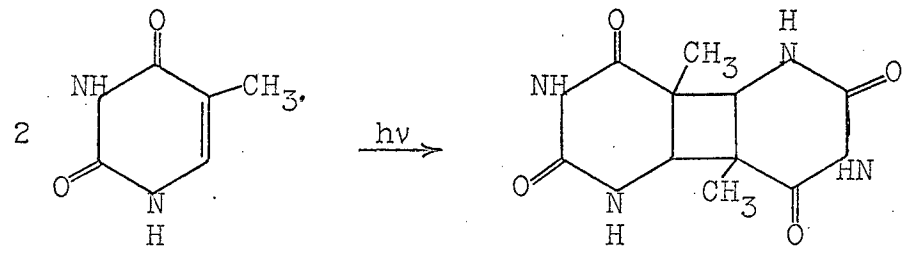



Figure 65. Optical absorption of thymine in ice.

we show below.




Wang found that as he added alcohol to a thymine solution, dimer formation from UV irradiation of frozen solutions progressively decreases, presumably due to the breaking up of the highly aggregated puddles of thymine.[3,62] When Sinsheimer froze thymine into an EPA glass (ethanol, ethyl ether, isopentane), and took the absorption spectrum, it was much closer to the aqueous solution spectrum than was that in his thin films. We took an absorption spectrum of a sample of a 10% ethanol solution of thymine, frozen into an ice. The spectrum appeared quite similar to that taken by Sinsheimer in EPA glass (Fig. 66).

Wang found a good deal of photodimer formation in samples of uracil UV irradiated while in frozen solutions.[3] These, again seem attributable to the highly aggregated state of the molecules under these circumstances. The spectral change of frozen samples of uracil are not nearly so dramatic as are those exhibited by frozen samples of thymine (Fig. 67). Sinsheimer found a different spectrum for his sublimed uracil film than we found for our frozen sample.[62] After he let his film stand for a number of days, it came to resemble ours much more closely.

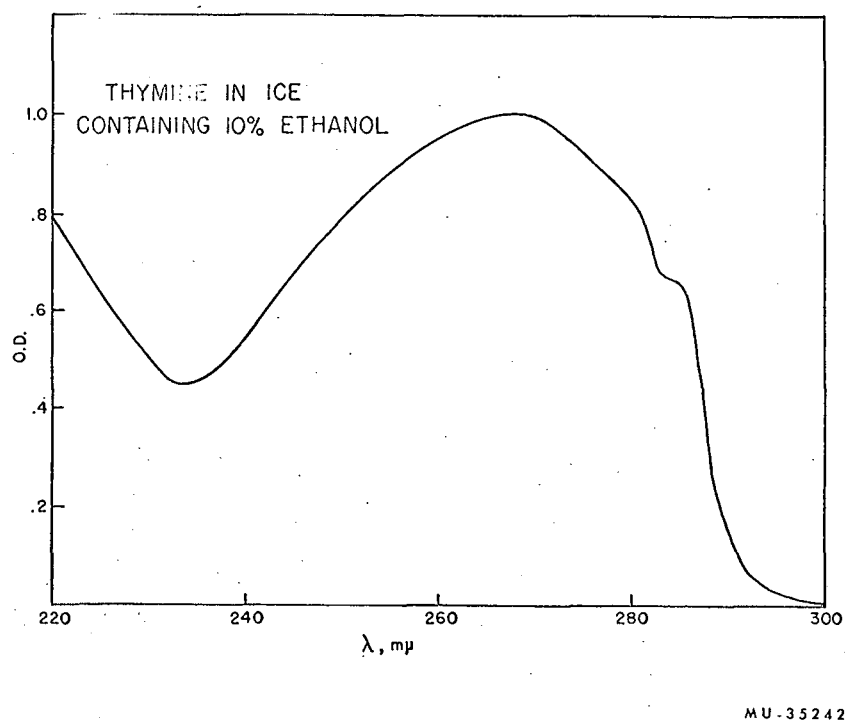



Figure 66. Optical absorption of thymine in ice, containing 10% ethanol.

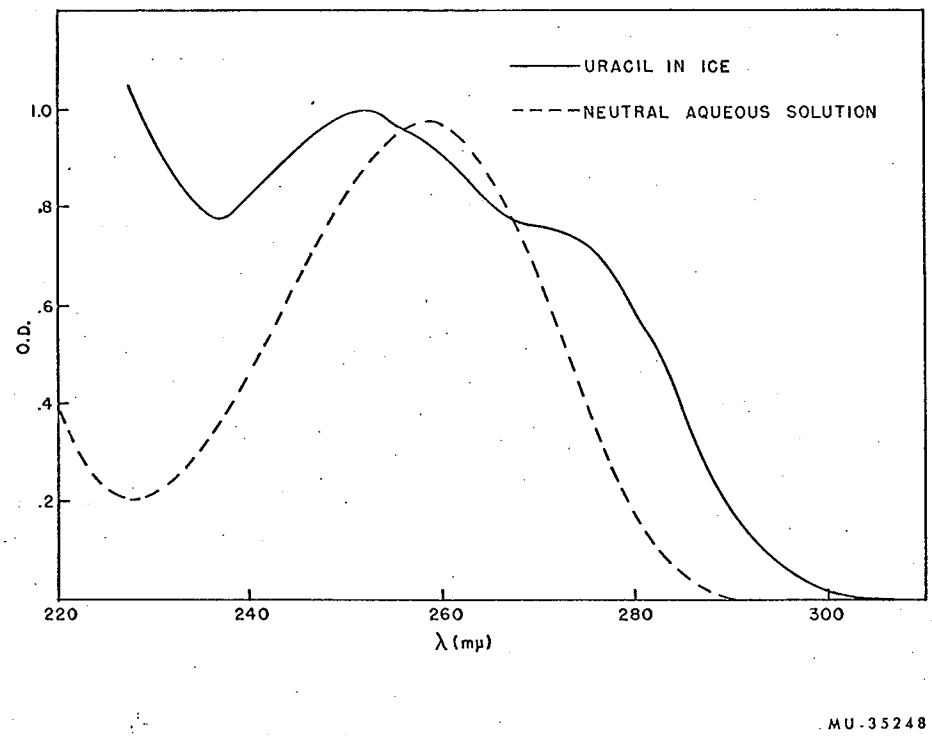



Figure 67. Optical absorption of uracil in ice.

Wang found that frozen solutions of uridylic and thymidylic acids would also participate in photodimer formation when irradiated with UV light, though not to the degree of the free base. Absorption spectra of the two compounds under these circumstances show considerable changes from those in neutral aqueous solutions (Fig. 68 and 69).

The spectrum of adenine taken from a frozen sample, likewise shows a large change from that in solution (Fig. 70). It is very much like the spectrum obtained by Sinsheimer from his thin sublimed film of adenine.

The probable highly aggregated state of thymine necessary for photodimer formation gives us a pretty good clue to why we are unable to discern spectral differences between Ap and ApAp under the conditions we used for observation. The general spectral broadening of all the substances we looked at, which likewise probably result from aggregation of the molecules, reinforce that clue. The intermolecular interactions of Ap molecules, under conditions of high aggregation could be as strong as the intramolecular interactions of the chromophores in ApAp. One would then not expect to see much difference in the spectra of these two substances.

<u>Summary</u>

Solutions of nucleotides, frozen into thin samples of ice possess quite broadened spectra, probably resulting from highly aggregated states of the molecules.

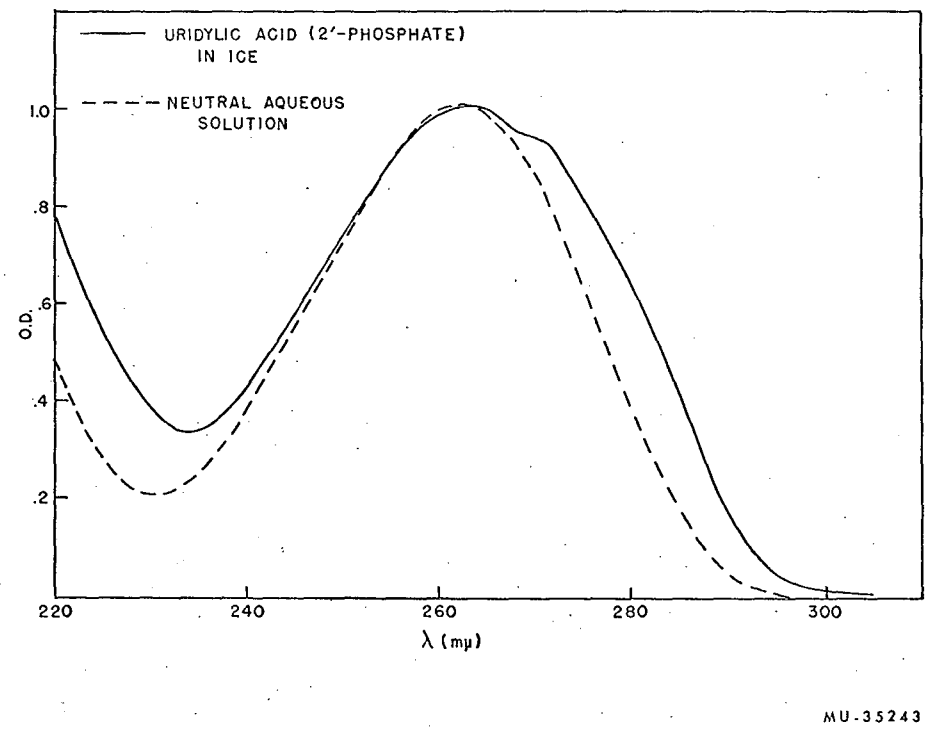



Figure 68. Optical absorption of uridylic acid in ice.

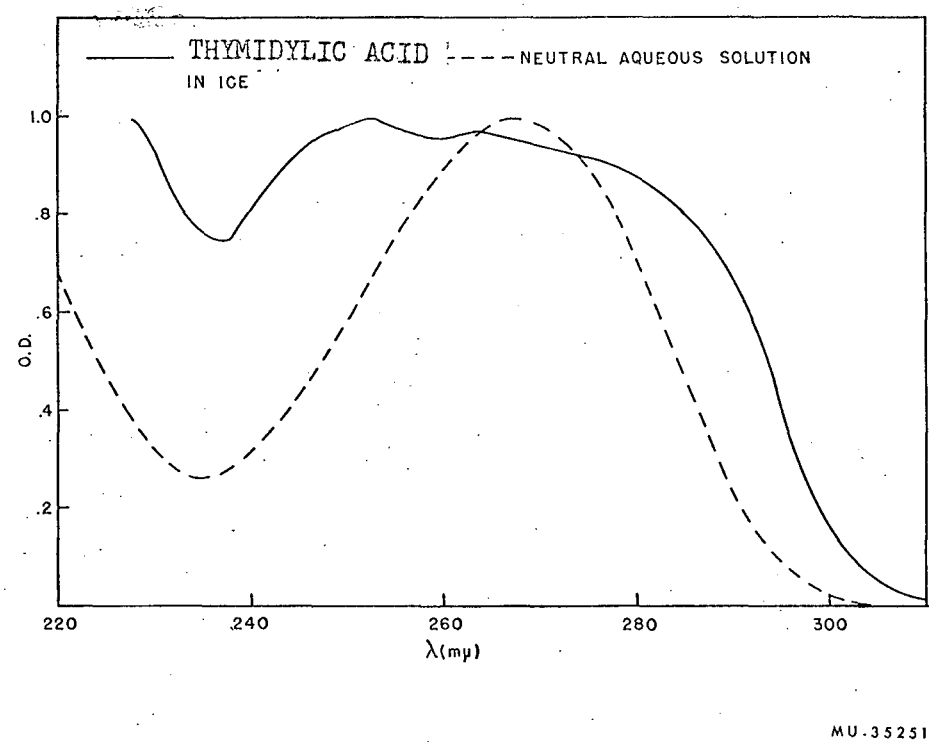



Figure 69. Optical absorption of thymidylic acid in ice.

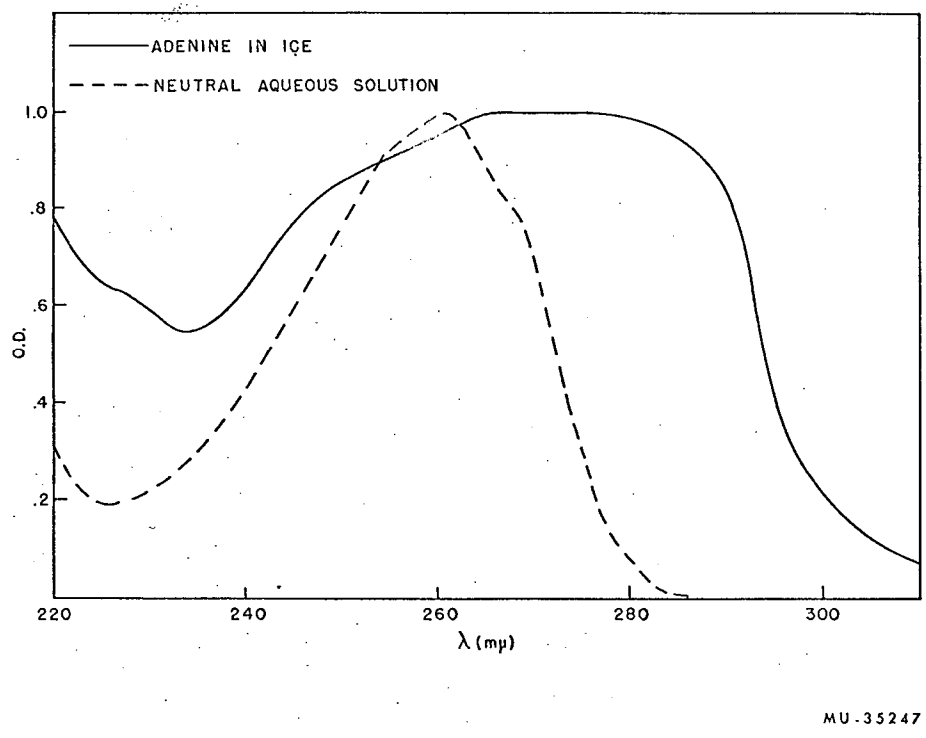



Figure 70. Optical absorption af adenine in ice.

An attempt to see enhanced base-base interaction in ApA, dissolved in ethanol, described in the next section, failed because ethanol weakens the conformation of the molecule necessary for such interaction.

Contrariwise, in frozen blocks of ice, the aggregation of the molecules is so close that intermolecular interactions in Ap may become quite as strong as intramolecular interactions in ApAp with resulting spectral indistinguishability.

## Light Absorption from Ethanol Solutions at Low Temperatures

In an early experiment, we brought ethanol solutions of adenylic acid and its dimer molecule to low temperatures (-120°C). We had hoped that the dimer spectrum recorded under these conditions would exhibit evidence of augmented base-base interaction. But ethanol, as well as most organic solvents weaken such base-base interaction as exists in the dimer in aqueous solution. This experiment was likely doomed from the start; however, we were able, with the same procedures, to observe the vibrational structures of the UV absorption bands for a number of molecules of interest.

### Methods

In taking low temperature measurements, we used a cryostat based upon the design of Jones and Willard.[65] Cuvettes, for optical absorption measurements may be placed in the light path of a spectrophotometer. We can hold their temperature at a fixed value ($\pm 2°$ between -50 and -140°C). The coolant is liquid nitrogen. The cold chamber holds two cuvettes. One is filled with a sample solution, the other with solvent alone. In the reference compartment one has a solvent blank at room temperature. The measurement of cold solvent against that at room temperature enables corrections to be made for changes in absorption characteristics of the solvent with temperature. The cryostat was used with either a Cary 14 or Cary 15 spectrophotometer.

Changes in solvent density with temperature, with a resultant increase in concentration of solute molecules, could be compensated for by correction factors from reference tables.[66] It was not so easy to correct for the occasional condensation of water vapor upon the cuvettes in the cryostat. This caused some degree of light scattering, and consequent changes in the base line of the recorded spectra. Indeed, this was the major problem in the experimental procedure.

The solvent for all the spectra herein recorded was absolute ethanol, though no precautions were taken to insure its dryness. The alcohol was used either alone, gassed with HCl, or as a solution with KOH or $CH_3ONa$, depending upon the acidity or basicity desired. The alcohol, with no dissolved acid or base, tends to form a glass which cracks at about -120°C. According to Ronayne absolute alcohol which is maintained thoroughly dry (e.g., over sodium metal) may be distilled directly into a cuvette and forms a glass which resists cracking, even at liquid nitrogen temperatures.[67]

The AM(5')P we used was a commercial preparation from Pabst laboratories. The ApA was a gift from Dr. Myron Warshaw. The 9-methylhypoxanthine was commercially available from the Cyclo Chemical Corporation.[68] The other compounds were commercial preparations from Cal Biochem.[50]

The spectra of compounds, even at the low temperatures contain too few vibrational peaks to make extensive correlations with the IR spectra; however, the splittings we do observe fall within reasonable values expected from the IR data.

APA and AMP

There is some difference in the low temperature spectra of AMP and its dimer, ApA (Fig. 71). We have arbitrarily set the height of the two spectral curves equal. We cannot say definitely whether the differences between the two curves are real, or the result of scattering problems and changes in base line as previously mentioned. The only features of the spectra we trust completely are the prominent peaks and shoulders they exhibit. These do not appear to differ significantly in the monomer and the dimer, see the table below. The accuracy in reading our spectra leads to an uncertainty in tabulated wave number values of perhaps ±50 $cm^{-1}$.

Splittings in Adenylic Acid Spectrum
(and APA Spectrum)

| Wavelength mμ | Wave number $cm^{-1}$ | Wave number Difference | Energy Difference in ergs |
|---|---|---|---|
| 252.5 | 39604 | 994 | $1.98 \times 10^{-13}$ |
| 250 | 38610 | 1227 | 2.44 |
| 267.5 | 37383 | | |

An infrared peak in the 900-1000 $cm^{-1}$ range is characteristic of most purine molecules.[69] A peak in this range appears very prominently in the IR spectrum of adenine.[70] Such a peak persists in the spectrum of adenosine.[16] It is likely

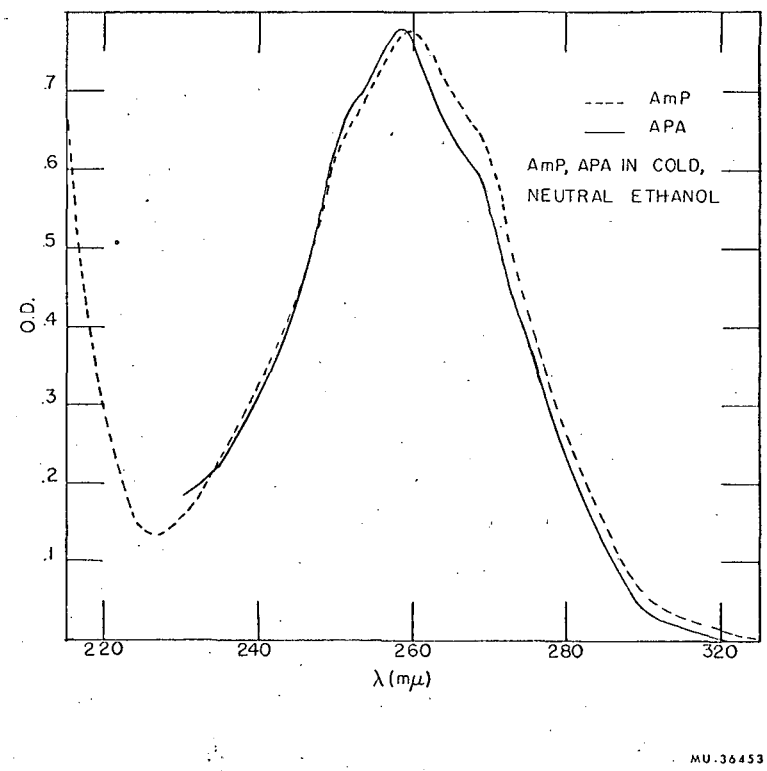


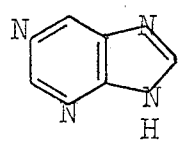

Figure 71. Amp, ApA in cold, neutral ethanol.

characteristic of a ring vibration of the purine structure. The same explanation seems to hold for the 1227 $cm^{-1}$ splitting.

Other IR peaks appear in these regions due to sugar and phosphate groupings, but these could not couple to an electronic transition. Very strong IR peaks for adenosine and adenylic acid are due to the deformation of the amino groups and appear near 1700 $cm^{-1}$ in the IR spectrum.[72] This vibrational mode despite its strength, does not appear coupled to the electronic absorption spectrum. Ethanolic solutions of ApA and adenosine appear much the same as one another at room temperature, and neither exhibits vibrational structure (Fig. 38).

Spectra of Other Purines

The simplest of the purine molecules, purine itself, is indicated below.




Its near UV absorption spectrum possesses a maximum at 264 mμ. This band seems composed of two overlapping bands. The second and smaller is observable as a large bump on the blue tail of the absorption band. Cooling an ethanol solution of purine to -100°C adds no discernable vibrational structure to the absorption spectrum, nor does it alter the shape of the spectrum (Fig. 72). The spectrum in ethanol is identical to that in neutral aqueous solution. The spectrum of purine in ethanol, saturated with sodium methoxide is shifted in wavelength and shape just as in basic water solution. The pK for ionization

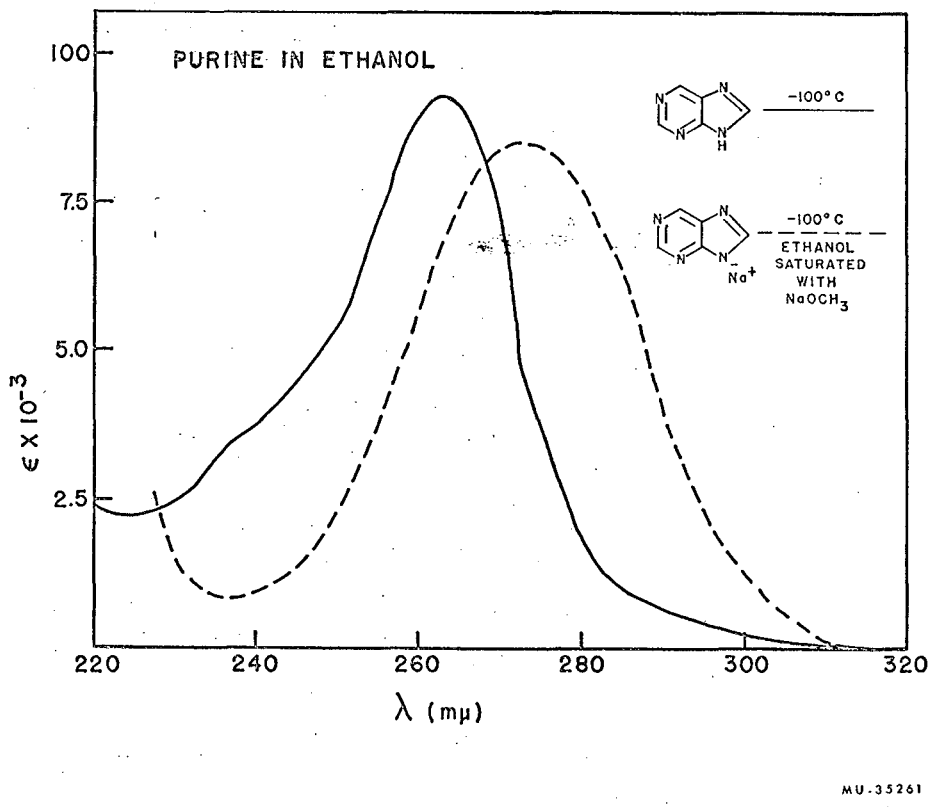
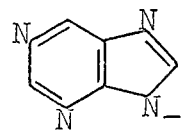



Figure 72. Purine at low temperatures in ethanol, neutral and basic.

in water is 8.9.[53] Again, no vibrational structure becomes apparent upon cooling the basic solution to -100°C. The probable structure of the ionized molecule (again in analogy to adenine) is shown below.



Spectra of purine in ethanol (.05-.9M in HCl) possessed identical shapes. This is consistent with a pK for protonation of 2.3 in water. This spectrum contained some vibrational detail, but this was not increased on cooling the solution. The shape of the spectrum did change when the solution was cooled, however (Fig. 73). In analogy with adenine, the purine is probably protonated upon the $N_1$ position.

Shoulders and Peaks of Purine

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| | Protonated 20°C | |
| 260 | 38400 | 700 |
| 265 | 37700 | 1000 |
| 272.5 | 36700 | |
| | Protonated -100°C | |
| 261.5 | 38200 | 500 |
| 265.5 | 37700 | 800 |
| 271 | 36900 | |

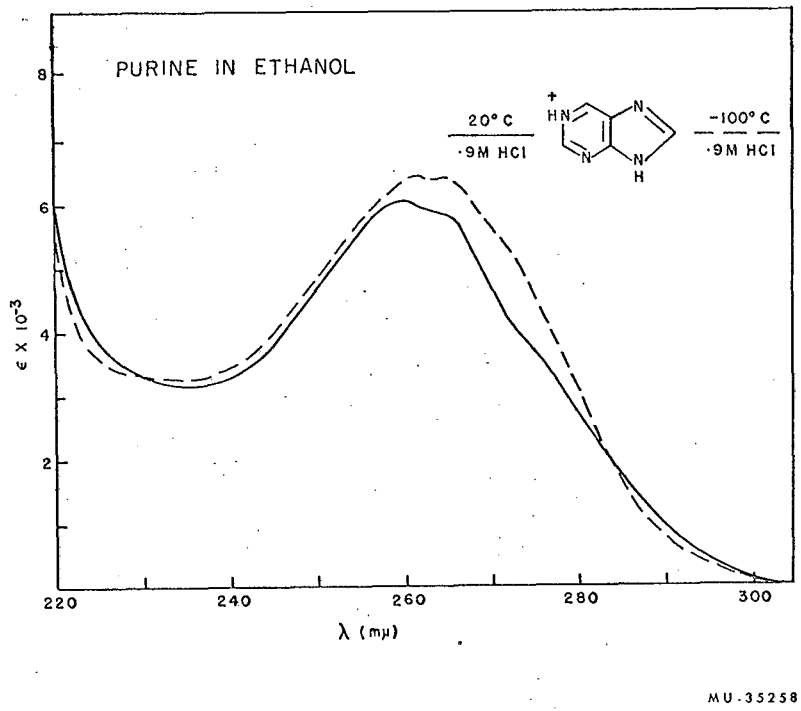



Figure 73. Purine in ethanol, acidic.

Why the vibrational structure of the spectra shows so much more clearly in the protonated than in the unprotonated or ionized forms of purine is hard to say.

In ethanol as a solvent, the near UV spectrum of adenine differs from that in water only by lacking such a prominant shoulder at 267.5 mμ. There is some vestige of this shoulder, however. Other than accentuating this shoulder, the spectrum at low temperature possesses no additional structural detail. The molecule remains unprotonated with HCl concentrations of less than $10^{-4}$ M (Fig. 74). With spectra taken in ethanol, $10^{-2}$-$10^{-3}$ M in HCl, we appear to have the singly protonated adenine molecule. When we raise the HCl concentration above .2M the spectral changes indicate that yet another (diprotonated) form of adenine predominates. This spectrum remains the same even when we increase the HCl concentration to 2M. Both the protonated forms possess some vibrational detail when cooled to -100°C (Fig. 75).

The most interesting spectrum in this study is that of the ionized adenine molecule, structure shown below. At room temperature, in either water or ethanol made basic, there is a shoulder on the red limb of the spectrum at about 280 mμ. On cooling in ethanol, this shoulder develops into a very prominant spike-like structure. In addition, the major spectral peak develops considerable vibrational detail (Fig. 76). For reference, the pK's of adenine in water are: less than zero, 4.22, and 9.8.[53]

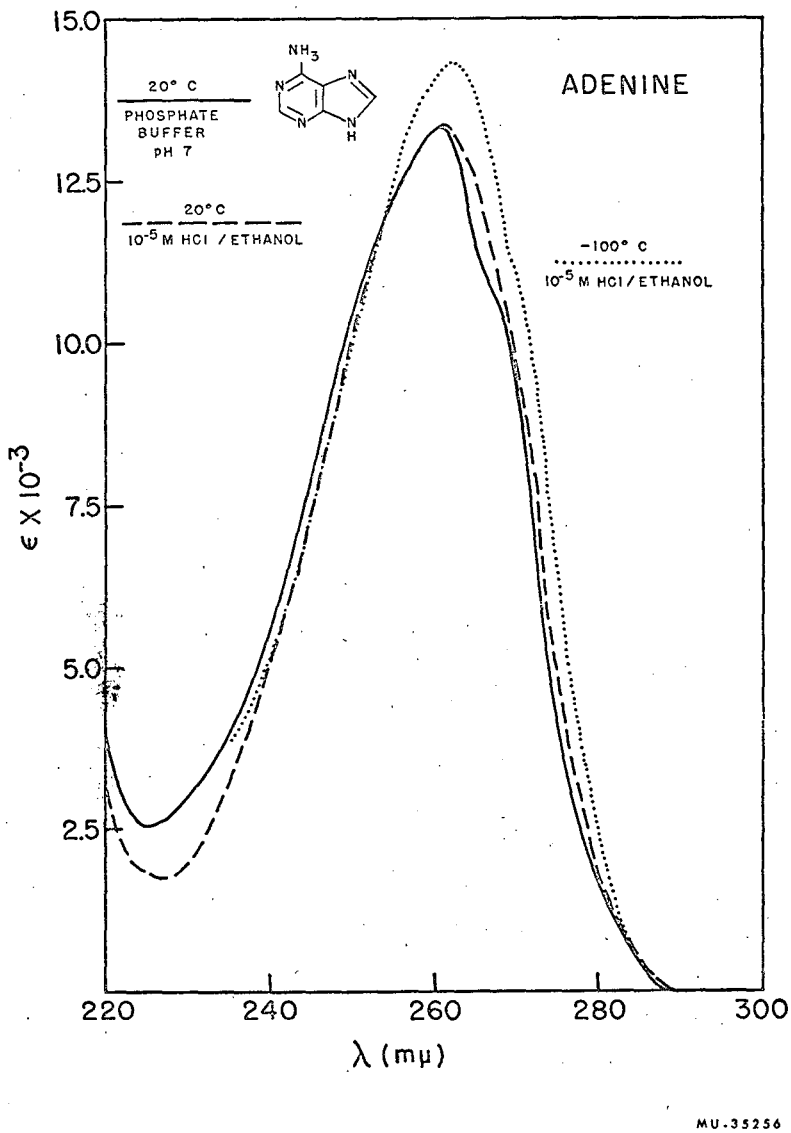



Figure 74. Adenine in ethanol at low temperatures, neutral.

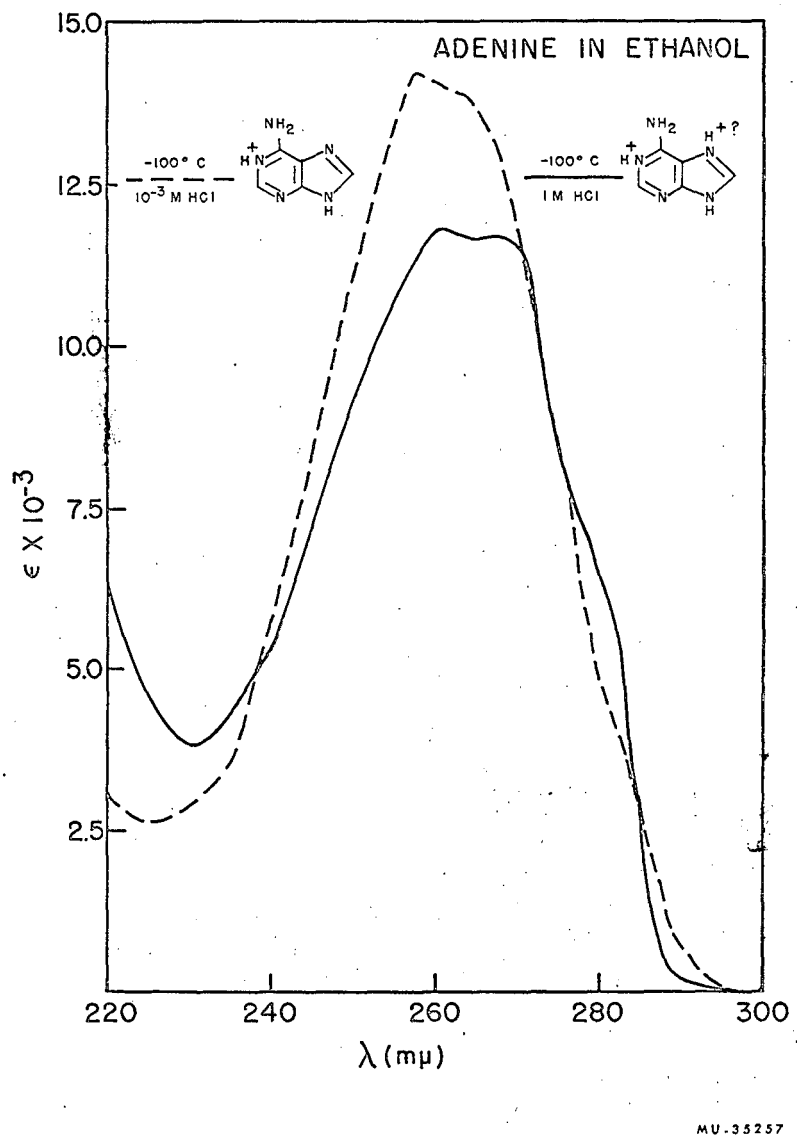



Figure 75. Adenine in ethanol at low temperature, acidic.

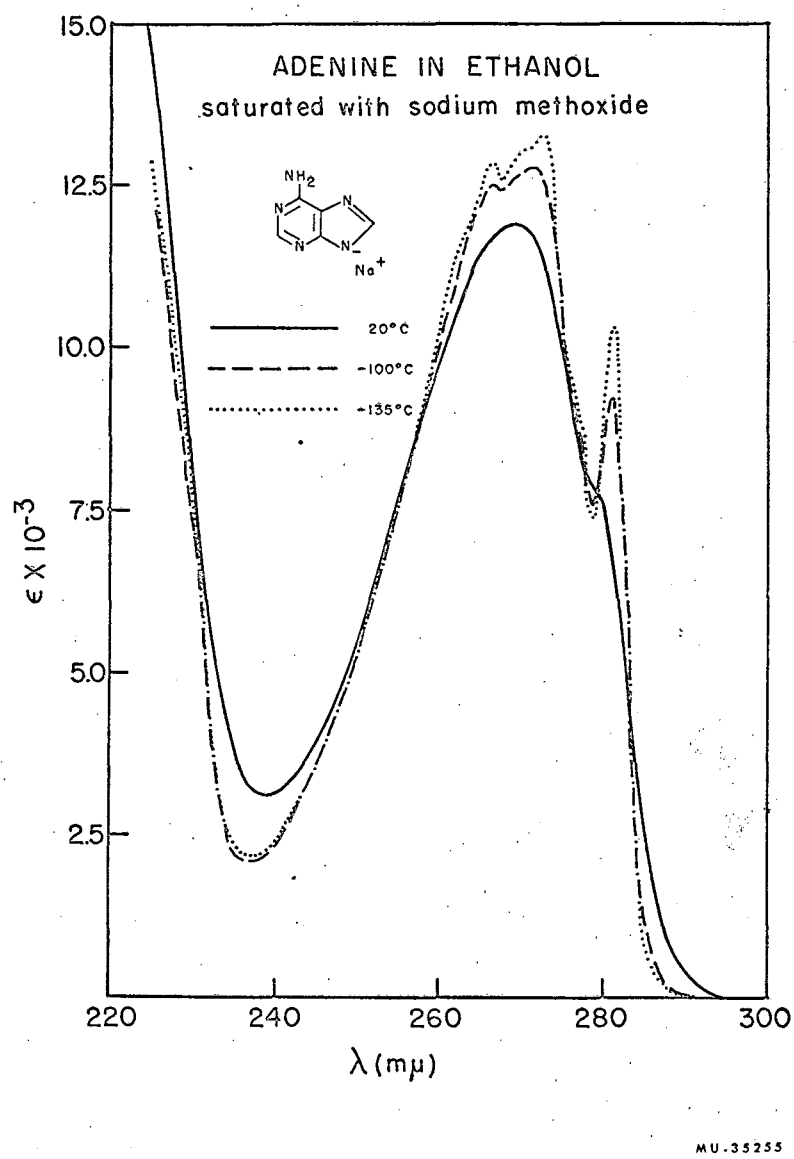
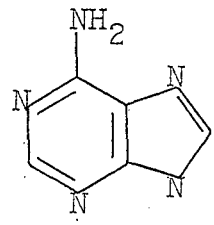



Figure 76. Adenine in ethanol at low temperature, basic.




Peaks and Shoulders of Adenine UV Absorption Spectra of Adenine in Ethanol

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| | Neutral (-100°C) | |
| 262.5 | 38100 | 1100 |
| 270.5 | 37000 | |
| | $10^{-3}$ M HCl (-100°C) | |
| 258.5 | 38500 | 650 |
| 264.0 | 37850 | 2620 |
| 284.0 | 35200 | |
| | 1M HCl (-100°C) | |
| 261.0 | 38300 | 950 |
| 266.5 | 37550 | 1350 |
| 276.5 | 36200 | 550 |
| 280.5 | 35650 | |
| | $NaOCH_3$ saturated ethanol (-100°C) | |
| 263.5 | 37900 | 400 |
| 266 | 37500 | 500 |
| 270.0 | 37000 | 300 |
| 272.5 | 36700 | 500 |
| 276.0 | 36200 | 750 |
| 281.5 | 35450 | |

The apparent splittings for ionized adenine are rather smaller than the cut off for recording of most IR spectra, and correlation is not possible.

The strange absorption spectral properties of adenine in basic ethanol at low temperatures are matched by its properties of fluorescence. In basic (although also in neutral ethanol) adenine at -100°C fluoresces more (by an order of magnitude) than any other naturally occurring nucleic acid base, nucleoside or nucleotide.[73]

The protonated form of 9-methylhypoxanthine exhibits considerably less vibrational structure at -100°C in ethanol than does the neutral species (Fig. 77). In the protonated molecule, the main absorption band exhibits two maxima, one at 246, and the other at 251 mμ. This splitting is some 900 $cm^{-1}$. The probable molecular structures of the neutral and protonated forms of 9-methylhypoxanthine are shown below. Like 9-methylhypoxanthine so also guanosine in the neutral form possesses more absorption band vibrational detail than does the protonated form. Lowering the temperature does not greatly effect the spectra of either form. The maxima in the guanosine appear at 250, and 256 mμ, again a difference of 900 $cm^{-1}$ (Fig. 78). Given the structural similarities of the protonated and neutral forms of guanosine to similar forms of the 9-methylhypoxanthine, similarities in their spectra should not be unanticipated. See below.[16]

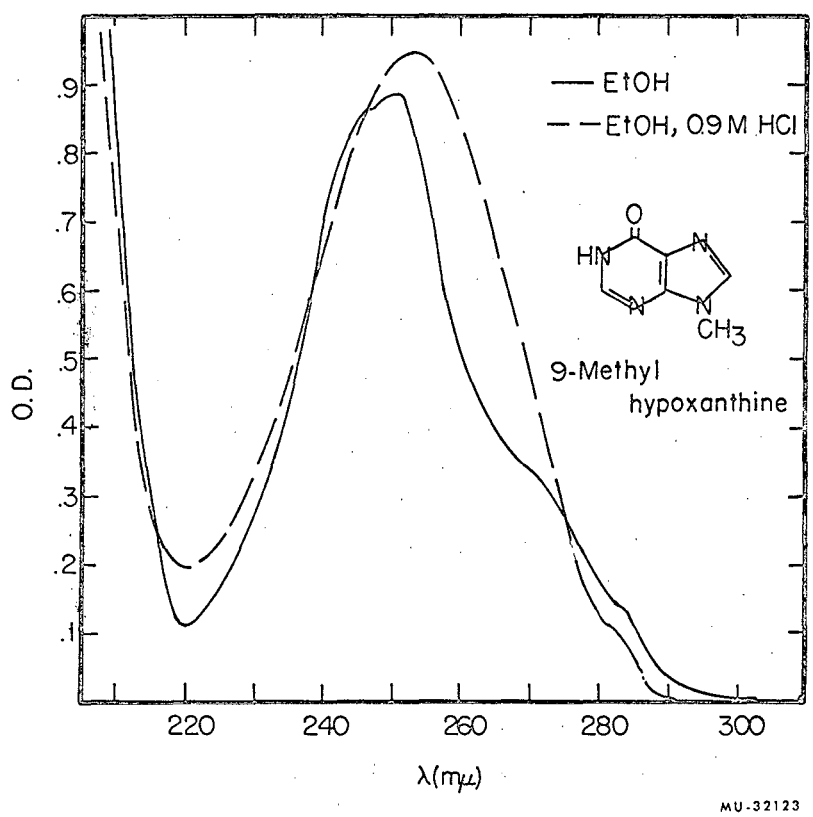



Figure 77. 9-methylhypoxanthine in ethanol at low temperature (-100°C.), neutral and acidic.

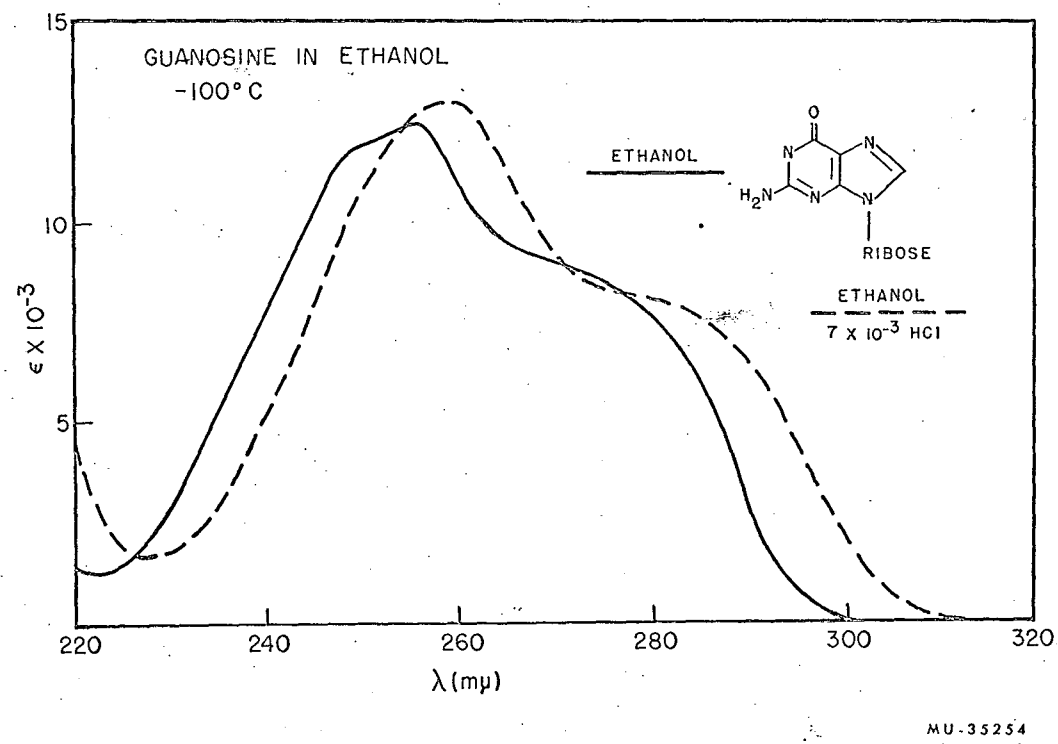
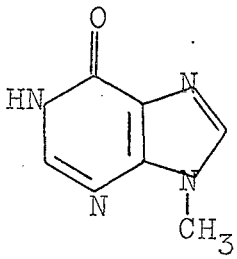
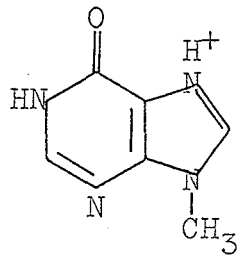


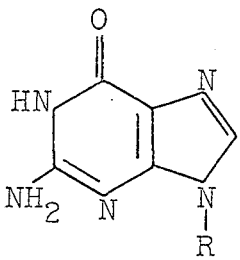

Figure 78. Guanosine in ethanol at low temperature, neutral and acidic.



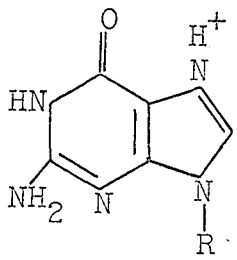
9-methylhypoxanthine



9-methylhypoxanthine
(protonated)

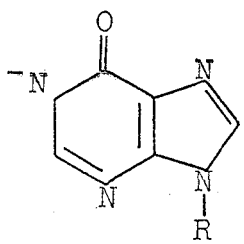


guanosine



guanosine
(protonated)

The ionized form of guanosine shows vibrational details at -100°C when dissolved in basic ethanol. Indeed, the vibrational interaction is considerably greater here than in the neutral or protonated forms of guanosine (Fig. 79).



guanosine
(ionized)

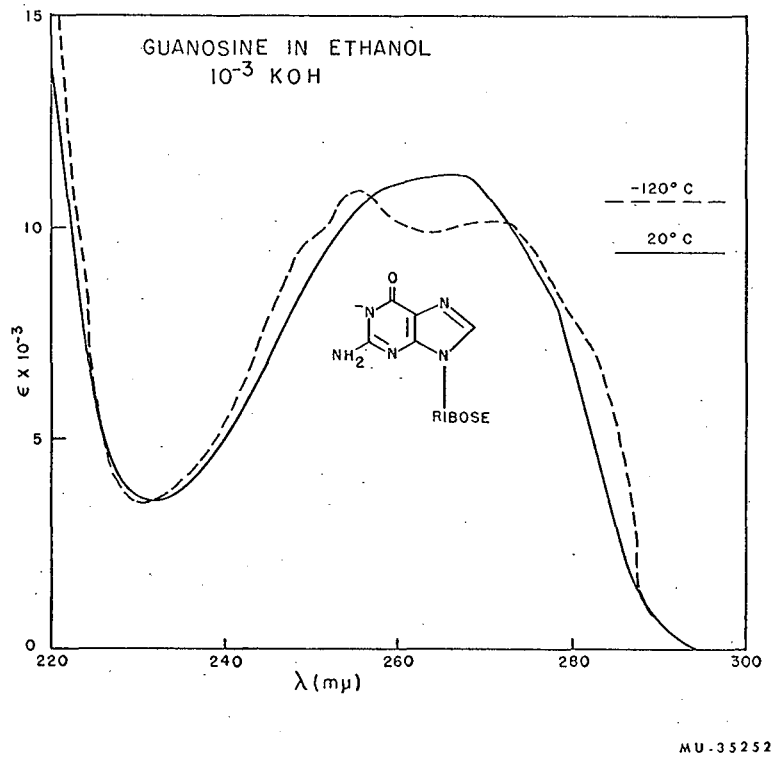



Figure 79. Guanosine in ethanol at low temperature, basic.

Peaks and Shoulders in Ionized
Guanosene Ethanol (-100°C)

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| 251 | 39700 | 500 |
| 255 | 39200 | |
| 272.5 | 36800 | 1100 |
| 280 | 35700 | |

Angell has taken the IR spectrum of guanine hydrochloride, but does not discuss it in his paper.[72] However, guanine (the neutral form) a strong IR absorption at 1172 cm$^{-1}$. Our spectrum of guanine hydrochloride at -100°C possesses four peaks and shoulders as given below (Fig. 80).

Peaks and Shoulders of Protonated
Guanine (-100°C Acidic Ethanol)

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| 246 | 40600 | 1100 |
| 260 | 38500 | 1200 |
| 268.5 | 37300 | 1200 |
| 277 | 37100 | |

The protonation of guanine likely occurs, as in guanosine, upon the $N_7$ ring nitrogen.

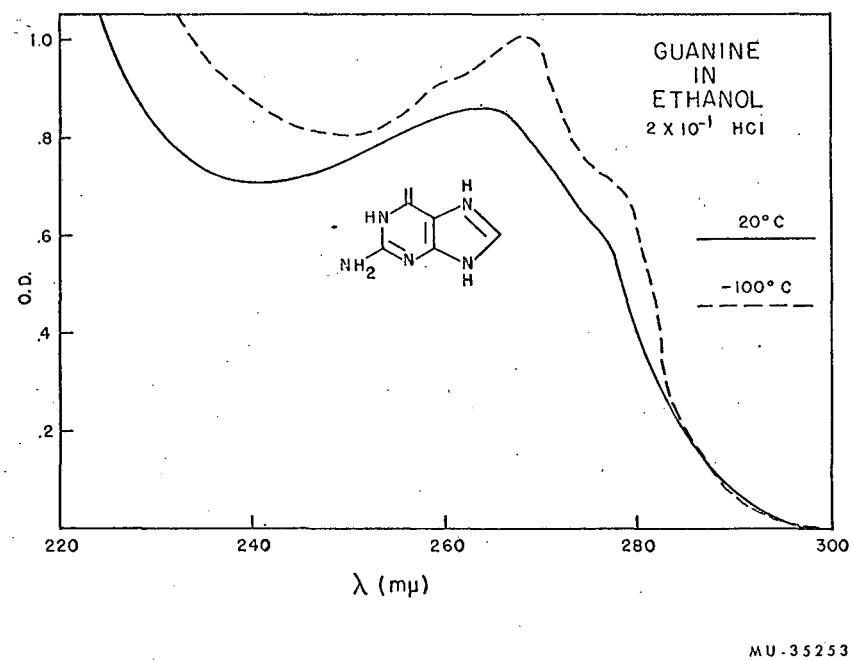
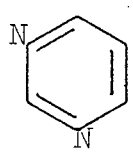



Figure 80. Guanine in ethanol at low temperature, acidic.

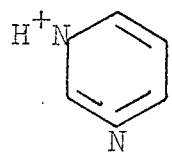
Spectra of Some Pyrimidines

The spectrum of pyrimidine exhibits two distinct absorption bands in the near UV. One of these is centered near 240 mμ. The other, weaker peak is located at 282.5 mμ. The latter peak possesses a large amount of vibrational structure in the gas phase.[74] It appears only as a single, smooth band in an alcohol solvent. Neither by lowering the temperature to -100°C, nor by saturating the ethanol with $NaOCH_3$ do we change the spectrum of pyrimidine significantly, although cooling the sample slightly sharpens the already evident vibrational peaks.

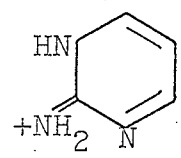
In acid solution (.7M HCl in ethanol) a lot of the structural detail is eliminated, and a new, very weak transition appears which is centered at 320 mμ (Fig. 81). Mason shows that the protonated form of 1,2 dihydro,2 iminopyrimidine possesses a similar sort of spectrum.[75]




pyrimidine




protonated pyrimidine




1,2 dihydro,2-imino-pyrimidine

There is a prominent peak near 800 $cm^{-1}$ in the IR spectrum of pyrimidine.[75] This peak likely corresponds to the benzene vibrations which absorb at 849 $cm^{-1}$ in the IR.[76]

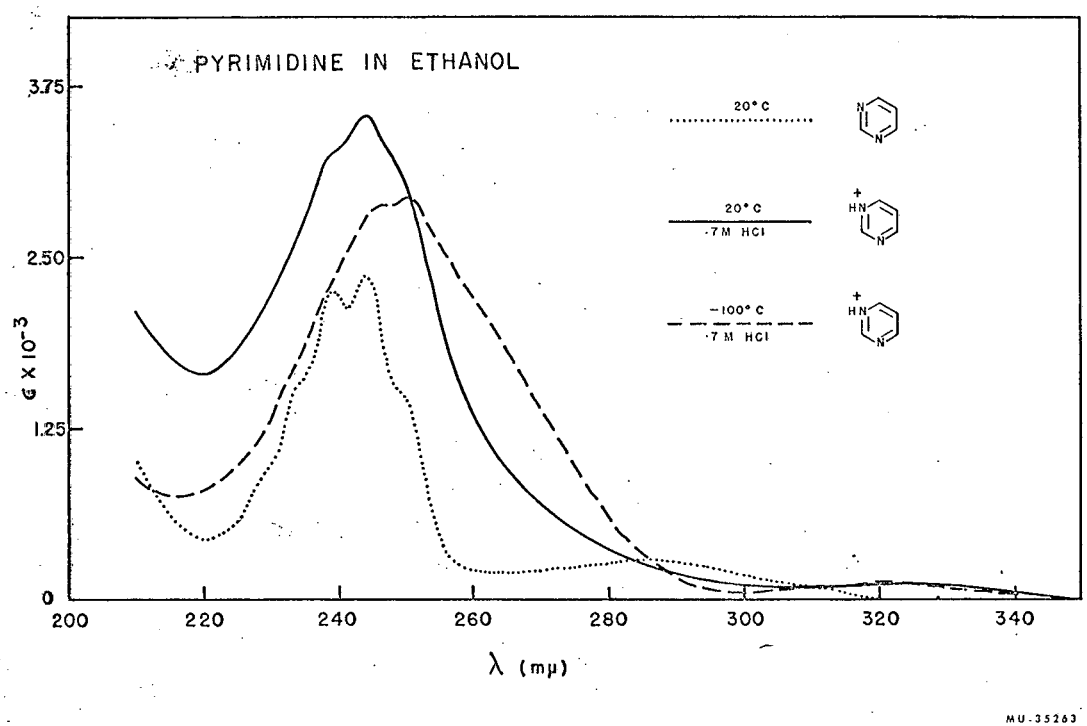
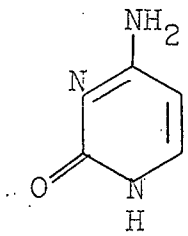



Figure 81. Pyrimidine in ethanol at low temperature, neutral and acidic.

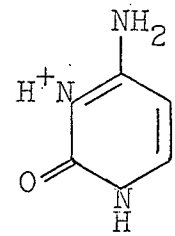
Vibrational Detail in UV Spectrum of Pyrimidine

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| | Ethanol (20°C) | |
| 234 | 42600 | 800 |
| 239 | 42800 | 800 |
| 244 | 41000 | 900 |
| 249 | 40100 | |
| | HCl .7M in Ethanol (20°C) | |
| 239 | 41800 | 800 |
| 244 | 41000 | 1000 |
| 250 | 40000 | |
| | HCl .7M in Ethanol (-100°C) | |
| 239 | 41800 | 1000 |
| 245 | 40800 | 800 |
| 250 | 40000 | |

The room temperature, ethanolic spectrum of cytosine matches, very closely that of the molecule in neutral aqueous solution.[77] At -100°C, the spectrum shows a fair amount of vibrational detail. The spectrum in ethanol, 1M in HCl is shifted to the red some 40 m$\mu$ from that of the corresponding protonated from in water. When cooled to -100°C it exhibits a pattern of vibrational detail similar to the nonprotonated molecule. The neutral and protonated forms shown below follow the structures mentioned by Tsubol.[16]

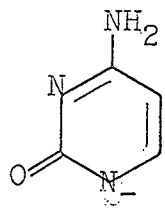



cytosine




protonated cytosine

The ionized form of the cytosine molecule formed by solution in ethanol 1M in KOH likewise has vibrational structure when cooled to -100°C, and its probable structure is shown below. (Fig. 82).




ionized cytosine

The pK's for the protonation and ionization of cytosine are 4.6 and 12.16, respectively.[53]

Pyrimidine, as well as substituted pyrimidines exhibit a vast number of absorption bands in the frequency range below 1000 $cm^{-1}$, although there appears to be no specific data available for cytosine in the region below 700 $cm^{-1}$.

At room temperature, the spectrum of uracil in ethanol is just as in neutral aqueous solution, with an extinction maximum at 259.5 mμ. In ethanol acidified with .6M HCl the peak maximum is shifted to 260 mμ. This is the same as occurs in water, where the pK for protonation is .5. Just where the

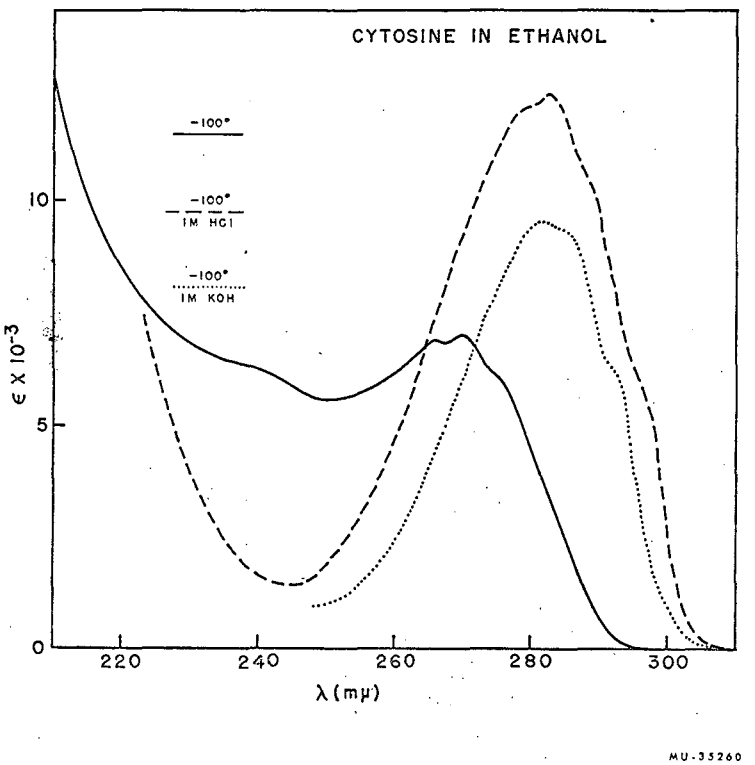



Figure 82. Cytosine in ethanol, at low temperature, acidic neutral, and basic.

Cytosine Vibrational Detail

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| | Ethanol (-100°C) | |
| 266 | 37600 | 600 |
| 270 | 37000 | 600 |
| 275 | 36400 | 800 |
| 281 | 35600 | |
| | 1M HCl in Ethanol (-100°C) | |
| 278.5 | 35900 | 550 |
| 283 | 35350 | 600 |
| 288 | 34750 | 900 |
| 295.5 | 33850 | |
| | 1M KOH in Ethanol (-100°C) | |
| 281 | 35600 | 650 |
| 286 | 34950 | 650 |
| 291.5 | 34300 | |

proton goes is not quite so obvious as in some of the other pyrimidines. At -100°C these two species cannot be spectrally distinguished from each other. Both seem to possess a peak at 261 m$\mu$ and a shoulder at 267 m$\mu$. There is a bit of additional vibrational structure on the red limb of the absorption band (Fig. 83). When the uracil is dissolved in a $10^{-3}$ M ethanolic solution of KOH, and cooled to -100°C, a good deal of vibrational structure becomes evident. The neutral and ionized forms of the uracil molecule are likely as shown below, although the $N_1$ rather than the $N_3$ could be the site of proton loss.

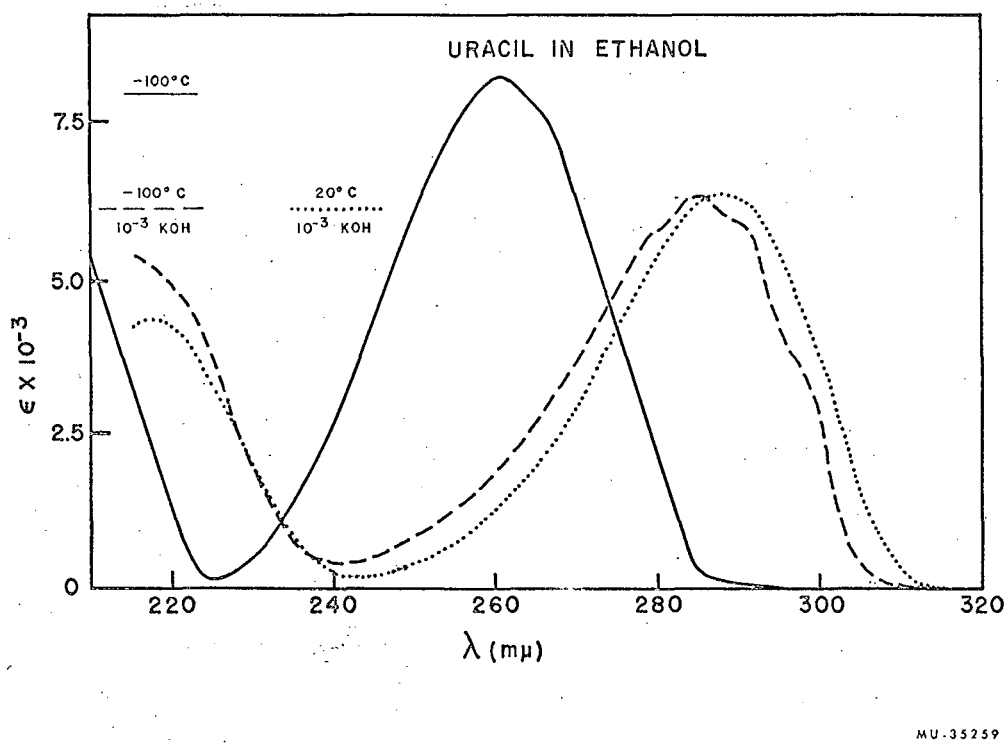
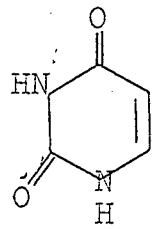
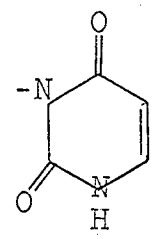



Figure 83. Uracil in ethanol at low temperatures.



uracil



ionized uracil

Vibration Structure in Uracil $10^{-3}$ M
KOH in Ethanol (-100°C)

| $\lambda$(m$\mu$) | $\nu$(cm$^{-1}$) | $\Delta\nu$(cm$^{-1}$) |
|---|---|---|
| 279.5 | 35800 | 700 |
| 285.0 | 35100 | 700 |
| 290.5 | 34400 | 700 |
| 296.5 | 33700 | |

Uracil itself does not possess an IR absorption at 700 cm$^{-1}$, although it does possess bands at 600 and 800 cm$^{-1}$; however, the ionization of the molecule could certainly lead to changes in the frequencies of some of the modes of vibration.[71]

The spectrum of thymine in ethanol is as in aqueous solution. Acidifying with 1M HCl causes no discernable change in spectrum. Cooling to -100°C causes a slight shift in peak maximum, but the prominent shoulder on the red limb, visible in uracil is not visible in thymine under these circumstances (Fig. 84). Thymine in $10^{-3}$ M KOH ethanol solution seems to possess the same sort of vibrational peaks as in uracil under

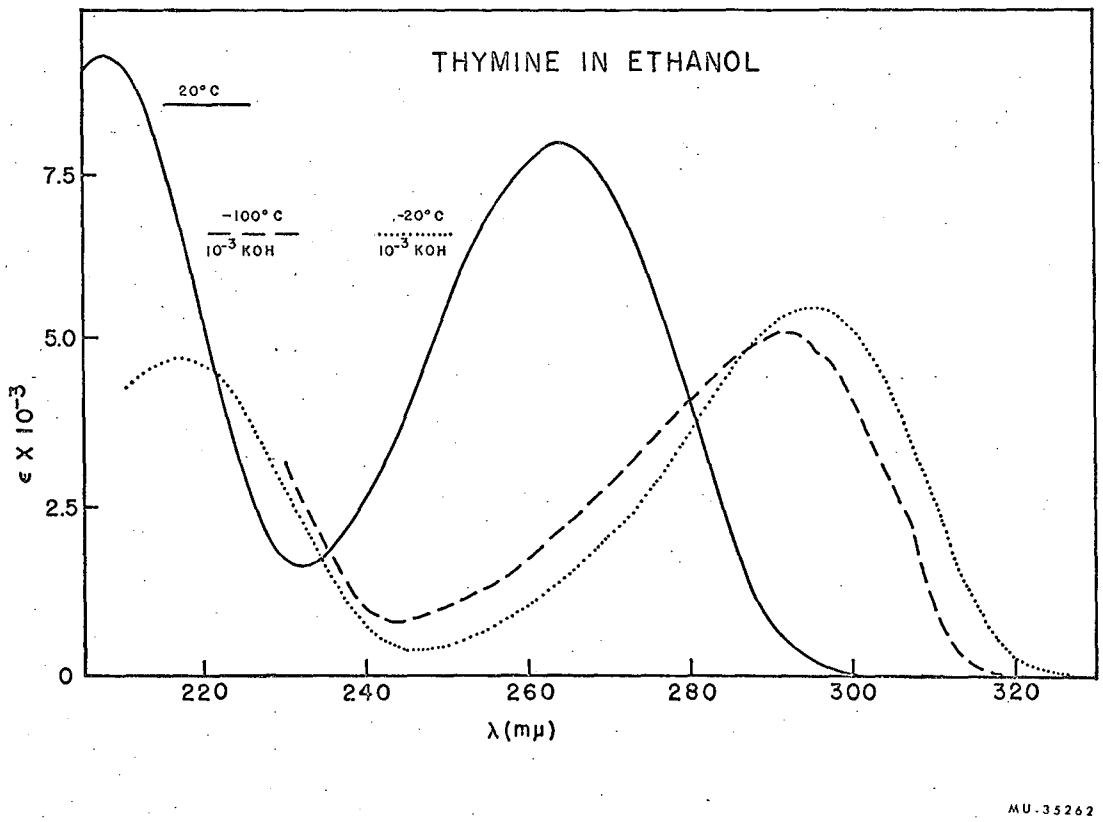



Figure 84. Thymine in ethanol at low temperature.

the same circumstances, but they are not nearly so prominent. This is probably due to the destruction of some symmetry properties which occur with the introduction of the methyl group at $C_5$.

In some cases, the extinction coefficients upon our spectra were based on gravimetric determinations of solute dissolved into the ethanol solvents. They are probably accurate to only 10%. In other cases, where the spectrum in neutral ethanol resembled that in neutral aqueous solution very closely, we arbitrarily set the extinction of the peak of the former equal to a reference value for the latter. Cold temperature spectra, or spectra in acidified or basic ethanol were then read relative to the spectrum in neutral ethanol at room temperature.

Summary

Low temperature spectra in ethanol provide only a glimpse of the vibrational modes of the purine and pyrimidine molecule. It would be interesting to look at the same compounds at even lower temperatures. The greatest difficulty to this is the cracking of an impure ethanol glass below 120°C.

# CONCLUSIONS

The function of nucleic acids in biological systems results not only from their primary structure (the sequence of monomer units which makes up the polymer), but also from the native steric conformations(s) which the polymers assume. To study the nature of these conformations, it would be very helpful to have as much knowledge as possible of the forces which can act between monomer units of nucleic acid polymers.

The simplest model system in which to study such interactions would be a dimer molecule, with both units the same. The model system we used was the adenylic acid dimer, ApAp, and in some instances, ApA, the dimer lacking a terminal phosphate group.

Nucleic acid polymers react strongly with mercuric chloride, but the individual monomer units react quite weakly. We have found that as we consecutively go from the monomer to the dimer, to the trimer, to the polymer of adenylic acid, the reaction constant of mercuric chloride-adenylic acid complex formation increases by orders of magnitude. This we have attributed to the monomer's weak reaction at the third coordination site of the mercuric chloride, as opposed to the ability of the oligomers to form chelates with the mercuric chloride, occupying two or perhaps even more coordination sites.

It would be interesting to study the binding properties of a number of metals to oligonucleotides, as the polymer chain is consecutively lengthened from monomer to long polymer. It would

be especially interesting to compare reactivities as a function of chain length for two classes of metal ions: those which are known to react preferentially with the phosphate groups of the nucleotides, and those which react preferentially with the purine and pyrimidine rings.

Native nucleic acids possess a high degree of conformational asymmetry, indeed some possess a helical structure. Because of this, they also have a very high specific optical rotation per nucleotide residue. The specific optical rotations of the individual nucleotides are much smaller, and are due only to structural asymmetry of the atoms about carbons of the ribose sugar groups. As one changes the solvent environment of a nucleotide, the change in optical rotatory dispersion is very small. On the other hand, when one changes the solvent environment of a helically ordered nucleic acid polymer, the changes in the ORD spectrum are truly huge.

The ApAp dimer has optical rotatory properties which are of a magnitude between those of the adenylic acid monomer and those of polyadenylic acid. In addition to the optical rotation of the dimer which comes from the structural asymmetry of the molecule, a large contribution to the rotation must come from conformational asymmetry. As one adds organic solvents to solutions of ApAp or of ApA, the conformation giving rise to

the augmented optical rotation is changed, and the rotation is reduced to the magnitude (and spectral characteristics) of the monomer. The hypochromism exhibited by nucleic acids is likewise exhibited by the ApAp dimer. This feature of the optical absorption spectrum is reduced or eliminated as organic solvent is added to neutral aqueous solutions of ApAp or of ApA.

We treated the breakdown of conformation in ApAp, as ethanol is added to a water solution of this molecule, according to the theory of Sinanoglu and Abdulnur. The experimental results are consistent with their theory, but are not conclusive enough to rule out other explanations for the denaturation.

According to theory, the absorption spectrum of ApAp, or ApA should be split into two bands due to coulombic interaction between the two nucleotide bases. This splitting is not observable at room temperatures. We tried two techniques to resolve this predicted splitting, both involving low temperature spectroscopy. (In the case of polyadenylic acid, theory predicts that an absorption band would be broken into n bands if there were n residues present in the polymer. These splittings would be too finely spaced to be observable under any circumstances.)

We first froze water solutions of ApAp, and looked for resolvable splittings in the absorption spectrum, or at least differences between this spectrum and that of the adenylic acid monomer measured under the same conditions. We found none. Additional experiments showed us that we had likely aggregated the ApAp molecules so greatly that intermolecular interactions between Ap residues became as great in magnitude as intramolecular

interactions, thus destroying the conditions under which the absorption spectrum would be split into two and only two bands.

The second procedure we used was to dissolve ApA in ethanol, and to lower the temperature to near -100°C. We found no splitting in the absorption spectrum. As mentioned, we found that organic solvents greatly reduce base-base interactions between nucleotide bases. Had we known this result earlier, we would not have anticipated any splitting in ethanol solutions of ApA, even at low temperatures.

To find the anticipated splittings, it will be necessary to find a solvent which neither leads to the aggregation of ApAp molecules nor weakens the interactions between the Ap residues. In such a solvent, at low enough temperatures, we might have a chance of observing spectral splittings.

Aside from the failure to find splittings, the study of properties of a dimer molecule does, indeed, provide us with a simple model system to study factors important to the properties of the longer nucleic acids.

# APPENDIX A

## Chromatographic Preparation of Adenylic Acid Oligomers

The ApA, ApApA, and $(Ap)_3A$ used in our experiments was prepared and given to us by Dr. Myron Warshaw, to whom the author is most grateful. For a number of experiments, with Dr. Warshaw's help and advice, we prepared a set of adenylic acid oligomers, possessing terminal phosphate groups. The methods involved in this column chromatographic preparation were first developed and utilized by Tomlinson and Tenor[78] and Staehelin et al.[79]

The preparation of the oligomers, starting from commercially available polyadenylic acid[10] is best described in several steps.

(1) The hydrolysis of the polyadenylic acid by KOH, and the neutralization of this hydrolysate.

(2) The fractionation of this hydrolysate on a DEAE cellulose column by a gradient elution technique, with sodium chloride in 7M urea.

(3) The removal of the urea and the NaCl from the fractions on a DEAE cellulose column by elution first with dilute, and then with concentrated $(NH_4)_2CO_3$.

(4) Lyophilization of the urea free, NaCl free oligomer, which contains only carrier $(NH_4)_2CO_3$, and evaporation of the ammonium carbonate at 45°C in a vacuum oven.

(1) Hydrolysis. 250 mg of the potassium salt of polyadenylic acid are dissolved in 25 mg of water, and this solution mixed with 225 ml of 1M KOH. This gives a 1 mg/ml solution of polyadenylic acid in .9M KOH. This is allowed to digest for six hours, hydrolysing the base labile phosphodiester linkages of the polymer. At the end of this time $H^+$ charged Dowex 50 ion exchange resin was added in an amount necessary to neutralize the .225 equivalents of KOH present. The reaction with the ion exchange resin is simply

$$K^+ + OH^- + H^+ \cdot\cdot Resin \rightleftharpoons K^+ \cdot\cdot Resin + H_2O$$

This has the dual advantage of stopping further hydrolysis as well as considerably reducing the ionic strength of the solution. This latter is necessary for a proper fractionation to occur. Neutralization of the hydrolysate with a mineral acid would have necessitated its dilution by a large factor to adequately lower the ionic strength.

It is necessary to monitor the final addition of the resin with a pH meter to insure that the solution does not become too acidic. The final pH is adjusted (if necessary with a small amount of KOH) to a value near 8. The hydrolysate is filtered free of the resin.

There was, in our second experiment, a tendency for a large amount of poly A to become adsorbed to Dowex AG 50. Mixing filtered resin with a small amount of pH 12 water, and rinsing it with nearly 500 cc of water at pH 10 was necessary to remove the last traces of highly UV absorbant material. A fairly

small amount of freshly acidified resin served to return the total rinse material to neutrality. The pooled hydrolysate containing solutions, were lyopilized till the volume was reduced to 400 ml.

In the first preparation we did not think to wash the resin, and ended up with a total hydrolysate solution after lyophization of 125 ml.

To each 40 ml of final hydrolysate was added 1 ml of a .1M tris phosphate buffer which had been previously adjusted to a pH of 7.8 with HCl. The hydrolysates as they were then finally prepared for application to the separation columns were approximately .0025 of tris phosphate buffer, and possessed a pH close to 7.8.

a. Preparation of Dowex AG 50 Ion Exchange Resin
   The resin is rinsed, and then soaked in 1-2 M HCl. It is washed free of excess acid with distilled $H_2O$ until the pH of the wash water no longer is acidic.

b. Preparation of Tris Phosphate Buffer .1M
   A pH 7.8 buffer can be prepared from 25 ml of .2M tris phosphate, 17.5 ml of .2M HCl, and adequate water to bring the mixture to 50 ml.

(2) Gradient elution of hydrolysates. Commercial DEAE cellulose must be sized and washed. Finally, one makes a slurry of this cleaned and graded material with a solution 7M in urea, and 1M in NaCl. This same solution is used to fill a 2x40 cm chromatography column roughly 1/3 full. Into this solution,

one pours the resin slurry (doing so slowly). The resin is allowed to lightly pack, and then further increments are added. When the column is adequately filled, and the resin sufficiently packed, the resin is washed with 7M urea, sufficient to remove the sodium chloride. (Adequate removal is indicated by a negative silver chloride test.) All urea solutions used (with or without NaCl) were .0025 M in tris phosphate, adjusted to a pH of 7.8.

The column is loaded with concentrated hydrolysate solutions (125 ml in our first preparation, 400 ml in the second). We rinsed the column until the nucleoside fragment came off, and then set up a gradient elation system. In the first preparation this system gave a gradient of 0 to .2M NaCl in a total of 4 liters. In this preparation, when the salt concentration of the eluant reached .14M NaCl, it was necessary to refill the reservoirs and reset the gradient so that it would run from .14M to .28M NaCl in an additional 4 liters of flow. In the second preparation of oligomers we used a gradient running from 0 to .3M NaCl in 8 liters, with the necessity of refilling the reservoirs just midway in the elution.

In both preparations, we were able to resolve chromatographic peaks through the nonamer. Both preparations purging the column at the culmination of a fractionation with 1M NaCl removed considerable UV absorbing material. Apparently, the hydrolytic preparation leaves some of the polymer in rather long chains.

a. Washing and Sizing of DEAE Cellulose (Warshaw Preparation). Using DEAE cellulose .55 me/gm, prepare a medium water slurry. Add sufficient NaCl to make the slurry 3-4 M in this salt, and acidify to .1M in HCl. Soak this overnight, and then rinse, in a Buechner funnel with water until the NaCl and HCl are removed. Make a thick slurry of the resin in 1-2 M $(Na)_2CO_3$. Soak for 1-2 hours, and rinse with water in a Buechner funnel. Repeat the washing procedure, but with sodium carbonate in place of sodium bicarbonate. In each soaking procedure, stir the slurry often. Wash out the sodium carbonate, and resuspend the resin to make a medium slurry .1-.5 M in NaOH. The resin should soak in this for from 1/2 to 1 hour. Wash out the sodium hydroxide, and resuspend the resin in 95% ethanol for 1/2 hour. Wash in a Buechner funnel with several volumes of ethanol, followed by a thorough rinsing with water. This resin preparation may be stored in water.

Because the original material is inhomogeneous in particle size, and because there is some degree of breakdown of the particles of cellulose in the cleaning procedures, it is necessary to separate the smaller fragments, otherwise referred to as "fines". To do this the equivalent of a bottle of DEAE cellulose is added to 8 liters of distilled water, and stirred until it is thoroughly suspended It is allowed to settle for a period of six minutes. What remains suspended are the "fines". This suspension is decanted. The "fines" may be collected and saved, for they are useful in the preparation of the 7M urea solutions described below.

b. Preparation of 7M Urea Solutions. Solutions of 7M urea, both with and without added NaCl serves as the eluent for the gradient fractionation of the oligoadenylic acids. After the urea has been dissolved with adequate stirring, it is mixed with clean "fines" of DEAE cellulose. These hopefully adsorbs contaminants in the urea which would otherwise be deposited on the resin in the column during the chromatography procedures. The "fines" are filtered from the urea, and any NaCl which is necessary is added. Finally to each two liters of urea solution 50 ml of pH 7.8 tris phosphate buffer is added.

(3) Purification of the oligomer fractions. The separated fractions had to be cleansed of both urea and of sodium chloride. A DEAE cellulose column which had first been flushed with .1M ammonium carbonate, and then with .0025 M ammonium carbonate was used. Each sample was applied to the column, and washed with enough of the dilute (.0025 M) ammonium carbonate to remove all traces of chloride. Presumably all the urea was also removed in this procedure. Each fraction was flushed from the column by using more concentrated solutions of ammonium chloride. We should have concentration somewhat greater than that of the NaCl eluent which washed the particular oligomer from the column during the initial fractionation procedure. We actually employed concentrations which were somewhat less.

The total collection volume for each purified fraction was somewhere between 250 ml and one liter.

(4) Lyophilization of the purified fractions and removal of carrier ammonium carbonate. Each of the fractions was lyopilized on a commercial freeze drying unit. The dry fractions were put in a vacuum oven at 45°C, appropriately trapped with liquid nitrogen. They were dried to constant weight, with the removal of carrier ammonium carbonate.

The final material was not pure in the sense that weight concentrations came out higher than the molar concentrations (the latter determined spectrophotometrically). Whether the contaminants were $H_2O$, some unremoved ammonium carbonate, or yet some other materials, we could not ascertain. The contaminants did not seem to adversely effect the relative spectral properties of the molecules.

a. Determination of Purity of Samples. By hydrolysing a given sample of oligonucleotide with weak base (.1M NaOH) the oligonucleotide can be quantitatively converted into a mixture of adenylic acids, of known spectral properties. In this way, from a given weight concentration of oligonucleotide, we can determine the molar concentration, the effective molecular weight, and hence the purity. According to the catalog of the Pabst company,[51] the experimentally determined average molecular weight of adenylic acid (probably determined to constant dryness) is 369, which includes the average water of hydration. This is for the acid form of adenylic acid. Since we isolated our adenylic acid in the form of the ammonium salt, we must take this change of molecular weight into account, in ascertaining the purity of our samples. Starting with 369 as the MW of

the acid form of the monomer, the diammonium salt should possess a molecular weight of 403, the dimer an MW of 787, and the $(Ap)_3$ trimer with its four ammonium groups, should have an MW of 1171.

In the following table, we give the total yield, in mg of the oligomers, dried in the vacuum oven to a constant weight, and where we possess the data, we give the percent of purity, based upon the above molecular weights.

| | Chromatography I | | Chromatography II | |
|---|---|---|---|---|
| Oligomer | mg | % purity | mg | % purity |
| Monomer | 22.5 | 62 | 49.7 | |
| Dimer | 17.5 | 85 | 51.9 | |
| Trimer | 8.0 | 85 | 15.4 | 79 |
| Tetramer | 6.7 | | 4.7 | |
| Pentamer | 18.4 | | 2 | |
| Hexamer | 12.8 | | | |
| Hexamer | 4.7 | | | |
| Octamer | 4.9 | | | |
| Nonamer | 9.5 | | | |

In each of the two chromatographic preparations, we started with 250 mg of poly A as the potassium salt. Our yields were none too good. The problem seems to lie in two places. In the hydrolysis, apparently a fairly sizable portion of the oligomers remains adherent to the Dowex AG 50. Especially obvious in the second preparation is the fact that the hydrolysis went a bit too far, giving way more of the small oligonucleotides than were desirable.

A second source of loss, again in the second preparation may have been in the column purification of the higher oligomers. There is a good possibility that beginning with the pentamer, we did not elute with a sufficiently concentrated solution of ammonium carbonate to remove all the oligomer from the column.

# APPENDIX B

## Characterization of Some Adenylic Acid Oligomers

In the pursuit of our experimentation, we collected, sometimes purposefully, sometimes incidentally, data on various of the adenylic acid oligomers. We seek in this appendix, to compile these data in as useful a form as possible. The spectral characteristics of some oligomers appear in Figs. 85, 86, 87 and 88.

### Extinction Coefficients of Oligomers

Analysis of the extinction coefficients of our oligomers followed, generally, the procedures of Michelson, though with some added precautions.[80] We performed these analysis with the ApA, ApApA, and $(Ap)_3A$ given to us by Dr. Warshaw, as well as with the dimer and trimer molecules of adenylic acid possessing terminal phosphate groups. In contrast to Michelson's chemically synthesized oligomers, ours were obtained by base hydrolysis of poly A containing exclusively 3'-5' phosphodiester linkages.[10]

The samples were dissolved in sodium hydroxide (.2N except .9N for the ApAp hydrolysis). We heated the ApAp for 48 hours at 37°C, the ApApAp for 30 hours at 39°C, and the remainder of the oligomers at 37°C for 36 hours. In each set of samples there was a preparation of adenylic acid dissolved in a like concentration of base. This allowed us to compensate for the

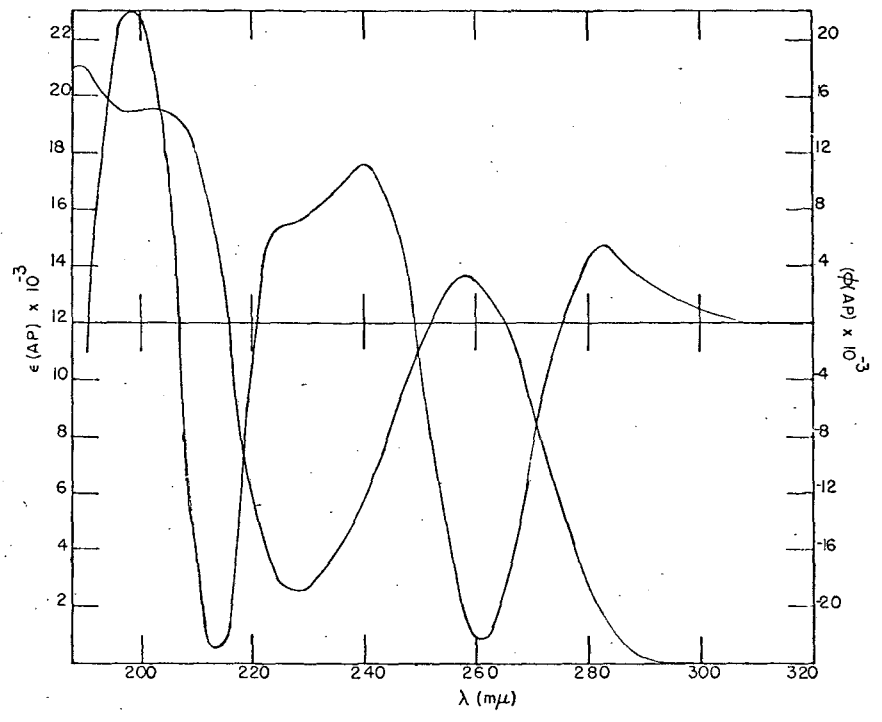



Figure 85. Absorption and rotation of light by ApAp at pH 5.7.

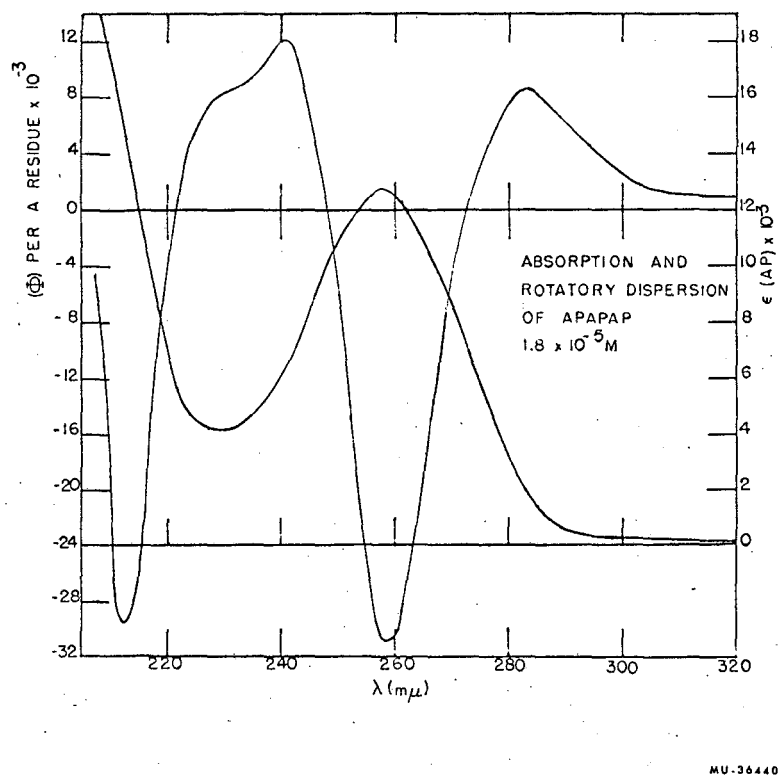



Figure 86. Absorption and rotation of light by ApApAp

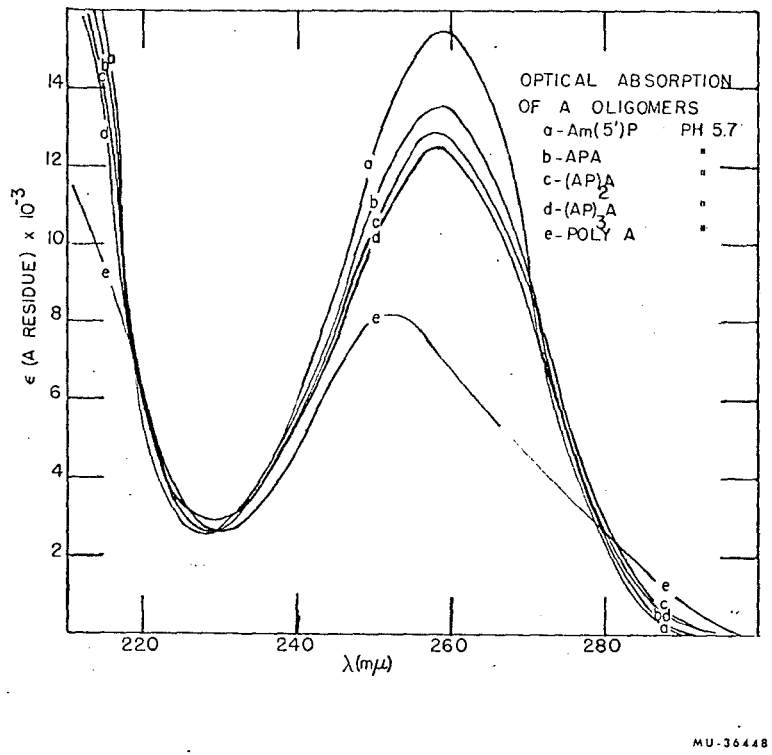



Figure 87. Optical absorption spectra of some adenylic acid oligomers.

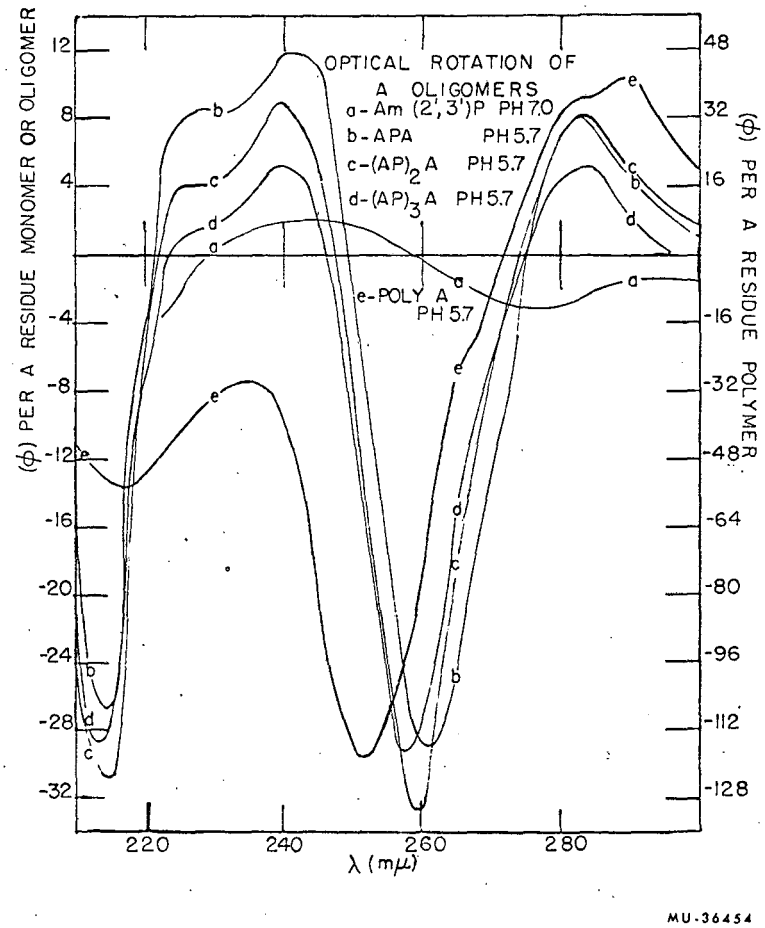



Figure 88. Optical rotatory dispersion of some adenylic acid oligomers.

small amount of sample degradation of the oligomer. (Assuming that the same percent of degradation will occur in the monomer as in the oligomer samples.)

Our best results, with the least degradation of sample, occurred at an NaOH concentration .2N, and at 39°C for 30 hours. In this case, in fact, none of the monomer sample, used as a control, was degraded.

The differences in absorption maxima of ApAp at pH 7 and 13.9 are trivial. On this basis we assumed a like behavior between peak maxima at pH 7 and pH 13 with the other oligomers analysed.

Likewise, the extinction maximum of the monomer varies only slightly with the change in pH from neutral to basic solutions. From these analyses, we found the extinction coefficient maxima for the oligomers. These are listed in the table below. Values obtained for adenylic acid oligomers by Michelson also appear in the table, and are denoted by an asterisk, (*).[37]

[illegible] (hetero[illegible]), and of the double stranded hydrogen [illegible] poly [illegible] acid. The value of the extinction coefficient of the double stranded poly A is taken from Jordon.[21]

From the program prepared by Tinoco,[55] and modified by Warshaw[56] we evaluated some parameters for the 260 mμ electronic absorption bands of these oligomer molecules. All the values are on a per residue of adenosine basis.

Extinction Coefficient Maxima of Some Adenylic Acid Oligomers (per monomer unit)

| Oligomer | Extinction Coefficient Maximum | Extinction Maximum (monomer) / Extinction Maximum (oligomer) (in basic solutions) |
|---|---|---|
| Adenylic Acid | 15400 | - |
| ApA 3'-5' phosphate | 13700 | 1.12 |
| *ApA 3'-5' phosphate | 13600 | 1.13 |
| *ApA 2'-5' phosphate | 13000 | 1.18 |
| *ApA mixed 2'-5', 3'-5' phosphate | 13400 | 1.15 |
| ApAp 3'-5' phosphate | 13600 | 1.13 |
| $(Ap)_2A$ 3'-5' phosphate | 12900 | 1.19 |
| $(Ap)_3$ 3'-5' phosphate | 12800 | 1.20 |
| *$(Ap)_2A$ mixed 2'-5', 3'-5' phosphate | 12500 | 1.23 |
| $(Ap)_3A$ 3'-5' phosphate | 12500 | 1.23 |
| *$(Ap)_3A$ mixed 2'-5', 3'-5' phosphates | 11800 | 1.31 |
| *$(Ap)_4A$ mixed 2'-5', 3'-5' phosphate | 11600 | 1.33 |

The values of these various properties depend upon the integrated area of under the absorption band, and it was necessary to extrapolate the blue limb of this absorption band to zero. We did this in an admittedly arbitrary manner. Since the total area under an extrapolated portion of the absorption band is small compared to the area under the entire

absorption band, the error involved in the extrapolation is small. These parameters are listed in Table IX.

Table IX. Some Integrated Optical Properties of Adenylic Acid Oligomers

| | Adenosine pH 7 | Adenylic Acid (5') pH 5.7 | ApA pH 5.7 | ApAp pH 7 | ApApA pH 5.7 | ApApAp pH 5.7 | ApApApA pH 5.7 | Poly A pH 5.7 |
|---|---|---|---|---|---|---|---|---|
| Oscillator Strength | .322 | .319 | .290 | .302 | .283 | .290 | .276 | .199 |
| Transition Moment (debyes) | 4.18 | 4.16 | 3.97 | 4.04 | 3.92 | 3.96 | 3.86 | 3.28 |
| Dipole Strength $(debyes)^2$ | 17.44 | 17.30 | 15.74 | 16.33 | 15.36 | 15.72 | 14.92 | 10.72 |
| Average Frequency $(cm)^{1-1}$ | 39202 | 39110 | 39138 | 39220 | 39098 | 39113 | 39174 | 39315 |
| Average Wavelength (mμ) | 255 | 256 | 255 | 255 | 256 | 256 | 255 | 254 |
| Second Moment $x10^{-10}$ $(cm)^{-2}$ | .154 | .153 | .154 | .154 | .153 | .153 | .154 | .155 |

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## Figure Captions

Fig. 1. Optical absorption of adenylic acid, with mercuric as a complexing agent, and alone.

Fig. 2. Optical absorption of ApA, with and without mercuric chloride as a complexing agent.

Fig. 3. Optical rotatory dispersion of ApA, with and without mercuric chloride as a complexing agent.

Fig. 4. Optical absorption of ApApA, with and without mercuric chloride as a complexing agent.

Fig. 5. Optical rotatory dispersion of ApApA, with and without mercuric chloride as a complexing agent.

Fig. 6. Optical absorption of $(Ap)_3A$, with and without mercuric chloride as a complexing agent.

Fig. 7. Optical rotatory dispersion of $(Ap)_3A$, with the without mercuric chloride as a complexing agent.

Fig. 8. Optical absorption of polyadenylic acid, with and without mercuric chloride as a complexing agent.

Fig. 9. Optical rotatory dispersion of polyadenylic acid, with and without mercuric chloride as complexing agent.

Fig. 10. Optical absorption of mercuric chloride.

Fig. 11. Optical absorption of adenylic acid with increasing concentrations of mercuric chloride.

Fig. 12. Optical rotatory dispersion of adenylic acid with increasing concentrations of mercuric chloride.

Fig. 13. Optical absorption of adenylic acid dimer with increasing concentrations of mercuric chloride.

Fig. 14. Optical rotatory dispersion of adenylic acid dimer with increasing concentrations of mercuric chloride.

Fig. 15. Optical absorption of $(Ap)_3$ with increasing concentrations of mercuric chloride, very low concentrations of mercuric.

Fig. 16. Optical absorption of $(Ap)_3$ with increasing concentrations of mercuric chloride, with intermediate amounts of mercuric.

Fig. 17. Optical absorption of $(Ap)_3$ with increasing concentrations of mercuric chloride, at high concentrations of mercuric.

Fig. 18. Optical rotatory dispersion of $(Ap)_3$ with increasing concentrations of mercuric chloride.

Fig. 19. The interaction of mercuric with aniline.

Fig. 20. The interaction of mercuric with pyridine.

Fig. 21. Interaction of mercuric perchlorate with adenylic acid: change in absorption spectrum.

Fig. 22. Interaction of mercuric perchlorate with adenylic acid: change in rotatory dispersion.

Fig. 22a. Reciprocal plot of ApAp-$HgCl_2$ complex formation.

Fig. 23. Interaction of mercuric perchlorate with ApA: change in absorption spectrum.

Fig. 24. Interaction of mercuric perchlorate with ApA: change in rotatory dispersion.

Fig. 25. Absorption spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of reactants changes.

Fig. 26. Absorption spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of reactants changes. (Expanded absorption at red end of absorption band.)

Fig. 27. Rotatory dispersion spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of reactants changes.

Fig. 28. Rotatory dispersion spectra of ApAp + $HgCl_2$; spectra at same total concentration for both reactants as ratio of reactants changes. (Expanded scale at red end of rotatory dispersion band.)

Fig. 28a. ApAp-$HgCl_2$ complex formation as a function of constituent ratio.

Fig. 29. Interaction of mercuric chloride with uridylic acid. Absorption spectra.

Fig. 30. Interaction of mercuric chloride with an equal parts mixture of uridylic acid and adenosine. Absorption spectra.

Fig. 31. Interaction of mercuric chloride with an equal parts mixture of uridylic acid and adenosine. Rotatory dispersion spectra.

Fig. 32. Interaction of mercuric chloride with ApU dimer. Absorption spectra.

Fig. 33. Interaction of mercuric chloride with ApU dimer. Rotatory dispersion spectra.

Fig. 34. Interaction of mercuric chloride with equal parts of cytitylic acid and adenosine.

Fig. 35. Interaction of mercuric chloride with ApC dimer. Absorption spectra.

Fig. 36. Interaction of mercuric chloride with ApC dimer. Rotatory dispersion spectra.

Fig. 36a. Reciprocal plot of ApAp-$HgCl_2$ complex formation.

Fig. 37. Omitted.

Fig. 38. Absorption spectra of AMP and ApA in ethanol and water.

Fig. 39. Rotatory dispersion spectra of ApA in methanol and in water.

Fig. 40. Denaturation of ApAp by ethanol. Optical absorption spectra.

Fig. 41. Denaturation of ApAp by ethanol. Optical rotatory dispersion spectra.

Fig. 42. Unstacking of ApAp by ethanol. Equilibrium constants.

Fig. 43. Unstacking of ApAp by ethanol. Energetics.

Fig. 44. ApA in water and in trimethyl phosphate. Optical absorption spectra.

Fig. 45. ApA in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

Fig. 46. Denaturation of ApAp by trimethyl phosphate. Optical absorption spectra.

Fig. 47. Denaturation of ApAp by trimethyl phosphate. Optical rotatory dispersion spectra.

Fig. 48. Uridine in water and in trimethyl phosphate. Optical absorption spectra.

Fig. 49. Uridine in water and trimethyl phosphate. Optical rotatory dispersion spectra.

Fig. 50. Guanosine in water and in trimethyl phosphate. Optical absorption spectra.

Fig. 51. Guanosine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

Fig. 52. Cytidine in water and in trimethyl phosphate. Optical absorption spectra.

Fig. 53. Cytidine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

Fig. 54. Adenosine in water and in trimethyl phosphate. Optical absorption spectra.

Fig. 55. Adenosine in water and in trimethyl phosphate. Optical rotatory dispersion spectra.

Fig. 56. Spectra of adenylic acid and its dimer, ApA.

Fig. 57. (No title particularly).

Fig. 58. Resolution of band splitting in optical absorption spectra.

Fig. 59. Ice film sample holder.

Fig. 60a. Several spectra of adenylic acid, taken in an ice sample.

Fig. 60b. Several spectra of ApAp taken in an ice sample.

Fig. 61. Optical absorption of guanosine, recorded from ice sample.

Fig. 62. Optical absorption of cytidine, recorded from ice sample.

Fig. 63. Optical absorption of uridine, recorded from ice sample.

Fig. 64. Optical absorption of adenosine, recorded from ice sample.

Fig. 65. Optical absorption of thymine in ice.

Fig. 66. Optical absorption of thymine in ice, containing 10% ethanol.

Fig. 67. Optical absorption of uracil in ice.

Fig. 68. Optical absorption of uridylic acid in ice.

Fig. 69. Optical absorption of thymidylic acid in ice.

Fig. 70. Optical absorption of adenine in ice.

Fig. 71. AMP, ApA in cold, neutral ethanol.

Fig. 72. Purine at low temperature in ethanol, neutral and basic.

Fig. 73. Purine at low temperatures in ethanol, acidic.

Fig. 74. Adenine in ethanol at low temperature, neutral.

Fig. 75. Adenine in ethanol at low temperature, acidic.

Fig. 76. Adenine in ethanol at low temperature, basic.

Fig. 77. 9-Methylhypoxanthine in ethanol at low temperature (-100°C), neutral and acidic.

Fig. 78. Guanosine in ethanol at low temperature, neutral and acidic.

Fig. 79. Guanosine in ethanol at low temperature, basic.

Fig. 80. Guanine in ethanol at low temperature, acidic.

Fig. 81. Pyrimidine in ethanol at low temperature, neutral and acidic.

Fig. 82. Cytosine in ethanol, at low temperature, acidic neutral, and basic.

Fig. 83. Uracil in ethanol at low temperature.

Fig. 84. Thymine in ethanol at low temperature.

Fig. 85. Absorption and rotation of light by ApAp at pH 5.7.

Fig. 86. Absorption and rotation of light by ApApAp.

Fig. 87. Optical absorption spectra of some adenylic acid oligomers.

Fig. 88. Optical rotatory dispersion of some adenylic acid oligomers.

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