U.D.C. 547.438.1

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18. Tyunosin Ukita, Kinzo Nagasawa, and Masachika Irie : Organic Phosphates. II.[8] Studies on Hydrolysis of Several Cyclic Phosphates.

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From the results of their detailed investigations on acid and alkaline hydrolysis of L-α-glycerylphosphorylcholine and L-α-lecithin, Baer and Kates[1,2] proposed mechanisms of these reactions as shown in Chart 1. Thus both in acid and alkaline conditions in the main pathway of the reactions, lecithin[3] at first liberates the fatty acid moieties to give α-GPC[4] (I) which, via cyclic glycerylorthophosphorylcholine (CGPC) (II) by reversible migration of the phosphoryl group, is converted to β-GPC (III). In the next stage, hydrolysis occurs to eliminate the choline groups from (I) and (III) giving, as the final products, α-GP (IV) and β-GP (V), respectively. Further, in acid condition especially, (IV) and (V) can isomerise alternatively by reversible migration of the phosphoryl group via cyclic ortho-GP (VI). In alkaline hydrolysis, the amounts of α-GP and β-GP which might be produced from (I) and (III) after liberation of choline depend on the equilibrium among (I), (II), and (III) at the given pH, thus CGPC is the only intermediate for the phosphoryl migration. This mechanism was supported by the results of their kinetic studies, showing that the decrease of the optical rotation, when

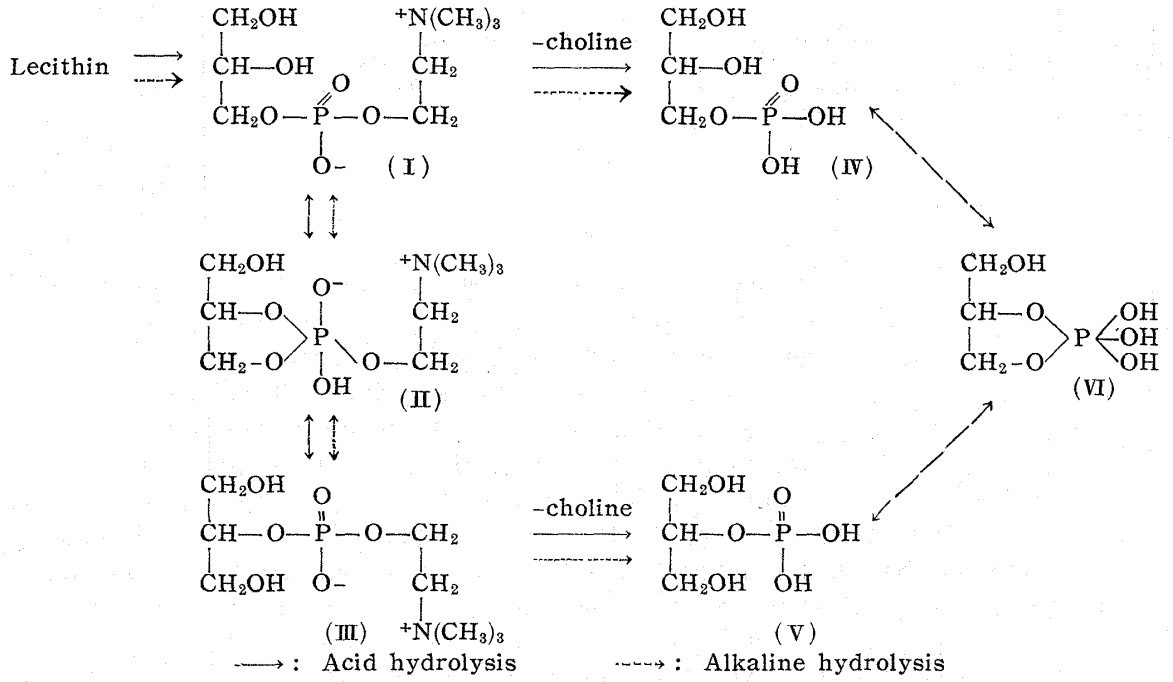

Chart. 1.

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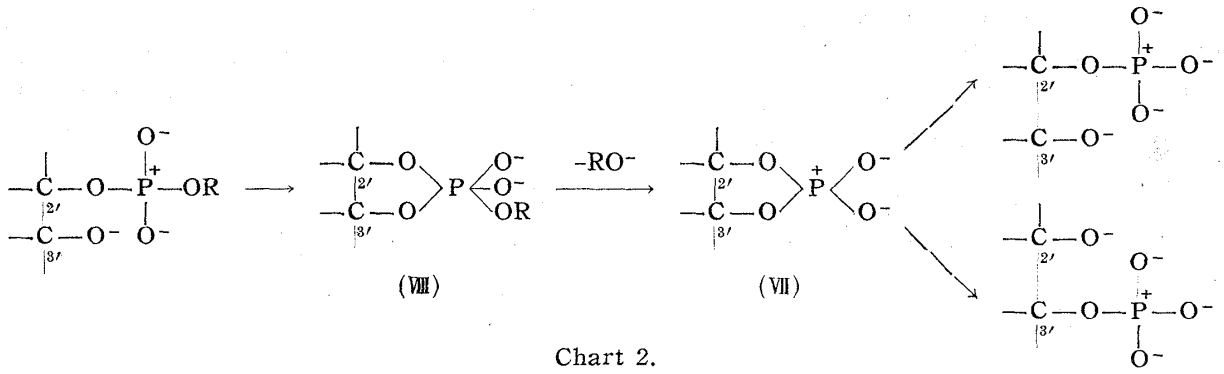
1) E. Baer, M. Kates : J. Biol. Chem., **175**, 79(1948).
2) E. Baer, M. Kates : *Ibid.*, **185**, 615(1950).
3) Chart 1 does not include the problems concerning optical properties of the products which occur when L-α-lecithin is hydrolysed.
4) The following abbreviations are used : GCP=glycerol cyclic phosphate, PCP=1,2-propanediol cyclic phosphate, ECP=ethyleneglycol cyclic phosphate, BCP=2,3-butanediol cyclic phosphate, GPC=glycerylphosphorylcholine, CGPC=cyclic ortho-glycerylphosphorylcholine, GP=glycerol phosphate PP=1,2-propanediol phosphate (preceded by cation substituted or Greek letter indicating the position of hydroxyl group concerned with phosphoryl ester where necessary).

L-α-GPC was hydrolysed, was much faster than the liberation of choline during the reaction.[5]

According to their reports,[1] after 100 hrs. in 1*N* hydrochloric acid at 37°, (I) gave 90% and 10% of (IV) and (V), respectively, and a solution of (I) in 1*N* sodium hydroxide, after being kept at 37° for 10 hrs. gave 44% and 56% of (IV) and (V), respectively, as the final proportions of the products.

Recently, Lipkin, Talbert, and Cohn[6] using $H_2{}^{18}O$ for hydrolytic reaction, proved that in the alkaline hydrolysis of ribonucleic acid the direct precursor which gives 2'- and 3'-nucleotide must be a transition state derived from cyclic 2',3'-nucleotide (VII) instead of the quinquevalent phosphorus compound as represented by (VIII). Thus an intermediate (VIII) is converted to a cyclic diester prior to the formation of 2'- and 3'-nucleotides.

Since Ukita, Bates, and Carter[7] observed a spot corresponding to synthetic GCP on the paper chromatogram of the alkaline hydrolysate of L-α-GPC, although they did not succeed in its isolation, it is not inconsistent to suppose that also in the alkaline hydrolysis of GPC a reaction similar to that for ribonucleic acid is involved (Chart 3) and that the immediate precursor to α- and β-GP should be GCP instead of the diesters, α- and β-GPC, as proposed by Baer and Kates.



(VIII) (VII)

Chart 2.

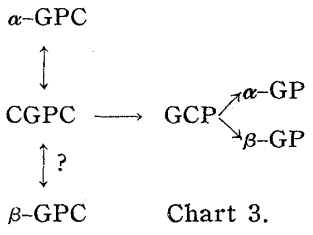
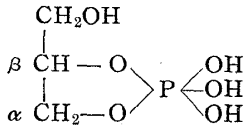


Chart 3.

From these viewpoints, it is of interest to apply acid and alkaline hydrolysis to synthetic GCP and to observe the resulting α- and β-GP, both qualitatively and quantitatively.

The synthetic GCP was hydrolysed under conditions comparable to those used by Baer and Kates and the products were tested by both paper chromatography and periodate oxidation. The results obtained are summarized in Table I. As shown in this table, although GCP was not detected on paper chlomatogram after one hour's incubation, the amount of α-GP resulting from acid hydrolysis increased gradually and reached the constant value of ca. 80% after 96 hours, which is a value similar to that observed by Baer and Kates for the amount of α-GP after hydrolysis of GPC under the same conditions.



(IX)

This result could be interpreted by assuming that, GCP, at first, was converted to (IX) which was promptly protonated and suffered P-O fission to give

5) More recently, Todd, *et al.* (D.M. Brown, D.I. Magrath, A.H. Neilson, A.R. Todd: Nature, **177**, 1124(1956)) reported that during alkaline hydrolysis of alkyl 3'-nucleotide, alkyl 2'-nucleotide, the migration product of alkyl phosphate residue, was not detected, although a remarkable amount of the latter appeared during acid hydrolysis. Thus they invalidated the occurrence of β-GPC during the alkaline hydrolysis of α-GPC suggested by Baer and Kates.

6) D. Lipkin, P.T. Talbert, M. Cohn: J. Am. Chem. Soc., **76**, 2871(1954).

7) T. Ukita, N.A. Bates, H.E. Carter: J. Biol. Chem., **216**, 867(1955).

Table I. Acid and Alkaline Hydrolysis of GCP

Results obtained by		Hydrolysis with 1N HCl at 37°C (hrs.)				Hydrolysis with 1N NaOH at 37° (hrs.)		
		1	6	20	96	1	6	20
Paper chromatography	GCP ($Rf^{8)}$ 0.60)	−	−	−	−	−	−	−
	GP ($Rf^{8)}$ 0.23)	+	+	+	+	+	+	+
Periodate oxidation	Content of α-GP (%)	48.0	64.0	79.6	80.6	46.3	45.4	45.0

+ indicates that the phosphorus spot with the corresponding Rf value was found and − indicates that no spot with corresponding Rf value was observed on paper chromatograms.

ca. 50% of α- and β-GP. In the next stage, as the given condition was favored to cause a migration of the phosphoryl group to give α-GP from β-GP via (IX), the most probable intermediate, the latter was converted into the former gradually to reach an equilibrium state of both compounds.

The similar conversion of β-GP into α-GP was observed for a sample of a β-GP-rich mixture of both isomers (containing 72% of β-GP); thus when the latter was treated under the same conditions, the amount of α-GP was found to be ca. 80% after incubation.

In the case of alkaline hydrolysis, GCP gave ca. 45% of α-GP under a given conditions. As an alkaline condition has well been known not to cause the phosphoryl migration for 1,2-alkanediol-1 phosphate, the observed data reveal that the GCP used for the reaction was hydrolysed to yield ca. 45% of α-GP and ca. 55% of β-GP which suffered no further phosphoryl migration.

The similarity of the data for the amount of α-GP obtained by the alkaline hydrolysis of GCP this time, to that observed for similar hydrolysis of GPC by Baer and Kates, leads one to an assumption that the direct precursor which gives α-GP and β-GP in the pathway of the alkaline hydrolysis of GPC will be GCP.

This could be further supported by both the observations of Lipkin *et al.*[6] and Ukita *et al.*[7] cited above. Thus the mechanism of the reaction could be represented by Chart 3.

The rapid decrease of optical rotation before the liberation of choline during alkaline hydrolysis of L-α-GPC, as observed by Baer and Kates, could only be interpreted by the occurrence of β-GPC during the reaction, although it was invalidated by Todd, *et al.*[5] from the result of the analogous reaction for an alkyl nucleotide.

Irrespective of the occurrence of β-GPC in the pathway to GCP, the present result strongly suggests that, in alkaline hydrolysis of GPC, α- and β-GP were produced from the latter, instead of α- or β-GPC.

Acid and alkaline hydrolysis, under conditions similar to those used for GCP, were tested on PCP, the structure of which differs from the former by the absence of a free hydroxyl group on the vicinal position to one of the phosphate bonds.

From the results of synthetic research reported in the previous paper of this series,[8] two compounds, α-PP and β-PP, which are expected as the hydrolysis products of PCP, are known to give respective Rf values of 0.27 and 0.36 on paper chromatogram.[9] PCP was hydrolysed under acid and alkaline conditions similar to those used for GCP hydrolysis and aliquots were withdrawn from the hydrolysates at intervals to be submitted to paper chromatographic detection of phosphorus compounds. The results are summarized in Table II which shows that, differing from the case of GCP, the only product of both acid and alkaline hydrolysis of PCP was α-PP and that the

8) Part I: T. Ukita, K. Nagasawa, M. Irie: This Bulletin, **5**, 121(1957).
9) The solvent system used for paper chromatography was *iso*-PrOH : 5N NH_4OH (2 : 1).

Table II. Acid and Alkaline Hydrolysis of PCP

hrs. \ Rf[8]	Hydrolysis at 37°C with 1N HCl			Hydrolysis at 37°C with 1N NaOH			Control		
	PCP 0.63	α-PP 0.27	β-PP 0.36	PCP 0.63	α-PP 0.27	β-PP 0.36	PCP 0.63	α-PP 0.27	β-PP 0.36
1	−	+	−	−	+	−	+	+	+
3	−	+	−	−	+	−	+	+	+
25	−	+	−	−	+	−	+	+	+
50	−	+	−	−	+	−	+	+	+
150	−	+	−						

+ Phosphorus spot with the corresponding Rf value found on paper chromatogram. − No spot with corresponding Rf value observed.

latter showed no further phosphoryl group migration to give β-PP even after being kept in the given acid environment at 37° for 150 hours.

From these results, it is obvious that some differences are seen in the mode of the hydrolysis reaction of 1,2-diol cyclic phosphates, largely depending upon the structure of the substrates used, and further, that the tendency in the migration of the phosphoryl group attached to one of the hydroxyl groups of 1,2-diols to the other in an acid condition also differs largely with the structure of the 1,2-diol phosphates used.

For the other two 1,2-diol cyclic phosphates, ECP and BCP, reported in the previous paper,[8] there is no question of the migration of the phosphoryl group, because they have symmetric structures with respect to the phosphate moieties. Thus the respective products are ethyleneglycol-1 and 2,3-butanediol-2 phosphate.

The stability of four cyclic phosphates, GCP, PCP, ECP, and BCP against hydrolysis in various pHs at 30° were examined. The test compounds were kept in buffers at that temperature and aliquots were withdrawn to be submitted to paper chromatographic detections. Table III shows the result indicating that, between pH

Table III. Stability Tests of GCP, PCP, ECP and BCP in Several pHs at 30°C

Substrate \ hrs.		0.25	0.5	1	2	4	8	24	48	72
GCP	Control	−	−	−	−	−	−	−	−	−
	pH 1.5	###	###	###	###	###	###	###	###	###
	pH 2.5	−	−	−	−	−	±	##	###	###
	pH 3.5~9.5	−	−	−	−	−	−	−	−	−
	0.1N NaOH	−	−	+	+	+	##	###	###	###
PCP	Control	−	−	−	−	−	−	−	−	−
	pH 1.5	###	###	###	###	###	###	###	###	###
	pH 2.5	−	−	−	−	−	∓	±	###	###
	pH 3.5~9.5	−	−	−	−	−	−	−	−	−
	0.1N NaOH	−	−	−	±	+	##	###	###	###
ECP	Control	−	−	−	−	−	−	−	−	−
	pH 1.5	###	###	###	###	###	###	###	###	###
	pH 2.5	−	−	−	−	−	±	##	###	###
	pH 3.5~9.5	−	−	−	−	−	−	−	−	−
	0.1N NaOH	−	+	+	+	##	###	###	###	###
BCP	Control	−	−	−	−	−	−	−	−	−
	pH 1.5	###	###	###	###	###	###	###	###	###
	pH 2.5	−	−	−	−	−	−	±	##	###
	pH 3.5~9.5	−	−	−	−	−	−	−	−	−
	0.1N NaOH	−	−	−	−	−	±	##	##	##

Signs indicate the grades of decomposition observed on paper chromatograms, −, ∓, ±, +, ##, ###, ### indicating nil, ca. 5%, ca. 10~15%, ca. 25%, ca. 50%, ca. 75%, and 100% decomposition.

3.5~9.5, all test compounds did not suffer hydrolysis, but in more acid circumstances, at pH 2.5 for instance, all compounds showed similar lability and gradual decomposition started to occur after 8 hours.

However, in alkaline condition, in 0.1*N* sodium hydroxide, the four compounds showed somewhat different stabilities. GCP and PCP, which contain similar cyclic phosphate groups with regard to two hydroxyl groups concerned with the phosphate bonds, showed similar stability. ECP, having the cyclic phosphate bonds attached to two primary hydroxyl groups, showed a predominant lability compared to BCP, which contains two secondary hydroxyl groups bound to phosphate bonds.

From the results observed, although it is necessary to have more detailed experimental data in order to deduce the regularity in the mode of reactions and stabilities of 1,2-diol cyclic phosphates for hydrolysis, the difference in properties of these compounds in this reaction were proved to be largely dependent on the slight differences of the structure and not to be so simple as hitherto regarded.

Experimental

Acid and Alkaline Hydrolysis of GCP—Each 3 mg. of Ba-GCP was accurately weighed into a series of tubes. To two sets of 4 tubes each was added 0.04 cc. of 1*N* HCl or 0.04 cc. of 1*N* NaOH per tube, respectively.

All the tubes were incubated at 37°. A pair of tubes which contained acid and alkali were taken out at intervals and the content of each was detected by periodate oxidation.

To each of the tubes were added 1 cc. of distilled water and a saturated aqueous solution of $(NH_4)_2SO_4$. The precipitate of $BaSO_4$ was centrifuged off and the precipitate was washed twice with 1 cc. of distilled water. The washing was combined with the supernatant. To this solution, 5 cc. of periodate solution (0.6250 g. of KIO_4 in 500 cc. of 0.1*N* H_2SO_4) was added and the mixture was made up to 10 cc. with water. Each 1 cc. of the solution was taken out at intervals and titrated with 0.004*N* $Na_2S_2O_3$ solution up to the constant consumption of the reagent.[10]

The consumptions of the reagent were recalculated to the amounts of α-GP and are given in Table I.

Another series of tubes prepared similarly to above for periodate oxidation was incubated under the same conditions and used for paper chromatographic detections.

Acid and Alkaline Hydrolysis of PCP—Ca. 10 mg. of Ba-PCP was dissolved in 0.5 cc. of 1*N* HCl and another ca. 10 mg. of Ba-PCP was dissolved in 0.5 cc. of 1*N* NaOH and the solutions were incubated at 37°. 0.02 cc. was withdrawn from both solutions at intervals and applied to paper chromatography after decationization with Amberlite IR-120 (H-type). The results observed are summarized in Table II.

Stability Tests of GCP, PCP, ECP, and BCP in various pHs—Each 2 mg. of Ba-salt of the test compounds was dissolved in 0.2 cc. of the buffer and 0.1*N* NaOH. The buffers used were those reported by Clark[11] for pH 1.5, 2.5, 3.5, and 6.5, and by Michaelis[12] for pH 8.0 and 9.5. The solutions were kept at room temperature (30°) and 0.01 cc. each of the solutions was taken out at intervals, spotted on Toyo Roshi No. 3 filter paper, and run ascendingly for 15 hrs. at 10°. The solvent system used for chromatography was *iso*-PrOH : 5*N* NH_4OH (2 : 1). The results obtained are given in Table III.

Summary

Synthesized glycerol 1,2-cyclic phosphate (GCP) was hydrolysed to glycerol-1 and -2 phosphate under both acid and alkaline conditions and from the quantitative estimation of the resulting glycerol-1 phosphate, the mode of hydrolytic reaction of glycerophosphorylcholine was discussed. Synthetic 1,2-propanediol cyclic phosphate (PCP) was found to give only 1,2-propanediol-1 phosphate after hydrolysis under tested acid and

10) L. Volis, G. Ellis, L. A. Maynard : J. Biol. Chem., **133**, 492(1940).
11) W. M. Clark : "The Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, U. S. A.(1920).
12) L. Michaelis : J. Biol. Chem., **87**, 33(1930).

alkaline conditions. GCP, PCP, synthetic ethyleneglycol cyclic phosphate (ECP), and 2,3-butanediol cyclic phosphate (BCP) were tested for their stabilities to hydrolysis at various pHs.

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19. Zen-ichi Horii, Tatsuo Sakai, and Yasumitsu Tamura : A Modified Leuckart Reaction. III.[1] Rearrangement in the Leuckart Reaction.

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In the preceding papers[1,2] we reported that a mixture of urea or 1,3-dimethylurea and formic acid can be used in the place of formamide or N-methylformamide in the Leuckart reaction. Subsequently, this method has been successfully extended to the synthesis of N-alkylamino compounds from corresponding carbonyl compounds by using 1,3-dialkylurea (Table I).

Table I.

Carbonyl compd.	$CO(NHR)_2$ R	Product: Formula	Yield (%)	b.p. (°C/mm.)	Hydrochloride: m.p. (°C)	Found C%	Found H%	Calcd. C%	Calcd. H%
$C_6H_5COCH_3$	C_2H_5	$C_6H_5CH(NHC_2H_5)CH_3$	47	60/3	199	64.68	8.68	64.75	8.85
$C_6H_5COC_6H_5$	C_2H_5	$C_6H_5CH(NHC_2H_5)C_6H_5$	35	160/15	245	72.71	7.32	72.54	7.55
$C_6H_5COCH_3$	n-C_4H_9	$C_6H_5CH(NHC_4H_9)CH_3$	40	72～74/4	153	67.42	9.43	67.25	9.53
C_6H_5CHO	n-C_4H_9	$C_6H_5CH_2NHC_4H_9$	<10	80/3	240	66.14	9.08	66.20	9.15

In order to extend this method to the synthesis of N-arylamino compounds, we have now investigated the reaction of a mixture of 1,3-diphenylurea and formic acid with benzophenone. Unexpectedly, it was found that the reaction does not proceed normally, giving a mixture of *o*- and *p*-aminotriphenylmethane instead of the expected product, i.e. N-benzhydrylaniline. We now wish to discuss the chemical structures of the reaction products and to propose a possible mechanism for this reaction.

A mixture of benzophenone, 1,3-diphenylurea, and formic acid was treated according to the general procedure proposed by us.[1,2] From a reaction mixture we obtained a substance having a wide melting range of 82～95° which was somewhat higher than that of the expected N-benzhydrylaniline, m.p. 53～54°. It had a basic property and gave a positive diazo reaction. On chromatographic purification there were obtained two pure crystalline compounds which melted at 127～127.5° and 84～84.5°, respectively. The former m.p. was identical with that of *o*-aminotriphenylmethane and the latter m.p. with that of *p*-aminotriphenylmethane. The identity of the reaction products with the authentic specimens was established by direct comparison of m.p., mixed m.p., and ultraviolet absorption spectra.

The same reaction products were also obtained by the normal Leuckart reaction using a mixture of benzophenone, formanilide, and formic acid. On heating a mixture of 1,3-diphenylurea and formic acid at 180° for 1 hour, formanilide was obtained in a good yield (88%). From this result it is assumed that formanilide is also a reactant in

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1) Part II : This Bulletin, **3**, 159(1955).

2) Z. Horii, Y. Tamura, Y. Murakami : J. Pharm. Soc. Japan, **72**, 1208(1952).