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54. Morio Ikehara, Seiichi Uesugi, and Toshikazu Fukui: Studies of Nuclesides and Nucleotides. XXXI.*1 Reaction of Nitrous Acid with Mono- and Di-esters of Phosphoramidate.

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Reaction of mono- and di-esterified phosphoramidate with nitrous acid or isoamyl nitrite was investigated. While in the monoesterified phosphoramidate the reaction enhanced intensively by both nitrous acid and isoamyl nitrite, in the case of dibenzylphoramidate nitrous acid did not bring about hydrolysis reaction. Order of the rate of the reaction with diesterified phosphoramidate indicates direct attack of NO^+ on NH_2. Various intermediates of the reaction of thymidine 5′-phosphoramidate with isoamylnitrite were isolated by the DEAE-cellulose chromatography.

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The chemistry of phosphoramidate was investigated extensively with the intention of the synthesis of pyrophosphate linkage.[1,2] Recently, the mechanism of the hydrolysis of inorganic phosphoramidic acid in the presence of nitrous acid was reported.[3] Jencks, *et al.* postulated a metaphosphate intermediate, which could be employed to the synthesis of internucleotidic linkage as stated in the case of polymerization of mononucleotide with dicyclohexyl carbodiimide (DCC).[4] This paper is dealing with the study of the reaction of mono- and di-esterified phosphoramidate with nitrous acid or with isoamyl nitrite.

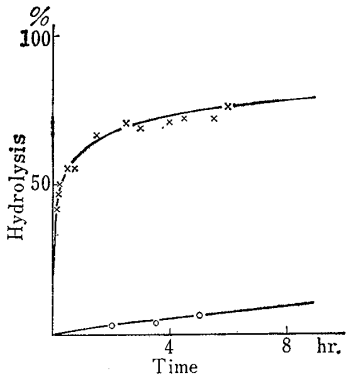

Fig. 1. Hydrolysis of Monobenzylphosphoramidate

×-×-×-×: In the presence of HONO. After 72 hr. Though it is not shown in the figure, 87% was hydrolyzed after 72 hr.

○-○-○-○: Without HONO. 19.5% of the starting material was hydrolyzed after 72 hr. and 26.6% was hydrolyzed at 288 hr. reaction.

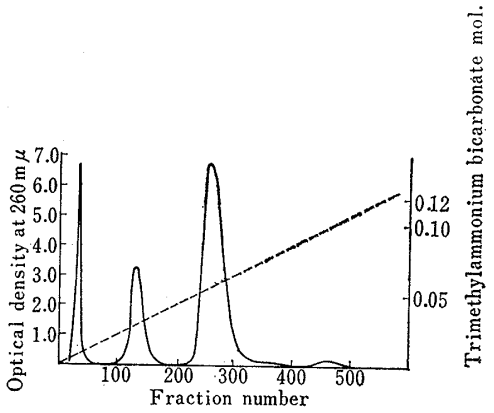

Fig. 2. Chromatography of Large Scale Experiment of the Reaction of TMP-NH_2 with Isoamyl Nitrite

*1 Part XXX. M. Ikehara, H. Tada: This Bulletin, **14**, 46 (1966).

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1) V. M. Clark, G. W. Kirby, A. R. Todd: J. Chem. Soc., **1957**, 1497.

2) R. W. Chambers, H. G. Khorana: J. Am. Chem. Soc., **80**, 3749 (1958); J. G. Moffatt, H. G. Khorana: *Ibid.*, **80**, 3756 (1958); R. W. Chambers, P. Shapiro, V. Kurkov: *Ibid.*, **82**, 970 (1960).

3) P. Jencks, M. Gilchrist: *Ibid.*, **86**, 1410 (1964).

4) G. Weimann, H. G. Khorana: *Ibid.*, **84**, 4329 (1962).

The reaction of monobenzyl phosphoramidate[1] with sodium nitrite in acidic media of various conditions was investigated by paper chromatography and paper electrophoresis. Generally, the rate of reaction to afford benzyl dihydrogenphosphate was promoted intensively than in the absence of nitrous acid. As shown in Fig. 1, the hydrolysis of benzylphosphoramidate was over 70% of completion after 8 hours and very much faster than in the usual acidic hydrolysis. The results summarized in Table I suggested the hydrolysis rate increased with the acid concentration. The product formed was P^1,P^2-dibenzyl pyrophosphate and monobenzyl dihydrogenphosphate. The reaction proceeded similarly with isoamyl nitrite and only trace amount of benzyl-isoamylphosphate was detected even in the case of 10 fold excess of isoamyl nitrite or in the various ratios of alcohol-water mixture. This fact was in contrast with the results obtained in the acid catalyzed alcoholysis of nucleoside phosphoramidate.[5]

In the case of thymidine 5′-phosphoramidate[6] (TMP-NH_2), the reaction was mainly carried out in anhydrous dimethylformamide (DMF) with isoamyl nitrite. Analysis of the reaction mixture by paper chromatography showed the presence of 4~5 spots other than thymidine 5′-phosphate (TMP) as described in the Experimental. A large scale reaction mixture was then analyzed by DEAE-cellulose column chromatography.[7] Major three peaks were obtained according to the charge of phosphate residue as shown in Fig. 2. Peak A contained the nucleotidyl materials A_1, A_2 and A_4 in addition to thymidine (A_3). The chromatographic behaviors of these compounds were in Experimental. Although the structure of these compounds was not elucidated as yet, A_1 and A_2 has the absorption properties characteristic of thymidine and A_4 has λ_{max} 259 mμ, which indicates the transformation of thymine moiety.*3

Second peak B is corresponding to mono-dissociated phosphate and contained isoamyl ester of TMP (B_1) and another nucleotide B_2, which has similar behavior on the paper chromatography in two solvent systems with thymidine 3′,5′-cyclic phosphate.[9]

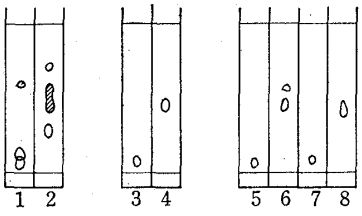


Fig. 3. Paper Chromatography of Fraction C_3

Chromatogram Nos. 1, 3, 5 and 7 were in solvent A and 2, 4, 6 and 8 were in solvent B. No. 3 showed the rechromatography of the middle spot (shadowed) of No. 2 extracted with water. No. 5 and 6 showed the ethanol extract of the upper half of the middle spot of No. 2. 7 and 8 showed the lower half of the middle spot in No. 2. extracted with ethanol.

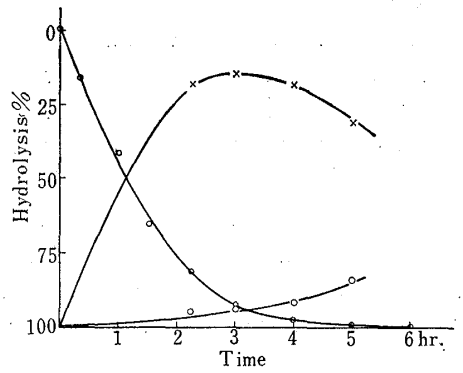


Fig. 4. Hydrolysis of Dibenzylphosphoramidate with Isoamyl Nitrite

○-○-○-○ : % of hydrolysis of dibenzylphosphoramidate
×-×-×-× : % of appearance of dibenzylphosphate
□-□-□-□ : % of appearance of monobenzyl dihydrogenphosphate

*3 The position of the elution from the column suggested the non-dissociated phosphate for A_1 and A_2. The phosphotriester[8] formation would be recalled.

5) J. G. Moffatt, H. G. Khorana : J. Am. Chem. Soc., **83**, 649 (1961).
6) J. Baddiley, N. L. Blumson : Biochem. J., **81**, 115 (1961).
7) H. G. Khorana, J. P. Vizsolyi : J. Am. Chem. Soc., **83**, 675 (1961).
8) J. Nagyvary : Biochemistry, **5**, 1316 (1966).
9) G. M. Tener, H. G. Khorana : J. Am. Chem. Soc., **80**, 6223 (1958).

The structure of B_1 was confirmed by the comparison with an authentic sample of isoamyl-TMP synthesized by the reaction of thymidine phosphoramidate with isoamyl alcohol saturated with hydrochrolic acid. Compound B_2 was further investigated by the hydrolyses in various conditions and compared with the reaction of 3′,5′-TMP. When B_2 was hydrolyzed with 0.1*N* hydrochloric acid at 100° for 4 minutes, TMP was the product. In the same condition 3′,5′-TMP was hydrolyzed to afford thymine. Hydrolysis of B_2 with 1*N* hydrochloric acid at room temperature gave TMP and TMP-NH_2 in the ratio of 1:1. In the alkaline hydrolysis (1*N* sodium hydroxide at 100° for 1 hour) compound B_2 also gave thymidine phosphoramidate and TMP, although in this condition 3′,5′-TMP was not changed. From these evidences and the absorption characteristic to thymidine (and different from 3′,5′-TMP), compound B_2 assumed to be P^1,P^2-dithymidine 5′-pyrophosphate monoamidate. The occurrence of this type of compound as the intermediate of the hydrolysis of phosphoramidate and as the precursor of P^1,P^2-dithymidine 5′-pyrophosphate (TppT)[9] was previously suggested by Clark, *et al.*[1] and recently by Hamer.[10]

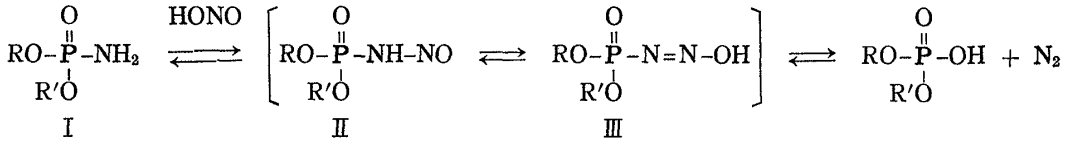
Peak C contained a nucleotide C_1, TMP and TppT. The structure of the latter two nucleotides were confirmed by the comparison with the authentic samples. Compound C_1 has the thymidine chromophor and very labile even at room temperature in neutral condition. Rechromatography of this compound separated by paper chromatography showed the similar Rf with TMP (see Fig. 3). The paper electrophoresis showed the similar migratory distance with both at pH 7.5 and 4.5. When this compoued was treated with hydrochloric acid, it gave a characteristic spot on the paper chromatography. TMP gave only inorganic phosphate and thymidine in the same condition. Considering the nature of the compound C_1 described as above, together with the fact that C_1 has no characteristic absorption band at 375~380 mμ[10] assigned for nitrosoamidate compound, we postulate for this compound thymidine phosphoro-hydroxyazo structure, which might occur as an intermediate of TMP-NH_2 to TMP reaction.[11]

Compound separated after three major peaks (ca. 5% of the total nucleotidyl material) were analyzed as having 2 or 3 phosphate residues by the electrophoresis. Although the position of the elution from the column chromatography indicated di- and tri-nuelcotides,[7] the possiblity of pyrophosphate linkage between thymidyl units could not be eliminated.

The reaction of diesterified phosphoramidate with nitrous acid was then investigated. When dibenzylphosphoramidate was reacted with nitrite in acetic acid solution, the reaction proceeds very slowly. Even after 10 days at room temperature, very slight degradation of the starting material was observed. Contrary to this, dibenzylphosphoramidate reacted with isoamyl nitrite in anhydrous media smoothly and gave dibenzylphosphate as the main product. The rate of the reaction was investigated more precisely using diphenyl-, dibenzyl- and di-*p*-nitrobenzylphosphoramidate. A typical pattern of the distribution of the products plotted against time was shown in Fig. 4. In the case of former two phosphoramidates, the main products formed were diphenyl- and dibenzyl-phosphate accompanied by a small amount of phenyl- and benzyl-dihydrogenphosphate. In the case of di-*p*-nitrobenzyl phosphate, again the main product was diesterified phosphate and a by-product having an unknown structure (not mono-*p*-nitrobenzyl phosphate) was produced. The rate of the decomposition calculated by the pseudo-first order equation was in the decreasing order : benzyl >*p*-nitrobenzyl >phenyl.

10) N. K. Hamer : J. Chem. Soc., **1964**, 1961.
11) P. J. Bunyan, J. I. G. Cadogan : J. Chem. Soc., **1962**, 1304.

The fact that the amount of isoamyl nitrite in the initial reaction mixture affected the rate of the reaction, suggests the reaction proceeds according to the multiple order kinetics. The addition of lithium chloride showed some enhancement of the rate of reaction and this fact suggests that the debenzylation reaction[12,13] had occurred in due course of the reaction, but not as the main pathway. If we assume that the phosphoramidate is exsisting as the neutral form (I) in the weakly acidic media, the attacking species NO^+ may enter the NH_2-nitrogen to form the nitrosamine derivative (II). This attack of the nitrosyl cation on the nitrogen rather than on phosphorus could be supported by the comparison of the reaction rate of three types of diesterified phosphoramidates described above. In these cases the intermediate II would be very unstable and further reaction may proceed either to the left or the right hand side of the equation, according to the nature of the environment. In the case of the TMP-NH_2, an intermediate presumably having the structure of II (or its rearranged type (III) is fairly stable enabling its isolation at low temperature. Intermediate II would then undergo intramolecular rearrangement to afford diazohydroxide compound (III), which was decomposed to phosphoric acid derivatives. The fact that in the reaction with alcohol the sole product was the phosphate rather than the alkylated ester supports this view. The "metaphosphate" intermediate should be excluded from the above results and especially in the case of the diesterified phosphoramidate. Although detailed mehanism has not yet been reported, the formation of phosphate from dibenzyl N-alkylphosphoramidate with isoamyl nitrite[14] could be explained by the mechanism proceeding via an intermediate of the type II. The formation of the pyrophosphate derivative could also be explained by the attack of TMP-NH_2 to the intermediate II or III. The occurrence of the minor product such as A_1, A_2 or the higher nucleotides in the case of TMP-NH_2 may be explained by the same mechanism.



Experimental

Paper Chromatography——Solvent A : isopropanol-ammonia-water (7:1:2), solvent B : *n*-butanol-acetic acid-water (5:2:3), solvent C : *n*-propanol-ammonia-water (55:10:35), solvent D : ethanol-1*M* ammonium acetate (pH 5) (7:3). All chromatographies were performed by descending technique on Toyo Filter Paper No. 51 A.

Paper Electrophoresis——Buffer A : triethylammonium bicarbonate (0.05*M*, pH 7.5), buffer B : 0.05*M* ammonium acetate (pH 4.5). All electrophoreses were performed on Toyo Filter Paper No. 51 A at 20 v/cm. for 1 hr.

Detection of the Spots on Chromatogram——Phosphate : molibdate spray,[15] followed by irradiation with a mercury lamp.[16] Ultraviolet absorbing spot : detected under the ultraviolet lamp.

Reaction of Monobenzylphosphoramidate with Sodium Nitrite in Aqueous Acetic Acid——a) Comparison of the reaction in various concentrations of acid : Monobenzyl phosphoramidate[1] (lithium salt, 80 mg.) was dissolved in 6 ml. (30 equiv.) of approriate concentration of acetic acid, followed by the addition of 80 mg. of sodium nitrite (3 equiv.). The mixture was kept sealed at room temperature. Results are shown in Table II.

12) V. M. Clark, A. R. Todd : J. Chem. Soc., **1950**, 2050; S. S. H. Christie, D. T. Elmore, G. W. Kenner, A. R. Todd, F. J. Weymouth : *Ibid.*, **1953**, 2947.
13) M. Ikehara, T. Ueda, K. Watanabe : This Bulletin, **8**, 663 (1960).
14) W. J. Hopwood, P. D. Regan, J. A. Stock : Proc. Chem. Soc., **1964**, 394.
15) C. S. Haynes, F. A. Isherwood : Nature, **164**, 1107 (1949).
16) R. S. Bandursky, B. Axelrod : J. Biol. Chem., **193**, 405 (1951).

Table I.

Acetic acid concentration	Time required for total decomposition of monobenzylphosphoramidate	Acetic acid concentration	Time required for total decomposition of monobenzylphosphoramidate
2*N*	15 min.	0.1*N*	more than 24 hr.[a]
1*N*	10 min.	none	almost no reaction
0.2*N*	4 hr.		

a) 50% degradation occurred in 2 hr.

Table II. Rf Values of Phosphates

	Paper chromatography			Paper electrophoresis	
Solvent	A	B	C	Buffer A	B
Benzyl P	0.46			1.00[a]	
Dibenzyl P	0.83	0.81		0.43[a]	
Benzyl P-NH_2	0.80				
Dibenzyl P-NH_2	0.84	0.93		0.01[a]	
Dibenzyl PP	0.90				
Dibenzyl PP-NH_2	0.98				
Diphenyl P	0.86			0.42[b]	
Phenyl P	0.25			1.00[b]	
Diphenyl P-NH_2	0.91			0.02[b]	
Di-nitrobenzyl P	0.92				
Di-nitrobenzyl P-NH_2	0.97				
TMP	0.11	0.37	0.37	1.00[c]	1.00[c]
TMP-NH_2	0.25	0.28	0.50	0.60[c]	
T-3′,5′-P	0.39	0.39			
TppT	0.14	0.29		0.82[c]	1.40[c]
TppT-NH_2	0.37	0.35		0.60[c]	
TMP-NNOH	0.07	0.50			
TMP-amyl	0.72	0.75		0.58[c]	
Thymidine	0.61	0.66	0.69	0.31[c]	0.19[c]

a) $R_{benzyl\text{-}P}$ *b*) $R_{phenyl\text{-}P}$ *c*) R_{TMP}

b) Quantitative estimation of the rate of reaction : Monobenzylphosphoramidate (15 mg.) was dissolved in 3/20*N* acetic acid (0.8 ml.), followed by the addition of 15 mg. of sodium nitrite (3 equiv.). Reaction was carried out at room temperature. An aliquot was withdrawn at proper intervals and applied to paper chromatography in two solvent systems. Spot having Rf 0.25 (solvent A) of monobenzylphosphate was located by molibdate spray. Appropriate spot was then excised, digested with perchloric acid and hydrogen peroxide, molibdate reagent was added, and the absorption at 830 mμ was measured.[17,18] Results are shown in Fig. 1 with the results without addition of sodium nitrite.

Reaction of Monobenzylphosphoramidate with Sodium Nitrite in the Presence of Ion-exchange Resin——Monobenzylphosphoramidate (5 mg.) and sodium nitrite (20 mg.) were dissolved in 1 ml. of water, followed by the addition of Amberlite IRC-50 (H^+ form) resin (500 mg.). Reaction was carried out at room temperature with occasional shaking. After 15 min. the spot of monobenzylphosphoramidate disappeared. Monobenyl dihydrogenphosphate and P^1,P^2-dibenzyl pyrophosphate were detected together wiith the spot presumably of P^1,P^2-dibenzyl pyrophosphroamidate, which disappeared after 26 hr.

Reaction of Monobenzylphosphoramidate with Sodium Nitrite in Anhydrous Methanol——Monobenzylphosphoramidate (10 mg.) was dissolved in 2 ml. of anhydrous methanol containing 1*N* glacial acetic acid. Even after 4 days, a half of the starting material remained unchanged. Products were monobenzyl phosphate and P^1,P^2-dibenzylpyrophosphate in the ratio of 2:1. None of the benzylmethylphosphate was detected.

17) R. G. Bartlett : J. Biol. Chem., **234**, 466 (1959).
18) D. A. Usher : J. Chromatog., **12**, 262 (1963).

Reaction of Monobenzylphosphoramidate with Ethyl Nitrite in Ethanol——Monobenzylphosphoramidate (10 mg.) was dissolved in anhydrous ethanol (2 ml.) containing 1*N* glacial acetic acid, followed by the addition of 0.5 ml. of ethyl nitrite. Reaction was carried out at room temperature for 24 hr. Examination of the reaction mixture by paper chromatography showed the presence of monobenzyl phosphate as the main product accompanied by a thin spot corresponding to benzylethylphosphate.

Reaction of Dibenzylphosphoramidate with Sodium Nitrite——Dibenzylphosphoramidate (20 mg.) was dissolved in DMSO (0.5 ml.) containing 1*N* acetic acid, followed by the addition of sodium nitrite (50 mg., 10 equiv.). After standing at room temperature for 2 days, no reaction occurred. The reaction involving water in various ratios showed also no degradation of phosphoramidate.

Reaction of Dibenzylphosphoramidate with Isoamyl Nitrite——a) In the presence of water: Dibenzylphosphoramidate (0.1 mmole) was dissolved in a mixture (0.4 ml.) of DMSO and water (3:1, v/v) containing 1*N* acetic acid, followed by the addition of isoamyl nitrite (5 equiv.). After the reaction at 37° for 3.5 hr., 95% of the starting material disappeared. After 4 days dibenzyl phosphate was the main product accompanied by 16% monobenzyl dihydrogenphosphate, which arose by the acidic debenzylation of the dibenzylphosphate.

b) In anhydrous DMSO: Dibenzylphosphoramidate (0.1 mmole) was dissolved in DMSO (0.2 ml.) containing 1*N* acetic acid, followed by the addition of isoamyl nitrite (0.5 mmole) and kept standing at 37° in a sealed tube. At the appropriate intervals (each 1 hr.) an aliquot was withdrawn from the reaction mixture and appllied to paper chromatography. After 3 hr. amount of dibenzylphosphate reached a maximum of 88% (see Fig. 4). Rate constant calculated by the pseudo-first order rate expression[19] was $k=0.185\ \mathrm{hr}^{-1}$. After 5 hr.' reaction, 10% of monobenzyl dihydrogenphosphate appeared.

When the reaction was carried out with excess (50 equivalents) of isoamyl nitrite, the reaction almost completed in 40 min. at 37°.

Reaction of Diphenylphosphoramidate with Isoamyl Nitrite——a) In anhydrous DMSO: Diphenylphosphoramidate (0.1 mmole) was dissolved in DMSO (0.2 ml.) containing 1*N* acetic acid, followed by the addition of isoamyl nitrite (5 equiv.), and kept for standing at 37°. After 30 hr. 86% of the starting material disappeared. Rate constant calculated by pseudo-first order rate equation was $k=0.020\ \mathrm{hr}^{-1}$. The products were diphenylphosphate and small amount of phenyl dihydrogenphosphae (2%).

When the reaction was carried out with 50 equiv. of amyl nitrite, the reaction completed after 8 hr.

b) In water-DMSO mixture: Conditions were exactly the same as in a), except that the solvent was altered to DMSO-water (3:1, v/v) mixture. After 28 hr.' reaction, 82% of diphenylamidate decomposed and 36% of phenyl dihydrogenphosphate was formed.

Reaction of Di-*p*-nitrobenzyl Phosphoramidate——a) In anhydrous DMSO: Di-*p*-nitrobenzylphosphoramide (0.05 mmole) was dissolved in DMSO (0.2 ml.) containing 1*N* acetic acid, followed by the addition of 10 equivalents of isoamyl nitrite, and the mixture was incubated at 37°. The pseudo-first order rate constant was $k=0.106\ \mathrm{hr.}^{-1}$ At the end of the reaction (hr.) di-*p*-nitrobenzylphosphate produced was 70% and the product having unknown structure was formed (22%).

When the amount of isoamyl nitrite in this reaction was increased to 100 equivalents, reaction completed within 90 min.

b) Addition of lithium chloride: Lithium chloride (2 equiv.) was added to the reaction mixture in a). The reaction completed after 6 hr. and showed that the reaction was slightly enhanced by the addition of lithium chloride. The amount of *p*-nitrobenzyl dihydrogenphosphate (54%) and unknown product (37%) also varied from the results of a).

Reaction of TMP-NH_2 with Isoamyl Nitrite in the Neutral Condition——a) In dimethyl formamide: TMP-NH_2 (16 mg.) and isoamyl nitrite (0.02 ml., 4 equiv.) were dissolved in 0.5 ml. of DMF. After 12 days at room temperature only slight decomposition of amidate occurred. When this reaction mixture was heated at 70° for 3 hr., TMP-NH_2 totally converted to TMP.

b) In ethanol: TMP-NH_2 (20 mg.) and amyl nitrite (0.05 ml.) were dissolved in anhydrous ethanol. After the reaction for 7 hr. at 25°, no decomposition of amidate occurred. Heating of this mixture at 70° for 4 hr. gave a samll amount of TMP-OEt together with TMP and starting material (ca. 60%).

c) In aqueous DMF: TMP-NH_2 (20 mg.) and amyl nitrite (0.05 ml., 10 equiv.) were dissolved in a mixture of water (0.03 ml.) and DMF (0.17 ml.). After the overnight reaction at room temperature TMP-NH_2 converted completely to TMP.

Reaction of TMP-NH_2 with Amyl Nitrite in Acetic Acid——a) In DMF with resin: TMP-NH_2 (20 mg.) was dissolved in 0.2 ml. of DMF, followed by the addition of IRC-50 (H^+ form) resin (6 mg.) and isoamyl nitrite (0.05 ml., 10 equiv.). After one day, ca. 80% of the starting material converted to TMP.

b) In ethanol: Solvent was changed to ethanol (0.2 ml.) in the reaction mixture in a). After 3 days the reaction proceeded less than 50%. Two new spots (solvent A) having Rf 0.58 (thin) and Rf 0.41 corresponding to TMP-OEt and TppT-NH_2 were detected.

Reaction of TMP-NH_2 with Isoamyl Nitrite in the Presence of Hydrochloric Acid——TMP-NH_2 (8 mg.) was dissolved in isoamyl alcohol (1 ml.) followed by the addition of isoamyl alcohol (1 ml.) previously

19) K. J. Laidler: "Chemical Kinetics," McGraw-Hill Co., Ltd., New York, p. 7 (1950).

saturated with dry hydrogen chloride at 0°. After standing at 3~4° in a refrigerator for 24 hr., the spot corresponding to TMP-NH_2 disappeared. Reaction mixture (TOD_{267}=32) was applied to a column (0.7×11 cm.) of DEAE-cellulose. The column was eluted with 0~0.125*M* triethylammonium bicarbonate by the linear gradient technique. Fractions corresponding to isoamyl-TMP were combined and evaporated to dryness *in vacuo*. A white powder was obtained (75% recovery). Rf's were summarized in Table II.

Large Scale Experiment of the Reaction of TMP-NH_2 with Isoamyl Nitrite——TMP-NH_2 (566 mg., 1 mmole) was dissolved in 3 ml. of dry pyridine and evaporated *in vacuo* under 30°. This process was repeated five times to remove the traces of water. The residue was taken up in 0.5 ml. of DMF followed by the addition of DMF solution (0.5 ml.) of acetic acid (2*N* in DMF). Into this mixture isoamyl nitrite (1.34 ml.) was added. The mixture was stirred for 1 day at room temperature to obtain a homogeneous solution. Reaction was continued for 5 days and finally the solvent was removed *in vacuo* The residual thick syrup was taken up in small amount of water and filtered. Filtrate and washings were combined and adjusted to pH 9 with ammonia and applied to a column (4×37 cm.) of DEAE-cellulose.[7] After washing with water, nucleotides were eluted with 4 L. of 0.125*M* triethylammonium bicarbonate and 4 L. of water by linear gradient elution. Flow rate was 30 ml./hr. and the eluent was collected in 15 ml. fractions. Pattern of the chromatography was shown in Fig. 1. The distribution of the nucleotides among various peaks were listed in Table III.

Table III.

	TOD_{267}	Yield (%)
Peak A (fraction No. 25~43)	8.7 ×15	2.2
Peak B (fraction No. 118~157)	75.0 〃	19.4
Peak C (fraction No. 238~312)	288.2 〃	74.4
Peak D	4.6 〃	1.2
Peak E	10.3 〃	2.7

Table IV.

	$\lambda_{max}^{H^+}$ mμ	$\lambda_{max}^{H_2O}$ mμ	$\lambda_{max}^{OH^-}$ mμ
A_1	265	266	266
A_2	267	267	266
A_4	259	259	260

i) Peak A (fraction No. 25~43): An aliquot of peak A was analyzed by paper chromatography (solvent A). Spot having Rf's 0.77 (A_1), 0.72 (A_2), 0.60 (A_3) and 0.17 (A_4) were detected. Yield was 0.3, 0.3, 1.4 and 0.2% of the starting material used, respectively. Compound A_3 has the characteristic absorption properties of thymidine and identified with authentic sample of thymidine by chromatography (solvent B, Rf 0.68). Although the structure of A_1, A_2 and A_4 (these spots revealed a blue color reaction with molibdate spray) was not clarified as yet, UV absorption properties were listed in Table IV.

ii) Peak B (fraction No. 118~157): Paper chromatography (solvent A) of this peak showed the presence of four compounds having Rf's: 0.67 (B_1), 0.36 (B_2), 0.24 (B_3) and 0.09 (B_4). B_1 revealed a characteristic pale blue color by the molibdate spray and had UV absorption properties: $\lambda_{max}^{H_2O,H^+}$ 268 mμ, $\lambda_{max}^{OH^-}$ 267.5 mμ. B_1 was not decomposed by the heating with 0.1*N* hydrochloric acid at 100° for 4 min. The comparison of this compound with an authentic specimen of TMP-isoamyl ester synthesized as described above, showed complete identity in paper chromatography (Rf (A) 0.72, Rf (B) 0.75), paper electrophoresis and optical properties. B_2 showed the similar Rf values with thymidine 3′,5′-phosphate in the solvent A and B. However the λ_{max} of this material ($\lambda_{max}^{H_2O}$ 268 mμ) was not identical with the reported value ($\lambda_{max}^{H_2O}$ 264.8 mμ). Acidic hydrolysis of this material gave only TMP (0.1*N* hydrochloric acid at 100° for 4 min.), and did not give thymine, which was detected in the hydrolysate of 3′,5-TMP in the same condition. When B_2 was hydrolyzed in 1*N* sodium hydroxide at 100° for 1 hr., TMP and TMP-NH_2 were obtained. The values of P^1,P^2-dithymidine 5′-pyrophosphoroamidate were shown in Table II.

B_3 was identical with the starting material, TMP-NH_2 in all respects. B_4 was shown to be identical with TMP by the comparison with authentic sample. These two compounds may arise from TppT-NH_2 in the evaporation process of the pooled eluting fractions.

iii) Peak C (fraction No. 238~312): This peak contains three compounds shown by the examination by paper chromatography. C_1 was identical with thymidine, which appeared by the decomposition of TMP.

Second substance C_2 was identical with TppT, which was synthesized after Khorana's procedure.[9] Paper electrophoresis in pH 7.5 and 4.5 showed the presence of two monodissociated phosphates. The ratio, base–phosphate=1:1 and $\varepsilon(P)=8.26\times10^3$. Acidic hydrolysis gave TMP other then the spot of the starting material. C_3 was identical with TMP in paper chromatography in solvent A. However in the solvent B it clearly showed the existence of an accompanied substance, which runs after TMP as described in Fig. 3. The upper part of this spot was cut out from the paper and rechromatographed in the same solvent. This time TMP was the only spot detected. The water solution of this compound after prolonged (about one month) storage at room temperature contained only TMP. These facts showed the compound C_3 is very labile and give rise to TMP after hydrolysis. Hence, this peak is containing TMP and a labile presursor of TMP.

iv) Peak D and E : There appeared two minor peaks (D and E) after three major peaks (A~C). Compound D showed $A_{260}/A_{280}=1$, which showed the modification of thymine moiety. Peak E appeared at the elution position corresponding to linear di- or cyclic tri-nucleotide of thymidine. However, the structure of this compound has not yet been elucidated. The possibility of the existence of pyrophosphate linkage in this compound could not be excluded.