Oligonucleotide Studies. III. Optical Rotatory Dispersion of Seven Trinucleotides Obtained from Ribonuclease T_1 Digests[1]

Yasuo Inoue, Shohei Aoyagi, and Koji Nakanishi

Contribution from the Department of Chemistry, Tohoku University, Sendai, Japan.
Received July 7, 1967

Abstract: The optical rotatory dispersion and hypochromism were measured at pH 1, 7, and 11 for seven trinucleotides, ApApGp, ApCpGp, ApUpGp, CpApGp, CpCpGp, UpApGp, and UpUpGp, which were obtained from Taka-Diastase ribonuclease T_1 digestion of yeast ribonucleic acids. The ORD and hypochromicity criteria for a trinucleotide conformation indicate that ApApGp, ApCpGp, CpApGp, CpCpGp, and UpApGp exist in the stacked conformation at pH 7; trimers having one or no protolytic base (uridylyl or guanylyl residue) still exist in the stacked form at pH 11 whereas those having two or three protolytic bases are present in random conformations at this pH. CpCpGp seems to be the only trinucleotide of the compounds studied here to exhibit appreciable retention of the stacking effect at pH 1. The importance of a 3′-terminal phosphate group in the conformation of a dinucleotide is suggested from the observation that the agreement between the ORD curves made up from nearest neighbor calculations, assuming a negligible effect of a 3′-terminal phosphate of a trinucleotide on the ORD, and those obtained experimentally is rather poor.

Optical rotatory dispersion (ORD) is regarded as the most powerful physical method in the characterization of the molecular geometry of oligo- and polynucleotides of different base composition and nucleotide sequence. Consequently, many recent reports have appeared where ORD studies of oligonucleotides have been used to shed light on their conformational effects.[2-7] Although the work on the dinucleoside phosphates is quite extensive and covers the 16 possible combinations made up from the four major bases of ribonucleic acids (RNA),[7] the study of higher oligonucleotides has been restricted to those obtained either from bovine pancreatic ribonuclease IA (EC 2.7.7.16) digestion of RNA or from a partial degradation of synthetic polynucleotides. Recently, we have succeeded in the isolation in pure form of seven trinucleotides (ApApGp, ApCpGp, ApUpGp, CpApGp, CpCpGp, UpApGp, and UpUpGp)[8] out of nine possible trinucleotides[9] produced on Taka-Diastase ribonuclease T_1 (EC 2.7.7.26) digestion of high molecular weight RNA.[10] Since RNase T_1 is a guanylic acid specific endoribonuclease,[11] the terminal residue of the oligonucleotides produced is exclusively guanylic acid. Therefore, a wider variety of trinucleotides being classified by three categories of base content ratios of pyrimidine to purine, *i.e.*, 0.5(py-pu-pu, pu-py-pu), 0(pu-pu-pu), and 2(py-py-pu), can be obtained from RNase T_1 digests, whereas the corresponding base content ratio is limited to 0.5(pu-pu-py) in trinucleotides from RNase IA digestion.

Thus, the purpose of this paper is to report the results of an ORD study of seven trinucleotides in the light of identification of these oligonucleotides by means of ORD and thereby to increase our understanding of RNA conformations.

Experimental Section

1. Preparation of Trinucleotides. Yeast RNA (600 mg) was digested with RNase T_1 (A_{278} = 14) at 37°. After removal of proteins, the digest was chromatographed on a DEAE Sephadex A-25 column (3.9 × 56 cm) in the presence of 7 *M* urea according to the method of Rushizky, *et al.*[12] The portion of trimer peak was freed from urea and salts and again chromatographed on a Dowex 1 × 2 column (2.6 × 75 cm) at pH 2.2 with an increasing NaCl concentration gradient. A typical elution pattern is reproduced in Figure 1. The details of the identification and purity of each trinucleotide were the same as those reported in a previous paper.[10] The separation of a pair of sequence isomers, CpUpGp and UpCpGp, has been tried under several different conditions, but has not yet been successful.

2. ORD Measurements. The salt-free solid samples were dissolved in about 15 ml of 0.1 *M* NaCl to give a solution with $A_{260} \simeq 1.1$. Exact absorption measurements were made with a Hitachi Perkin-Elmer 139 spectrophotometer. The pH settings at 1, 7, and 11 (or 11.5) were made with a pH meter Model TTT1b (Radiometer) by adding concentrated acid (70% $HClO_4$) or alkali (10 *N* NaOH) through a microsyringe, the volume change caused by this procedure being less than 0.45% of the total solution.

ORD measurements were made at 20° on a JASCO Model ORD/VU-5 spectropolarimeter with a 1-cm quartz cell. To ascertain the reproducibility, measurements were repeated at least twice for each sample. Blanks were determined just before or after measurement of each sample.

3. Treatment of the Results. Net values of optical rotation were read at 2-mμ intervals after averaging the noise of ORD curves and blanks. Rotations were expressed in molar rotation per residue, $[\phi]$, defined as

$$[\phi] = \frac{[M]}{n} = \frac{1}{n}[\alpha]\frac{\text{molecular weight}}{100}$$

where n is the number of base residues in a molecule, [M] the molecular rotation, and $[\alpha]$ the specific rotation. Molarity of the sample solutions were determined by the A_{260} at neutral pH and ϵ_{260} value for each trinucleotide.

(1) Part II in this series: Y. Inoue, S. Aoyagi, and K. Nakanishi, *Tetrahedron Letters*, 3575 (1967).
(2) M. M. Warshaw, C. A. Bush, and I. Tinoco, Jr., *Biochem. Biophys. Res. Commun.*, 18, 633 (1965).
(3) M. M. Warshaw and I. Tinoco, Jr., *J. Mol. Biol.*, 13, 54 (1965).
(4) C. R. Cantor and I. Tinoco, Jr., *ibid.* 13, 65 (1965).
(5) J. Brahms, A. M. Michelson, and K. E. Van Holde, *ibid.*, 15, 467 (1966).
(6) J. N. Vournakis, H. A. Scheraga, G. W. Rushizky, and H. A. Sober, *Biopolymers*, 4, 33 (1966).
(7) M. M. Warshaw and I. Tinoco, Jr., *J. Mol. Biol.*, 20, 29 (1966).
(8) Abbreviations used: ApApGp, adenylyl-(3′–5′)-adenylyl-(3′–5′)-guanosine 3′-phosphate; ApCpGp, adenylyl-(3′–5′)-cytidylyl-(3′–5′)-guanosine 3′-phosphate; ApUpGp, adenylyl-(3′–5′)-uridylyl-(3′–5′)-guanosine 3′-phosphate; etc.
(9) A pair of sequence isomers, CpUpGp and UpCpGp, are not yet resolved.
(10) S. Aoyagi and Y. Inoue, *J. Biol. Chem.*, in press.
(11) F. Egami, K. Takahashi, and T. Uchida, *Progr. Nucleic Acid Res.*, 3, 59 (1964).
(12) G. W. Rushizky, E. M. Bartos, and H. A. Sober, *Biochemistry*, 3, 626 (1964).

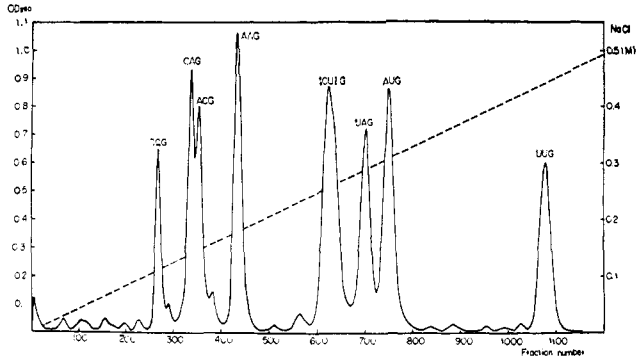

Figure 1. Chromatography of trinucleotide fraction obtained from RNase T_1 digest. Desalted sample (1800 A_{260} unit) was applied to a column (2.6 × 75 cm) of Dowex 1 × 2 (200–400 mesh) in chloride form. The column was then connected to a 12,000-ml linear gradient of 0.00–0.53 *M* NaCl in 0.01 *N* HCl and developed at a flow rate of approximately 160 ml/hr (fraction volume, 9.3 ml).

The data used for the determination of interaction parameters and nearest neighbor calculations were quoted from the literature: nucleosides and 5′-mononucleotides, from ref 13; dinucleoside phosphates, from ref 7.

Results and Discussion

ORD Criteria for Identification of Nucleotide Sequence. Although the absorption spectra are, in general, not sensitive to base sequence, in some cases, a detailed examination of the spectra at different pH's can be easily identified by absorption spectral measurements alone.[10] However, in many cases, especially in pairs of sequence isomers, the absorption spectra at certain pH's are so similar that determination of the nucleotide sequence by these spectra alone is difficult. However, the ORD criteria for the identification of sequence isomers are more conveniently used as was demonstrated in a pair of sequence isomers, ApGpU and GpApU.[4] These ORD criteria are also valid for the two pairs of sequence isomers obtained for the first time in this study, ApCpGp and CpApGp, and ApUpGp and UpApGp (Figure 2).

Figure 3 shows that the ultraviolet absorption curves of CpCpGp, for example, are very different in going from acidic to alkaline through neutral pH's, since nucleotide components undergo protonation and deprotonation equilibria. At the corresponding pH's, the anomalous ORD curves also exhibit drastic changes both in magnitude and shape of the Cotton effects. Since the rotational strength is expressed in terms of the dot product of the electric dipole and magnetic dipole moments for a certain transition,[14] a decrease in the rotational strength at 289 mμ with an increase in ultraviolet absorption observed in going from neutral to acidic pH's should be attributable to changes in the conformational asymmetry of the secondary structure of CpCpGp (Figure 3 and Table I). Values of the molecular rotation per residue at selected peaks and troughs at three pH's are given in Table I with those of

Table I. Absorption and ORD Extrema of Seven Trinucleotides

pH	Trinucleotide	λ_{max} mμ	λ_{max} $\epsilon \times 10^{-3}$	pk_1 mμ	pk_1 $[\phi] \times 10^{-3}$	tr_1 mμ	tr_1 $[\phi] \times 10^{-3}$
	ApApGp	258	39.7	272	0.7	250	−2.9
	ApCpGp	261	30.2	286	1.6	265	−2.6
	ApUpGp	260	34.0	280	1.9	261	−2.0
1	CpApGp	259	30.4	286	3.0	270	−0.9
	CpCpGp	278	30.6	297	7.6	275	−5.9
	UpApGp	259	34.0	281	3.2	250	−1.8
	UpUpGp	261	28.9	280	3.1	255	−7.8
	ApApGp	258	35.3	284	5.5	260	−18.9
	ApCpGp	259	29.6	286	6.8	260	−11.7
	ApUpGp	259	33.6	283	2.2	259	−3.2
7	CpApGp	258	30.3	288	5.6	265	−12.0
	CpCpGp	267	23.3	289	12.1	260	−16.3
	UpApGp	258	32.9	282	7.2	267	−0.9
	UpUpGp	259	29.0	283	3.2	257	−8.3
	ApApGp	259	36.4	282	4.1	258	−16.8
	ApCpGp	262	29.7	286	7.0	258	−10.3
	ApUpGp	261	31.9	278	1.6	247	−6.0
11[a]	CpApGp	261	30.4	284	5.8	260	−11.0
	CpCpGp	269	25.0	289	12.3	259	−12.3
	UpApGp	260	31.9	284	2.4	244	−4.3
	UpUpGp	262	24.7	275	4.7	245	−10.2

[a] Absorption data are at pH 13.

together with hypochromicity measurements makes it possible to determine nucleotide sequences of di- and trinucleotides.[10] In the case of the di- and trinucleotides obtained from RNase T_1 digests, markedly characteristic spectra were observed for ApGp, CpGp, ApApGp, and CpCpGp due to changes in base composition and chain length, and thus these nucleotides molar extinction coefficient at absorption maxima for the seven trinucleotides.

Molecular Geometry of Trinucleotides. Base–Base Interactions. pH dependence of ORD curves is representatively shown for ApApGp and UpUpGp in Figure 4. The rotational strength of ApApGp is greatly reduced at pH 1 relative to that at pH 7, whereas the

(13) J. T. Young, T. Samejima, and P. K. Sarkar, *Biopolymers*, 4, 623 (1966).

(14) C. Y. Lin, D. W. Urry, and H. Eyring, *Biochem. Biophys. Res. Commun.*, 17, 642 (1964).

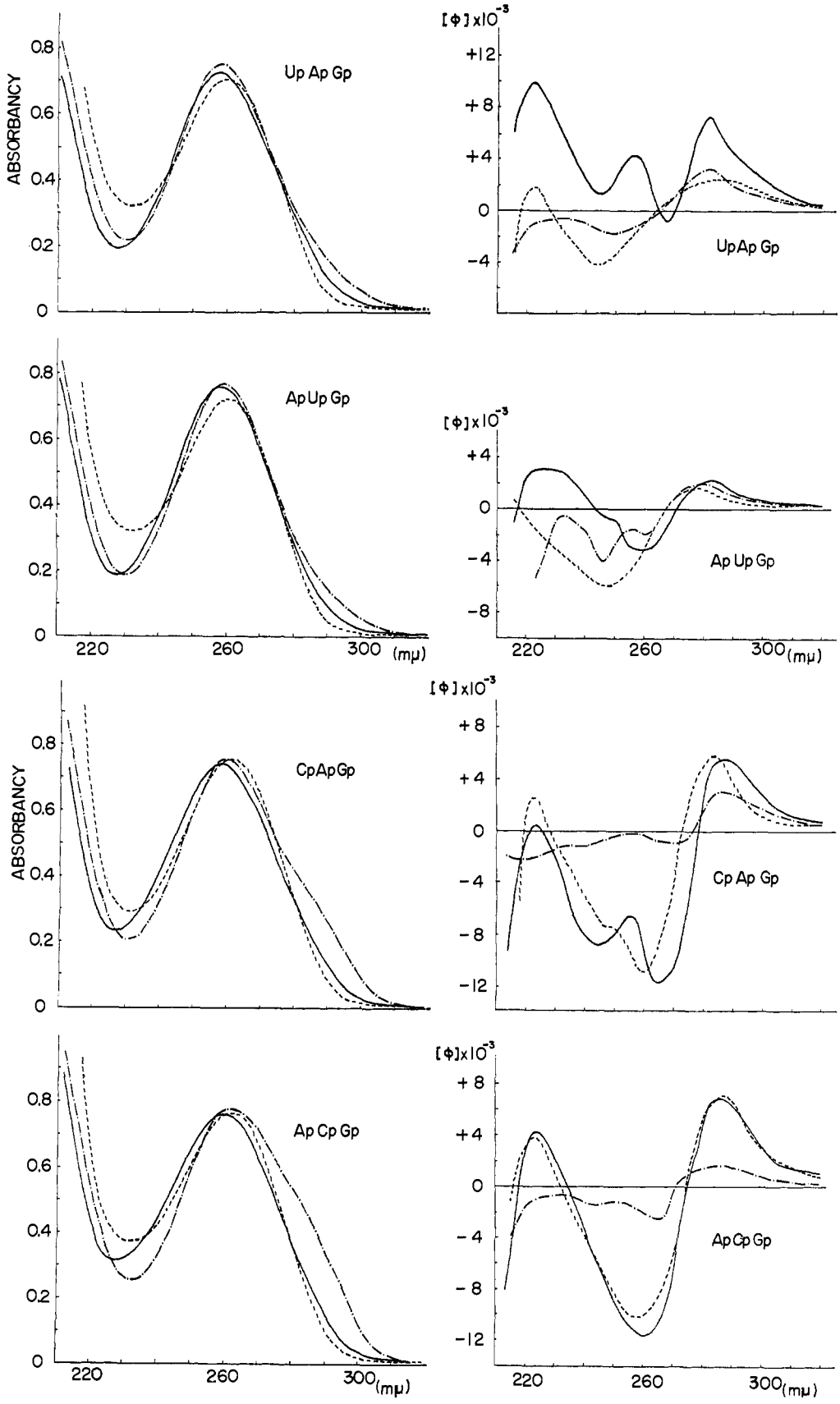

Figure 2. Ultraviolet absorption and ORD (in molecular rotation per residue) of two pairs of sequence isomers of trinucleotides, ApCpGp and ApUpGp, CpApGp and UpApGp, at pH 1, — · —; 7, ———; and 11, - - - - (absorption data, at pH 13). The ionic strength is 0.1.

ORD of UpUpGp is rather similar at these pH's. Furthermore, the ORD of ApApGp at pH 1 and the curves of UpUpGp at pH 1 and 7 are found to be quite similar to the corresponding curves made up of the sum of the molecular rotations of the component mononucleotides, indicating that at pH 1 both ApApGp

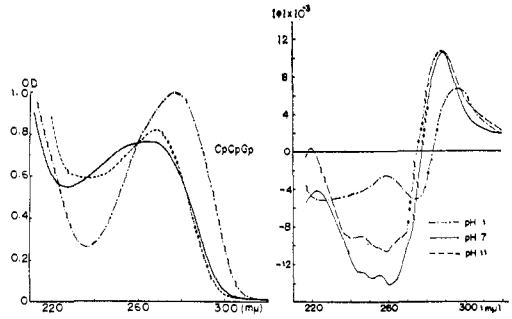

Figure 3. Absorption and molecular rotation per residue of CpCpGp at pH 1, — · —; 7, ———; and 11, - - - - (absorption data, at pH 13); and an ionic strength of 0.1.

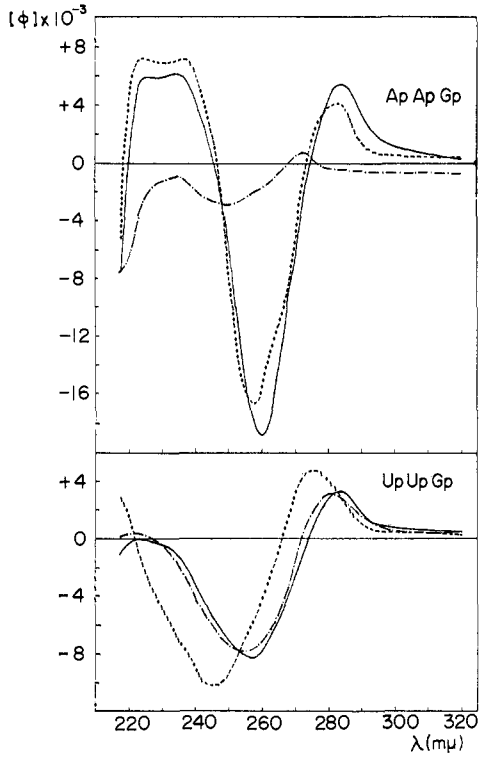

Figure 4. pH dependence of the ORD of ApApGp and UpUpGp: pH 1, — · —; pH 7, ———; pH 11, - - - -.

and UpUpGp are substantially in random conformation. Minor change in the ORD and absorption spectra observed for UpUpGp in going from neutral to acidic pH may be attributable to the fact that UpUpGp has random conformation even at pH 7 and is not much protonated at pH 1 (basic p*K*: adenylic acid, 3.7; cytidylic acid, 4.2; guanylic acid, 2.4; uracil, *ca.* 0.5). The hard-to-be-protonated nature of UpUpGp is consistent with the observation that on fractionating the trinucleotide fraction from RNase T_1 digest according to the sequence, UpUpGp is eluted in the last peak under the acidic elution condition used (see Figure 1).

At pH 11, the ORD curves of ApApGp, ApCpGp, CpApGp, and CpCpGp are not much different from those at pH 7, letting us predict that these trinucleotides are still appreciably stacked at this pH.

Although at pH 7 the ORD of ApUpGp does not much differ from the sum of the molecular rotation of its component monomers, its sequence isomer,

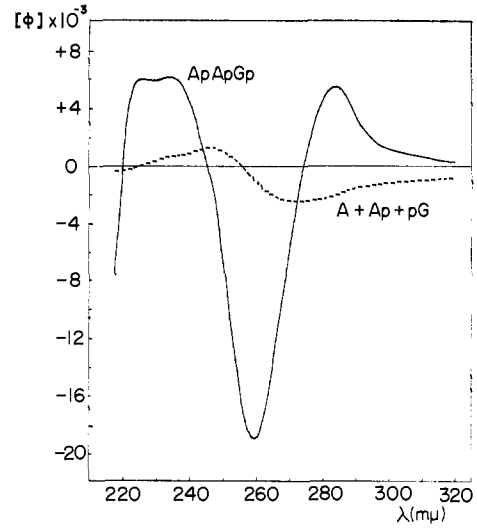

Figure 5. The ORD (in molecular rotation per residue) of ApApGp and its corresponding components (A + pA + pG) at pH 7 and an ionic strength of 0.1.

UpApGp, shows considerable difference in the ORD curve. Thus, we expect that ApUpGp is unstacked whereas UpApGp is stacked.

Since it has been reported[4,7] that a 3′-terminal phosphate does not substantially change the conformation of a trinucleoside diphosphate, the absolute value of maximum difference in molecular rotation between trinucleotide, say, ApCpGp, and its component monomers, A + pC + pG, for wavelengths longer than 230 mμ, *viz.*, $|[M]_t - \Sigma[M]_m|_{max}$, was employed as ORD criterion of stacking (see also ref 4).

For simplicity, only the results for ApApGp at pH 7 are shown graphically in Figure 5. Values of $|[M]_t - \Sigma[M]_m|_{max}$ at pH 7 are given in Table II with hypochromicity data.

Table II. Per Cent of Hypochromicity at 260 mμ and $|[M]_t - \Sigma[M]_m|_{max}$

Trinucleotide	$\lvert[M]_t - \Sigma[M]_m\rvert_{max}{}^a \times 10^{-4}$ (mμ)	h,[b] % pH 1	pH 7	pH 13
ApApGp	5.38 (259)	1.9	17.9	13.0
ApCpGp	2.70 (261)	8.2	13.6	13.8
ApUpGp	0.83 (280)	4.8	9.3	6.5
CpApGp	2.49 (268)	7.4	13.1	11.5
CpCpGp	3.18 (267)	9.9	11.2	14.5
UpApGp	2.24 (257)	5.7	11.7	6.5
UpUpGp	0.86 (264)	8.2	7.8	6.5

[a] $|[M]_t - \Sigma[M]_m|_{max} = |[M]_{XpYpZp} - ([M]_X + [M]_{pY} + [M]_{pZ})|_{max}$; at pH 7. [b] $h = [(A_{Xp} + A_{Yp} + A_{Zp} - A_{XpYpZp})/(A_{Xp} + A_{Yp} + A_{Zp})] \cdot 100 = 100[1 - (\epsilon_t/\Sigma\epsilon_m)]$; at 260 mμ.

Tentatively, we have assigned trinucleotides having a $[M]_t - \Sigma[M]_m|_{max}$ value greater than 1×10^4 to those having "stacked" conformation (S), those having $\sim 1 \times 10^4$ to "partially stacked" conformation ($^1/_2$S), and those having less than 1×10^4 to "unstacked" conformation (U). A similar classification of conformations can also be made tentatively on the basis of the hypochromicity at 260 mμ by assuming that the hypochromicity at 260 mμ is taken as a measure of the conformation of these trinucleotides, that is, a semiquantitative measure of the degree of stacking of bases

[h, % (conformation)]: >10, (S); ~10, ($^1/_2$S); <10, (U). In Table III the results concerning the conformation of seven trinucleotides are summarized.

Table III. Conformation of Seven Trinucleotides from RNase T_1 Digest

Trinucleotide	pH 1	pH 7	pH 11
ApApGp	U	S	S
ApCpGp	U	S	S
ApUpGp	U	U	U
CpApGp	U	S	S
CpCpGp	$^1/_2$U	S	S
UpApGp	U	S	U
UpUpGp	U	U	U

Protonation on the bases of these trinucleotides causes destacking and the formation of disordered structures, but CpCpGp seems to be the only trinucleotide of the compounds studied here to exhibit appreciable retention of the stacking effect at pH 1. In alkaline media in which complete ionization of the guanylic acid portion (acidic pK of guanylic acid, 9.3) is attained, the unstacking effect is not so marked and in ApApGp, ApCpGp, CpApGp, and CpCpGp the stacking conformations are retained at pH 11. However, UpApGp presumably forms an extra negative charge on the uridylyl residue (acidic pK of uridylic acid, 9.4) to exist in a hexaanion at pH 11, and, thus, a repulsion of the two negative charges on the bases causes destacking. When the present observations are combined with the previous results by Cantor and Tinoco of seven trinucleoside diphosphates obtained from RNase IA digest, one can safely say that trimers having one or no protolytic base (uridylyl or guanylyl residue) still exist in the stacked form at pH 11, and trimers having two or three protolytic bases are present in random conformation at this pH.

Stacking of the nearest bases in the pair of sequence isomers, ApCpGp and CpApGp, are more or less comparable at pH 1, 7, and 11, since both the hypochromicity and $|[M]_t - \Sigma[M]_m|_{max}$ values are quite similar in both isomers, whereas in another pair of the isomers, ApUpGp and UpApGp, the former is less stacked at pH 7 than the latter. These findings are unique for the trinucleotides from RNase T_1 digests, and may be compared to the previous observation by Ts'o, *et al.*,[15] *i.e.*, insertion of a uridyl residue into the purines exerts a destacking effect. Thus, for the two pairs of sequence isomers, the stacking at neutral pH decreases in the order of ApCpGp $\gtrsim$ CpApGp > UpApGp > ApUpGp.

In Figure 6, the results of nearest neighbor calculations of the ORD curve is shown for CpApGp as an example. The calculations were made by using eq 9 in ref 4. On applying this equation to the trinucleotides studied in this work, we assumed that the effect of a 3′-terminal phosphate on the ORD is negligible, *i.e.*

$$[\phi]_{XpYpZp} \simeq [\phi]_{XpYpZ} = \frac{2[\phi]_{XpY} + 2[\phi]_{YpZ} - [\phi]_Y}{3}$$

(15) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, 87, 524 (1965).

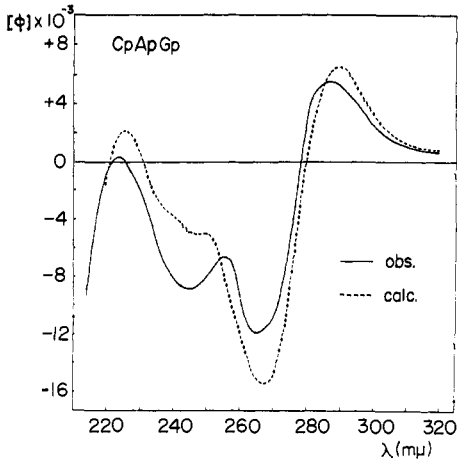

Figure 6. Calculated and measured ORD curves of CpApGp at pH 7 and an ionic strength of 0.1.

Although the shapes of the ORD curves are similar in both calculated and observed spectra, the agreement between calculation and experiment is rather poor. At present, however, we cannot draw any definite conclusion as to whether the disagreement is enhanced in trinucleotides more than in trinucleoside diphosphates. Nevertheless, we feel that a 3′-terminal phosphate is a significant factor in the formation of ordered conformations in oligonucleotides, and consequently to their ORD and hypochromicity, and that the importance of the 3′-phosphate group may diminish with increasing chain length of oligonucleotides, that is, as the ratio of base to phosphate (B/P) approaches unity in n-nucleoside (n − 1)-phosphate. The fact that in naturally occurring RNA the B/P ratio is unity strongly indicates that the dinucleotide (B/P = 1) should be used as a unit structure in the nearest neighbor calculations rather than dinucleoside phosphate (B/P = 2), provided that the effect of the terminal phosphate on the ORD is not small in oligonucleotides.[16] In fact, the order of the stacking effect in the dinucleotides from RNase T_1 digest was found to be ApGp > CpGp > UpGp at pH 7 from the hypochromicity at 260 mμ and the ORD criteria (S. Aoyagi, unpublished results). This order differs from the order which can be drawn from hypochromicity data and the ORD data reported[7] by Warshaw and Tinoco for the corresponding dinucleoside phosphates, ApG $\gtrsim$ UpG > CpG, indicating that the additional 3′-terminal phosphate residue does have an effect on the conformations. The presence of the terminal phosphate effect on the conformation seems to have been observed also by Davis in California.[17] With a view to understanding this terminal phosphate

(16) For the nearest neighbor calculations of trinucleotides and trinucleoside diphosphates, the following equations should be used instead

$$[\phi]_{XpYpZp} = \frac{2[\phi]_{XpYp} + 2[\phi]_{YpZp} - [\phi]_Y}{3}$$

and

$$[\phi]_{XpYpZp} = \frac{2[\phi]_{XpYp} + 2[\phi]_{YpZ} - [\phi]_Y}{3}$$

(17) Quoted from C. R. Cantor and I. Tinoco, Jr., *J. Mol. Biol.*, **13**, 72 (1965).

effect, we are now undertaking the preparation and measurement of the ultraviolet ORD of 16 possible dinucleotides to compile base–base interaction parameters at any wavelength. Furthermore, for a better understanding of the molecular geometry of RNA of known nucleotide sequence through extention of the nearest neighbor calculations,[18] it seems essential to obtain some basic information on the conformations of minor base-containing oligonucleotides, since, for example, alanine-specific *t*-RNA and serine-specific *t*-RNA contain 13 and 15% minor nucleotides per molecule,[19,20] and pseudouridine exhibits a small negative Cotton effect while uridine shows a positive Cotton effect.[21]

Acknowledgments. We wish to thank Professor F. Egami for his interest and encouragement throughout this work. We wish also to thank Sankyo Co., Ltd., Tokyo, for the availability of purified RNase T_1. This study was in part supported by a grant from the Ministry of Education of Japan.

(18) See C. R. Cantor, S. R. Jaskunas, and I. Tinoco, Jr., *J. Mol. Biol.*, **20**, 39 (1966).

(19) R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick, and A. Zamir, *Science*, **147**, 1462 (1965).

(20) H. G. Zachau, D. Dutting, and H. Feldmann, *Angew. Chem.*, **78**, 392 (1966).

(21) T. L. V. Ulbricht, T. R. Emerson, and R. J. Swan, *Biochem. Biophys. Res. Commun.*, **19**, 643 (1965).

Communications to the Editor

The Formation of $Ru(NH_3)_5N_2^{2+}$ in Aqueous Solution by Direct Action of Molecular Nitrogen

Sir:

Since the discovery[1] of $Ru(NH_3)_5N_2^{2+}$ and the elaboration of the class of N_2 complexes,[2] it has been a goal of our own research on reactions of molecular nitrogen to find a reagent which will combine spontaneously with it in water solutions under mild conditions. The isolation of compounds containing N_2 in combination and formed by the reaction of N_2 with metal complexes in nonaqueous media has been accomplished,[3-5] and these results have encouraged us in our pursuit of the stated goal.

We here report evidence that N_2 reacts spontaneously with $Ru(NH_3)_5H_2O^{2+}$ [6] in aqueous solution at room temperature to form Allen and Senoff's ion. This ion shows a strong absorption at 221 mμ with ϵ 1.3 ± 0.1 × 10^4. A solution containing $Ru(NH_3)_5H_2O^{2+}$ at *ca.* 10^{-3} *M* was prepared by the reduction of $Ru(NH_3)_5Cl_3$ in 0.1 *M* H_2SO_4 by amalgamated Zn and was then separated from the reducing agent. When N_2 is bubbled through the solution the absorption peak at 221 mμ develops, and in several hours the absorption corresponds to *ca.* 50% conversion to Allen and Senoff's ion. The rise in absorption itself does not, of course, prove that molecular nitrogen has been brought into reaction. Proof that reaction with N_2 has indeed taken place was provided by the two lines of evidence which are herewith outlined.

A solution 0.10 *M* in $Ru(NH_3)_5Cl_3$ and 0.1 *M* in H_2SO_4 was treated with amalgamated Zn, and argon was passed through. After 45 min the solution was separated from the residual metal, taking care to exclude air; at this stage it showed no significant absorption in the region of interest. A stream of N_2 was now passed through; absorption in the ultraviolet region began to develop promptly, the peak at 221 mμ first appearing as a shoulder on much stronger absorption at 260 mμ. After 6 hr at room temperature, further increase in peak heights was slow. The stream of N_2 was now replaced by a stream of He, and after 45 min an excess of Ce(IV) was added. Nitrogen, identified by gas chromatography, was liberated. The amount was measured and it was found that between 0.5 and 0.6 molecule of N_2 had been trapped for each mole of Ru(II) which was used. Earlier experiments have shown that Ce(IV) in excess releases N_2 rather cleanly from Allen and Senoff's ion.

In another experiment, amalgamated zinc was added to a solution 0.067 *M* in $Ru(NH_3)_5Cl_3$ and 0.1 *M* in H_2SO_4, and N_2 was passed through for 10 hr. Aqueous ammonia was added to bring the pH to *ca.* 9, and the solution was filtered. After several hours a concentrated solution of sodium tetrafluoroborate was added, whereupon a light yellow solid was obtained. The solid was washed with water, acetone, and ether and dried, and the infrared spectrum was taken. The spectrum closely resembled that of genuine $Ru(NH_3)_5N_2(BF_4)_2$ prepared by the method of Allen and Senoff, and in particular a strong, sharp peak at 2130 ± 10 cm^{-1} was observed. The solid material obtained from reaction with molecular N_2 also was found to release N_2 on being oxidized by Ce(IV). The amount of N_2 corresponded to that which would be expected on the basis of the intensity of the absorption at 221 mμ using the molar extinction coefficient measured for Allen and Senoff's ion to calculate the concentration of $Ru(NH_3)_5N_2^{2+}$ in the direct preparation and making allowance for the liberation of N_2 also by the small amount of the species absorbing at 260 mμ which was present in the solid.

The nature of the species showing strong absorption at 260 mμ is not yet clear to us. We have found that

(1) A. D. Allen and C. V. Senoff, *Chem. Commun.*, 621 (1965).
(2) J. P. Collman and J. W. Kang, *J. Am. Chem. Soc.*, **88**, 3459 (1966).
(3) A. Yamamato, S. Kitazume, L. S. Pu, and S. Ikeda, *Chem. Commun.*, 79 (1967).
(4) A. Sacco and M. Rossi, *ibid.*, 316 (1967).
(5) The absorption of N_2 by a solution of $RuCl_3$ or $RuCl_3OH$ in THF and treated with Zn has been reported: A. B. Shilov, A. K. Shilova, and Yu. G. Borod'ko, *Kinetika i Kataliz*, **7** (4), 768 (1966); *Chem. Abstr.*, **65**, 19655*b* (1966).
(6) The aquo ion may be partly converted to the sulfato complex in the presence of sulfate ion. The equilibrium between the two forms is labile and will not greatly affect the observations.