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# Metal(II) Ion Catalyzed Transphosphorylation of Four Homodinucleotides and Five Pairs of Dinucleotide Sequence Isomers†

Hiroshi Ikenaga and Yasuo Inoue*

ABSTRACT: The great rate enhancement in $Zn^{2+}$-catalyzed transphosphorylation was observed for four homodinucleotides, XpXp(3′) in which X represents adenosine (A), cytidine (C), guanosine (G), or uridine (U) relative to XpXp(2′). The relative reactivity has been found to decrease in the order: UpUp(3′) > ApAp(3′) > CpCp(3′) > GpGp(3′), which is the reverse of the order of the affinity of the base moiety toward the metal ion (G > C > A ≥ U). The base-specific nature of the $Zn^{2+}$-catalyzed degradation of dinucleotides has been further examined with five pairs of sequence isomers, such as ApUp(3′) and UpAp(3′). The present results provide new evidence regarding the origins of the base and nucleotide-sequence specificity in the zinc-cleavage reaction at a neutral pH; *i.e.*, the base binding of $Zn^{2+}$ as well as the stacking interaction between bases plays a controlling role in the observed differential rate enhancement. The effects of the pH and of the $Zn^{2+}$/P level on the rate of the $Zn^{2+}$-catalyzed transphosphorylation of ApUp(3′) have been interpreted in terms of a mechanism in which the dinucleotide, which is coordinated to $Zn^{2+}$ to form a reactive 1:1 complex through the *bridging* of $Zn^{2+}$ in a prior equilibrium, undergoes a cleavage reaction. Supporting evidence for this interpretation has been provided by observing the change in the optical properties of selected dinucleotides during $Zn^{2+}$ titration. For comparison, the rates of the $Cu^{2+}$-, $Co^{2+}$-, and $Ni^{2+}$-catalyzed cleavage reactions of four homodinucleotides have also been determined.

The first to investigate the catalytic effect of metal ions on the degradative reactions of RNA were Dimroth *et al.*, who found that nucleosides and nucleotides could be obtained by the degradation of RNA in the presence of $Pb^{2+}$ (Dimroth *et al.*, 1950) and $Zn^{2+}$ (Dimroth *et al.*, 1959; Dimroth and Witzel, 1959), respectively. It has since been shown that the increase in rate of the RNA and synthetic homopolyribonucleotide degradations is caused by the presence of $Cd^{2+}$, $Bi^{3+}$, $Al^{3+}$, $La^{3+}$, $Mn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, and $Co^{2+}$ (Witzel, 1960; Eichhorn and Butzow, 1965; Farkas, 1968; Eichhorn *et al.*, 1971). However, until recently no detailed kinetic study has been made with defined oligoribonucleotides (Butzow and Eichhorn, 1971). Butzow and Eichhorn analyzed their data by considering the effects of the charge of an adjacent phosphate group and the nature of the nearest-neighbor base; the rates of the zinc-cleavage of the internal phosphodiester bonds of adenosine dimers and a trimer were found to decrease in this order: ApAp(3′) (100 ~ 150) ≫ Ap|ApA(~7) > ApAp(2′) (~3) ≥ ApAp > p(2 ~ 3) ≥ ApAp|A(~2) > ApA(~1).

With a view to studying the structure–reactivity correlation in oligonucleotides, a preliminary report on the base-catalyzed transphosphorylation of adenylate dinucleotides has appeared (Koike and Inoue, 1972); this work forms an extension of similar studies of divalent metal ion (mainly $Zn^{2+}$)-catalyzed reactions of various dinucleotides. We have thus examined: (a) the effect of the terminal phosphate group, (b) the effect of the nature of the neighboring base, and (c) the base sequence dependence, if any, on the rate of zinc ion-catalyzed transphosphorylation by using XpX, XpXp(2′), XpXp(3′), and five pairs of sequence isomers, represented by XpYp(3′) and YpXp(3′).

## Experimental Section

*Materials.* Poly(rA) was purchased from Boehringer, Mannheim. Dinucleotides, ApCp(3′), ApUp(3′), GpCp(3′), and GpUp(3′), were obtained from pancreatic ribonuclease digest of yeast RNA in a manner similar to that described by Aoyagi and Inoue (1968a). ApGp(3′), CpGp(3′), and UpGp(3′) were also prepared by the ribonuclease $T_1$ catalyzed degradation of RNA (Aoyagi and Inoue, 1968b). The other dinucleotides used in this study, ApAp(2′), ApAp(3′), CpCp(2′), CpCp(3′), GpGp(2′), GpGp(3′), UpUp(2′), UpUp(3′), CpAp(3′), GpAp(3′), and UpAp(3′), were prepared by a controlled alkaline hydrolysis of RNA in 0.2 N NaOH, followed by column chromatographic separations with DEAE-Sephadex A-25 and Dowex 1-X2 anion exchangers. The details of the procedures are substantially the same as those reported in a previous paper (Satoh and Inoue, 1969). Dinucleoside monophosphates, ApA and UpU, were prepared by the enzymatic dephosphorylation of ApAp and UpUp with *Escherichia coli* alkaline phosphatase at 36° and at a pH of about 8. Ribonuclease $T_1$ and $T_2$ were gifts from Dr. H. Okazaki (Sankyo Co. Ltd.), while ribonuclease IA and alkaline phosphatase were from Boehringer, Mannheim. All the other chemicals, including $Zn(NO_3)_2$, were analytical grade.

† From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo, Japan. *Received July 10, 1973.*

TABLE I: Pseudo-First-Order Rate Constants, $k_{obsd}$, for the $Zn^{2+}$-Catalyzed Transphosphorylation of Homoribodinucleotides and Dinucleoside Monophosphates at 62.1°.

| Substrate | $k_{obsd} \times 10^3$ ($hr^{-1}$) | $k_{XpXp(3')}/k_{XpXp(2')}$ |
|---|---|---|
| ApAp(2′) | 11.5 | 15.5 |
| ApAp(3′) | 178 | |
| ApA | 2.0 | |
| UpUp(2′) | 6.4 | 32.3 |
| UpUp(3′) | 207 | |
| UpU | 1.1 | |
| GpGp(2′) | 7.9 | 4.3 |
| GpGp(3′) | 33.9 | |
| CpCp(2′) | 5.4 | 17.1 |
| CpCp(3′) | 92.1 | |

*Methods.* The initial reaction mixtures for the divalent metal ion catalyzed transphosphorylations of dinucleotides and dinucleoside monophosphates were made by mixing $M(NO_3)_2$ and a substrate to a final substrate concentration of $5.0 \times 10^{-4}$ M [*i.e.*, $1.0 \times 10^{-3}$ M per residue], and with $[Zn^{2+}]/[XpY(p)] = 2.0$; then the pH was adjusted to 7.0 at room temperature with the substrate itself by adding an NaOH solution through a microcapillary tube. No buffer was used, as was the case in a similar study by Eichhorn and his coworkers. The zinc-containing solutions of dinucleotides were made up immediately before use. Metal ion catalyzed reactions were carried out at 62.1°. The constancy of the temperature during the reactions was maintained by the use of a Haake thermostat FS unit. The kinetic measurements and the procedure were similar to those previously reported (Koike and Inoue, 1972). Reactions were stopped at appropriate time intervals by lowering the temperature to 0°, as Butzow and Eichhorn (1971) did in their study. The reaction mixture was then charged on a column (usually 0.9 × 20 cm in size) of DEAE-Sephadex A-25 (chloride form) and eluted with a linear gradient (300 ml, 0–0.3 M NaCl) at pH 7.6 (adjusted with 0.01 M Tris-Cl). Virtually no reaction of the unreacted dinucleotide occurred during the chromatographic procedure, and the unreacted nucleotide, XpYp, and the product (X>p, and Xp plus Yp) were eluted in well-resolved peaks. The fractions of these nucleotides were determined from the areas under the peaks by spectrophotometric measurements at a particular wavelength(s) either at a pH below 1 or after the treatment of the dimer fraction with ribonuclease $T_2$; the breakdown of dinucleotides and dinucleoside monophosphates was thus confirmed to follow pseudo-first-order kinetics. The observed values of the rate constant, $k_{obsd}$, are correct to ±5%. Conditions were chosen so that the results could be compared with those previously reported by Eichhorn and his coworkers. For ApUp(3′) and ApAp(3′), the values of $k_{obsd}$ were determined under varying conditions of pH and zinc ion concentrations.

*Instrumentations.* The light absorption was measured on a Hitachi Perkin-Elmer spectrophotometer, Model 139. The optical rotatory dispersion (ORD) measurements were made on a Jasco spectropolarimeter, Model ORD/UV-5.

## Results and Discussion

*$Zn^{2+}$-Catalyzed Transphosphorylation of Four Homodinucleotides.* EFFECT OF THE TERMINAL PHOSPHATE GROUP. With a view to studying the structure–reactivity correlation in oligonucleotides, we have determined the rate constants of zinc-cleavage reactions for various homodimers in order to ascertain the effects of the presence of the terminal phosphate group first observed by Butzow and Eichhorn (1971). The values of $k_{obsd}$ are given in Table I. The observed rate constants for transphosphorylation of dimers are, in principle, composite because of the dynamic equilibrium between corresponding pairs of metal ion–substrate complexes and free substrate molecules. However, the non-metal ion catalyzed reaction is so slow at the neutral pH ($k_{H_2O} < 10^{-3}$ $hr^{-1}$; Koike, 1973) where the reaction is studied that the uncatalyzed (or water-catalyzed) transphosphorylation can be neglected in discussing the stoichiometry of the reaction.

A comparison of $k_{obsd}$ shows that the introduction of a 3′-terminal phosphate group into XpX causes large differences in the rate, whereas that of a 2′-terminal phosphate enhances the rate to a smaller extent. Thus, the values of $k_{XpXp(3')}/k_{XpXp(2')}$ decrease in this order: uridylate dimers > cytidylate dimers > adenylate dimers > guanylate dimers. The greater rate enhancement observed for XpXp(3′) relative to XpXp(2′) can be interpreted in terms of a mechanism: XpXp(3′) + $Zn^{2+} \rightleftharpoons$ XpXp(3′)·$Zn^{2+}$ complex; XpXp(3′)·$Zn^{2+}$ complex → Xp(3′) + X>p + $Zn^{2+}$, in which the dinucleotide, which is coordinated to the zinc ion to form a reactive complex in a prior rapid equilibrium, undergoes transphosphorylation. That is, XpXp(3′) seems to fit, better than XpXp(2′) does, the structural requirements for a catalytically active $Zn^{2+}$–substrate complex, presumably for geometric reasons. This situation was anticipated in the work of Inoue and Satoh on the effect of a terminal phosphate on a dinucleotide conformation (Inoue and Satoh, 1969). These results, as well as others not discussed here, suggest that $Zn^{2+}$ interacts primarily with the two phosphate groups by bridging between the 3′-neighboring terminal phosphate and the phosphodiester group, and that the secondary association is also present between the base moiety of XpXp(3′) and $Zn^{2+}$. An inspection of space-filling CPK models of a bridged complex also supports these geometric insights into the effect of the terminal phosphate group on the reactivity.

The pseudo-first-order rate constants at 62.1° give the $Zn^{2+}$-catalyzed reactivity of homodimers as UpUp(3′) > ApAp(3′) > CpCp(3′) > GpGp(3′); this order is the reverse of the order of the affinity of the base moiety toward metal ions, as judged from the relative stability order of the complexes formed between various nucleotides and $Cu^{2+}$ (Fiskin and Beer, 1965). Thus, a differential rate enhancement should result from the mode of attachment of the substrate to the catalyst and the requirements for a particular spacing between $Zn^{2+}$ and the sites of the substrate, which causes not only a simple but effective electrostatic neutralization of the phosphate diester, promoting the nucleophilic attack of the 2′-OH group on the phosphorus of the polarized phosphoryl moiety, but may also induce a strain in the $Zn^{2+}$-complexed dinucleotide. The base specific nature of the $Zn^{2+}$-catalyzed degradation of dinucleotides will be discussed in a later section.

*Relative Effect of Divalent Ions ($Cu^{2+}$, $Co^{2+}$, and $Ni^{2+}$) on the Transphosphorylation of Homodinucleotides.* The rate of the $Cu^{2+}$-, $Co^{2+}$-, and $Ni^{2+}$-catalyzed degradation of four homodinucleotides was determined under the same conditions as we used in the case of $Zn^{2+}$ catalysis. The values of $k_{obsd}$ are listed in Table II. It has again been found that $Zn^{2+}$ is most effective in bringing about internucleotide cleavage, as was previously observed in the course of the degradation of poly(A) ($Zn^{2+} \gg Co^{2+} > Ni^{2+} \geq Cu^{2+}$) (Butzow and Eichhorn, 1965).

TABLE II: Pseudo-First-Order Rate Constants, $k_{obsd}$, for $Cu^{2+}$-, $Co^{2+}$-, and $Ni^{2+}$-Catalyzed Degradations of Homodinucleotides at 62.1°, $[Mn^{2+}] = 1 \times 10^{-3}$ M, $[XpXp]/[M^{2+}] = 2$, pH 7.0, $k_{obsd} \times 10^3$ in $hr^{-1}$.

| Substrate | $Cu^{2+}$ | $Co^{2+}$ | $Ni^{2+}$ |
|---|---|---|---|
| ApAp(2′) | 4.0 | 20 | 8.0 |
| ApAp(3′) | 17.5 | 26 | 10 |
| UpUp(2′) | 4.3 | 5.0 | 4.5 |
| UpUp(3′) | 107 | 7.2 | 4.6 |
| GpGp(3′) | 7.8 | 22 | 5.4 |
| CpCp(3′) | 20.0 | 20 | 10.5 |

First, it should be noted that the value of $k_{obsd}$ for $Co^{2+}$- and $Ni^{2+}$-catalyzed reactions showed only a slight dependence on the position of a terminal phosphate group. In contrast to this, for a $Cu^{2+}$-catalyzed reaction quite a large "3′-terminal phosphate effect" was found, as was the case with the $Zn^{2+}$ reaction. In the case of $Cu^{2+}$ catalysis, solutions of $Cu^{2+}$–dinucleotide complexes other than diuridylic acid showed a tendency to precipitate, so that the values of $k_{obsd}$ might have been somewhat greater than those listed in Table II for the $Cu^{2+}$-catalyzed degradation of ApAp, CpCp, and GpGp.

Next, although it can be seen that the rates of the degradation of XpXp(3′) fall with the divalent cation in the order: $Zn^{2+} > (Cu^{2+}) > Co^{2+} > Ni^{2+}$, we have to await further study in order to understand the factors governing such a variation in catalytic activity. Incidentally, a similar order has also been observed for the base-catalyzed hydrolysis of ethyl glycinate–metal complexes—$M(NH_2CH_2COOEt^{2+}$—(Hix and Jones, 1966) and the metal ion catalyzed hydrolysis of ethyl oxalate (Johnson and Angelici, 1971).

In any attempt to elucidate what factors influence the value of $k_{XpXp(3')}/k_{XpXp(2')}$, steric effects (geometrical factors and the size of the metal ion) must be considered. However, considerations of these factors are severely handicapped by the lack of knowledge of the structures involved. As in the present case with bi- and multidentate ligands, where the ligand can fill more than one coordination site round the metal, the most stable structures are those formed when the orbitals of the $M^{2+}$ can point in directions which overlap the two phosphate group oribtals in XpXp(3′) so as to minimize the mutual repulsions between their charges without serious distortion. It seems reasonable that $Zn^{2+}$ ions do not have any possibility of crystal-field stabilization energies stabilizing one configuration with respect to others (Basolo and Pearson, 1958), so that, depending only on size and electrostatic forces, the zinc ion can adopt any of three configurations (tetrahedral, planar, or octahedral); the tetrahedral case seems to be most probable for an XpXp(3′)–$Zn^{2+}$ complex, judging from model building with space-filling CPK atomic models.

*Specificity in the $Zn^{2+}$-Catalyzed Transphosphorylation of Dinucleotides.* With a view to understanding the main factors affecting zinc cleavage specificity [$k_{UpUp(3')} > k_{ApAp(3')} > k_{CpCp(3')} > k_{GpGp(3')}$] and, further, examining whether or not $Zn^{2+}$-catalyzed degradation is nucleotide sequence dependent, we have initiated a set of studies by making use of five pairs of sequence isomers. Table III summarizes the results of our measurements of the reaction rates for these pairs of isomers, together with those for four homodinucleotides [We were unable to prepare a pair of isomers, CpUp(3′) and UpCp(3′), in their pure form.].

TABLE III: First-Order Rate Constants ($hr^{-1}$) for the $Zn^{2+}$-Catalyzed Transphosphorylation of Fourteen Dinucleotides, XpYp(3′).[a]

| | A | U | C | G |
|---|---|---|---|---|
| A | 178 | 347 | 194 | 128 |
| U | 372 | 207 | | 157 |
| C | 139 | | 92.1 | 35.2 |
| G | 214 | 147 | 44.0 | 33.9 |

[a] $[XpYp(3')]/[Zn^{2+}] = 2.0$; temperature, 62.1°; pH 7.0.

It is clear from these data and those of others that $Zn^{2+}$ interacts with the base moieties of XpXp molecules, apparently in the order of preference for sites on G > C > A ≥ U, since the reactivity decreases in the order of UpUp > ApAp > CpCp > GpGp. The rate constants for the $Zn^{2+}$-catalyzed cleavage of dinucleotides, XpYp(3′), including heterodimers follow similar trends: *e.g.*, for GpXp(3′) and XpGp(3′), GpUp > GpAp > GpCp > GpGp and ApGp > UpGp > CpGp > GpGp. The next logical question is whether the sequence-dependent nature of the presently observed reactivity is simply due to the interaction of metal ions with bases as well as with phosphate in XpYp(3′), or whether the base-stacking or single-stranded dinucleotide conformation is responsible for the $Zn^{2+}$ ion catalyzed reactivity (*cf.* Eichhorn *et al.*, 1971; Butzow and Eichhorn, 1971). In order to answer this question, let us consider the data listed in Table III for five pairs of sequence isomers. The ratios of $k_{XpYp}$ to $k_{YpXp}$ are: $k_{ApUp}/k_{UpAp} = 1.07$, $k_{GpUp}/k_{UpGp} = 1.07$, $k_{CpGp}/k_{GpCp} = 1.25$, $k_{CpAp}/k_{ApCp} = 1.40$, and $k_{ApGp}/k_{GpAp} = 1.67$. In the first three cases, the difference in rate between XpYp and YpXp is small, indicating that the rate of $Zn^{2+}$ cleavage is apparently not sequence dependent and is dependent only upon the base compositions of the dimers. This seems quite reasonable because for these three pairs of isomers, no stacking can be expected under the reaction conditions; thus, only the base binding of $Zn^{2+}$ plays a controlling role in the observed differential rate enhancement. By contrast, somewhat greater differences are observed in the cases of two pairs of sequence isomers, CpAp(194) > ApCp(139) and ApGp(214) > GpAp(128). These sequence-dependent differences can be explained on the basis of the possible sequence effect on stacking interactions between bases in a molecule. [In a study of the base-catalyzed transphosphorylation of a series of adenylyl dimers, ApXp, we have noticed a small but definite variation in the rate; the stacking interaction tendency of these dimers, as measured by the difference ORD, is in agreement with the reverse order of the reactivity (Koike and Inoue, 1972).] The unusual rate enhancement in ApGp should be ascribed to the unique conformational feature of this sequence, as judged from the drastically different patterns in the CD spectra of two sequence isomers, GpAp and ApGp. A more quantitative correlation does not seem possible at the present time. However, the present results provide new evidence which supplements the recent work of Eichhorn and his associates regarding the origins of the base and the nucleotide-sequence specificity in zinc-cleavage reactions.

*Effect of pH on the Rate of $Zn^{2+}$-Catalyzed Transphosphorylation.* The pH–rate profile for the cleavage of ApUp-

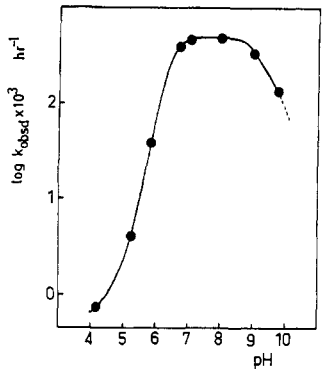



FIGURE 1: pH–reaction rate profile at 62.1° for the zinc cleavage of ApUp(3′). Substrate concentration is $5.0 \times 10^{-4}$ M [*i.e.*, $1.0 \times 10^{-3}$ M per base], and $[Zn^{2+}]/[ApUp(3')] = 2.0$ [*i.e.*, $[Zn^{2+}]/[ApUp(3')] = 1.0$ per base residue].

(3′) in the presence of $Zn^{2+}$ is shown in Figure 1. The influence of $Zn^{2+}$ on the observed rate increases in going from $Ap_{(-1)}Up_{(-1)}$ to $Ap_{(-1)}Up_{(-2)}$, but it decreases with the ionization of the metal complex above pH 9 [*cf.* the fact that the p$K$ of hydrolysis is approximately 9.7 for $Zn(H_2O)_4^{2+} + HO^- \rightleftharpoons Zn(H_2O)_3OH^+ + H_2O$]. In the pH range of 7 and 8, $Zn^{2+}$ at a level of $[Zn^{2+}]$:[ApUp] = 2:1 leads to a maximum acceleration. The ionization of the secondary phosphate group of a 3′-terminal phosphate must be necessary for the formation of a highly catalytically active $Zn^{2+}$–substrate complex in order to explain the much greater rates for XpYp(3′) than the rates for the corresponding XpY and XpYp(2′); this is in line with the previous observation of the zinc cleavage of $Ap_{(-1)}$ | $Ap_{(-1)}A$ and $Ap_{(-1)}Ap_{(-2)}$ (Butzow and Eichhorn, 1971). For other selected dinucleotides we have also found that the optimal pH for the reaction lies in the same pH range, as is demonstrated in Figure 1 for ApUp(3′). This is the reason why we have made all our measurements at pH 7.

*Evidence for the Formation of a Reactive Complex.* Figure 2 shows that, at the substrate concentration of $1 \times 10^{-3}$ M, the rate of degradation increased with the increase in the $Zn^{2+}$ concentration until it reached approximately an equimolar concentration in $Zn^{2+}$/2P. When $[Zn^{2+}]$ became greater, the rate decreased with $[Zn^{2+}]/[ApUp]$. This initial change appears to be associated with the stoichiometric formation of the reactive 1:1 complex. At the level of $[Zn^{2+}]/[ApUp] > 1$, *i.e.*, $[Zn^{2+}] > 2[P]$, the formation of less reactive higher order complex(es), probably the 1:2 complex, starts to occur. Thus, the amount of $Zn^{2+}$ required to promote the maximum velocity has been found to be a 1:1 equivalence with a substrate, indicating that the transiently formed 1:1 $Zn^{2+}$–substrate complex is most reactive. Comparative studies of the effect of a stepwise addition of $Zn^{2+}$ on the rate of zinc cleavage for other dinucleotides, XpYp(3′), have proved that an extremum (maximum) in the log $k_{obsd}$ *vs.* log $[Zn^{2+}]/[XpYp]$ plot appears at approximately $[Zn^{2+}] = [XpYp]$.

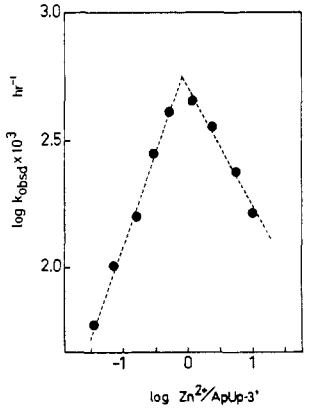
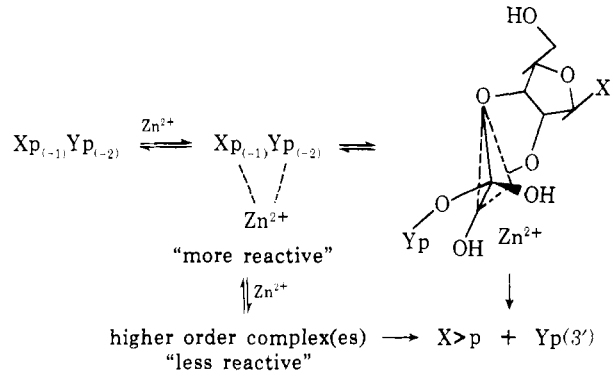



FIGURE 2: The effect of $Zn^{2+}$ concentration on rate of $Zn^{2+}$-catalyzed degradation of ApUp(3′) at 62.1°. [ApUp] was kept constant at $5.0 \times 10^{-4}$ M. The abscissa is the logarithm of $[Zn^{2+}]/[ApUp]$, the ratio of zinc and substrate in molar concentration, *i.e.*, $[Zn^{2+}]/2[P]$.

On the basis of all of the above kinetic data, we propose that the zinc ion catalyzed transphosphorylation of XpYp(3′) proceeds by a mechanism in which the formation and breakdown of the pentacovalent phosphorus intermediate is $Zn^{2+}$ catalyzed. Thus, the reaction at a neutral pH may be seen in Scheme I. The juxtapositon of additional binding site(s) of

SCHEME I




base moieties thus results in a lowering of the catalytic activity of $Zn^{2+}$, probably because an unfavorable geometric disposition for the nucleophilic attack of the 2′-OH is forced as a result of the simultaneous chelation to the base moiety. (The tetrahedral relationship between the chelating ligands is presumably required for the $Zn^{2+}$ coordinated with four ligands.)

Since most of the dinucleotides used in this study are in the dissymmetric conformation characteristic of a single-stranded helical stacking geometry, it can be expected that the ORD of such compounds will depend upon the state in which these molecules exist. The $Zn^{2+}$ effects on the ORD of ApAp(3′), GpCp(3′), and poly(rA) are shown in Figure 3. The addition of $Zn^{2+}$ at a lower level until $[Zn^{2+}] = [ApAp]$, *i.e.*, $[Zn^{2+}] = 2[P]$, resulted in a definite increase in the rotatory power of the first Cotton effect due to the excition interaction of the $B_{2u}$ transition on one base with the excited state of the other base, whereas the further addition of $Zn^{2+}$ to the solution caused a decrease in the rotation measured as $[\phi]_{283} - [\phi]_{260}$. This indicates that the molecular geometry of the species constructing the transient 1:1 coordination complex seems to have a more ordered or stacked conformation, but that the molecular constitution formed at higher levels of $Zn^{2+}$ is probably formed in such a way as to counteract the strengthening of the rotatory power. In addition, the appearance of a minimum in the $[\phi]_{283} - [\phi]_{260}$ *vs.* $[Zn^{2+}]/[ApAp]$ plot at about 2 indicates that the formation of the 1:2 complex occurs almost quantitatively at the sites of the base residue. The further addition of $Zn^{2+}$ presumably leads to the formation of a polymerized coordination complex (*cf.* Rifkind and Eichhorn, 1972). The above ORD and hypochromicity measurements have revealed that, in line with what has been proposed

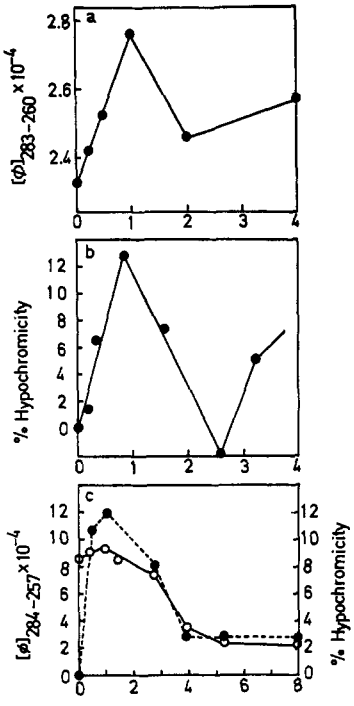



FIGURE 3: Changes induced on the absorption and/or ORD spectra of ApAp(3′), GpCp(3′), and poly(rA) at pH 7.1, temperature 20° by $Zn^{2+}$: (a) the plot of the amplitude, $[\phi]_{283} - [\phi]_{260}$, in molar rotation per residue, of the first Cotton effect of ApAp against $[Zn^{2+}]/[ApAp]$, *i.e.*, $[Zn^{2+}]/2[P]$. (b) Plot of per cent hypochromicity at 280 nm relative to the absorption of GpCp(3′) at $[Zn^{2+}] = 0$ against $[Zn^{2+}]/[GpCp]$, *i.e.*, $[Zn^{2+}]/2[P]$. (c) Dependence of the rotation measured as $[\phi]_{284} - [\phi]_{257}$ (open circle) and the absorption intensity measured as per cent hypochromicity at 258 nm relative to the intensity of poly(rA) at $[Zn^{2+}] = 0$ (closed circle) on $[Zn^{2+}]/[poly(rA)]$ in phosphorus concentration, *i.e.*, $[Zn^{2+}]/[P]$.

from the data based on the effect of a step-by-step addition of $Zn^{2+}$ on the rate, one zinc ion binds a molecule of XpYp(3′) to form a reactive complex through *bridging*, as is shown in Scheme I. By contrast to the case with dinucleotides, the per cent hypochromicity change observed during the addition of $Zn^{2+}$ to the poly(A) solution was found at first to increase up to a maximum at about $[Zn^{2+}]/[P] = 1$, followed by a gradual decrease with the further addition of $Zn^{2+}$. A corresponding change in the optical rotatory power, defined as $[\phi]_{284} - [\phi]_{257}$ (in degrees per residue), was also observed, though it was not so drastic as was the hypochromicity change. These results suggest that, contrary to the case with ApAp, $Zn^{2+}$ binds the phosphate monoanion but not by bridging, giving the complex shown in Scheme II. The present results are also in line with

SCHEME II

$$\text{poly(rA)} + Zn^{2+} \overset{K_1}{\rightleftharpoons}$$

$$\begin{matrix} Ap_{(-1)}Ap_{(-1)}\cdots \\ | \quad | \\ Zn^{2+}\ Zn^{2+}\cdots \end{matrix} + Zn^{2+} \overset{K_2}{\rightleftharpoons} \text{higher order complex(es)}$$

the observed effects of $Zn^{2+}$ on $T_m$ of DNA when the levels of $Zn^{2+}$ increase (Eichhorn and Shin, 1968). It should be noted that the value of the second equilibrium quotient, $K_2$, in Scheme II is estimated to be about $2.5 \times 10^3$ $M^{-1}$ from the data based on the ORD as well as the hypochromicity measurements.

*Unambiguous Identification of UpUp(2′) and UpUp(3′).* As a possible utilization of the present study, that a 3′-terminal phosphate group greatly enhances the rate of the $Zn^{2+}$ degradation of dinucleotides has been suggested as a diagnostic tool in identifying a pair of isomers, XpYp(2′) and XpYp(3′), especially when they are otherwise difficult to identify, as is the case with UpUp(2′) and UpUp(3′). It was previously reported that the 2′-position isomers were always eluted in earlier peaks than the 3′ isomers when the dinucleotide fraction obtainable by the controlled alkaline hydrolysis of RNA was subfractionated by chromatography on Dowex 1-X2 ($Cl^-$ form) at an acidic pH of, say, 2.4 (Satoh and Inoue, 1969). Although this is correct for XpAp, XpCp, and XpGp, the conclusion concerning the identity of UpUp(2′) and UpUp(3′) was less direct because, at the monomer level, 2′-UMP and 3′-UMP are difficult to separate from the mixture of 2′ and 3′ isomers. The previous conclusion concerning UpUp(2′) and UpUp(3′) was simply based on analogy with other conclusions drawn unambiguously for XpAp, XpCp, and XpGp.

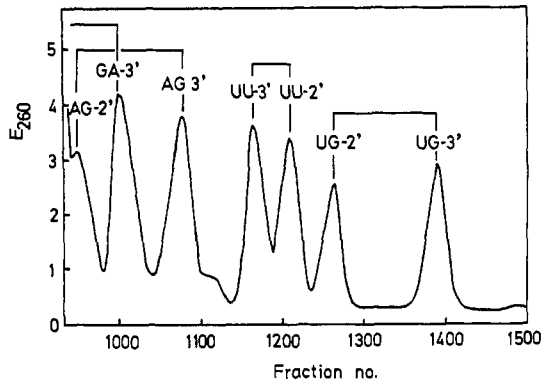



FIGURE 4: A part of chromatogram of a dinucleotide mixture (17,500 $E_{260}$ units) on Dowex 1-X2 ($Cl^-$ form). For a detail, see a previous paper (Satoh and Inoue, 1969). Peak assignment is now revised for UpUp(3′) and UpUp(2′).

The authentic samples of UpUp(2′) and UpUp(3′) have been prepared by the deamination of CpCp(2′) and CpCp(3′) according to the method of Shapiro and Pohl (1968). It has been ascertained that no migration of a 3′-terminal phosphate group to the 2′ terminus was observed during the deamination of CpCp(3′). The pure sample of UpUp(3′) thus obtained was subjected to zinc degradation, and the rate constant of this authentic sample was found to be quite close to that of UpUp wrongly assigned to UpUp(2′) in the elution diagram in a previous paper (Satoh and Inoue, 1969). In a similar way, the rate constant of the authentic UpUp(2′), prepared by the deamination of CpCp(2′), was identical with that ($6.4 \times 10^{-3}$ $hr^{-1}$) of UpUp, which had been eluted in the second peak of a pair of isomers in the chromatogram. The agreements warrant the use of these samples for the further study of oligonucleotides. A part of the revised chromatographic elution profile is reproduced in Figure 4. This very apparent anomaly in the elution order of a pair of isomers, UpUp(2′) and UpUp(3′), may be due to the unique molecular forms of UpUp which are involved in the interaction with the surface of ion-exchange resin.

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# Specific Purification of Eel Serum and *Cytisus sessilifolius* Anti-H Hemagglutinins by Affinity Chromatography and Their Binding to Human Erythrocytes†

Isamu Matsumoto and Toshiaki Osawa*

ABSTRACT: Eel serum and *Cytisus sessilifolius* anti-H hemagglutinins were purified by affinity chromatography using specific adsorbents prepared by cross-linking the corresponding inhibitory sugar to insoluble starch. The preparations were homogeneous by ultracentrifugal analysis and disc electrophoresis. The sedimentation constants ($s_{20,w}$) and the molecular weights were estimated to be 7.2 S and 140,000 for eel serum anti-H hemagglutinin and 6.8 S and 110,000 for *C. sessilifolius* anti-H hemagglutinin. The binding studies using $^{125}$I-labeled eel serum and *C. sessilifolius* hemagglutinins revealed that there are approximately the same number of receptor sites (1.7–1.9 $\times$ $10^6$ per cell) for both anti-H hemagglutinins on human group O erythrocytes. Furthermore, the presence of one anti-H hemagglutinin effectively inhibited the binding of the other anti-H hemagglutinin to group O erythrocytes. Although these two anti-H hemagglutinins possess different sugar specificities (Matsumoto, I., and Osawa, T. (1971), *Vox Sang. 21*, 548), the results described above indicate that they bind to different but overlapping portions of the same H-determinant structure on the cell surface. Human peripheral lymphocytes were also found to contain 0.4 $\times$ $10^6$ receptor sites per cell for eel serum anti-H hemagglutinin.

Numerous heterologous anti-H hemagglutinins have been found in eel serum and the extracts of certain plant seeds. These anti-H hemagglutinins can be subdivided into two groups on the basis of the inhibition assays using simple sugars as inhibitors: one is a group of eel serum type anti-H hemagglutinins which includes eel serum hemagglutinin, *Lotus tetragonolobus* hemagglutinin, and *Ulex europeus* hemagglutinin I (Matsumoto and Osawa, 1969), and the other is a group of *Cytisus* type anti-H hemagglutinins which includes *Cytisus sessilifolius* hemagglutinin, *Laburnum alpinum* hemagglutinin, and *Ulex europeus* hemagglutinin II (Matsumoto and Osawa, 1970). Since eel serum type anti-H hemagglutinins are inhibited by L-fucose (Springer and Williamson, 1962; Springer *et al.*, 1964; Matsumoto and Osawa, 1969), they are assumed to bind with $\alpha$-L-fucopyranosyl residue in the H-determinant structure of human erythrocyte surface or soluble blood group substances. On the other hand, *Cytisus* type anti-H hemagglutinins are inhibited by di-*N*-acetylchitobiose and the other oligosaccharides having a terminal nonreducing $\beta$-*N*-acetylglucosaminyl residue (Watkins and Moran, 1962; Osawa, 1966), the structure of which is, however, not found in the carbohydrate chain of blood group determinant structure. Thus, the structure which *Cytisus* type anti-H hemagglutinins actually recognize on the human erythrocyte surface has not been clarified, even though $\beta$-*N*-acetylglucosaminyl linkage has been found at the internal position in the carbohydrate chains of blood group H glycolipids (Stellner *et al.*, 1973) and soluble blood group substances (Watkins, 1972). In the previous papers (Matsumoto and Osawa, 1970, 1971), we demonstrated that the *Cytisus* type anti-H hemagglutinins could not agglutinate human group O erythrocytes previously treated with purified H-decomposing enzyme ($\alpha$-L-fucosidase) from *Bacillus fulminans* (Iseki *et al.*, 1962), and they were more effectively inhibited by 2′-fucosyllactose and lacto-*N*-fucopentaose I than by the corresponding fucose-free oligosaccharides, *i.e.*, lactose and lacto-*N*-tetraose. It has been, therefore, assumed that *Cytisus* type anti-H hemagglutinins recognize the *O*-$\alpha$-L-fucopyranosyl-(1→2)-*O*-$\beta$-D-galactopyranosyl-(1→3 or 4)-$\beta$-D-*N*-acetylglucosamine sugar sequence in the carbohydrate chain of blood group determinant structure. Thus, it can be inferred that eel serum type and

† From the Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Received September 17, 1973.* This investigation was supported by a research grant from the Ministry of Education of Japan.