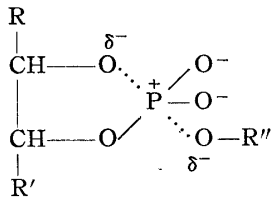
UDC 547.635'118.5

57. Tyunosin Ukita and Akira Hamada : Organic Phosphates. XIII.*2 Studies on Alkaline Hydrolysis of Cyclic Phosphotriesters.*3

*(Faculty of Pharmaceutical Sciences, University of Tokyo*1)*

Results of several investigations on the alkaline hydrolysis of naturally occurring or synthetic phosphodiester-type compounds, which contain a 1,2-alkanediol 1-phosphate group, showed that a five-membered cyclic phosphate is formed as an intermediate compound to give the final phosphomonoesters. Thus, in the case of the hydrolysis of α-glycerophosphorylcholine, the final product α- and β-glycerol phosphate was shown to be produced via glycerol cyclic phosphate as their direct precursor,[1] and in a similar reaction of alkyl 3'-ribonucleotide, 2',3'-cyclic phosphate was also proved to be the precursor of the final product, 2'- and 3'-ribonucleotides.[2]

As for the occurrence of such intermediate cyclic phosphates, a previously proposed reaction mechanism involving a cyclic phosphotriester[3] has recently been revised involving a transition state represented by (A)[2] which occurs by atttack of phosphorus center in the starting diester by the hydroxylate anion produced from hydroxyl group in the diol moiety.[4]



(A)

However, no observation has ever been reported on the behavior of five-membered cyclic phosphotriesters in similar alkaline circumstance to that used in the above-mentioned hydrolysis conditions.

In the previous paper of this series the authors attempted and succeeded in synthesizing two kinds of cyclic phosphotriesters, 2-(benzyloxycarbonylamino)ethyl hydrobenzoin cyclic phosphate and 1,2,3,4-tetra-O-acetyl-β-D-glucose 6-hydrobenzoin cyclic phosphate.*2 As reported in that paper, these triesters were found to be sufficiently stable in water and available for the examination of their behavior in alkaline hydrolysis. In the present work, the mode of the decomposition of one of the above two triesters, 2-(benzyloxycarbonylamino)ethyl hydrobenzoin cyclic phosphate (I) was investigated and its results are described.

In order to identify the alkaline hydrolysates of (I), paper chromatography and paper electrophoresis were employed. The four phosphates were synthesized and used as standards; 2-(benzyloxycarbonylamino)ethyl hydrobenzoin phosphate (II) and hydrobenzoin cyclic phosphate[5] (III), which are supposed to be the intermediates, and hydrobenzoin phosphate[5] (IV) and 2-(benzyloxycarbonylamino)ethyl phosphate (V), the probable final

---

*1 Hongo, Tokyo (浮田忠之進, 浜田 昭).

*2 Part XII : This Bulletin, **9**, 363 (1961).

*3 From the thesis of Akira Hamada for the degree of Doctor of Pharmaceutical Sciences, University of Tokyo, 1959.

1) T. Ukita, K. Nagasawa, M. Irie : This Bulletin, **5**, 127 (1957).
2) D. M. Brown, D. I. Magrath, A. H. Neilson, A. R. Todd : Nature, **177**, 1124 (1956).
3) D. M. Brown, A. R. Todd : J. Chem. Soc., **1952**, 52.
4) D. Lipkin, P. T. Talbert, M. Cohn : J. Am. Chem. Soc., **76**, 2871 (1954).
5) T. Ukita, K. Nagasawa, M. Irie : *Ibid.*, **80**, 1373 (1958).

products in alkaline hydrolysis of (I). These standard compounds were submitted to combined run by paper chromatography in horizontal and paper electrophoresis in vertical directions. The Rf values and mobilities (M) observed for each standard compound are shown in Fig. 1. The two phosphomonoesters (IV and V), which were found overlapping in both Rf and M values, were differentiated by subsequent catalytic hydrogenolysis of the extract obtained from the cutting of the overlapped spot. Thus, after hydrogenolysis, inorganic phosphate and phosphorylethanolamine were respectively detected from (IV) and (V) by electrophoresis.

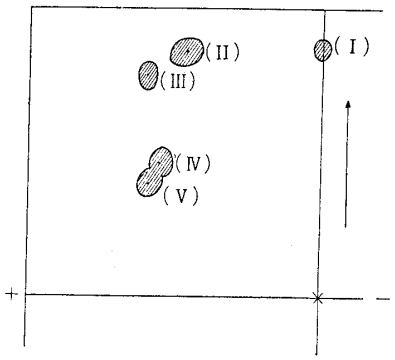


Fig. 1.

Two-dimensional Chromatogram of Combined Paper Electrophoresis with Ascending Chromatography

(I)	Rf 0.87	M 0
(II)	Rf 0.86	M 0.60
(III)	Rf 0.77	M 0.76
(IV)	Rf 0.47	M 0.69
(V)	Rf 0.40	M 0.71

Solvents applied : see text

Table I. Alkaline Hydrolysis of 2-(Benzyloxycarbonylamino)ethyl Hydrobenzoin Cyclic Phosphate (I) by NaOH at 37°

NaOH		hr. 0	0.5	1	3	6	24
0.01*N*	I	⫼	⫼	⫼	⫼	⫼	⫽
	II	±	+	+	+	+	⫽
	III	−	−	−	−	−	−
	IV	−	−	−	−	−	−
	V	−	−	−	−	−	−
0.1*N*	I	−	−	−	−	−	−
	II	⫼	⫼	⫼	⫼	⫼	⫼
	III	−	−	−	−	−	−
	IV	−	−	−	−	−	−
	V	−	−	−	−	−	−

NaOH		hr. 0	0.5	1	3	6	24
0.5*N*	I	−	−	−	−	−	−
	II	⫼	⫼	⫼	⫼	⫼	⫽
	III	−	−	−	−	−	−
	IV	−	−	∓	+	+	⫽
	V	−	−	−	−	−	−
1*N*	I	−	−	−	−	−	−
	II	⫼	⫼	⫽	⫽	+	−
	III	−	−	−	−	−	−
	IV	−	+	⫽	⫽	⫼	⫼
	V	−	−	−	−	−	−

Signs indicate the grades of the sizes and tones of phosphate spots observed on the filter papers, −, ∓, ±, +, ⫽, ⫼, ⫼ indicating nil, ca. 2~5%, ca. 10~15%, ca. 25%, ca. 50%, ca. 75%, and ca. 90~100% phosphorus of total phosphorus in the test mixture.

Stability of the triester (I) in various concentrations of sodium hydroxide at 37° is shown in Table I. This compound (I) was found to be very unstable in alkaline medium and a part of it was decomposed immediately by 0.01*N* sodium hydroxide to the corresponding acyclic diester (II), and after 30 minutes about 20% of (I) was found to be converted into (II). In 0.1*N* sodium hydroxide solution 100% of (I) was immediately decomposed to (II), which was found stable even after 24 hours in the same medium. The diester (II) produced by hydrolysis of (I) began to be hydrolyzed in 0.5*N* sodium hydroxide after 3 hours' incubation at 37° to form hydrobenzoin monophosphate (IV). Similar hydrolysis of the diester to the monoester was observed after 30 minutes' contact of the former with *N* sodium hydroxide solution.

It should be noted that in the alkaline hydrolysis of the diester (II) no 2-(benzyloxycarbonylamino)ethyl phosphate (V) but hydrobenzoin monophosphate (IV) was found. Such mode of alkaline hydrolysis in which a phosphodiester involving a 1,2-alkanediol phosphate moiety gives phosphomonoester of the 1,2-alkanediol is commonly observed for such

Table II. Stability of Hydrobenzoin Cyclic Phosphate (III) in NaOH Solution at 37°

NaOH		hr. 0	0.5	1	2	4	6	24
0.1N	III	⫲	⫲	⫲	⫲	+	−	−
	IV	−	∓	±	+	⫲	⫲	⫲
0.5N	III	⫫	+	±	−	−	−	−
	IV	⫫	⫲	⫲	⫲	⫲	⫲	⫲
1N	III	−	−	−	−	−	−	−
	IV	⫲	⫲	⫲	⫲	⫲	⫲	⫲

Signs are the same as in Table I.

types of naturally occurring or synthetic phosphodiester as ribonucleic acid,[4,6] alkyl ribonucleotide,[2,7] alkyl glycerophosphate,[3,8,9] and alkyl cyclohexanediol phosphodiester.[10]

As in the above experiments on detection of the intermediate products in hydrolysis of (I), hydrobenzoin cyclic phosphate (III) was not detected, the stability of (III) in similar medium was tested using an authentic specimen. As shown in Table II, (III) was found to be completely decomposed to (IV) after 6 hours' incubation in 0.1N sodium hydroxide, 2 hours' in 0.5N sodium hydroxide, and immediately on contact with N sodium hydroxide. These results show that in the minimum concentration of alkali, in 0.5N sodium hydroxide, which is necessary for the hydrolysis of (II) to (IV), the cyclic phosphate (III) could not be accumulated in sufficient amount to be detected but decomposed to the monophosphate (IV), even if (III) could be produced as an intermediate from the diester (II) to monoester (IV).

Table III. Alkaline Hydrolysis of 2-(Benzyloxycarbonylamino)ethyl Hydrobenzoin Cyclic Phosphate (I) and 2-(Benzyloxycarbonylamino)ethyl Hydrobenzoin Phosphate (II) in conc. NH_4OH

	(I) at 37° hr. 1	6	24		(I) at 100° hr. 5 min.	0.5	1		(II) at 37° hr. 1	6	24		(II) at 100° hr. 2	4
I	⫲	⫲	−	I	−	−	−	II	⫲	⫲	⫲	II	⫲	⫲
II	+	⫲	⫲	II	⫲	⫲	⫲	III	∓	∓	∓	III	±	±
III	∓	∓	∓	III	∓	∓	∓	IV	−	±	+	IV	+	⫲
IV	−	−	−	IV	−	−	∓	V	−	−	−	V	−	−
V	−	−	−	V	−	−	−							

Signs are the same as in Table I.

In order to have a more precise information on the alkaline hydrolysis of the triester (I), the reaction was carried out in ammonia solution in which the cyclic phosphate (III) was assumed to be more stable than in alkali hydroxide solution. As shown in Table III, about 20% of the phosphotriester (I) added was found to be converted into the diester (II) after 1 hour's incubation at 37° in ammonia with simultaneous production of ca 2~5% of cyclic phosphate (III). At 100° in the same medium, (I) was completely decomposed to (II) after 5 minutes with the production of 2~5% of cyclic phosphate (III), and after 1 hour's prolonged incubation at 100° the final hydrolysis product, phosphomonoester (IV), was began to be detected. On similar incubation of (II) with ammonia at 37°, the reaction mixture contained a small amount (ca. 2%) of (III) which was still detectable with monophosphate (IV) after 6 hours' prolonged incubation. In these experiments, the amount of

6) R. Markham, J. D. Smith : Biochem. J., **52**, 552 (1952).
7) D. M. Brown, A. R. Todd : J. Chem. Soc., **1952**, 44,
8) E. Baer, M. Kates : J. Biol. Chem., **175**, 79 (1948); **185**, 615 (1950).
9) D. M. Brown, G. E. Hall, R. Letters : J. Chem. Soc., **1959**, 3547.
10) D. M. Brown, H. M. Higson : *Ibid.*, **1957**, 2034.

cyclic phosphate (III) detected gradually increased with prolonged incubation time, within the yield of 2~5% of the starting material used. In hydrolysis of (II) in ammonia at 100°, after 2 hours a marked amount of (III)(15%) was detected with simultaneous production of phosphomonoester (IV).

The results of these experiments indicate that alkaline hydrolysis of phosphodiester (II) to phosphomonoester (IV) involved cyclic phosphate (III) as an intermediate compound.

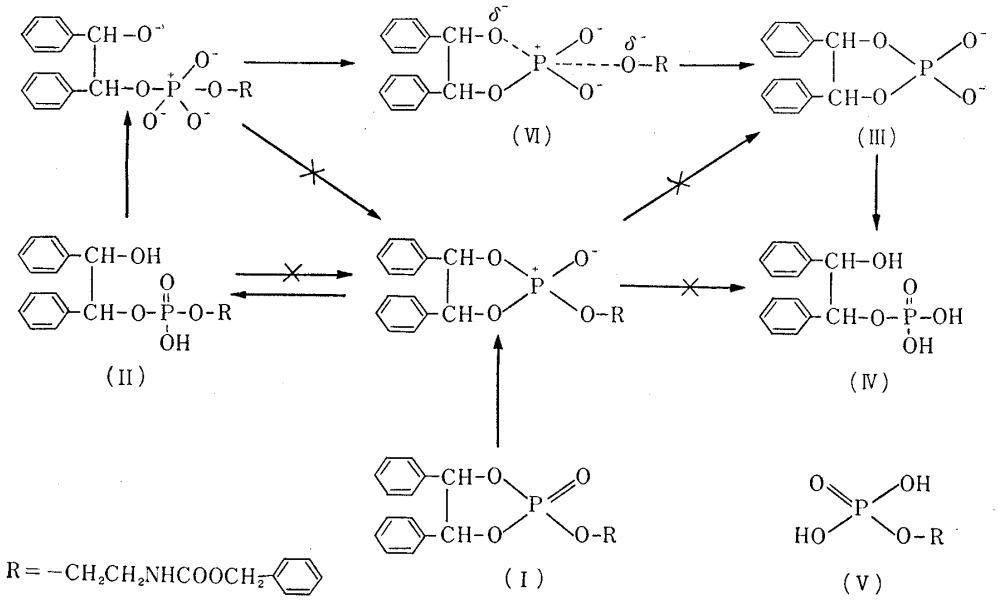


Chart 1.

Chart 1 schematically represents the fate of phosphotriester (I) in alkaline media. The triester (I) which on exposure in alkaline medium polarizes to cyclic orthophosphotriester should be converted to the phosphodiester (II) in 0.01*N* sodium hydroxide. The latter is fairly stable in this concentration of alkali but begins to decompose in 0.5*N* sodium hydroxide after 3 hours' incubation at 37° to phosphomonoester (IV) via cyclic phosphate (III). As the phosphotriester (I) is more unstable than phosphodiester (II) to alkali, possibility of the conversion of (II) to (I) should not be expected in any of the concentrations of alkali used.

The pathway of the triester (I) to form cyclic phosphate (III) directly could be excluded, because 30 minutes' incubation of (I) in 0.1*N* sodium hydroxide, a condition which converts all of the latter to (II), did not give any evidence of cyclic phosphate (III) produced while (III) was found to be stable after 30 minutes' incubation in the same concentration of sodium hydroxide at the same temperature.

Furthermore no possibility of the direct formation of (IV) from (I) is expected from the results shown in Table I.

The observations obtained here furnished the route of alkaline hydrolysis of the phosphotriester (I) to follow the product (II), (III), and (IV) in that order. As for the mechanism of the formation of cyclic phosphate (III) from the diester (II) in the critical concentration of alkali, in which sufficient concentration of hydroxylate anion formed from the hydroxyl group of the hydrobenzoin moiety in (II) is accumulated, its nucleophilic attack of the phosphorus center should form a transition state (VI) prior to the production of the cyclic phosphate (III). Thus, in the alkaline hydrolysis of (II) to (IV), no evidence was found for the possible rôle of (I) as an intermediate compound, and Lipkin, *et al.*[4] indicated a similar evidence in the formation of cyclic nucleotide.

Experimental

Paper Chromatography and Paper Electrophoresis——i) Paper chromatography : Samples were applied on Toyo Roshi No. 53 filter paper and run ascendingly for 15 hr. with a solvent system of iso-PrOH-conc. NH_4OH-H_2O (7:1:2). P was detected by the method of Bandurski and Axelrod.[11]
ii) Paper electrophoresis : The materials were applied on strips (40×10 cm.) of Toyo Roshi No. 53 filter paper (start line was placed 12.5 cm. from edge of the paper set on cathode side) which were moistened with buffer solution of pH 5.6 (BuOH-pyridine-AcOH-H_2O (20:10:2:968)). These strips were subjected to electrophoresis at a potential of 25 v/cm. for 120 min. P-containing spots were detected on paper as above, the mobility (M) for each P spot was represented by the ratio of the distance of the spot from the start line to that for DNP-glutamic acid used as standard.
iii) Two-dimensional chromatograms by combined paper electrophoresis with ascending paper chromatography : The materials loaded on Toyo Roshi No. 53 filter paper (40×40 cm.) were separated first by electrophoresis and, after drying, the paper was applied to ascending chromatography.

Potassium 2-(Benzyloxycarbonylamino)ethyl Hydrobenzoin Phosphate (II)——To a solution of 36 mg. of crude 2-(benzyloxycarbonylamino)ethyl hydrobenzoin cyclic phosphate*2 (I) in 2 cc. of *tert*-BuOH, 0.2*N* KOH was added and the mixture was incubated at 37° for 1 hr. Paper electrophoresis at this stage showed complete conversion of (I) to the diester (II). The solution was neutralized with Amberlite IR-120 (H^+ form), extracted with Et_2O, and the aqueous layer, after removal of the resin, was lyophilized. The residue was dissolved in 3 cc. of H_2O and the insoluble material was separated by filtration. The filtrate was concentrated *in vacuo* to a small volume and added with Et_2O. After standing overnight in a refrigerator, the colorless powdery precipitate that separated was collected by centrifugation to obtain a very hygroscopic product. *Anal.* Calcd. for $C_{24}H_{29}O_7NKP$: P, 6.28. Found : P, 6.25.

Ammonium 2-(Benzyloxycarbonylamino)ethyl Phosphate (V)——A solution of 2.2 g. of phosphorylethanolamine[12] in 8 cc. of 2*N* NaOH was placed in a three-necked flask provided with a mechanical stirrer and two dropping funnels. The flask was cooled in an ice bath and 2.7 g. benzyloxycarbonylchloride dissolved in toluene[13] and 4 cc. of 4*N* NaOH were simultaneously added with vigorous stirring over a period of 10 min. The mixture was stirred for additional 20 min. in an ice bath and 30 min. at room temperature. The toluene layer was separated and the aqueous layer was lyophilized to furnish 5.2 g. of a residue. A solution of 1.0 g. of the residue thus obtained dissolved in 10 cc. of H_2O was filtered and the clear filtrate was applied on the top of a column (3×15 cm.) prepared by pouring a suspension of Toyo Roshi cellulose powder in a mixed solvent of iso-PrOH-conc. NH_4OH-H_2O (7:1:2). The column was eluted with the same solvent mixture to collect 10-cc. fractions. The fractions which gave a single spot with Rf 0.40 were combined and lyophilized. Slightly yellow solid (0.7 g.) thus obtained was dissolved in 15 cc. of H_2O, the solution was passed through a column of Amberlite IRC-50 (NH_4^+ form), and the resin was washed twice with 20-cc. portions of H_2O. The residue obtained on lyophilization of the effluent and washings was dissolved in 30 cc. of warm MeOH and filtered. The filtrate was concentrated to 10 cc., added with equal volume of Et_2O, and kept in a refrigerator. The inorganic impurity that precipitated with the oily product was separated by filtration and washed with Et_2O. The filtrate and washing were combined, concentrated to 5 cc., and 5 cc. of AcOEt and 30 cc. of Et_2O were added to the concentrate. The crystals that separated on cooling were collected and recrystallized from EtOH to hygroscopic fine needles, m.p. 121~122°; yield 520 mg. (60%). The sample for analysis was dried over P_2O_5 *in vacuo* at room temperature for 5 hr. *Anal.* Calcd. for $C_{10}H_{17}O_6N_2P$: C, 41.10; H, 5.89; N, 0.59; P, 10.60. Found : C, 40.96; H, 6.04; N, 9.30; P, 10.32.

Alkaline Hydrolysis of 2-(Benzyloxycarbonylamino)ethyl Hydrobenzoin Cyclic Phosphate (I)——i) With various concentrations of NaOH : To 4 tubes each containing 12 mg. of (I) dissolved in each 0.13, 0.13, 0.40, and 0.40 cc. of *tert*-BuOH, 0.65 cc. of 1.2*N* NaOH, 0.65 cc. of 0.6*N* NaOH, 0.40 cc. of 0.2*N* NaOH, and 0.40 cc. of 0.02*N* NaOH were respectively added to make the final alkalinity of *N*, 0.5*N*, 0.1*N*, and 0.01*N* in each tube.

All the tubes were incubated at 37°. Each 0.05 cc. was withdrawn from the 4 solutions at intervals and after neutralization with CO_2, applied to paper electrophoresis and succeeding paper chromatography. The results observed are summmarized in Table I.
ii) With conc. NH_4OH : Each one of two sets of sealed tubes which contained 2 mg. of (I) and 0.2 cc. of conc. NH_4OH was incubated at 37°, and 100°, respectively. The tube was taken out at intervals and after removal of NH_3 by aeration, the product was applied to paper electrophoresis and sub-

*4 The insoluble unreacted (I) which was separated from the supernatant was dissolved in *tert*-BuOH and applied to similar detection.

11) R. S. Bandurski, B. Axelrod : J. Biol. Chem., **193**, 405 (1951).
12) H. N. Christensen : *Ibid.*, **135**, 399 (1940).
13) M. Bergmann, L. Zervas : Org. Syntheses, **23**, 13.

sequent paper chromatography.*4 The results are given in Table III.

Hydrogenation of Phosphomonoester produced by Alkaline Hydrolysis of (I)——To a solution of 30 mg. of (I) in 0.2 cc. of *tert*-BuOH, 1 cc. of *N* NaOH was added and the solution was incubated at 37° for 14 hr. The resulting solution was streaked on Toyo Roshi No. 53 filter paper (40×10 cm.) and run ascendingly at room temperature for 15 hr. The single P band observed was cut out and the cutting was extracted with a minimum volume of H_2O (ca. 30 cc.). The extract was hydrogenated for 6 hr. over 200 mg. of Pd-C (10% Pd) in H_2 atmosphere at room temperature, the catalyst was filtered off, and parcholated with 0.1*N* HCl. The filtrate and washing were separately concentrated *in vacuo* to a small volume, and submitted to both paper chromatography and paper electrophoresis. The common P product obtained from the filtrate and washing was identified as inorganic phosphate at Rf 0.06 and M 1.08, no phosphorylethanolamine was detected.

Alkaline Hydrolysis of Potassium 2′-(Benzyloxycarbonylamino)ethyl Hydrobenzoin Phosphate (II) with conc. NH_4OH——The reaction procedure and analytical method were the same as in the case of the hydrolysis of (I) with conc. NH_4OH, except that 1.5 mg. of the test compound and 0.15 cc. of conc. NH_4OH were used. The results obtained are shown in Table III.

Alkaline Hydrolysis of Sodium Hydrobenzoin Cyclic Phosphate (III)——To 0.4 cc. of *N*, 0.5*N*, and 0.1*N* NaOH, 4 mg. of (III) was added. The mixture was kept at 37° and 0.05 cc. each of the reaction mixtures was taken out at intervals, and, after neutralization with CO_2, tested by paper chromatography. The results observed are given in Table II.

A part of the expenses of this work was supported by the Grant-in-Aid for Scientific Research provided by the Ministry of Education to which the authors' thanks are due. Thanks are also due to Mr. Ohata, Iatrochemical Institute of Pharmacological Research Foundation, and Misses Iwanaga and Ohno, Institute for Infectious Diseases, University of Tokyo, for the microanalyses.

Summary

2-(Benzyloxycarbonylamino)ethyl hydrobenzoin cyclic phosphate (I), 2-(benzyloxycarbonylamino)ethyl hydrobenzoin phosphate (II), and hydrobenzoin cyclic phosphate (III) were reacted with various concentrations of aqueous alkali and concentrated ammonia, and the hydrolysis products of these compounds were examined at intervals. From the results obtained by this kinetic study, it was concluded that the triester (I) does not take part as an intermediate in the alkaline hydrolysis of (II) to the final product of hydrobenzoin phosphate (IV).

(Received August 4, 1960)