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EFFECT OF NUCLEOPHILIC CATALYST ON THE DEGRADATIVE OLIGOMERIZATION OF THYMIDINE 5'-p-NITROPHENYLPHOSPHATE

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Abstract—Degradative oligomerizations of thymidine 5'-p-nitrophenylphosphate(PNP-pT) were studied in the presence of imidazole, its derivatives, and metal ions. PNP-pT was degraded to give several kinds of thymidylic acid oligomers such as pTpT, TppT, cyclic-pTpT. The linearities of the structures of the obtained oligomers depended on the linearities of the structures of imidazole derivatives. Metal ions favoured formation of the cyclic dimer.

A transesterification technique is one of the most useful methods to obtain a polyester-type polymer. Recently, we observed that thymidylic acid oligomers were given from thymidine 5'-p-nitrophenylphosphate(PNP-pT) *via* a degradative condensation even in an aqueous solution. In general, catalysis by imidazole, one of the most nucleophilic moieties in many enzymes as histidine residue, is of great importance in nucleophilic catalysis. The imidazole moiety may play important roles in condensation reactions by enzymes. In addition, divalent metal ions have a significant contribution to reactions of nucleotides.

In this paper, we report effects of imidazole, its derivatives as well as divalent metal ions on the degradative condensation of PNP-pT. Different thymidylic acid oligomers were obtained, reflecting structures of intermediates in the reactions. Roles of added materials were discussed in terms of so-called matrix effect.

RESULTS AND DISCUSSION

From an aqueous solution of PNP-pT, thymidylic acid oligomers as well as hydrolyzed materials such as thymidylic acid(pT) and p-nitrophenol were formed. On the other hand, no pT oligomers formed from an aqueous solution of pT. This means that the p-nitrophenol moiety activates an electrophilic P atom of PNP-pT and facilitates the attack of nucleophiles such as phosphate anion of pT and 3'-O atoms of pT and PNP-pT to P atom.

Figure 1 shows the time-conversion curves for the oligomer formation *via* the degradation of PNP-pT with the alkaline. As the degradation proceeded, amounts of pT and pT oligomers increased. At an initial stage of the reaction, TppT was the dominant product, and then it decreased. The time-conversion curve of the TppT had the optimum. These results imply that an initial stage of the reaction, phosphate anion of pT attacks the P atom of PNP-pT rather

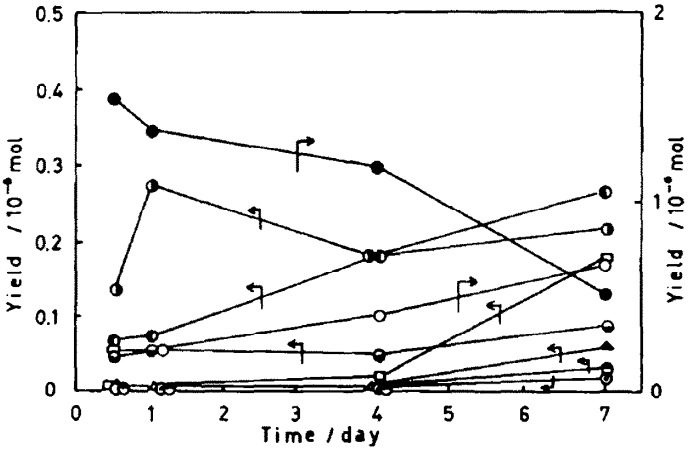
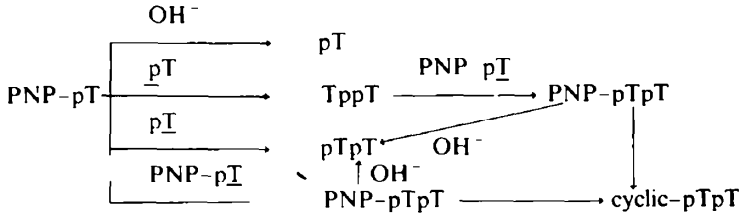


Fig. 1. Time-conversion curves for oligomer formation in the degradative oligomerization of PNP-pT with alkaline. [PNP-pT] = 5 × 10^{-4} mol/l, [KOH] = 1 mol/l, water: dioxane = 3:1 (v/v), room temperature, ●, PNP-pT; ○, pT; ◑, TppT; ◐, cyclic-pTpT; ◒, pTpT; ⊙, cyclic-pTpTpT; ◓, pTpTpT; ▲, PNP-pTpT; □, other adducts.

than 3'-O atoms of pT and PNP-pT, and then the formed TppT was converted into other oligomers consecutively. As time proceeded, the amount of the degree of oligomerization of the product gradually increased. This shows that a successive condensation took place in the reaction.

When the concentration of nucleotide in the reaction solution was made to increase by addition of pT, the composition of the oligomer products was changed. As is shown in Table 1, conversions of TppT and cyclic-pTpT increased, whereas conversions of pTpT and $(pT)_n$, which means longer oligomers and other ones, decreased by the addition of pT. This shows that the elongation of the oligomers was suppressed by the added pT and that the reaction between pT and PNP-pT took place in the degradative oligomerization *in situ*. From these results, a tentative mechanism is shown as follows:



(Reaction moieties are underlined.)

TppT is considered to be a reactive intermediate. Cyclic-pTpT was formed from PNP-pTpT, which was formed by the reaction of PNP-pT with PNP-pT or TppT.

Additives such as imidazole, its derivatives, and divalent metal ions in the reaction systems did not remarkably affect the rate of the degradative oligomerization. However, the composition of the products was changed. Table 2 shows the results of several degradative oligomerizations of PNP-pT at pH 7-8. Without additives (No. 1), the main product was TppT(57%). This means that the phosphate anion of pT attacked P atom of PNP-pT more dominantly than 3'-O atom of pT. Addition of imidazole and its derivatives largely affected the oligomer formation. In the addition of Im(No.1), the yield of cyclic-pTpT (42%) largely increased compared with the exp. No. 1, while the yield of TppT (10%) decreased. These results

Table 1. Effect of additive thymidylic acid on the degradative oligomerization of PNP-pT*

pT /equiv.	Ratio/mol% cyclic-pTpT	TppT	pTpT	$(pT)_n$**
0	20	48	15	17
0.5	24	56	8	12
1.0	29	59	6	6

*[PNP-pT]=5.0×10^{-4}M, [KOH]=1M, H_2O/Dioxane=3/1(v/v), 4 days, r.t.

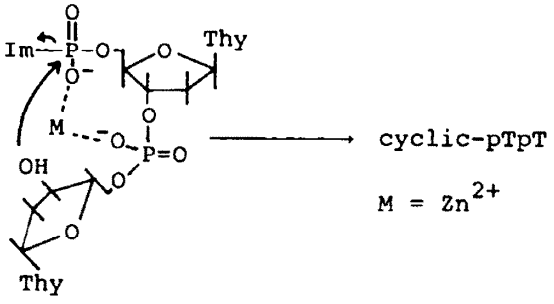
**$(pT)_n$ means the summation of longer oligomers and other oligomers.

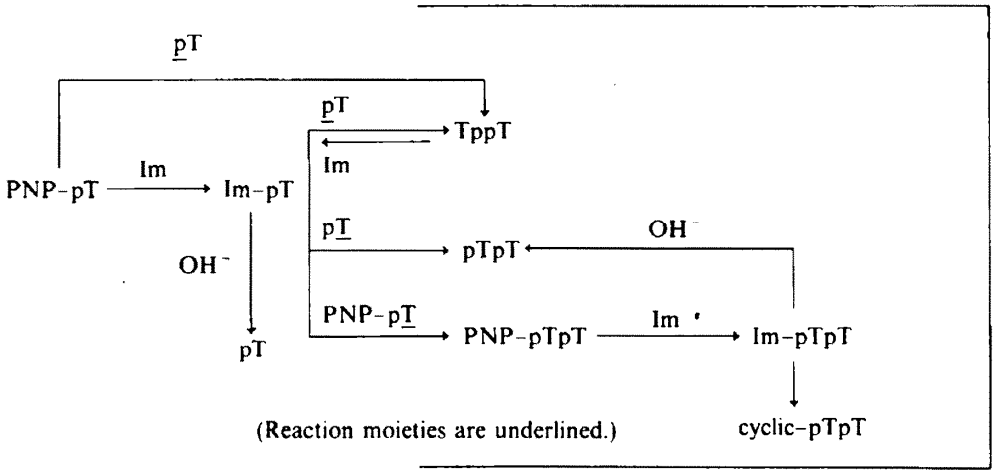
Table 2. Effect of imidazole derivatives on the degradative oligomerization of PNP-pT on the absence or presence of divalent metal ions*

Exp. No.	System	Ratio/mol% cyclic-pTpT	TppT	pTpT	cyclic-pTpTpT	PNP-pTpT	pTpTpT	others
1	none	13	57	15	1	3	3	8
2	Im	42	10	11	4	5	9	16
3	Im--Zn^{2+}	74	16	2	tr	1	3	4
4	PNIm	11	43	31	1	1	5	8
5	PNIm--Mg^{2+}	34	22	34	1	3	3	3
6	PLHis	28	37	25	1	2	3	4

*[PNP-pT]=2.5×10^{-2}M, [Im]=[PNIm]=[$ZnCl_2$]=[$MgCl_2$]=2.5×10^{-2}M, [PLHis]=9.7×10^{-3}M, 5-7 days, r.t.

suggest the formation of more active intermediate than PNP-pT, in the course of the oligomerization. It has been reported that imidazole likely reacts with phosphate or pyrophosphate.[1,2] So it is considered that PNP-pT and TppT react with Im to form an active intermediate, Im-pT,[3] which is more reactive than PNP-pT, and that almost oligomers were formed *via* Im-pT.[3] When Im-pT is attacked by several nucleophiles, (a) OH^-, (b) phosphate anion of pT, and (c) 3'-O of pT and PNP-pT, oligomers are formed. These reactions seem to take place in parallel to the reactions without additive (No. 1). As TppT likely reacts with Im to form Im-pT, it is considered that the conversion of TppT decreases. Further, the increase of cyclic-pTpT is elucidated by the increase of PNP-pTpT which is likely converted into Im-pTpT and then into cyclic-pTpT. A tentative mechanism of these oligomer formation is shown as follows:





On the other hand, in the addition of polymeric imidazoles such as poly(N-vinylimidazole) (PNIm) and poly(L-histidine) (PLHis) (No. 4 and No. 6), the conversion of TppT decreased whereas that of pTpT increased, compared with exp. No. 1. It is suggested that these reactions took place *via* active intermediates such as PNIm-pT and PLHis-pT. In addition, the conversion of cyclic-pTpT decreased and those of TppT and pTpT increased compared with No. 2. The result is similar to no additive system (No. 1). Though these results show that the mechanisms of the oligomerizations are similar, reactivities of PNIm-pT and PLHis-pT are less than that of Im-pT. The steric hindrance and topochemical effects control the property of the activated P atom and lead to different oligomer compositions. The decrease of cyclic-pTpT is elucidated by the steric hindrance which suppresses the intramolecular cyclization reaction, and the increase of pTpT is due to the topochemical effect, which makes the internucleotide bond formation between neighboring activated pT easy. These results lead to a significant conclusion that the polymeric catalysts have a tendency to produce relatively linear oligomers.

When divalent metal ions such as Zn^{2+} and Mg^{2+} were added in these systems, the kinds and the conversions of oligomers were largely changed. Especially, the conversion of cyclic-pTpT remarkably increased. As to the metal-nucleotide interaction, metal ions likely interact with phosphate moiety and/or base moiety.[4,5] In the present reaction systems the increase of cyclic-pTpT might be elucidated by the chelation effect. When the chelation between phosphate moieties of Im-pTpT and metal ion takes place, the reaction between 3'-OH of thymidine and the activated P atom is sterically easy as follows:

These results give a new concept of matrix of catalyst which has a contiguous catalytic site on the oligomerization of nucleotides.

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EXPERIMENTAL

Materials

Thymidine 5'-p-nitrophenylphosphate ammonium salt, (PNP pT), imidazole (Im), poly(*N*-vinylimidazole) (PNIm), and poly-(L-histidine) (PLHis) were purchased from Bochlinger Manheim Gmbh, Nakarai Chemical Ltd., Sigma Chemical Co., and BASF, respectively. They were used without further purification.

High pressure liquid chromatography (HPLC)

HPLC was carried out with a Shimadzu-Dupont 830 (column; Parmaphase AAX, 1 m × 2.1 mm$^{\varnothing}$) by an exponential gradient of 0.002 M KH_2PO_4 (pH 3.35) to 0.5 M KH_2PO_4 (pH 4.5) at the gradient rate of 3%/min. Authentic samples for HPLC were obtained by the condensation of thymidine 5'-phosphate and by the cocondensation of PNP-pT and pT using dicyclohexylcarbodiimide as the

Table 3. Retention volumes of thymidylic acid oligomers in the high pressure liquid chromatography*

oligomer	retention volume** /ml
pT	2.2
cyclic-pTpT	3.0
PNP-pT	3.4
TppT	4.7
pTpT	5.0
cyclic-pTpTpT	6.0
PNP-pTpT	6.9
pTpTpT	9.1

*Shimadzu-Dupont 830 type, column;Parmaphase AAX, 1mx2.1mm$^{\phi}$.

**0.5ml/min. exponential gradient of 0.002M KH_2PO_4(pH 3.35) to 0.5M KH_2PO_4 at a gradient rate of 3%/min.

condensing reagent. The retention volumes of the authentic samples are listed in Table 3.

Reactions

Time-conversion curves. The solution containing 0.5 mM PNP-pT and 1M KOH was prepared using a water-dioxan mixture (3/1, v/v) as a solvent. The solution was allowed to stand at room temperature and the small aliquots were analyzed by HPLC from time to time after neutralization by addition of Dowex 50W (H form) resin.

Effects of additive pT. To the solution containing 0.5 mM PNP-pT and 1M KOH using a water-dioxane mixture (3/1, v/v) as a solvent, pT was added at the concentration of a) 0.25 mM (0.5 equivalent for PNP-pT) b) 0.5 mM (1.0 equivalent for PNP-pT). The solution was kept to stand at room temperature for 4 days and the small aliquots were analyzed by HPLC after neutralization by addition of Dowex 50W (H form) resin.

Effects of additive imidazole derivatives and metal ions. Degradative oligomerizations of PNP-pT were carried out in aqueous media in the following solution systems. The reaction mixtures were prepared as follows: (a) 25 mM PNP-pT, (b) 25 mM PNP-pT and 25 mM Im, (c) 25 mM PNP-pT, 25 mM Im, and 25 mM $ZnCl_2$, (d) 25 mM PNP-pT and 25 mM PNIm, (e) 25 mM PNP-pT, 25 mM PNIm and 25 mM $MgCl_2$, and (f) 25 mM PNP-pT and 9.7 mM PLHis. pHs of the reaction solutions were not adjusted to avoid any effect of buffering materials on the oligomerizations. The pH range of reaction solutions was *ca.* 7–8. The reaction solutions were allowed to stand at room temperature for 5–7 days. The small aliquots were analyzed by HPLC and compared with authentic samples.

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