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STRUCTURAL IMPLICATIONS OF MINOR NUCLEOSIDES IN RNA:
INOSINE AND DIHYDROURIDINE

Carl Joseph Formoso
(Ph.D. Thesis)

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DEDICATION:

This thesis is dedicated to two groups of people too easily ignored and forgotten: those who refrained from helping me -- thus allowing my independence and self confidence to develop; and those who did not hinder me -- thus enabling my creativity and imagination to grow.

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Structural Implication of Minor Nucleosides in RNA:

Inosine and Dihydrouridine

Carl J. Formoso

ABSTRACT

Using dinucleoside phosphates as models for single-stranded RNA, the effects of the minor nucleosides inosine and dihydrouridine have been investigated. A nitrosyl chloride deamination of ApA has been used to obtain some of the dinucleoside phosphates containing inosine. A photo-reduction with sodium borohydride has been used to produce the dimers containing dihydrouridine. The stacking interactions of these dimers has been studied at a variety of conditions by means of circular dichroism, optical rotatory dispersion, and ultraviolet absorption spectroscopy.

The results indicate that IpA, ApI and IpI stack, while IpU stacks very little. Studies with varying concentrations of IpI in low salt and 1 M NaCl indicate that the stacking geometry of this compound is sensitive to ionic strength of solution. The CD of poly I is presented and compared to the data for IpI. Little change was found in the CD of poly (G,I) (1:1) with change of salt concentration, and we conclude that, unlike poly I, there is no major structural change. From the CD of poly (G,I), IpI, and GpG, the CD of IpG plus GpI is calculated using

the nearest neighbor approximation. From the calculated spectrum, we tentatively conclude that there is stacking in one or both of IpG and GpI.

The results show that HpA is strongly stacked, but that ApH, GpH, and probably HpH are not stacked at room temperature. The temperature dependence of the CD suggests that the stacking is unusual. The results with dimers containing open-ring forms of dihydroU indicate that there is more intramolecular interaction when the ring is open than when it is closed.

The tetramer rTp ψ pCpGp was studied and compared to the calculated CD and ORD for UUCG. It is suggested that T ψ CG possesses a structure different from that of UUCG.

An attempt is made to relate the results to tRNA structure and function.

Two appendices are included: the first lists four subroutines written for a PDP 8/S computer; the second describes a simple analysis of the kinetics of the NaBH₄ reduction and attempts to relate the kinetics to stacking in dimers.

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TABLE OF CONTENTS

	<u>Page</u>
DEDICATION	1
ABSTRACT	11
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	vi
ABBREVIATIONS	viii
I. INTRODUCTION	1
A. Biological Background	3
B. Optical Theory	6
II. EXPERIMENTAL MATERIALS AND METHODS	13
A. Chemicals	13
B. Nucleic Acid Materials	13
C. Separation Techniques	14
D. Optical Techniques	16
III. INOSINE	19
A. Preparation of ApI	19
B. The Deamination of ApA	19
C. Separation of the Deamination Reaction Mixture	29
D. Results and Discussion	36
IV. DIHYDROURIDINE	60
A. The Reduction of Uridine	60
B. Separation of Reaction Mixture	62

	<u>Page</u>
C. Results and Discussion	66
V. T ψ CG	85
A. Results and Discussion	85
VI. CONCLUSIONS	90
APPENDIX A. SUBROUTINES FOR THE PDP 8/S COMPUTER . .	97
APPENDIX B. KINETICS OF THE NaBH ₄ REDUCTION	113
REFERENCES	118

ABBREVIATIONS

A,U,C,G	the ribonucleosides adenosine, uridine, cytidine, and guanosine, respectively (abbreviations used for minor nucleosides are given in Figure 1)
β	β -ureidopropanol riboside; the product of the reduction of dihydrouridine with sodium borohydride
β'	β -ureidopropionic acid riboside; the product of the ring opening (hydrolysis) of dihydrouridine with NaOH
N or X	any ribonucleoside
Np	a 3' (or 2') ribonucleotide
pN or NMP	a 5' ribonucleotide
NpX	a 3'-5' dinucleoside phosphate
poly N	a polynucleotide of N
poly(G,I)(1:1)	a random copolymer containing equal amounts of G and I
ϵ_N^λ	the extinction coefficient (liters/mole cm) of N at wavelength λ (m μ)
OD_N^λ	the optical density of N at wavelength λ
OD $^\lambda$ unit	the amount of material that will give an OD of 1.0 at the specified wavelength in 1.0 ml of solution with a 1.0 cm light path
CD	circular dichroism
ORD	optical rotatory dispersion
UV	ultraviolet

tris a buffer; 2-amino-2-(hydroxymethyl)-1,3-
propanediol, adjusted to the given pH
with HCl

pDAB p-dimethylaminobenzaldehyde

I. INTRODUCTION

Since the announcement in 1953 of the structure of DNA by Watson and Crick¹ an immense amount of research has been done and much knowledge has been gained concerning the functions and inter-relationships of DNA, RNA, and proteins. But, while the basic scheme of genetic action is well laid out and solidly founded in experimental results, many of the precise details of this action are still unknown. For example, the exact way in which DNA is duplicated in vivo is uncertain. Though this is a dramatic gap in our knowledge there are, of course, many others less dramatic. The work presented here is an attempt to fill some of these gaps concerning RNA.

For the most part RNA is a polymer made up of four subunits: adenosine, uridine, guanosine, and cytidine. But there occur in some RNA's other nucleosides which are modified forms of the above, or, as they are sometimes called, minor nucleosides. These occur to the extent of approximately 10% in transfer RNA,^{2,3} 3% in ribosomal RNA,^{4,5} have been reported to compose 27% of chromosomal RNA,⁶ and possibly also appear in other RNA's. The minor components in ribosomal RNA are pseudouridine, base-methylated nucleosides, and ribose-methylated nucleosides.⁷⁶ Those reported in chromosomal RNA have been restricted to reduced pyrimidines.^{6,82} Transfer RNA contains the most diverse selection of minor nucleotides. Figure 1 presents

Figure 1. Some of the minor nucleosides found in tRNA
 (R $\equiv$ ribose).

a fairly complete list of the minor nucleosides in transfer RNA.

The primary interest of this study is to learn more about how the minor bases might influence the structure of transfer RNA. As models for single stranded RNA the simplest units, dinucleoside phosphates, have been used. If an understanding of how minor bases affect the structure of these simple compounds can be obtained, then a basis exists for predicting how a minor base might influence the secondary structure of the region of RNA in which it occurs.

The techniques used for the study of the structures of the dinucleoside phosphates were optical rotatory dispersion and/or circular dichroism spectroscopy, and ultraviolet absorption spectroscopy.

I-A. Biological Background

In this section an outline of the basic scheme of genetic action will be given, and some specific applications to minor bases pointed out.

The making of protein according to the master plan laid down by DNA can be divided into three steps: (1) DNA replication, (2) transcription to RNA, (3) translation of the RNA message into protein. The first step maintains the genetic message by creating more DNA. This is accomplished by virtue of the specificity of base pairing. Adenine pairs only with thymine, and guanine pairs only with cytosine. In this way an accurate copy of the genetic message is made.

In transcription messenger RNA is formed on the DNA template. Again accuracy is guaranteed by the specificity of base pairing. The final step, translation, requires two more units, ribosomes and amino-acylated transfer RNA's, to be available. Ribosomes are essentially RNA packaged up in protein. Their structure is complex and much remains to be learned about it. The ribosomal RNA is, of course, formed on a DNA template. The ribosome is made up of two subunits. Messenger RNA is bound to the smaller subunit and tRNA interacts with this mRNA by the base pairing of three nucleotides and also interacts with the larger subunit of the ribosome in a manner which is not yet entirely clear. There are two sites on the ribosome; an entry site and a polymer site. Each tRNA carries a specific amino acid and as such is bound to the entry site. After that amino acid is joined to the polypeptide already formed, the tRNA, now carrying the polymer, is moved to the polymer site and a new tRNA with its amino acid is bound at the entry site. Three nucleotides in the mRNA thus indicate that a specific amino acid is to be added to the polypeptide. The list of the 64 possible trinucleotides and the corresponding amino acids (and punctuation) constitutes the genetic code.

Transfer RNA is also formed on a DNA template. The minor bases are generally thought to be formed after this stage by the action of enzymes which alter the appropriate bases. There is conclusive evidence of this for sulfur containing bases,⁷ methylated bases,⁸ and isopentenylated bases.²⁰ All

known tRNA's do contain minor bases. Thus it is justified to conclude that minor bases are necessary to the function of tRNA. This leads to the expectation that each minor base should have some unique biological property (or properties) differing from the corresponding property of any normal base. This will perhaps be clearer if we consider a minor nucleoside B. If A, G, U, or C could replace B and not disrupt the functions of tRNA, there would be an evolutionary advantage for this happening. For the appearance of a normal base in tRNA only one DNA base pair need be expressed, while for the appearance of B many more than one must be expressed for the synthesis of the necessary enzymes which convert the appropriate normal base to B. If there were nothing special about B, organisms which could use A, G, U, or C in B's place would be selectively favored.

While this argument may or may not be valid, there is some support gained by considering the methylated bases, particularly N,N-dimethyl G, N,N-dimethyl A, 1-methyl G, etc. These have the obvious property of being unable to base pair in the Watson-Crick configuration. This, of course, is untrue of the normal bases, and could have a decided effect on the structure of a tRNA molecule. The proposed structures for tRNA³ all show these methylated bases in single strand regions.

That minor bases do affect the function of tRNA has been shown in a number of ways. The effects of methylation have been studied by use of methyl-deficient tRNA from a mutant

strain of E. Coli.^{77,8} This methyl-deficient tRNA has been compared with normal tRNA in both the acylation and transfer functions. While methylation does appear to have an effect, different laboratories have obtained contradictory results. An excellent discussion of the experimental results is given in a recent review article.⁷⁹ Some of the reported results concerning dihydrouridine and pseudouridine will be discussed in Section VI.

The specificity of each tRNA interacting with the correct amino-acyl synthetase and with the ribosome-mRNA complex must be related to its structure. And that structure must certainly be influenced by the minor bases. The study of structure, then, is most likely a good place to begin looking for unique properties in the minor bases.

I-B. Optical Theory

The theories of optical activity and hypochromism will briefly be given and applied to dinucleoside phosphates. The presentation will be by no means complete and most of the mathematical manipulations will be omitted. There are sufficient works⁹⁻¹⁸ where the theory can be found so that it need not be presented in detail here.

The rotational strength, R_a , is a measure of the intensity of optical activity for a transition a and in general is

$$R_a = \text{Im } \vec{\mu}_{0a} \cdot \vec{m}_{a0} \quad (1)$$

where Im means the imaginary part, μ_{0a} is the transition

electric dipole moment of the transition $0 \rightarrow a$, and m_{a0} is the transition magnetic dipole moment of the transition $a \rightarrow 0$. In order to keep the presentation simple only interactions between electric dipoles will be considered, and for a model, a dimer whose monomers have only one transition is used. With these assumptions

$$R_a = \left(-\frac{\pi \nu_a}{2c} \right) \vec{r} \cdot \vec{\mu}_b \times \vec{\mu}_a \quad (2)$$

where ν_a is the frequency of dimer transition, a , $\vec{r}$ is the distance from monomer 1 to monomer 2, and μ_a and μ_b are the electric dipole moments of transition a and b . This can be written in terms of monomer properties ν_1 , ν_2 , μ_1 , and μ_2 . Thus

$$R_a = \left(-\frac{2\pi}{hc} \right) \left(\frac{\nu_1 \nu_2}{\nu_2^2 - \nu_1^2} V_{12} \right) \vec{r} \cdot \vec{\mu}_2 \times \vec{\mu}_1 \quad (3)$$

where V_{12} is the electrostatic interaction potential and can be written explicitly as

$$V_{12} = \frac{1}{r^3} \left(\vec{\mu}_1 \cdot \vec{\mu}_2 - 3 \frac{(\vec{\mu}_1 \cdot \vec{r})(\vec{\mu}_2 \cdot \vec{r})}{r^2} \right) \quad (4)$$

This expression assumes that the separation of the dipoles is large which may not be valid for dinucleoside phosphates. The expression is sufficiently accurate to fit our purposes here, however, and when V_{12} is replaced

$$R_a = \left(-\frac{2\pi}{hc} \right) \left(\frac{\nu_1 \nu_2}{\nu_2^2 - \nu_1^2} \right) \left(\frac{1}{r^3} \right) \left[\vec{\mu}_1 \cdot \vec{\mu}_2 - 3 \frac{(\vec{\mu}_1 \cdot \vec{r})(\vec{\mu}_2 \cdot \vec{r})}{r^2} \right] \vec{r} \cdot \vec{\mu}_2 \times \vec{\mu}_1 \quad (5)$$

If we now apply unit vector notation using $\hat{k}_1$ as the unit vector along $\hat{u}_1$, $\hat{k}_2$ as the unit vector along $\hat{u}_2$, and $\hat{j}$ as the unit vector along $\hat{r}$ we have

$$R_a = \left(-\frac{2\pi}{hc}\right) \left(\frac{v_1 v_2}{v_2^2 - v_1^2}\right) \mu_1^2 \mu_2^2 \frac{[\hat{k}_1 \cdot \hat{k}_2 - 3(\hat{k}_1 \cdot \hat{j})(\hat{k}_2 \cdot \hat{j})] \hat{j} \cdot \hat{k}_2 \times \hat{k}_1}{r^2} \quad (6)$$

Thus R_a has been factored into a term which depends only on the magnitudes of various physical parameters and a term which depends only on the geometrical arrangement of the dimer. If the geometry is restricted such that $\hat{k}_1$ and $\hat{k}_2$ are perpendicular to $\hat{j}$ some predictions can easily be made which will be useful in interpreting the CD of dinucleoside phosphates. With this restriction the geometrical term will become

$$\frac{(\hat{k}_1 \cdot \hat{k}_2)(\hat{j} \cdot \hat{k}_2 \times \hat{k}_1)}{r^2} = \frac{\cos \theta \sin \theta}{r^2}$$

with θ the angle between $\hat{k}_1$ and $\hat{k}_2$. Three conditions can immediately be seen which would lead to zero rotational strength: $\theta = 0^\circ$, $\theta = 90^\circ$, and a large value of r . Also, if the dimer can take on many conformations some giving positive rotational strengths and others giving negative rotational strengths, the net observable result may well be close to zero. If the indices in Equation (6) are exchanged and R_b constructed, it can be seen that $R_a = -R_b$. Thus if v_a is very close to v_b (degenerate transitions are discussed below) there may be a large amount of cancellation. But if there is a preferred orientation, with the bases

close together, v_a not close to v_b and $\theta \neq 0^\circ, 90^\circ$, then there will be an observable rotational strength. We could remove our geometrical restriction and add more transitions to the model to get a more realistic (and more complicated) picture, but we would arrive at the same type of conclusions as above. When there is an interaction between the bases which limits the range of structures available to the dimer there will usually be optical activity. Certain conformations, however, will give little optical activity either because of cancellation (close juxtaposition of positive and negative rotational strengths) or low rotational strengths. In general, therefore, we can say that if there is optical activity it indicates a secondary structure, whereas the lack of optical activity does not necessarily mean the lack of secondary structure.

One further complication arises when we go to the real dimers. The monomers are themselves optically active. We must therefore compare the optical activity of the dimers to that of the monomers. This has been done for all of the normal-base dimers.^{19,72} However, the CD of the monomer is determined by the conformation of the base relative to the sugar. If this conformation changes radically on formation of the dimer a misinterpretation of CD and ORD results might occur. The best evidence to date indicates that such changes do not occur.⁷⁸

The treatment of homodimers is slightly different than that given above. Obviously Equation (6) cannot be used since

$\nu_1 = \nu_2$ leading to an infinite R_a . In general, however, $\nu_a \neq \nu_b \neq \nu_1$ because of resonance splitting causing one transition to occur at a higher frequency, ν_+ , and one at a lower frequency, ν_- , given by $\nu_{\pm} = \nu_1 \pm V_{12}$. Then using our simple model,

$$R_{\pm} = \pm \left(\frac{\pi \nu_1}{2c} \right) (\vec{r} \cdot \vec{\mu}_1 \times \vec{\mu}_2) = \pm \left(\frac{\pi \nu_1}{2c} \right) (\mu_1^2) r \sin \theta$$

This appears to have a different geometrical dependence than Equation (6) but in reality it does not. Though $R_{\pm}$ would not be zero at large values of r and $\theta = 90^\circ$ there would be no optical activity observable because V_{12} becomes zero and $\nu_+ = \nu_- = \nu_1$.

The rotational strength can also be obtained experimentally from a CD spectrum by an integration if the spectrum can be resolved into individual bands. Thus in principle an accurate knowledge of the geometry of stacking interactions could be obtained from the study of optical activity. In practice this is never possible. An insurmountable problem is the resolution of the CD spectrum into individual bands. And there are further problems, such as uncertainty in monomer properties. Even if these problems could be perfectly solved there would be no unique solution to the geometrical problem unless some restrictions could be made or assumed. This is why optical activity calculations are usually done in the opposite direction. A geometry is assumed and a CD spectrum is calculated and compared to the experimental spectrum.

This all means that the interpretation of the CD of dinucleoside phosphates cannot be expected to give definite results. While it can usually be determined if there is an interaction between the bases, not much can be said about the geometry of that interaction without a great deal more effort.

Hypochromism¹⁶⁻¹⁸ is another property which can be related to structure. It is a measure of changes in intensity of ultraviolet absorption. The property is called hypochromism when the intensity decreases, and hyperchromism when there is an increase. For nucleic acids the low energy transitions (260 mμ) are usually hypochromic.

Though the total intensity of absorption cannot change there can be a shifting of intensity between bands. For bands that closely overlap these shifts are not seen. A simple model to consider, therefore, is a dimer formed of monomer 1 with a transition in the 260 mμ region and monomer 2 with a transition in the 200 mμ region. The intensity is measured by the oscillator strength, f_D in the dimer and f_M in the monomer. Hypochromism is given by $H = 1 - f_D/f_M$. And

$$\begin{aligned} H_1 &= \frac{4}{h\nu_1\mu_1^2} \left(\frac{\nu_1\nu_2}{\nu_2^2-\nu_1^2} \nu_{12} \vec{\mu}_1 \cdot \vec{\mu}_2 \right) \\ &= \frac{4}{h\nu_1\mu_1^2} \left(\frac{\nu_1\nu_2}{\nu_2^2-\nu_1^2} \right) \frac{1}{r^3} \left(\vec{\mu}_1 \cdot \vec{\mu}_2 - 3 \frac{(\vec{\mu}_1 \cdot \vec{r})(\vec{\mu}_2 \cdot \vec{r})}{r^2} \right) \vec{\mu}_1 \cdot \vec{\mu}_2 \\ &= \frac{4\mu_2^2}{h} \left(\frac{\nu_2}{\nu_2^2-\nu_1^2} \right) \frac{(\vec{k}_1 \cdot \vec{k}_2 - 3(\vec{k}_1 \cdot \vec{j})(\vec{k}_2 \cdot \vec{j})) \vec{k}_1 \cdot \vec{k}_2}{r^3} \end{aligned}$$

where H_1 is the hypochromism of transition 1.

Again a geometrical term is factored. If $\vec{k}_1$ and $\vec{k}_2$ are constrained perpendicular to $\vec{j}$ it becomes

$$\frac{\cos^2 \theta}{r^3}.$$

Thus the geometrical term is always positive or zero (at $\theta = 90^\circ$ or large r). With this constraint on the model H_1 is likewise always positive or zero, and H_2 , the hypochromism of transition 2, is seen to be always negative or zero (exchange indices). The low energy transition can only be hypochromic and the high energy transition can only be hyperchromic.

Other geometries need not be considered here. The above is sufficient to show that hypochromism can be used as an index of stacking. Primarily it is a measure of the proximity of the bases (the effect of the r^{-3} dependence). The angular dependence can only be important when the bases are close to each other. The largest value observed for hypochromism in a dimer is approximately 10%.¹⁹

II. EXPERIMENTAL MATERIALS AND METHODS

II-A. Chemicals

Dimethylformamide was reagent grade from Matheson, Colman, and Bell. Nitrosyl Chloride was obtained from Matheson Gas Co. Trizma Base from Sigma was used. p-Dimethylaminobenzaldehyde (pDAB) was from Eastman Kodak. Sodium borohydride was from Ventron Corp. All other chemicals used were reagent grade from either Mallinckrodt or Baker and Adamson.

II-B. Nucleic Acid Materials

It seems simplest to present this section in tabulated form. Many of the commercially obtained dimers were used as starting materials to obtain the dimers prepared in this lab. These preparations are described in detail in Sections III and IV.

Commercial sources:

Calbiochem	ApA, rT, Hp, 6-chloropurine
Cyclo	dihydrouracil, H, Ap
Gallard Schlesinger	GpU
P-L Biochemicals	pA, A, pU, pC, pG, pI, I
Miles Labs	IpU, IpI, poly. I, poly (G, I), 1:1
Zellstoffabrik Waldhof	ApA, ApU, UpA, UpU

Gifts:

James Ofengand	rTp ψ pCpGp, ψ
Olke C. Uhlenbeck	UpApI

II-C. Separation Techniques

Throughout this work three separation or purification methods were used extensively: paper chromatography, paper electrophoresis, and column chromatography. The general procedure for each will be described here.

Water-washed Whatman 3 MM paper was used for paper chromatography. It was cut to the desired size and the starting material applied in as small a spot as possible. Then it was allowed to equilibrate with the solvent vapors in the closed chromatography tank for about 18 hours. All paper chromatograms were developed in descending fashion at 72°F. The duration of development varied with the particular use.

The same type paper was used for electrophoresis. The Savant Instruments Co. electrophoresis tank was used. Buffers were either 0.025 M ammonium formate, pH 3.6; or 0.05 M formic acid, pH 2.7. The paper was attached to the electrophoresis rack, saturated with buffer, and allowed to equilibrate in the electrophoresis tank for at least 1 hour. After this time the paper was removed from the tank, the sample applied as quickly as possible, and the paper returned to the tank. The electrophoresis proceeded at 2900 volts for 2 1/2 hours at 72°F.

After separation on paper the spots were detected under UV light, and the areas which contained the desired spots were cut from the paper and eluted with water. This generally gave a final volume of 0.1 ml or less. Usually there was background

contamination from the paper. This was not troublesome if the eluate was highly concentrated, i.e., from a very dark spot. The dilution necessary reduced the contamination to a negligible level. When this background contamination was troublesome, it was removed by ion-exchange chromatography.

Two types of column chromatography were used: gel-filtration and ion-exchange. Bio Gel P-2 (Calbiochem), a polyacrylamide resin, and Sephadex G-10 (Pharmacia), a cross-linked dextran, were used for gel-filtration. In this procedure the column, containing some water, was aligned vertically and a slurry of hydrated resin poured in. After allowing the resin to settle for a while, flow was started to pack the resin. The packed column was washed with 5-10 bed volumes of distilled water before use. To load the sample the water level in the column was drained to the top of the resin bed and the sample, in as small a volume as possible, applied to the top of the resin bed. Care was taken not to disturb the resin. The sample was then drained to the top of the bed, water added, and elution begun. These columns could be saved for re-use, since with the small pore sizes there is no compression of the resin. If the column is to be stored for very long it is advisable to add an agent, such as chloroform, to prevent bacterial growth.

In ion-exchange chromatography only the anion-exchange resins AG 1 $\times$ 2 (Calbiochem), a polystyrene resin lattice with basic quaternary ammonium groups, and DEAE Sephadex A-25 (Pharmacia), a cross-linked dextran with diethylaminoethyl

functional groups, have been used. The resin is prepared in the form desired and the column is packed in much the same way as the gel columns. The column is washed with at least 10 volumes of low ionic strength buffer. The sample, dissolved in the same buffer, is then applied and the negatively charged species will be bound to the resin. The column is then washed with 2-5 bed volumes of starting buffer. A linear salt gradient was always used for elution. These resins were discarded after one use.

The optical density of the column eluent was monitored in the UV with either the Beckman DB, Gilford Model 2000, or Isco Model UA-2 instruments. Fractions were collected using either the Gilson or Isco collectors.

II-D. Optical Techniques

Ultraviolet absorption spectra were taken with a Cary 15 recording spectrophotometer, and CD and ORD spectra with a Cary 60 recording spectropolarimeter with a 6001 CD attachment. Because the CD attachment was installed after the work had begun only ORD spectra are reported for some of the early work. Where there was a duplication, only the CD spectra were chosen for presentation. All absorption, ORD, and CD data presented are on the basis of one mole of base residue. The concentrations of all samples were determined from the UV absorption, using a known or assumed extinction coefficient.

Temperature control for the CD studies was obtained by use of a cell block designed by Dr. D. M. Gray. This block features Dynatech Frigistor heating-cooling modules and a

circulating water heat sink. The frigistors are controlled from a Hallikainen Thermotrol 1053A.

The data from both the Cary 15 and Cary 60 was reduced by an on-line PDP 8/S computer (Digital Equipment Corporation) using a program written by Drs. M. S. Itzkowitz and B. L. Tomlinson.^{21,22} This program smooths both high and low frequency noise, and records the data in both punched tape and printed format. The punched tape can then be used for further calculations on the spectral data. The routines to calculate oscillator strengths and rotational strengths were written by the present author and are given in Appendix A.

To determine extinction coefficients and oscillator strengths for the dimers, they were degraded to monomers and the known monomer extinction coefficients^{46,47} used. A digestion with venom phosphodiesterase (Worthington) was used for all dimers except IpI, where an alkaline hydrolysis was used. In the enzymatic method the following samples were prepared in triplicate: buffer; buffer + dimer stock; buffer + enzyme stock; dimer stock + enzyme stock. The dimer concentration in the second and fourth samples were, within experimental error, identical. The dimer stock and enzyme stock were in the same buffer so that the first sample was a blank for the second, and the third sample was a blank for the fourth. The samples were tightly sealed and the enzyme-containing samples were placed in a 37°C bath for 18 hours while the other samples were frozen. The UV spectra of all samples were then taken. With IpI, a stock solution was prepared and the UV spectrum taken. Then with triplicate samples and blanks 4 M NaOH was

added to give a final concentration of 0.5 M. After hydrolysis at 37°C for 18 hours the samples and blanks were neutralized with 4 M HClO₄ containing concentrated pH 7.3 buffer. The UV spectra of the resulting monomers thus gave, after correcting for dilution, the concentration of IpI in the stock solution. In all cases the CD of the hydrolysate was checked and was, within experimental error, that of the monomers.

The extinction coefficients and oscillator strengths of the dimers could then be calculated. From these values hypochromism was calculated as: $\% H = (1 - f_D/f_M)100$; and hypochromicity calculated as:

$$\% h = (1 - \frac{\epsilon_D^\lambda}{\epsilon_M^\lambda})100.$$

The wavelength chosen for the hypochromicity calculation is that where the maximum occurs in the UV spectrum, unless otherwise stated.

These values and others determined from the UV spectra are given in Tables 2 and 4 with mean deviation listed for % h and % H. Though the mean deviation in basic UV data (ϵ or f) was always less than 2%, and usually less than 1%, the nature of the calculation magnifies this basic error to the level given in the tables.

III. INOSINE

III-A. Preparation of ApI

The trimer UpApI, generously given by Dr. O. Uhlenbeck, had been enzymatically synthesized by a method previously described.⁴⁸ To obtain ApI the trimer was digested with RNAase A (Sigma) in the presence of phosphatase (Worthington), 0.1 M tris pH 8.2, and 0.01 M $MgCl_2$ for 45 minutes at 34°C. After digestion this mixture was diluted and loaded onto an AG 1 × 2 column, and separation accomplished by elution with a NaCl gradient at pH 3.6 (0.025 M NH_4HCO_2). The dimer peak was collected, lyophilized, and redissolved in pH 7.3 phosphate buffer for subsequent optical work.

III-B. The Deamination of ApA

The dimers IpA and IpI were obtained by deamination of ApA using nitrosyl chloride. The procedure of Sigel and Brintzinger²³ in slightly modified form was used. An ethylene glycol constant temperature bath with a circulating pump was used to maintain the reaction vessel at -8°C. The reaction is carried out in N,N-dimethylformamide (previously dried by refluxing at 153°C for 1/2 hour, and stored under dry N_2). One-tenth ml dimethylformamide is added to 3-5 mg ApA, and the slurry cooled to -8°C. Gaseous NOCl is added and will liquify at this temperature.²⁴ After adding NOCl, gas evolution can be noticed, and the slurry will begin to clear. For a partial reaction care must be taken to avoid

an excess of NOCl. This can easily be checked by noting the color of the reaction mixture because NOCl is a dark red-brown color. Anything darker than a light yellow indicates that too much NOCl has been added. A dark brown color in the reaction mixture should never be tolerated because it means that extensive degradation to monomers will occur. Excess NOCl can be removed by flushing the reaction vessel with dry N₂. After waiting 15 minutes, more NOCl is added. The second addition of NOCl can be omitted for a partial reaction. The reaction mixture is left at -8°C for approximately 1 hour for a partial reaction, or up to 3 hours for a fairly complete reaction. After this time the reaction mixture is slowly warmed to room temperature. While it is warming most of the unreacted NOCl can be removed by flushing with N₂. The mixture is then cooled to -8°C, 0.1 ml water added, and the mixture neutralized with solid KHCO₃. Sufficient KHCO₃ is added so that a few crystals remain on the bottom of the vessel. This will usually insure a pH between 7 and 8.

Deaminations of A and AMP were used for controls with the same procedure. It was found that the dimer reacted more slowly than the monomers.

The probable mechanism of the NOCl deamination is shown in Figure 2 along with other reactions in the procedure. Sigel and Brintzinger²³ report that a diazonium ion is not stable under the reaction conditions, and indeed gas evolution is seen soon after the addition of NOCl. Two expected

Figure 2. Probable reaction mechanism of the NOCl Deamination of Adenosine.

Possible Side Reactions:

Other Reactions:

side products from the reaction would be a chloropurine and a nitropurine. The reactions leading to these products are shown. In our work products were obtained which may have been one or both of these, but no attempt was made to identify any of the side products. One of the side products exhibited absorption peaks at both the 270 mμ region and the 250 mμ region. This may have been a nitropurine. Shapiro²⁵ has reported the isolation of a 2-nitropurine from the reaction of guanosine with nitrous acid. But apparently no side products are obtained in the deamination of A with HNO_2 .²⁶

The deamination using HNO_2 should be similar to NOCl deamination except that the attacking species is NO_2^- . The benefit of using NOCl is that a non-acidic medium can be used, shorter reaction times are necessary, and higher yields can be obtained.²³ Once the carbonium ion is formed the possibility of side products should be less with NOCl . Any anion should react with the carbonium ion, but only OH^- will give the desired product. Since the HNO_2 deamination is carried out in acidic medium the OH^- concentration must be small. There are other anions available for reaction in both cases. In the NOCl deamination there will be Cl^- and NO_2^- present. These ions may be particularly troublesome if the dimethylformamide is not dry (see Figure 2). Nevertheless, since the diazonium ion is not stable, the OH^- ion should be most proximate to the reaction site of the carbonium ion. This is true in both methods and is probably the reason the desired product, inosine, is produced in reasonable yield

by either method.

Since the NOCl deamination gives larger yields, there must be a greater number of carbonium ions formed during the reaction. Thus a higher yield of side products might also be expected. A higher yield of side products might also occur if the diazonium ion is more stable, hence giving more time for the OH^- ion to be displaced from its favorable position. Both of these factors are probably important in the NOCl deamination.

The amounts of IpA and IpI obtainable from the NOCl deamination of ApA vary considerably, while the yield of ApI, if any, is always small. Starting with 5 mg of ApA, less than 0.5 OD unit of ApI (see Section III-C) is recovered. By changing the reaction conditions the yield of IpI can be varied from less than 1 OD unit to milligram amounts. More NOCl and longer reaction times are necessary for larger yields of IpI, but this will also lead to more cleavage of the dimers. When large yields of IpI are obtained, no ApI or IpA will be produced. With light reaction conditions IpA is always obtained, sometimes as the sole product. We can write the following kinetic scheme in an attempt to explain this:

This could be possible if the conformation of the dimer during the reaction were such that the reaction site of the adenine on the 3' end were shielded. The rate-limiting step would

probably be the addition of NOCl to the NH_2 group. This addition should proceed better in the polar environment of the dimethylformamide solvent than in the relatively non-polar region surrounding the purine bases. One conformation in which shielding may happen is the DNA planar conformation. Figure 3 shows ApA in this conformation, where it can be seen that the NH_2 group on the adenine on the 3' end is more shielded from solvent than that on the 5' end. Of course, many other conformations could lead to shielding at this site; and we have no other information on the structure of ApA (or anything else) in a dimethyl formamide-liquid NOCl solvent. What can be said is that there is definite secondary structure to ApA in this solvent. The low yield of ApI, and also the fact that the monomer reaction proceeds more rapidly than does the dimer reaction, point out that there is an interaction between the bases in ApA.

In addition to the above products, some unexpected products were obtained. One of these was similar to ApA in absorption and ORD spectra but had a different mobility than ApA in both the paper chromatography and electrophoresis systems which are described below. This product was obtained only when a sizeable yield of IpA was produced; that is, small amounts of NOCl and short reaction times were necessary. Under harsher reaction conditions another product was found which may or may not be related to the above. This product had a UV absorption similar to IpI but a dissimilar CD spectrum. It was discovered when the reaction mixture was

Figure 3. ApA in DNA planar conformation showing that the NH₂ group of adenine on the 5' end is more exposed to solvent (adapted from Figure IV-2, reference 19).

separated by column chromatography on AG 1 x 2 resin at pH 3.6 (see Figure 4). The CD of this product, shown in Figure 10, seemed to have a large negative contribution in the 260 mμ region in addition to the normal IpI CD spectrum. After refrigeration for 2-1/2 months the CD was retaken and proved to be a normal IpI spectrum. A portion of the same reaction mixture had first been separated by paper chromatography and the material from the origin then subjected to column chromatography. The "complexed" IpI did not appear in this elution (Figure 4). There seems to be two possibilities: that a complex is formed as a result of the NOCl deamination, or that a physical aggregate of IpI is formed. During the reaction the concentration is certainly high enough (5 mg/0.1 ml) so that an aggregate might form. The dissociation of this aggregate would have to be a slow process. On the other hand, there is evidence of a "cross-linking" side reaction with the nitrous acid deamination of DNA.^{27,28,29} If this is happening in our case the site of "cross-linking" would have to be in the sugar or phosphate portion of the molecule. It seems highly unlikely that an attachment could be made to the bases themselves without affecting the UV spectrum. The attachment would also have to be unstable since the observed "complex" dissociates. Though there is insufficient evidence to eliminate either possibility, the formation of a physical aggregate seems more reasonable.

Figure 4a. The separation of the ApA deamination reaction mixture on AG 1 × 2 at pH 3.6 (partial reaction).

Figure 4b. The separation of the ApA deamination reaction mixture on AG 1 x 2 at pH 3.6 (extensive reaction):

—— total reaction mixture,
 ---- material from origin of paper chromatography.

III-C. Separation of the Deamination Reaction Mixture

After neutralization the reaction mixture is lyophilized and redissolved in a small volume of water. It is then spotted for chromatography. The solvent used is 99% n-butanol saturated with water + 1% concentrated NH_4OH ,³⁰ and the chromatogram is developed for about 5 days. This is an important step in the separation because compounds which contain inosine do not move in this system, while non-inosine compounds and salts are washed away. Here we may add that KHCO_3 appears to be the best means of neutralization; other salts do not wash away from the origin as well. Ammonium formate (pH 6.8), pH 7 phosphate buffer, and NaOH have been tried with unsatisfactory results. Some examples of the developed chromatogram are shown in Figure 5. The spot at the origin (a) contains IpA, ApI, IpI, and some monomer I and/or pI and/or Ip. The spot at 1.9 cm (b) contains the compound like ApA which is discussed above. The spot at 2.8 cm (c) contains ApA. The spots further from the origin (d, etc.) are faint and probably contain monomers and/or side products.

The first four spots were eluted and respotted on washed Whatman 3MM paper for electrophoresis. This was carried out at pH 3.6 in 0.025 M NH_4HCO_2 . A separation is affected as shown in Figure 6. This figure does not represent one particular electrophoresis, but is intended to demonstrate and summarize the results of several.

A pH of 3.6 for electrophoresis was chosen because the

Direction of development

Figure 5. Paper chromatography of the reaction mixture of deamination of ApA. Developed with n-butanol saturated with H_2O + conc. NH_4OH (99/1).

Figure 6. Electrophoresis at pH 3.6 of various components from the NOCl deamination of ApA (a,b,c,d from paper chromatography; see Figure 5).

largest charge difference between the sequence isomers IpA and ApI is expected to occur near the pK of either I (8.8) or A (3.5).³⁰ We have not tried varying the pH to see if a better separation could be achieved.

The movement of ApA towards the positive pole is in agreement with the titration data of Simpkins and Richards.³¹ They report that ApA is isoelectronic at pH 3.5. The spots which moved toward the negative pole were discarded since the desired products, IpA, ApI, and IpI, should all be negatively charged. These positively charged species are most likely nucleosides or bases. The material from spots (b) and (c) had similar UV and ORD spectra, and these spectra would ordinarily be sufficient to identify these compounds as ApA. The mobility of the material from spot (c) was identical to authentic ApA as seen in Figure 6. The material from spot (b) may have behaved differently because it was still "complexed," or because it had a slightly different salt concentration, or other unknown factors. If nothing else, at least this confirms that we must look to the material from spot (a) for our desired products. In our first attempt at identification of this material an alkaline hydrolysis was done. Only the first spot, a-1, gave conclusive results because of the small amount of material in the second and third spots. A paper chromatography in propanol/H₂O/conc.NH₄OH (55/35/10) was used for identification. The developed chromatogram is shown in Figure 7. Unfortunately, there was no Ip on hand at that time

Figure 7. Paper chromatography of alkaline hydrolysates and controls in propanol/H₂O/conc. NH₄OH (55/35/10); 21 1/2 hours of development.

(it is not commercially available), and, as can be seen from the figure, our sample of "authentic" Ap was not very good. Nevertheless, the alkaline hydrolysis of spot a-1 produced A and no I; and spot c-1, used for a control, produced A as well as Ap. From this, it can be argued that spot a-1 is most likely IpA. An alkaline hydrolysis was also done on a later preparation of this compound. After neutralization, the hydrolysate was separated on a Sephadex G-10 column. Only two peaks were eluted and they were in the order expected, that is, Ip first.^{49,50} No Ap or I was detected in the elution. It is quite certain that the material from spot a-1 is only IpA.

The identification of spot a-2 is inconclusive. Though it may be ApI, preliminary optical work did not agree with results with authentic ApI. Other possibilities are that it is a monomer, and/or contains one of the side products of the reaction. The UV spectra is argument against both of these possibilities. Because in the paper chromatography only I containing compounds are expected to remain at the origin, any monomer retained at the origin should be I. However, the UV spectrum of spot a-2 is not that of I. Nor does it appear to be the sum of the spectra of I and A. But the UV spectrum is not abnormal, having a maxima at about 255 mμ. It could not contain a nitropurine, but may contain a chloropurine. The UV spectrum of 6-chloropurine was taken, and has a maxima at 265 mμ. Thus spot a-2 may possibly contain ApI and a dimer containing 6-chloropurine.

Spot a-3 is IpI. It is identical to IpI purchased from Miles Labs. After enzymatic or alkaline hydrolysis the UV and CD spectra are identical to the monomer.

The last spot is monomer Ip and/or pI, as determined by UV and ORD spectra, and electrophoretic mobility.

Several attempts were made to obtain a separation by column chromatography. On AG 1 × 2 resin at pH 3.6 IpI is eluted first (see Figure 4). This is contrary to expectations since IpI is more negatively charged than ApA at this pH. This must mean that A is much more strongly adsorbed to the resin than is I. If this is the case there is little hope of obtaining a resolution of the sequence isomers IpA and ApI with this resin. The small difference in charge would be overshadowed by the adsorptive effects. There may be hope of a separation on DEAE Sephadex or DEAE Cellulose where adsorption is less.^{32,33,34} It was found that ApA is eluted before IpI at pH 3.6 on Sephadex A-25 but the two peaks were quite close together and this column separation has not been pursued.

The IpI obtained from the AG 1 × 2 column was quite pure, but the other products were contaminated by side products and by NO_2^- ion, which absorbs in the UV. To obtain only IpI, the column separation is the desirable method, and has been used for this purpose. To obtain IpA, however, the paper chromatography and electrophoresis procedure seems the best at present.

III-D. Results and Discussion

We have observed the CD spectrum of IpI under various conditions of temperature, ionic strength, and dimer concentration. It is well known that the structure of poly I is dependent on ionic strength; it is single stranded at low ionic strengths and becomes triple stranded as salt concentration approaches 1 M.^{35,36,37} IpI in essentially zero ionic strength solution (less than 10^{-8} M Cl^-) was obtained by gel-filtration on a Bio Gel P-2 column. A solution of IpI in 1 M NaCl was prepared by lyophilizing a portion of the zero-salt sample and redissolving it in 1 M NaCl solution. Neither of these samples was buffered, but this does not cause any difficulty because the pH of distilled water is always on the acid side of neutrality within a range where there is no protonation of I. The results were spot-checked with buffered (pH 7) samples and no differences were found. Figure 8 shows the CD spectra of the zero salt sample at various temperatures, and also the CD spectrum of the monomer pI for comparison. There was no change in the CD spectrum of the monomer with salt concentration in the range 0-1 M NaCl, with temperature between 0°C and 60°C, or with monomer concentration from 1-20 OD²⁴⁸ units/ml. Also the CD spectrum of I is identical to that of pI. The CD spectrum at various temperatures of the 1 M NaCl sample of IpI are shown in Figure 9. It can be seen that the CD of IpI is not like the CD of pI under any of the conditions studied. And, as has been reported for other dimers,^{42,43,44} the CD of IpI approaches that of

Figure 8. The CD of IpI in zero salt solution at

5°C ———,
 25°C ———; and
 40°C — · — · —, and
 60°C · · · · ·;
 and the CD of pI ———.

Figure 9. The CD of IpI in 1 M NaCl solution at

0°C ———,
 5°C ———,
 25°C ———,
 40°C ———, and
 60°C ———.

the monomer as the temperature is raised. Thus, we may conclude that the base-base interaction is considerable at room temperature (25°C) and below, while at 60°C the interaction is diminished, but not insignificant. Using the standard two-state analysis,⁴³ we determine ΔH° of unstacking to be 14 Kcal/mole, which is considerably higher than ΔH° values reported for normal dimers.⁴²

Observe also that ionic strength does affect the CD spectrum of IpI, indicating a structural change. This fact has also been reported for IpIp by Inoue and Satoh.³⁸ The CD in high salt solution is increased in magnitude, but of approximately the same shape. The increase in magnitude is about the same as would occur if the temperature of the low salt solution were lowered by about 20°C. The change in magnitude can also be appreciated by looking at the rotational strengths reported in Table 1. To learn more about this structural change we have observed the CD and/or ORD spectra of IpI over a 200 fold concentration range in both zero salt and 1 M salt solutions. For this purpose cylindrical quartz cells of path lengths 0.05 cm, and 10 cm were employed. Since the Cary 60 CD attachment will only hold up to a 3 cm path length cell, it was necessary to revert to ORD for measurements using the 10 cm pathlength cell. However, there was no change in the ORD for either the high salt or the low salt case in going from the 1 cm cell to the 10 cm cell (diluting from 8×10^{-5} M to 8×10^{-6} M in base residue). Obviously there would likewise

be no change in the CD.

Table 1
Rotational Strengths (esu-cgs)
Calculated from CD Spectra of IpI

T, °C	$R_{230}^{\dagger} \cdot 10^{38}$		$R^* \cdot 10^{38}$	
	zero salt	1 M NaCl	zero salt	1 M NaCl
0°	---	1.28	---	1.63
5°	0.60	1.30	1.00	1.48
25°	0.46	0.45	0.36	0.72
40°	-0.75	-0.68	-0.12	0.21
60°	-0.88	-1.42	-0.14	-0.17

$\dagger$ Integration from 350 m μ to 230 m μ .

* Integration over the region of the first positive band; 264 m μ to 244 m μ .

In low salt solution, using the 0.05 cm cell, there was no change in CD up to 39.0 OD²⁴⁸/ml (3.25×10^{-3} M). In high salt solution we believe that there is no change in CD using the 0.05 cm cell, but we did observe one exception. Previous to this, the IpI prepared by deamination was similar in all ways to that purchased commercially. But the 1 M NaCl sample prepared from the commercial IpI (concentration 1.77×10^{-3} M) showed an altered CD spectrum (Figure 10) while three samples prepared with our IpI, with concentrations 1.48×10^{-3} M (in 1 M NaCl), 1.84×10^{-3} M (1 M NaCl), and 2.70×10^{-3} M (1.7 M NaCl) showed no change in CD. There seemed to be little doubt about the validity of the CD spectrum which

Figure 10. The CD of possible aggregates of IpI: upper curve, IpI ($1.77 \cdot 10^{-3}$ M) in 1 M NaCl, 0.05 cm light path; lower curve, "complexed" IpI from column chromatography of reaction mixture (see Figure 4b).

did show a change. The spectrum was taken twice consecutively, and a baseline was taken both before the first spectrum and directly after the second spectrum. Thinking that the difference might be due to a trace amount of metal ion in the commercial sample, we added MgCl_2 to the remaining portion of our most concentrated high salt solution. With 10^{-2} M Mg^{++} , 1.4 M NaCl, and a base residue concentration of 2.32×10^{-3} M, there was no change in CD. We have since learned that the commercial sample was also prepared by deamination of ApA (probably using HNO_2), and should not have contained any heavy metal salts.⁵¹ There appears to be no clear explanation of the observed anomaly. If an aggregate actually was formed, it is not thermodynamically stable. Possibly the aggregate dissociation process is kinetically slow. Though the CD is vastly different (Figure 10), the slow dissociation of the "complexed" IpI from the NOCl deamination (see Section III-A) may be further evidence for a slow process. On the other hand, though it is unlikely, the anomaly may be a CD artifact.

We therefore must conclude that there is no stable aggregation of IpI in 1 M NaCl at ordinary optical concentrations. The structural change caused by the increase in ionic strength must thus be an internal one, i.e., a change in stacking geometry. We have reproduced the ORD data of Inoue and Satoh in Figure 11 for comparison with our data. It can be seen that the terminal phosphate on IpIp causes a sizeable change in ORD. This effect can not be due to

Figure 11. The ORD of IpI and IpIp: upper curves, IpI in 1 M NaCl — and zero salt ----; lower curves, IpIp in 1 M KCl — and 0.1 M KCl ---- from Inoue and Satoh.³⁸

steric factors since the ORD of IpIp in 1 M KCl becomes very like the ORD of IpI in low salt. This is probably because of electrostatic shielding by the K^+ ion. Thus the stacking geometry is also quite sensitive to the coulombic influence of the terminal phosphate group.

The hypochromism of 2.7% and the hypochromicity are consistent with the conclusion that there is a stacking interaction in IpI. While the hypochromicity is only 1.5% at 248 m μ (λ_{max}) a maximum difference in extinction of 550 is reached at 259 m μ which gives a hypochromicity of 7%. This closely agrees with the hypochromicity of 6% reported by Inoue and Satoh for IpIp at 260 m μ . The extinction coefficient of IpI in 1 M NaCl solution was determined by preparing solutions of equal IpI concentration in zero and 1 M NaCl, respectively. The results are given in Table 2. The hypochromism of 4.2% would imply that stacking is increased in 1 M NaCl.

Although ORD spectra of poly I have previously been reported,³⁷ we have taken the CD spectra (see Figures 12 and 13) for direct comparison with our IpI data. The polymer concentrations were determined by using an extinction coefficient of 10,200 for poly I at 248 m μ in 0.1 M NaCl solution,³⁷ and assuming it was identical for our low salt sample. The samples were prepared in such a way that the polymer concentration would be identical in both the low salt and high salt samples. This gave an extinction coefficient for multistranded poly I in 0.85 M NaCl of 6500,

Figure 12. The CD of poly I in low salt solution at

5°C ———,
 27°C ———,
 40°C ———, and
 60°C ———.

Figure 13. The CD of poly I in 0.85 M NaCl solution at

5°C ———, ,
 27°C ———, and
 50°C ———.

Table 2

Values Determined from Ultraviolet Absorption Spectra

Compound	Experimental Conditions	$\lambda_{\max}$ (m μ)	$\epsilon_{\max} \cdot 10^{-3}$	f_{230}^*	% H	% h †
pI ⁻	pH 7.3, 25°C	248	12.2	.261		
IpI	zero salt, 25°C	248	12.0	.254	2.7 $\pm$ 0.2	1.5 $\pm$ 0.2
IpI	1 M NaCl, 25°C	248	11.9	.250	4.2 $\pm$ 0.2	2.5 $\pm$ 0.2
pA + pI	pH 7.3, 25°C	252	12.3	.288		
IpA	pH 7.3, 25°C	251	11.0	.261	9.4 $\pm$ 0.9	10.6 $\pm$ 0.9
ApI	pH 7.3, 25°C	252	11.6	.281	2.5 $\pm$ 1.6	6.7 $\pm$ 2.9
pU + pI	pH 7.3, 25°C	251	9.7	.246		
IpU	pH 7.3, 25°C	251	9.6	.243	1.2 $\pm$ 0.4	0.8 $\pm$ 0.5

*Integration of UV spectrum was from 360 m μ to 230 m μ . † Calculated at $\lambda_{\max}$.

which is comparable to that obtained from other sources.^{36,39}

The CD data for poly I can be compared with that for monomer and dimer by the following equation:⁴⁵

$$\Delta\epsilon_{\text{poly I}} = 2 \Delta\epsilon_{\text{IpI}} - \Delta\epsilon_{\text{pI}}$$

If the secondary structure of dimer and polymer are identical, and if only nearest neighbor interactions contribute to the CD, then this equation will be a good approximation. In Figures 14, 15, and 16 we show the results of this calculation compared to the experimental CD of single-stranded poly I. For poly I at high temperature (50-60°C) the calculated CD is not too bad for either the high salt or low salt case. While the agreement between experiment and calculation is good for low salt at 27°C, it is poor for low salt at 5°C. This may mean that at 5°C in low salt multistand poly I is formed; however, no sharp transition is seen. It may alternatively mean that the temperature dependence of the structure of the polymer is different from that of the dimer. As we have stated above, I-I stacking interactions are sensitive to electrostatic environment. Certainly the electrostatic environment of the polymer is different from that which the dimer experiences. This may be sufficient to cause a difference in stacking geometry and temperature dependence. Perhaps it is only fortuitous that the secondary structures of dimer and polymer are approximately the same at room temperature. Both IpI and single-stranded poly I show unusually sharp melting profiles. This would

Figure 14. Comparison of experimental and calculated CD for poly I at high temperature (50°-60°C):

High salt, Measured ———, Calculated — — — —
 Low salt, Measured — · — ·, Calculated · · · · ·

Figure 15. Comparison of experimental and calculated CD for poly I at 27°C. in low salt:

Measured ———,
Calculated -----.

Figure 16. Comparison of experimental and calculated CD for poly I at 5°C in low salt:

Measured ———,
Calculated -----.

mean that slight differences in stability could lead to considerable differences in structure.

The CD of poly (G,I) (1:1) was observed in low salt and 1 M NaCl solution. The polymer concentration was estimated by assuming an extinction coefficient of 10^4 at 251 mμ ($\lambda_{\max}$). Since we were mainly interested in determining if multiple strands formed, this was sufficiently accurate for our purpose. The CD (Figure 17) is independent of salt concentration indicating that the strandedness of poly (G,I) is also independent of this factor. The CD is unlike that of poly I, and there is more of a similarity to that of poly G.⁵² Thus G-G interactions probably dominate the various contributions to the poly (G,I) CD spectrum.

Assuming a random sequence, one can estimate the I-G interaction by using the nearest neighbor formulation for circular dichroism:⁵³

$$\Delta\epsilon_{IpG} + \Delta\epsilon_{GpI} = 2\Delta\epsilon_{poly\ GI} - (\Delta\epsilon_{IpI} + \Delta\epsilon_{GpG}) + \Delta\epsilon_{pI} + \Delta\epsilon_{pG}$$

The result of this calculation is also shown in Figure 17 (CD data for GpG from Warshaw and Cantor).⁵⁴ Since the calculated spectrum is significantly different from the monomers spectrum, we conclude that at least one and perhaps both of the dimers, IpG and GpI, stack. This would not be surprising since purines usually do show a tendency to stack. The calculation was also made by using the CD data for poly I and poly G in place of that for IpI and GpG. While the CD calculated in this manner differed

Figure 17. The CD of poly (G,I) (1:1) in low salt or 1 M NaCl and calculated CD for IpG + GpI (per mole of base residue);

poly (G,I) ———,
 (IpG + GpI) - - - - -,
 monomers

significantly from that presented above, the conclusion concerning stacking in IpG and GpI is not altered. The differences between the two calculated spectra may be due to aggregation in poly G or to other unknown factors. Certainly, both calculations are subject to doubt because poly (G,I) may not be sufficiently random in sequence; and also it may not be completely single stranded.

The CD spectrum of IpA at three temperatures is shown in Figure 18. The CD spectrum of the monomers is also shown for comparison. It was found that ionic strength did not affect the ORD between 0 and 1 M NaCl. There was also no change in ORD in diluting the sample from 10^{-4} M (1 cm cell) to 10^{-5} M (10 cm cell). The base-base interaction in IpA is quite extensive as may be concluded from the CD data, hypochromism of 9.4% and the hypochromicity of 10.6% (Table 2). There is no change in structure with changes in salt concentration.

The CD of ApI is given in Figure 19. There was only a very slight increase in both positive and negative CD bands when the salt concentration was raised to 1 M NaCl. The CD results demonstrate that there is stacking in ApI, and this is supported by the hypochromism of 2.5% and the hypochromicity of 6.7% (Table 2). There is only a small change in CD with ionic strength. Thus the sensitivity of inosine to this factor may be restricted to self interaction.

The CD spectrum of IpU is reported at three temperatures, along with the monomers CD, in Figure 20. There was only a

Figure 18. The CD of IpA at pH 7.3;

5°C ———— ,
 25°C ———— ; and
 50°C ······ ;
 and monomers CD — · — · — .

Figure 19. The CD of ApI at pH 7.3, 0.1 M NaCl; 5°C — — —, 21°C ———, and 60°C - - - -.

Figure 20. The CD of IpU at pH 7.3, low salt; 5°C ———, 25°C ———, and 50°C ———, and monomers CD ———.

very small increase in CD in 1 M NaCl solution. The CD spectrum of IpU is not very different from the sum of the monomers CD at room temperature. But the difference that does exist indicates some degree of interaction. The hypochromism of 1.2% also indicates that there is some interaction. This is consistent with previous results^{17,18} with dimers containing U; they usually exhibit less stacking at room temperature than do other dimers.

A portion of the UpApI sample had been saved for CD measurements. The extinction coefficient of the trimer was calculated to be 10,600 at 254 mμ from the extinction of ApI and UpA¹⁹ using the nearest neighbor approximation.⁵³ The CD of the trimer was also calculated with this approximation using our data for ApI and Warshaw and Cantor's⁵⁴ data for UpA. The results are shown in Figure 21 with the sum of the monomers CD. While both calculated and experimental trimer CD's indicate considerable stacking, the agreement between the two is generally poor. Hence, either there is interaction between electronic transitions of U and I in the trimer, or relative positions of the bases differ in the trimer and the dimers, or both.

Figure 21. The CD of UpApI:

— experimental, pH 7.3,
----- calculated;
..... monomers.

IV. DIHYDROURIDINE

IV-A. The Reduction of Uridine

The selective photoreduction of uridine was first reported in 1965.⁵⁵ While A, G, or C do not react, U is reduced to dihydrouridine by sodium borohydride in the presence of UV light. There are also other reactions which can occur when uracil is irradiated with UV light in the presence of NaBH_4 . Reactions which do not depend on NaBH_4 are photodimerization and photohydration. These do not occur to any appreciable extent under the conditions used in this work.⁵⁶ However, once uracil has been reduced to dihydrouracil, there is further reaction with BH_4^- to produce the open-ring, β -ureidopropanol.* This reaction is independent of UV irradiation, and does occur at our conditions. There is also a side product to this dark reaction, but this is not extensive and the product has not been identified.⁵⁷

The reaction has been studied as a function of both pH and temperature during irradiation.⁵⁶ It was found that the optimum conditions for formation of dihydrouracil was about pH 9.5 and 50°C. While the reduction of U is slower at 50°C there is essentially no photohydration. The reaction is correspondingly faster at 40°C and 10°C, but with appreciable photohydration, especially at 10°C. The higher temperature also retards the dark reaction of dihydroU with BH_4^- . The

photoreduction has been shown to be irreversible, while the photohydration is not. The higher temperature favors the breakdown of the water addition product.⁵⁶

The mechanism of the primary photoreduction has been studied by the use of labeled reducing systems and nmr spectroscopy.⁵⁸ This study used thymidine, but the mechanism should be similar for uridine. Thymidine reacts more slowly than uridine, but dihydrothymidine cannot be isolated; it is a short-lived intermediate. The open ring (2 epimeric forms) 3-ureido-2-methylpropanol* is the major product. The reaction was carried out in $\text{NaBD}_4\text{-H}_2\text{O}$, $\text{NaBH}_4\text{-D}_2\text{O}$, and $\text{NaBD}_4\text{-D}_2\text{O}$ labeled systems. From the nmr of the products from these systems it was concluded that the hydrogen donated by NaBH_4 enters the molecule at C-5, but that hydrogen added to C-6 comes from the water solvent.

The dark reaction with NaBH_4 has also been extensively studied.⁵⁷ With both dihydrothymidine and dihydrouridine the two hydrogens which are added to C-4 come from BH_4^- . Under the conditions used the minor nucleosides N^4 -acetylcytidine and 4-thiouridine also reacted with NaBH_4 .

The general procedure used for the sodium borohydride reduction in the current work is quite simple. A solution of NaBH_4 in pH 9.5 NaOH is added to an equal volume of solution $2 \cdot 10^{-3}$ M in uracil residue. This is covered with a quartz window and irradiation began with a Hanovia

#30620 "Utility" mercury arc ultraviolet lamp. There are no filters used with the lamp. The irradiation is carried out at 50-55°C in a glove box with a nitrogen atmosphere. The glove box eliminates any danger of explosion from released H_2 , and also protects the eyes from stray UV light. After irradiation, excess borohydride is destroyed by adding an appropriate amount of pH 2 HCl. The reaction mixture is then neutralized and diluted for loading on an AG 1 × 2 column by adding 0.01 M NH_4HCO_2 , pH 6.8. The two variables in the general procedure are $NaBH_4$ concentration and duration of irradiation. In Table 3 the compounds being reduced are given with conditions used and yield of products. An equal volume of pH 2 HCl is used for quenching with the lower concentration $NaBH_4$ reactions, and twice the volume with the higher concentration. Some interesting inferences can be made from the product distributions observed, and these are discussed in Appendix B.

IV-B. Separation of Reaction Mixture

In most of the preparative reactions 1 ml of uracil solution was added to 1 ml of $NaBH_4$ solution. After irradiation and HCl addition, 100 ml of pH 6.8 0.01 M NH_4HCO_2 was added. To prevent problems in the subsequent column chromatography, this mixture was degassed under vacuum for 10-30 minutes. An AG 1 × 2 column of approximately 5 ml bed volume was used. After the sample was loaded, elution proceeded with either an NH_4HCO_2 or NaCl gradient at pH 6.8. With

Table 3

Starting material	NaBH ₄ concentration in reaction	Time (min) of irradiation	Relative Yield*		
			unreduced	containing H	containing β
U	$1 \cdot 10^{-2}$ M	15	0.5		
H	$2 \cdot 10^{-2}$	10	--	0.5	0.5
ψ	$1 \cdot 10^{-2}$	20	0.3		
ApU	$2 \cdot 10^{-2}$	40	0.6	0.4	0.01
UpA	$2 \cdot 10^{-2}$	40	0.25	0.75	0.003
GpU	$2 \cdot 10^{-2}$	40	0.7	0.2	0.1
UpU	$2 \cdot 10^{-2}$	40	0	0	1.0
UpU	$1 \cdot 10^{-2}$	10	undetermined		

* Estimated for monomers by monitoring the OD of the reaction mixture, and for dimers from the elution on AG 1 $\times$ 2.

either gradient the elution order was the same. It was quite easy to determine which peak contained the dihydroU dimer by adding NaOH to a sample portion of the peak. The characteristic ring opening of the reduced pyrimidine^{59,60,61} could be observed by monitoring the UV absorption at 235 mμ. Neither the normal dimers nor the β-ureidopropanol dimers show this behaviour. The ring opening in basic solution will be discussed in more detail in Section IV-C. The unreacted dimer could be detected by its known UV and/or CD spectrum. The remaining peak thus contained the open ring form. This was checked in the GpU reaction using a staining test sensitive to ureido groups.⁶² A portion of the peak was spotted on chromatography paper and sprayed with pDAB solution. The characteristic intense yellow stain was seen, proving the identity of the peak. For controls β' and urea, which gave the color reaction, and H, which gave none, were used.

The elution profile of an ApU reaction is shown in Figure 22, and is typical of the other dimer reactions, except that of UpU. After elution the desired peaks were lyophilized to remove NH_4HCO_2 , and redissolved in pH 7.3 phosphate buffer for optical studies. The ApH peak was checked for purity by dissolving a portion of the lyophilized powder in double distilled water and spotting it on paper for electrophoresis at pH 2.7. Only one spot resulted. The spot was eluted and the CD after electrophoresis was identical to that without electrophoresis. Since the other separations were similar the purity was not checked.

Figure 22. The separation of the ApU reduction reaction mixture on AG 1 x 2 at pH 6.8.

With the photoreduction of UpU, the separation of reaction products is considerably more complicated. There are potentially nine dimers to separate. In Figure 23 the elution pattern of one reaction is shown. The elution was monitored at both 260 mμ and 230 mμ using the Gilford Dual Wavelength Selector attachment. Since HCO_2^- absorbs light at 230 mμ, the linear gradient is seen in the record of the reference cell. The previous column results (see Figure 22) can be summarized: H containing dimers are eluted first, U containing dimers second, and β containing dimers third. From this it can be predicted with fair certainty that HpH will be eluted first and βpβ last. This does seem to be the case. Using the same reasoning the assignments in Figure 23 have been arrived at. All peaks (except that labeled βpβ, which was not tested) showed the characteristic ring-opening upon addition of NaOH. Thus it is certain that the third peak is not only Upβ and βpU. The HpH peak was lyophilized and redissolved in double distilled water for CD measurement. The peaks which absorbed at 260 mμ were used for CD measurement as they came off the column. The βpβ peak was not used.

IV-C. Results and Discussion

Though extinction coefficients have been reported for dihydroU,^{60,61,63} the values reported at pH 7 are few. To be certain that the absorption of H is negligible at the absorption maxima of the normal bases, and for use with the

Figure 23. The separation of the UpU reduction reaction mixture on AG 1 × 2 at pH 6.8.

HpH dimer, the extinction coefficient of dihydroU in neutral solution has been determined by two methods.

Using the borohydride reduction with a known starting concentration of U, the concentration of H was determined by measuring the concentration of U after reaction. This was done by taking 100 λ aliquots of the reaction mixture, quenching with 100 λ pH 2 HCl, then adding 1 ml pH 7 phosphate buffer and taking the UV spectrum. A blank was prepared in the same manner with H₂O used in place of the uridine stock. The extinction of dihydroU was then calculated as follows:

$$[H] = [U]_0 - [U] = \Delta OD^{262} / \epsilon_U^{262}$$

$$\epsilon_H^{230} = \frac{OD_H^{230}}{[H]} = \frac{OD^{230} - OD^{262} \frac{\epsilon_U^{230}}{\epsilon_U^{262}}}{[H]}$$

This assumes that only U absorbs appreciably at 262 m μ , later shown to be a fact, and that only dihydroU is produced in the reaction, which is reasonable in the early stages of the reaction. The extinction at 230 m μ was chosen since it is close to the minimum in the U absorption spectrum. After irradiation for ~5 minutes 20% of the U had been reduced and ϵ_H^{230} was determined to be 1650 ± 50 with duplicate samples.

The second method used the reported extinction coefficient of H at 235 m μ in 0.1 M NaOH.⁶⁰ One hundred λ 1 M NaOH was added to 0.90 ml of dihydroU in double distilled water and the UV absorption at 235 m μ monitored. When the NaOH is

added there is a sharp increase in absorption as H becomes ionized, then a slow decrease as ring opening occurs. By extrapolating to $t = 0$ the OD, and thus the concentration of the ionized species can be found. Adjusting for dilution, one can also determine the concentration and extinction of H in neutral solution. This method gave an extinction at 235 m μ of 1110 ± 30 for Hp and 1050 ± 3 for H with triplicate samples for each. The difference cannot be considered significant. One of the H samples was allowed to proceed to the entirely open ring form and thus an extinction coefficient of 140 at 235 m μ was determined for β -ureidopropionate riboside in 0.1 M NaOH. From the OD_{235}/OD_{230} ratio of the UV spectrum of H and Hp and the above values an extinction coefficient of 1700 at 230 m μ is calculated, in close agreement with the first method. The first value would be expected to be a little low because of the dark reduction. Using the 1700 value, the extinction of dihydroU is seen to be negligible (less than 100) above 250 m μ . The extinction of dihydroU was included in calculating the extinction of GpH (at 252 m μ) but neglected in the extinction of ApH and HpA.

In Figure 24 the room temperature CD of H, Hp, and β are presented. β was prepared by reduction of H with borohydride. β' was prepared by the alkaline ring opening reaction. The ORD of β' was negative with no Cotton effects above 200 m μ . Thus the CD of β' above 200 m μ is negligible. These data are used for the comparison of

Figure 24. The CD of dihydroU monomers:

— H₁,
- - - H_p,
- - - β (open ring form).

dimer and monomer properties. Published CD spectra⁶⁴ for dihydroU show a significant difference in magnitude between the 3' and the 5' nucleotides. Our CD results with H and Hp also show a significant difference. The CD of H was found to be sensitive to temperature, though the effect is small. Over the range 5°-70°C the total change in $\Delta\epsilon$ is of the same magnitude as the difference between H and Hp, with the 5°C curve being the larger.

The CD of ApH was taken at several temperatures and the results are presented in Figure 25 along with the sum of the monomers. The hypochromism of 0% and the hypochromicity of 2.7% were determined by enzymatic hydrolysis as described in Section II-D. Table 4 lists these and other values determined for the dimers in this section. While a hypochromicity of 7% has been reported⁶⁰ for ApHp at 260 m μ , the same report gives a hypochromicity of 11% for ApUp. We expect that their results are consistently high since other workers report a hypochromicity of 5% for ApU.⁷² Both our CD and UV results for ApH indicate that there is very little interaction of the 260 m μ transitions of A with the transitions of H. It is therefore expected that there is little stacking in ApH. The 5°C CD spectrum may be interpreted to mean that stacking does occur at lower temperatures.

Figure 26 presents the CD data for HpA, and Table 4 presents the UV data. The CD curve for the sum of the monomers is the same as that used in Figure 25. Since the position of the phosphate group influences the conformation

Figure 25. The CD of ApH at pH 7.3:

—— 5°C,
—— 21°C,
----- 75°C;
..... monomers.

Figure 26. The CD of HpA at pH 7.3:

——— 5°C,
 - - - 21°C,
 - - - 40°C,
 - · - 75°C;
 monomers.

of the monomer, the monomer conformation is probably not equivalent to that in the dimer. Thus it is likely that no monomer CD spectrum is exactly right for comparison with a dimer CD. I have simply chosen to be consistent. The differences which may exist should be small and would not change any of the conclusions.

Table 4

Values Determined from the
UV Spectra at 25°C, pH 7.3

Substance	$\lambda_{\max}$ (m μ)	$\epsilon_{\max} \cdot 10^{-3}$	f_{230}^*	% H	% h †
pA + H	259	7.7	0.162		
ApH	259	7.5	0.158	2.7 ± 0.3	0 ± 5
HpA	259	7.3	0.153	5.5 ± 1.4	7.7 ± 2.2
pG + H	252	6.9	0.194		
GpH	252	7.0	0.197	-1.4 ± 1.2	-2.8 ± 2.3

* Integration of the UV spectrum was from 360 m μ to 230 m μ .

† Calculated at $\lambda_{\max}$.

The CD of HpA indicates a strong interaction that is little changed from 5° to 75°C. The UV values of 5.5% hypochromicity and 7.7% hypochromism also suggest a strong interaction. For comparison, ApA shows a hypochromism of 6.8% and is thought to strongly stack.⁷² The change in CD in the 260 m μ region over the temperature range is very close to the limit of experimental error. Though CD changes with temperature below 225 m are larger, they are in the wrong direction. That is,

usually the dimer CD approaches the sum of the monomers CD as the temperature is raised. With HpA the CD below 225 m μ appears to approach the sum of the monomers as the temperature is lowered. Though the CD of H changes with temperature, the change is small and could not account for the observed behavior in the dimer. The phenomena is also seen to a certain extent in the ApH CD at low wavelength. This suggests that stacking in these compounds is unusual, and could not be considered by either the two-state model or the torsional oscillator model. The observed temperature dependence of the CD of HpA may be evidence for intramolecular hydrogen bonding, except that one would expect such bonding to be unstable at 75°C. Though the structure of HpA seems remarkably stable, it is certain that if any intramolecular bond exists, it is a weak bond. When the phosphodiester link is broken (by venom phosphodiesterase) the CD becomes just the sum of the monomers.

The CD of GpH is given in Figure 27 and the UV data in Table 4. While the CD of GpH above 240 m μ is only slightly larger than the sum of the monomers CD, it has two negative bands (except at 70°C) instead of one. Again the temperature dependence of the CD at lower wavelength seems anomalous. From the 21° spectrum there appears to be little stacking. Though the -2.8% hypochromism may belie this conclusion, the range of error of this value means that it cannot be considered strongly.

The results with the photoreduction of UpU have not been

Figure 27. The CD of GpH at pH 7.3:

--- 5°C,
 — 21°C,
 - · - 70°C;
 monomers.

entirely satisfactory. With HpH there was sufficient residual formate and/or scattering material to make it difficult to determine the optical density and thus the concentration of the sample used for the CD measurement. There was insufficient material to proceed with further purification. With the partially reduced dimers there is the problem of incomplete separation. The CD data that have been obtained, presented in Figure 28, indicate that there is little stacking in any of these compounds. The positive band of the HpH spectrum is of the same magnitude as that of H, but slightly shifted to lower wavelength. This may mean that there is some stacking, but it is really difficult to say anything conclusive with these results. The CD curves for the partially reduced dimers are quite close to the respective monomer curves (not shown), particularly the positive band centered at 270 m μ . The peak at 270 m μ is interesting because it indicates that there is very little UpU contained in these fractions. Warshaw and Cantor report the peak of the UpU CD at 277 m μ . There may be cancellations of the CD's of the various components of the fractions, but this does not seem likely.

The optical properties of some dimers containing open-ring forms of dihydroU have also been studied. The extinction coefficients of these dimers have not been determined; the extinction coefficient of the normal base was assumed. This should not introduce any appreciable error into the ORD and CD results. The β containing dimers were obtained from the NaBH₄ reduction.

The ORD of Ap β at several temperatures and in 100%

Figure 28. The CD of some of the products of the UpU reduction:

— — — HpH,
 ————— fraction #24

from the column chromatography
 (expected to be mainly UpH and HpU),

----- fraction #27 from the column
 chromatography (expected to be mainly
 Up β and β pU).

methanol is shown in Figure 29. Under all conditions the ORD is quite different from that of the sum of the monomers. For this comparison the ORD of β was obtained from a Kronig-Kramers transform of the CD using a program written by M. S. Itzkowitz. This dimer was treated with venom phosphodiesterase and the hydrolysate ORD was, within experimental error, that of the monomer. There can be no doubt that there is extensive interaction in this dimer, which I hesitate to call stacking. The meaning of the term is unclear when one is dealing with an open-ring structure. The results, particularly the spectrum in methanol, may indicate intramolecular hydrogen bonding.

To obtain $\text{Ap}\beta'$, 1 M NaOH was added to a solution of ApH so that ring opening occurred. The reaction was allowed to proceed for about 20 minutes and then the solution was neutralized. The mixture was loaded onto an AG 1 $\times$ 2 column and elution accomplished with a NH_4HCO_2 , pH 6.8 gradient. The $\text{Ap}\beta'$ peak was collected and lyophilized. The CD is given in Figure 30 compared to that for the monomers. This dimer also appears to "stack" well.

The ORD of βpA is compared in Figure 31 with the ORD of HpA . The sum of the monomers ORD for βpA is that shown in Figure 29. Certainly the large ORD for βpA would indicate interaction, especially since HpA is stacked.

When the CD of $\text{Gp}\beta$ is compared to the sum of the monomers (Figure 32) the conclusion is again that there is considerable interaction in the dimer.

Figure 29. The ORD of Ap β : In H₂O (pH $\sim$ 6) at

0°C ———,

25°C ———,

66°C — · — ·;

in 100% MeOH at 25°C — · — ·;

and the ORD of the monomers · · · · · .

Figure 30. The CD of Ap β' in distilled water, pH $\sim$ 6 —; and monomers

Figure 31. The ORD of β pA —, and HpA ----- at 25°C and pH $\sim$ 6.

Figure 32. The CD of Gpβ at pH 7.3:

----- 5°C,
———— 21°C;
..... monomers.

It seems to be a general conclusion that the open-ring dimers exhibit considerable "stacking." We can even go further and say that they show more interaction than do the corresponding closed-ring dimers with dihydroU. This is obviously the case with ApH and GpH, which show little stacking. With HpA vs. β pA, the larger ORD of β pA can reasonably be interpreted to mean more interaction in β pA. The electronic transitions of β are at higher energy than those of H, and further removed from the 260 m μ transitions of the normal bases. In substituting the electronic transitions of β for those of H the frequency term in the expression for rotational strength (see Section I-B) would become smaller. To maintain the same intensity of optical activity, or to increase it, some other term must become larger. This may well be the distance term. With the open ring some parts of the β or β' moiety may be able to get very close to the normal base moiety of the dimer.

V. rT ψ CG

The tetramer sequence T ψ CG is found in approximately the same position in all tRNA's that have thus far been sequenced.⁶⁵ While both rT and ψ are modified forms of U, they do exhibit properties different from U. Poly U is unique in its lack of secondary structure, but both poly ψ ^{66,75} and poly rT⁶⁷⁻⁷⁰ show considerable secondary structure. CD results reported by Zavilgelskij and Li⁷¹ indicate that there is considerable stacking in the dimers Ap ψ p and rTprT. The corresponding dimers containing U show little stacking.^{19,72}

V-A. Results and Discussion

The CD spectrum of rTp ψ pCpGp was observed between 5° and 60°C. Figure 33 shows these CD curves and presents also the sum of the monomers CD. These results agree with a previously reported CD for T ψ CG⁷¹ and, as expected, indicate extensive stacking in the tetramer. This is confirmed by the hypochromicity of 13.3% at 260 m μ (pH 7).⁷³ The CD of UUCG, calculated by the nearest neighbor formulation,^{53,54} is compared in Figure 34 with the CD of the sum of its monomers and with the CD of T ψ CG at 25°C. The large differences that exist between the CD of UUCG and its monomers indicate a good deal of stacking in this tetramer. There are also large differences between the calculated CD for UUCG and the CD of T ψ CG. To the extent that the nearest neighbor approximation can be trusted, this indicates extensive structural differences between the two tetramers. Certainly, the slight

Figure 33. The CD of rTpψpCpGp in distilled water, pH ~6:
 ----- 5°C, ————— 25°C, - - - - - 40°C, - . - . - 60°C;
 monomers.

Figure 34. The CD of TψCG compared to that of UUCG;

—— TψCG at 25°,
 ---- UUCG calculated;
 monomers (UUCG).

differences in energy and intensity of the UV transitions between U and ψ or T could not alone account for the CD differences. The 120° rotation of ψ relative to U may be important, however.

To carry the comparison between T ψ CG and UUCG further, we have calculated the temperature dependence of the ORD of UUCG. This was done by using temperature coefficients for dimers, which had been calculated by S. R. Jaskūnas⁷⁴ from the ORD data of R. C. Davis.⁴² The results are shown in Figure 35 with our data for T ψ CG. While the curve for T ψ CG is somewhat more steep, the difference is unremarkable. Thus the structural stabilities of the two tetramers may be rather similar.

Figure 35. The temperature dependence of the CD of TψCG and the calculated temperature dependence of the ORD of UUCG:

X — X TψCG at 260 mμ
 O --- O UUCG at 290 mμ
 + --- + UUCG at 262.5 mμ
 (- [φ] · 10⁻⁴ is plotted).

VI. CONCLUSIONS

What, finally, can be said about how these minor nucleosides might influence the function of tRNA? We can conclude from our studies that IpI and IpA stack considerably at room temperature, but that IpU does not. The stacking does not appear unusual unless I endows stability to a structure not allowed by other bases. This cannot be determined from CD studies without a great deal of calculation. Of course, the hydrogen bonding properties of I are unlike those of normal bases but to a good approximation I base pairs like G. What does seem important, and unique, is the sensitivity to ionic environment of the conformation of oligomers of I. While this may be influential in the single to triple strand conversion of poly I there is no clear interpretation in relation to tRNA structure since the sequence --IpI-- does not occur in any known tRNA. Perhaps in the geometrically specific environment of the ribosome complex or the synthetase complex this sensitivity of I may play an important role. Inosine seems to appear only in the anticodon loop in the "wobble"⁸⁰ position. Thus sensitivity of I to electrostatic environment might possibly be a mechanism through which "wobble" could occur.

Looking at specific sequences, -UpIpG- occurs in yeast alanine and serine³ tRNA's. while -UpIpA- occurs in valine³ and T. utilis isoleucine⁸¹ tRNA's. Thus of the nearest neighbor interactions involved we can only make a definite statement on IpA. But can we suppose that there will be

little stacking in UpI since there is little in IpU? In the results of Warshaw and Tinoco⁷² UpG shows evidence of stacking while GpU shows little. Thus UpI may be able to stack, while IpU does not. The calculated spectrum of IpG + GpI suggests that there is stacking of IpG. Thus we can tentatively say that I does not disrupt stacking in the anticodon loop, but allows the loop to maintain a rigid structure with the possibility of subtle structural variations, necessary for "wobble," caused by the sensitivity of I to coulombic environment.

Summarizing our results with dihydrouridine: HpA stacks, but ApH, GpH, and probably HpH stack little. The stacking appears unusual, particularly as reflected in the temperature dependence of the CD. While in the 260 mμ region the CD of the dimer approaches that of the monomer as the temperature is raised, at lower wavelength it does not. In HpA the change in CD above 230 mμ with temperature is very small, while below 230 mμ the change is larger. This suggests that temperature changes cause relative motion of the transition dipoles within a base. This may be possible for dihydroU. Since the reduced ring structure is not planar, there may be changes in ring puckering with temperature. While this would not necessarily have a large effect on monomer CD, it could have a large effect on dimer CD, where the electronic transitions of H are interacting with the transitions of A, for example. Puckering could lead to motion of the transition directions of H with respect to one another. Another possibility is that, as seen by the normal base, the transitions of H

appear to move with respect to one another. Figure 36 shows a mode of oscillation which could cause this. This type of oscillation would presumably be also possible for the normal bases, but the temperature dependence of the optical properties of normal base dimers does not indicate that it occurs. This motion might occur with dihydroU because of the nature of the intramolecular forces causing the interaction of the bases. Also, if the bonding at the glycosidic nitrogen of dihydroU is ammonia-like, and could possibly undergo configurational inversion, the oscillation described above would result. This inversion, similar to puckering, would probably cause in addition actual relative motion of the transitions of H. All this, of course, is pure speculation; but it does seem that there are several possibilities at the molecular level for explaining the observed CD data. Other physical methods, such as IR or NMR, would be useful for investigating these possibilities; and also the possibility of intramolecular hydrogen bonding. It has been found that dihydroU does not base pair with A, using poly A and partially reduced poly U;⁸³ and that dihydroU in partially reduced poly U has no template activity for binding aminoacyl-tRNA to ribosomes.⁸⁴ It is not readily apparent why H does not base-pair, but if stacking of dihydroU is unusual, it could lead to these results. For example, if dihydroU is most stable in the polymer with its plane parallel to the helix axis rather than perpendicular to it, base-pairing may well be impossible. The unusual stacking would also be a good recognition point when tRNA interacts with the synthetase or ribosome complex.

Figure 36. Possible oscillation of dihydrouridine in a dimer.

Ring-opening is another unique possibility with dihydroU. If an enzyme exists which could open and close the ring at the polymeric level, this could be structurally important in tRNA. Our results indicate that intramolecular interactions are stronger when the ring is opened. Several laboratories have studied the effect of ring-opening on tRNA by using the dark reduction reaction with NaBH_4 . The results, however, are contradictory. Molinaro, et al.⁶³ studied tRNA^{ser} , tRNA^{ala} , and tRNA^{val} for amino acid acceptance, and tRNA^{ser} for ribosome binding as a function of the amount of dihydroU destroyed. They conclude that there is no effect on either function. Cerutti⁸⁵ studied the aminoacylation of tRNA^{ser} and tRNA^{val} that had been treated with NaBH_4 . While he found no effect with tRNA^{ser} , he concluded that the acceptor activity of tRNA^{val} is decreased by the reductive cleavage of dihydroU residues. He suggests that this may be due to the H residue in the 3' half of tRNA^{val} which has no counter-part in tRNA^{ser} . No effect of the dark reduction of yeast tRNA with sodium borohydride on the acceptance of valine was observed by Roy and Tener.⁸⁶ It is difficult to relate this work to ours. Perhaps if dihydroU is important in the acylation or ribosome binding functions, it is the open-ring form that is involved. If there is an "open-ase" the dihydroU residues could serve as triggering points for structural changes in the tRNA molecule. With its various and specific functions, tRNA may have to take on different and specific conformations for each task. There is no evidence on this, and it would be difficult to obtain such evidence.

DihydroU has been found in only two regions of tRNA: the "dihydroU loop" and the "extra arm." The greatest dissimilarity between the fourteen sequenced tRNA's is in the "extra arm."⁶⁵ Of the loops, the dihydroU loop is the most varied, other than the triplet codon. Thus the dihydroU residues are in a region where they could influence the specificity of each tRNA molecule, and our results give some indication of how they might do that.

At best the results with T ψ CG suggest that the T and ψ residues may alter the structure of the loop in which they occur (compared to the U residues they supposedly replace). That the T ψ CG region is necessary to the function of tRNA is indicated by the loss of acceptor activity which results from cyanoethylation of the ψ residues in tRNA.^{87,88}

While it has been reported that ψ is photoreduced with NaBH₄ at a much slower rate than is U,⁸⁹ our results indicate that the rates are comparable (see Section IV, Table 3 and Appendix B). Unfractionated yeast tRNA has been treated with NaBH₄ under photoreductive conditions, and it was reported that only U residues of the tRNA were reduced.⁹⁰ If this is correct, it means that under conditions where a large fraction of the monomer would have been reduced, none, or very few, of the ψ residues in tRNA are reduced. Hence the ψ residues are probably protected by the tertiary structure of the tRNA. Previous work with cyanoethylation has also implied that ψ residues are protected in the native form tRNA's.^{88,91}

In summation then, the major findings of this study are:

for I, the sensitivity of stacking to electrostatic environment; for H, the unusual stacking properties, and the possibility of ring-opening being able to endow multiple structures to tRNA.

APPENDIX A

SUBROUTINES FOR THE PDP 8/S COMPUTER

Description

There are four subroutines listed which calculate integrals of UV or CD spectra using a Simpson's rule numerical integration.⁴⁰ There is no attempt at curve resolution made and the integration is performed on the unsmoothed data. The integrals calculated are:

oscillator strength, F (unitless)

$$F = 4.318 \cdot 10^{-9} \int \frac{\epsilon}{\lambda^2} d\lambda$$

dipole strength, D (debye²)

$$D = 9.18 \cdot 10^{-3} \int \frac{\epsilon}{\lambda} d\lambda$$

rotational oscillator strength, C (unitless)

$$C = 3.28 \cdot 10^{-13} \int \frac{[\theta]}{\lambda^2} d\lambda$$

and rotational strength, R (esu-cgs)

$$R = 6.959 \cdot 10^{-43} \int \frac{[\theta]}{\lambda} d\lambda$$

The first two are measures of the intensity of light absorption, and the second two are measures of the intensity of optical rotation.⁴¹ The values calculated by the subroutines will be F, D · 10⁸, C · 10¹⁰, and R · 10⁴⁹, respectively. The

subroutines are written in numerical format because that seemed the simplest way to make them compatible with the larger program²¹ of which they form a part. The meanings of the numerical format are described in Small Computer Handbook distributed by Digital Equipment Corp.

These subroutines destroy most functions of the main program, super spectrum. Most importantly, the program cannot be used for taking data or smoothing data while it contains these subroutines. There was a small patch written which restores super spectrum to its normal state, and it is also listed.

Operation

This write-up assumes a familiarity with the operation of super spectrum. The binary tape for the desired integration is loaded when super spectrum is in the computer. The subroutine occupies what is ordinarily the baseline storage array. The calculation is performed on data occupying the spectrum storage array from the starting wavelength to any given wavelength, with the restriction that there must be an even number of increments. The result of the integration is multiplied by E/OD and the appropriate constant. Operation is the same for all four subroutines:

- 1) Load the appropriate tape.
- 2) Start at 0200.
- 3) Enter monitor, type "G." The current heading will be typed out, then a lower limit of integration will be called for. This will be "LLIM(A)=."

- 4) Type in the desired lower limit of integration in angstroms, then hit the space bar. The computer will proceed with the calculation and type the desired value, unless an odd number of increments has been requested, when "NO" will be typed. After either case the monitor will automatically be entered.
- 5) Type "A" if a different lower limit is desired.
- 6) Type "S" if a different ID, OD, or E is desired.
- 7) Type "D" to read in another spectrum. This option operates normally.
- 8) When finished the patch which restores super spectrum to normal operation should be loaded.

PATCH TO SUPERSPECTRUM FOR CALCULATING OSCILLATOR STRENGTHS
(UNITLESS)

C. J. Formoso
April 10, 1969

```

*2231
2231 7300 7300
2232 3062 3062
2233 5634 5634
2234 4002 4002
*3431
3431 4000 4000
*4000
4000 7300 7300/OSCILLATOR STRENGTH
4001 5524 5524/LAMBDA= STRTEL, INCREM=INCR
4002 7300 7300
4003 1371 1371/ MESS: LLIM(A)=
4004 4501 4501
4005 6036 6036
4006 4405 4405
4007 4407 4407
4010 6333 6333
4011 5330 5330
4012 2330 2330/ OS ST = 0
4013 6330 6330
4014 0000 0000
4015 1163 1163
4016 1326 1326
4017 3010 3010
4020 4312 4312/ FIRST POINT
4021 4407 4407
4022 6330 6330
4023 5063 5063/INCREMENT LAMBDA
4024 1066 1066
4025 6063 6063
4026 0000 0000
4027 7300 7300/EVEN POINTS
4030 4312 4312
4031 4407 4407
4032 3344 3344/ X 4
4033 1330 1330/ + OSST
4034 6330 6330
4035 5063 5063
4036 1066 1066/INCREM LAMBDA
4037 6063 6063
4040 2330 2333
4041 7000 7000
4042 6336 6336
4043 0000 0000
4044 7300 7300/CHECK LLIM
4045 1336 1336
4046 7440 7440
4047 5267 5267/ GO TO ODD POINTS
4050 4312 4312/ LAST POINT

```



```

4051 4407 4407
4052 1330 1330
4053 3154 3154/X E
4054 4151 4151//OD
4055 3352 3352/ X -0.0004318
4056 3066 3066/ X INCREM
4057 4355 4355/ DIV BY 3
4060 0000 0000
4061 7300 7300
4062 1373 1373/ MESS: F=
4063 4501 4501
4064 7300 7300
4065 4406 4406
4066 5522 5522/GO TO MONITOR
4067 4312 4312/ODD POINTS
4070 4407 4407
4071 3347 3347/ X 2
4072 1330 1330/ + OSST
4073 6330 6330
4074 5063 5063
4075 1066 1066
4076 6063 6063
4077 2333 2333/ INC LAMBDA AND CHECK LLIM
4100 7000 7000
4101 6336 6336
4102 0000 0000
4103 7300 7300
4104 1336 1336
4105 7440 7440
4106 5227 5227/ GO TO EVEN POINTS
4107 1372 1372/ MESS: NO
4110 4501 4501
4111 5522 5522/ MONITOR
4112 0000 0000/ SUBROUTINE TO GET SPEC POINT
4113 1410 1410
4114 3045 3045
4115 3046 3046
4116 1327 1327
4117 3044 3044
4120 4407 4407
4121 7000 7000
4122 4063 4063
4123 4063 4063/ DIV BY LAMBDA SQ
4124 0000 0000
4125 5712 5712
4126 7777 7777/C-1
4127 0013 0013/C13
4130 0000 0000/ OS ST
4131 0000 0000
4132 0000 0000
4133 0000 0000/ LLIM
4134 0000 0000
4135 0000 0000
4136 0000 0000/ CHEK
4137 0000 0000
4140 0000 0000

```

4141	0000	0000/ SPEC
4142	0000	0000
4143	0000	0000
4144	0003	0003/FL 4
4145	2000	2000
4146	0000	0000
4147	0002	0002/ FL 2
4150	2000	2000
4151	0000	0000
4152	7765	7765/ FL -0.0004318
4153	4354	4354
4154	7161	7161
4155	0002	0002/ FL 3
4156	3000	3000
4157	0000	0000
4160	3414	3414/ MESS: LLIM(A)=
4161	1411	1411
4162	1550	1550
4163	0151	0151
4164	7500	7500
4165	1617	1617/ MESS* NO
4166	0000	0000
4167	3406	3406/ MESS: F=
4170	7500	7500
4171	4160	4160
4172	4165	4165
4173	4167	4167

PATCH TO SUPERSPECTRUM FOR CALCULATING DIPOLE STRENGTHS.
ANSWER WILL BE $D \times 10^8$ (DEBYE SQUARED)

C. J. Formoso
April 10, 1969

```

*2231
2231 7300 7300
2232 3062 3062
2233 5634 5634
2234 4002 4002
*3431
3431 4000 4000
*4000
4000 7300 7300/DIPOLE STRENGTH
4001 5524 5524/LAMBDA= STRTEL, INCREM=INCR
4002 7300 7300
4003 1371 1371/ MESS: LLIM(A)=
4004 4501 4501
4005 6036 6036
4006 4405 4405
4007 4407 4407
4010 6333 6333
4011 5330 5330
4012 2330 2330/ OS ST = 0
4013 6330 6330
4014 0000 0000
4015 1163 1163
4016 1326 1326
4017 3010 3010
4020 4312 4312/ FIRST POINT
4021 4407 4407
4022 6330 6330
4023 5063 5063/INCREMENT LAMBDA
4024 1066 1066
4025 6063 6063
4026 0000 0000
4027 7300 7300/EVEN POINTS
4030 4312 4312
4031 4407 4407
4032 3344 3344/ X 4
4033 1330 1330/ + OSST
4034 6330 6330
4035 5063 5063
4036 1066 1066/INCREM LAMBDA
4037 6063 6063
4040 2333 2333
4041 7000 7000
4042 6336 6336
4043 0000 0000
4044 7300 7300/CHECK LLIM
4045 1336 1336
4046 7440 7440

```

```

4047 5267 5267/ GO TO ODD POINTS
4050 4312 4312/ LAST POINT
4051 4407 4407
4052 1330 1330
4053 3154 3154/X E
4054 4151 4151//OD
4055 3352 3352/ X -0.0004318
4056 3066 3066/ X INCREM
4057 4355 4355/ DIV BY 3
4060 0000 0000
4061 7300 7300
4062 1373 1373/ MESS: F=
4063 4501 4501
4064 7300 7300
4065 4406 4406
4066 5522 5522/GO TO MONITOR
4067 4312 4312/ODD POINTS
4070 4407 4407
4071 3347 3347/ X 2
4072 1330 1330/ + OSST
4073 6330 6330
4074 5063 5063
4075 1066 1066
4076 6063 6063
4077 2333 2333/ INC LAMBDA AND CHECK LLIM
4100 7000 7000
4101 6336 6336
4102 0000 0000
4103 7300 7300
4104 1336 1336
4105 7440 7440
4106 5227 5227/ GO TO EVEN POINTS
4107 1372 1372/ MESS: NO
4110 4501 4501
4111 5522 5522/ MONITOR
4112 0000 0000/ SUBROUTINE TO GET SPEC POINT
4113 1410 1410
4114 3045 3045
4115 3046 3046
4116 1327 1327
4117 3044 3044
4120 4407 4407
4121 7000 7000
4122 4063 4063
4123 7000 7000/DIV BY LAMBDA
4124 0000 0000
4125 5712 5712
4126 7777 7777/-1
4127 0013 0013/13
4130 0000 0000/ OS ST
4131 0000 0000
4132 0000 0000
4133 0000 0000/ LLIM
4134 0000 0000
4135 0000 0000

```

4136	0000	0000/ CHEK
4137	0000	0000
4140	0000	0000
4141	0000	0000/ SPEC
4142	0000	0000
4143	0000	0000
4144	0003	0003/FL 4
4145	2000	2000
4146	0000	0000
4147	0002	0002/ FL 2
4150	2000	2000
4151	0000	0000
4152	0013	0013/FL - 918.0
4153	6152	6152
4154	0000	0000
4155	0002	0002/ FL 3
4156	3000	3000
4157	0000	0000
4160	3414	3414/ MESS: LLIM(A)=
4161	1411	1411
4162	1550	1550
4163	0151	0151
4164	7500	7500
4165	1617	1617/ MESS* NO
4166	0000	0000
4167	3404	3404/MESS: D=
4170	7500	7500
4171	4160	4160
4172	4165	4165
4173	4167	4167

PATCH TO SUPERSPECTRUM FOR CALCULATING ROTATIONAL STRENGTHS
ANSWER WILL BE R*10E+49 X (ESU-CGS UNITS)

C.J. FORMOSO
APRIL 10, 1969

```

*2231
2231 7300 7300
2232 3062 3062
2233 5634 5634
2234 4002 4002
*3431
3431 4000 4000
*4000
4000 7300 7300/ROTATIONAL STRENGTH
4001 5524 5524/LAMBDA= STRTEL, INCREM=INCR
4002 7300 7300
4003 1371 1371/ MESS: LLIM(A)=
4004 4501 4501
4005 6036 6036
4006 4405 4405
4007 4407 4407
4010 6333 6333
4011 5330 5330
4012 2330 2330/ OS ST = 0
4013 6330 6330
4014 0000 0000
4015 1163 1163
4016 1326 1326
4017 3010 3010
4020 4312 4312/ FIRST POINT
4021 4407 4407
4022 6330 6330
4023 5063 5063/INCREMENT LAMBDA
4024 1066 1066
4025 6063 6063
4026 0000 0000
4027 7300 7300/EVEN POINTS
4030 4312 4312
4031 4407 4407
4032 3344 3344/ X 4
4033 1330 1330/ + OSST
4034 6330 6330
4035 5063 5063
4036 1066 1066/INCREM LAMBDA
4037 6063 6063
4040 2333 2333
4041 7000 7000
4042 6336 6336
4043 0000 0000
4044 7300 7300/CHECK LLIM
4045 1336 1336
4046 7440 7440
4047 5267 5267/ GO TO ODD POINTS
4050 4312 4312/ LAST POINT

```

4051	4407	4407
4052	1330	1330
4053	3154	3154/X E
4054	4151	4151//OD
4055	3352	3352/ X -0.0004318
4056	3066	3066/ X INCREM
4057	4355	4355/ DIV BY 3
4060	0000	0000
4061	7300	7300
4062	1373	1373/ MESS: F=
4063	4501	4501
4064	7300	7300
4065	4406	4406
4066	5522	5522/GO TO MONITOR
4067	4312	4312/ODD POINTS
4070	4407	4407
4071	3347	3347/ X 2
4072	1330	1330/ + OSST
4073	6330	6330
4074	5063	5063
4075	1066	1066
4076	6063	6063
4077	2333	2333/ INC LAMBDA AND CHECK LLIM
4100	7000	7000
4101	6336	6336
4102	0000	0000
4103	7300	7300
4104	1336	1336
4105	7440	7440
4106	5227	5227/ GO TO EVEN POINTS
4107	1372	1372/ MESS: NO
4110	4501	4501
4111	5522	5522/ MONITOR
4112	0000	0000/ SUBROUTINE TO GET SPEC POINT
4113	1410	1410
4114	3045	3045
4115	3046	3046
4116	1327	1327
4117	3044	3044
4120	4407	4407
4121	7000	7000
4122	4063	4063
4123	7000	7000/DIV BY LAMBDA
4124	0000	0000
4125	5712	5712
4126	7777	7777/C-1
4127	0013	0013/C13
4130	0000	0000/ OS ST
4131	0000	0000
4132	0000	0000
4133	0000	0000/ LLIM
4134	0000	0000
4135	0000	0000
4136	0000	0000/ CHEK
4137	0000	0000
4140	0000	0000

4141	0000	0000/ SPEC
4142	0000	0000
4143	0000	0000
4144	0003	0003/FL 4
4145	2000	2000
4146	0000	0000
4147	0002	0002/ FL 2
4150	2000	2000
4151	0000	0000
4152	0016	0016/FL -6956.0
4153	6232	6232
4154	1000	1000
4155	0002	0002/ FL 3
4156	3000	3000
4157	0000	0000
4160	3414	3414/ MESS: LLIM(A)=
4161	1411	1411
4162	1550	1550
4163	0151	0151
4164	7500	7500
4165	1617	1617/ MESS* NO
4166	0000	0000
4167	3422	3422/MESS: R=
4170	7500	7500
4171	4160	4160
4172	4165	4165
4173	4167	4167

PATCH TO SUPERSPECTRUM FOR CALCULATING ROTATIONAL OSCILLATOR
STRENGTH. ANSWER WILL BE C*10 E+10 (UNITLESS)

C.J. FORMOSO
APRIL 10, 1969

```

*2231
2231 7300 7300
2232 3062 3062
2233 5634 5634
2234 4002 4002
*3431
3431 4000 4000
*4000
4000 7300 7300/ROTATIONAL OSCILLATOR STRENGTH
4001 5524 5524/LAMBDA= STRTWL, INCREM=INCR
4002 7300 7300
4003 1371 1371/ MESS: LLIM(A)=
4004 4501 4501
4005 6036 6036
4006 4405 4405
4007 4407 4407
4010 6333 6333
4011 5330 5330
4012 2330 2330/ OS ST = 0
4013 6330 6330
4014 0000 0000
4015 1163 1163
4016 1326 1326
4017 3010 3010
4020 4312 4312/ FIRST POINT
4021 4407 4407
4022 6330 6330
4023 5063 5063/INCREMENT LAMBDA
4024 1066 1066
4025 6063 6063
4026 0000 0000
4027 7300 7300/EVEN POINTS
4030 4312 4312
4031 4407 4407
4032 3344 3344/ X 4
4033 1330 1330/ + OSST
4034 6330 6330
4035 5063 5063
4036 1066 1066/INCREM LAMBDA
4037 6063 6063
4040 2333 2333
4041 7000 7000
4042 6336 6336
4043 0000 0000
4044 7300 7300/CHECK LLIM
4045 1336 1336
4046 7440 7440
4047 5267 5267/ GO TO ODD POINTS
4050 4312 4312/ LAST POINT
4051 4407 4407

```

4052	1330	1330
4053	3154	3154/X E
4054	4151	4151//OD
4055	3352	3352/ X -0.0004318
4056	3066	3066/ X INCREM
4057	4355	4355/ DIV BY 3
4060	0000	0000
4061	7300	7300
4062	1373	1373/ MESS: F=
4063	4501	4501
4064	7300	7300
4065	4406	4406
4066	5522	5522/GO TO MONITOR
4067	4312	4312/ODD POINTS
4070	4407	4407
4071	3347	3347/ X 2
4072	1330	1330/ + OSST
4073	6330	6330
4074	5063	5063
4075	1066	1066
4076	6063	6063
4077	2333	2333/ INC LAMBDA AND CHECK LLIM
4100	7000	7000
4101	6336	6336
4102	0000	0000
4103	7300	7300
4104	1336	1336
4105	7440	7440
4106	5227	5227/ GO TO EVEN POINTS
4107	1372	1372/ MESS: NO
4110	4501	4501
4111	5522	5522/ MONITOR
4112	0000	0000/ SUBROUTINE TO GET SPEC POINT
4113	1410	1410
4114	3045	3045
4115	3046	3046
4116	1327	1327
4117	3044	3044
4120	4407	4407
4121	7000	7000
4122	4063	4063
4123	4063	4063/ DIV BY LAMBDA SQ
4124	0000	0000
4125	5712	5712
4126	7777	7777/C-1
4127	0013	0013/C13
4130	0000	0000/ OS ST
4131	0000	0000
4132	0000	0000
4133	0000	0000/ LLIM
4134	0000	0000
4135	0000	0000
4136	0000	0000/ CHEK
4137	0000	0000
4140	0000	0000

4141	0000	0000/ SPEC
4142	0000	0000
4143	0000	0000
4144	0003	0003/FL 4
4145	2000	2000
4146	0000	0000
4147	0002	0002/ FL 2
4150	2000	2000
4151	0000	0000
4152	0013	0013/FL -328.0
4153	7270	7270
4154	0000	0000
4155	0002	0002/ FL 3
4156	3000	3000
4157	0000	0000
4160	3414	3414/ MESS: LLIM(A)=
4161	1411	1411
4162	1550	1550
4163	0151	0151
4164	7500	7500
4165	1617	1617/ MESS* NO
4166	0000	0000
4167	3403	3403/MESS: C=
4170	7500	7500
4171	4160	4160
4172	4165	4165
4173	4167	4167

PATCH WHICH RESTORES SUPERSPECTRUM TO NORMAL OPERATION

C. J. Formoso
April 10, 1969

		*2231
2231	7604	7604
2232	7010	7010
2233	7420	7420
2234	5267	5267
		*3431
3431	1544	1544

APPENDIX B
KINETICS OF THE NaBH_4 REDUCTION

In preparing the dimers containing dihydrouridine, it was noted that the product yield varied with the particular dimer. Though the reaction mechanism is unknown, these yields can be meaningfully compared since the reactions were carried out under equivalent conditions. From Table 3 (Section IV) it can be seen that, of the dimers, UpU is most extensively reduced, followed by UpA, ApU, and GpU in that order. Thus the reaction rate is sensitive to some property of the dimer. It would be interesting if this property was stacking or could be functionally related to stacking. Since the monomer U is reduced more rapidly than the dimers, and it cannot stack, it may be possible to do this. The yields may also reflect changes in the pK of the uracil residue, the ionization of U being apparently important in the photoreduction. Ionization of U may also reduce stacking, but a stacking interaction could in turn influence the pK of U. Thus stacking may be responsible for the observed yields.

Looking at the yields of the open-ring dimers the order $\text{Gp}\beta > \text{Ap}\beta > \beta\text{pA}$ is seen. This may again be related to stacking, but here it is harder to compare the monomer with the dimers. We can do this, and also get a rough estimate of the relative rates of reaction, by making a simple kinetic analysis. It should be pointed out that this analysis is being made on reactions designed for another purpose; the production of dihydrouridine. No effort was made to precisely control the

reaction conditions. However, the range of product yield appears too large to be entirely due to the small variations which may have existed in the reaction conditions. Since we do not know the kinetics of the reactions, let us assume for our purposes that both the photoreduction and the dark reduction follow second order kinetics and that the concentration of NaBH_4 is constant. This, in essence, means we are assuming first order reactions. The NaBH_4 concentration is ten or twenty times that of the uracil concentration, so using first order kinetics will not be much worse than using second order kinetics. Writing the reaction for ApU:

where $k_1 = [\text{BH}_4^-]k_1'$, and $k_2 = [\text{BH}_4^-]k_2'$. Writing the rate equation for ApU,

$$(1) \quad \frac{d(\text{ApU})}{dt} = -k_1(\text{ApU})$$

and integrating from $t=0$ to t gives:

$$(2) \quad \ln \frac{(\text{ApU})}{(\text{ApU})_0} = -k_1 t \quad \text{or} \quad \frac{(\text{ApU})}{(\text{ApU})_0} = e^{-k_1 t}$$

The rate equation for ApH,

$$(3) \quad \frac{d(\text{ApH})}{dt} = k_1 (\text{ApU}) - k_2 (\text{ApH}), \text{ when } k_2 (\text{ApH}) \text{ is assumed small (see values in Table 3)}$$

is integrated to

$$(4) \quad (\text{ApH}) = k_1 (\text{ApU})_0 \frac{1 - e^{-k_1 t}}{k_1} = (\text{ApU})_0 (1 - e^{-k_1 t}).$$

Using this expression in the rate equation for $Ap\beta$,

$$(5) \quad \frac{d(Ap\beta)}{dt} = k_2(ApH) = k_2(ApU)_o(1-e^{-k_1t})$$

and integrating gives:

$$(6) \quad (Ap\beta) = \frac{k_2}{k_1} (ApU)_o(k_1t + e^{-k_1t} - 1).$$

Using values from Table 3 and Equation (2), k_1 and k_1' can be determined. Once k_1 is known the value of k_2 can be found using Equation (6). The values for k_1' and k_2' are listed in Table 5. The analysis could not be carried out for UpU, but k_1' would obviously be larger than for the other dimers. The assumption made in Equation (3) may not be valid for GpU; with a steady state approximation the larger value of 1.5 for k_2' is obtained. The k_2' for H was calculated as:

$$k_2' = (2 \cdot 10^{-2} t)^{-1} \ln \frac{(OD_{230})_o}{OD_{230}}.$$

Table 5

Calculated Rate Constants for the $NaBH_4$ Reduction

Reactant	k_1' (liters mole ⁻¹ min ⁻¹)	k_2' (liters mole ⁻¹ min ⁻¹)
ApU	0.6	0.07
UpA	1.8	0.008
GpU	0.4	0.9 (1.5)
U	4.6	---
H	---	3.6
ψ	5.9	---

An ordering of rate constants can be seen, with the monomers having the largest values in both the photoreduction and the dark reduction. Both reactions look promising as tools for the investigation of stacking. A study of the statistical variation in reaction yields under carefully controlled conditions is needed; and also a more profound kinetic analysis would be necessary before anything could be said.

If we assume for the moment that the rate constants in Table 5 can be used as an index of stacking, we can compare the predicted stacking order to that determined by other methods. This is done in Table 6. Unfortunately, there is no previous work on stacking at pH 9.5 for comparison. There is some agreement seen, particularly that UpU stacks least by both optical properties and photoreduction. GpU stacks most at pH 11.5 by optical properties and at pH 9.5 by photoreduction. The order determined by ΔH° values does not agree with that predicted by the kinetics. The two methods probably measure different aspects of stacking: ΔH° reflects the energy required to displace the dimer from its equilibrium conformation; while the kinetics are influenced by other factors, such as ionization or shielding at a critical position. The order of stacking for the dihydroU dimers predicted by optical properties and by the rate constants shows fair agreement, considering the difference in pH.

Table 6
A Comparison of the Order of
Stacking Predicted by Various Methods

Conditions	Method	Order
pH 9.5, 50°C	photoreduction	GpU > ApU > UpA > UpU
pH 7, 25°C	optical properties ⁷²	ApU > UpA > GpU > UpU
pH 11.5, 25°C	optical properties ⁷²	GpU > UpA > ApU > UpU
pH 7	ΔH° ⁴²	ApU > UpU > GpU > UpA
pH 9.5, 50°C	dark reduction	HpA > ApH > GpH
pH 7.3, 25°C	optical properties*	HpA > GpH $\gtrsim$ ApH

* This work, Section IV.

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