

823. *Aryl-2-halogenoalkylamines. Part XI.* The Reaction of NN-Di-2'-chloroethyl-2-naphthylamine with some Phosphoric Acid Derivatives.*

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The reaction of *NN*-di-2'-chloroethyl-2-naphthylamine with some phosphoric acid derivatives has been studied. It has been shown that the competition factors of secondary monoester are much higher than those of primary diester phosphoryl groups. The stability and direction of fission of the diethyl and diphenyl phosphates of di-2'-hydroxyethyl-2-naphthylamine under acid and alkaline conditions have been examined.

BUTLER, GILBERT, JAMES, and ROSS (*Nature*, 1951, **168**, 985) suggested that the degradation of deoxyribonucleic acid preparations which occurs after treatment with "mustards" might be connected with the formation of unstable trisubstituted phosphoric esters by reaction of the primary phosphoryl groups in the nucleic acid with the reagents.

In view of the interest in the reaction of phosphoric acid groups with the cytotoxic agents we report the results obtained when *NN*-di-2'-chloroethyl-2-naphthylamine—a typical "aromatic nitrogen mustard" which has been the subject of clinical trials—reacts with the anions of ethyl, hydroxyethyl, hydroxypropyl, and α - and β -glyceryl dihydrogen phosphate and of diethyl, bishydroxypropyl, and diphenyl hydrogen phosphate.

EXPERIMENTAL.

Materials.—Barium ethyl phosphate. Ethanol (50 ml.) and metaphosphoric acid (20 g.) were heated under reflux for 6 days. The unchanged metaphosphoric acid dissolved when the mixture was poured into water (70 ml.) and the inorganic phosphate formed was precipitated on addition of an excess of barium hydroxide. Carbon dioxide was passed through the heated solution to remove the excess of barium, and the hot solution was filtered. The filtrate was evaporated to 70 ml. and then ethanol (70 ml.) was added. Barium ethyl phosphate separated in an amorphous form which changed into fine needles when left overnight in contact with the mother-liquor. The equivalent weight of the salt was found by titration with acid to phenolphthalein and methyl-orange (Found: equiv., 261. Calc. for $C_2H_5PO_3Ba$: equiv., 261). The method is similar to that of Langheld (*Ber.*, 1911, **44**, 2077) but modifications have been made in the isolation of the barium salt.

Barium tetraethyl diphosphate. Triethyl phosphate (90 g.), hydrated barium hydroxide (80 g.), and water (200 ml.) were heated under reflux for 1½ hours, treated whilst hot with carbon dioxide, and filtered. Barium diethyl phosphate separated on addition of ethanol to the concentrated filtrate. The barium salt was recrystallised by dissolution in the minimum quantity of water and treatment with ethanol (Found: Ba, 30.9. Calc. for $C_8H_{20}O_8P_2Ba$: Ba, 31.0%).

Barium hydroxyethyl phosphate. Tri-2-chloroethyl phosphate (65 ml.), hydrated barium hydroxide (250 g.), and water (700 ml.) were heated under reflux for 16 hours. When the slightly acid solution was rendered alkaline with ammonia a heavy precipitate formed. This was collected by filtering the hot solution and dissolved in cold water (600 ml.); the solution was clarified by addition of Celite and filtration through a Whatman No. 42 paper. Barium mono-hydroxyethyl phosphate separated when the solution was heated to boiling; it was filtered off from the hot solution and washed with methanol and ether. After several hours' drying at 90° the yield was 22 g. (Found: equiv., 279; Ba, 48.7%. Calc. for $C_4H_{10}O_8P_2Ba$: equiv., 277; Ba, 49.5%).

It was not found possible to prepare barium tetrahydroxyethyl diphosphate by Plimmer and Burch's method (*J.*, 1929, 279). The conditions described by these workers gave only the monohydroxyethyl ester.

Barium 2-hydroxypropyl phosphate. Propylene oxide was added to a hot solution of disodium hydrogen phosphate (250 g.) in water (200 ml.) under an efficient reflux condenser at such a rate that an excess was maintained throughout the 8 hours of the reaction. Water (800 ml.) and an excess of barium hydroxide were added and then the cold solution was filtered. Heating the filtrate caused a heavy precipitate to be formed; this was collected and redissolved in cold water (600 ml.). Heating to 95° caused separation of *barium 2-hydroxypropyl phosphate* which was

* Part X, preceding paper

collected and washed with methanol and acetone. The yield of material dried at 140°/20 mm. for 20 hours was 35 g. (Found: equiv., 291.5; Ba, 46.7%. $C_3H_7O_5P_2Ba$ requires equiv., 291; Ba, 47.1%).

Barium tetra-2-hydroxypropyl diphosphate. An excess of propylene oxide was added, cautiously at first, to orthophosphoric acid (15 ml.; d 1.75). After 24 hours most of the unchanged oxide was removed under reduced pressure and hydrated barium hydroxide (37.8 g.) in water (150 ml.) was added to the residue. The solution was allowed to evaporate slowly at room temperature in a vacuum-desiccator. Several weeks later the colourless gum was heated at 160°/0.5 mm. to remove propylene glycol. The residue was dissolved in the smallest quantity of dry ethanol and then poured into sodium-dried ether (800 ml.). The barium salt separated as a microcrystalline solid which was dried at 130°/5 mm. The product was mainly barium tetra-2-hydroxypropyl diphosphate but titration with methyl-orange and phenolphthalein, and also the barium content, showed it to be contaminated with about 6% of monohydroxypropyl ester (Found: Ba, 26.3. $C_{12}H_{28}O_{12}P_2Ba$ requires 24.4%).

The glycerophosphates used were commercial preparations, one being pure β -ester and the other containing 65% of α -ester (periodate titration).

Diphenyl hydrogen phosphate, m. p. 69—70°, from light petroleum (b. p. 60—80°) (Hoeflake, *Rec. Trav. chim.*, 1916, 36, 24, gives m. p. 70° for the anhydrous acid), was obtained by alkaline hydrolysis of diphenyl chlorophosphonate.

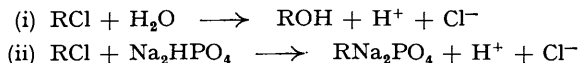
Bis(diethyl phosphate) of NN-di-2'-hydroxyethyl-2-naphthylamine. Diethyl chlorophosphonate (12 g.; McCombie, Saunders, and Stacey, *J.*, 1945, 380) was added during 15 minutes to a solution of NN-di-2'-hydroxyethyl-2-naphthylamine (5.8 g.) in 2:6-lutidine (12 ml.) at -5°. When the mixture had solidified it was allowed to reach room temperature. Next day water was added and the mixture extracted with ether. The ethereal extract was washed with water, 0.2N-acid, and water again, and finally dried and evaporated. A light brown oil remained, which decomposed on attempted distillation at 10⁻⁴ mm. Triethyl phosphate (b. p. 210—211°) and 1:4-di-2'-naphthylpiperazine (m. p. 230°, undepressed on admixture with a specimen of m. p. 238° prepared by the method of Davis and Ross, *J.*, 1949, 2831) were present in the decomposition products. Analysis of the original oil showed it to be the required *diethyl phosphate* (Found: C, 52.4; H, 7.1; N, 3.1; P, 12.4%; hydrolysis equiv., 254. $C_{22}H_{35}O_8NP_2$ requires C, 52.5; H, 7.0; N, 2.8; P, 12.3%; equiv., 252).

Bis(diphenyl phosphate) of NN-di-2'-hydroxyethyl-2-naphthylamine. To a solution of di-2'-hydroxyethyl-2-naphthylamine (6 g.) in 2:6-lutidine (10 ml.) cooled to -10° was added diphenyl chlorophosphonate (11 ml.). During the addition (15 minutes) the temperature rose to 0°. Later as the base hydrochloride began to separate the temperature reached 25°. After a further 4 hours in the ice-bath the mixture was left at room temperature overnight. Water (250 ml.) was added and the ester extracted with ether. The ethereal layer was washed thrice with water (300 ml.) and then with 0.2N-hydrochloric acid until the washings were neutral to Congo-red. The dried ethereal solution was evaporated and the semi-solid residue dissolved in a small quantity of dry benzene, and then 20 volumes of sodium-dried ether were added to the warmed solution. On cooling, flattened needles of the *diphenyl ester* formed, having m. p. 83° (Found: C, 65.6; H, 5.6; N, 2.0; P, 8.9%; hydrolysis equiv., 340. $C_{38}H_{35}O_8NP_2$ requires C, 65.7; H, 5.1; N, 2.0; P, 8.9%; equiv., 348).

Method.—Determination of competition factors. NN-Di-2'-chloroethyl-2-naphthylamine (536 mg., 0.002 mole) was dissolved in neutralised acetone (150 ml.), a solution of the phosphate ester in water (150 ml.) was added, and the mixture rapidly heated to 37° and kept at this temperature for 24 hours. The liberated hydrogen and chloride ions were then titrated with 0.1N-sodium hydroxide and silver nitrate, phenolphthalein and potassium chromate being used as indicators (the use of chromate was more generally applicable than that of dichlorofluorescein in the present work). The competition factors were calculated as described in Part III (*J.*, 1949, 2589). When the barium salts of the phosphoric esters were insoluble in the given volume of water they were converted *in situ* into the sodium salts by the addition of an exact equivalent of sodium sulphate.

The determination of the competition factor of the orthophosphate ion required a special method since in this case the acidity produced under the standard conditions was greater in the presence of the ion than in its absence—the increased rate of reaction being due to the presence of a negatively charged phosphate group on one side chain of the initial reaction product (cf. Davis, Thesis, London, 1950, p. 57)—and it was also impossible to titrate the liberated chloride ion in the presence of the phosphate ion. A normal 24 hour's hydrolysis was carried out and then the acetone was removed under reduced pressure and unchanged chloroethylamine extracted

with ether. The inorganic phosphate in the aqueous layer was determined by precipitation with magnesia mixture and ignition. This gave the amount of unchanged phosphate remaining and a blank run gave the amount originally present: the difference represents the amount of ester formed. The total acidity produced is derived from reactions:



The extent of reaction (ii) is now known and the extent of reaction (i) can be calculated and the competition factor, F, determined as follows:

$$F = \frac{\text{acidity produced by reaction with phosphate ion}}{\text{acidity produced by reaction with water} \times [\text{phosphate}]}$$

The concentration of anion has been expressed in all cases in the terms of normality with respect to dissociating hydrogen. For consistency this has been done even when it is certain that not all the hydrogen ions can have been dissociated at the same time in the reaction mixture (*e.g.*, in the case of the phosphate ion where the pH remained at about 9 throughout the reaction).

Rates of Hydrolysis of Phosphates of Di-2'-hydroxyethyl-2-naphthylamine.—The second-order velocity constants for alkaline and acid hydrolysis of the diethyl and diphenyl phosphates of di-2'-hydroxyethyl-2-naphthylamine in 80% acetone at 50° were determined as described in Parts VIII and IX (*J.*, 1950, 3056; 1951, 2706), with results given in Table 1.

TABLE 1. *Velocity constants for the hydrolysis of esters.*

Ester	10% in acid	10% in alkali
$2\text{-C}_{10}\text{H}_7\text{N}[\text{CH}_2\text{CH}_2\text{OPO}(\text{OEt})_2]_2$	0.33	0.5 *
$2\text{-C}_{10}\text{H}_7\text{N}[\text{CH}_2\text{CH}_2\text{OPO}(\text{OPh})_2]_2$	0.55	5.0 *
$2\text{-C}_{10}\text{H}_7\text{N}(\text{CH}_2\text{CH}_2\text{OAc})_2$	2.06	4800

* Approximate, probably being too high, since the consumption of alkali by side-reactions with the acetone becomes appreciable with these more slowly hydrolysed esters.

Direction of Fission during the Hydrolysis of the Bis(diethyl phosphate) of Di-2'-hydroxyethyl-2-naphthylamine.—(a) *Acid hydrolysis.* The ester (82.9 mg., 2×10^{-4} mole) was heated under reflux with 0.1N-sulphuric acid (5 ml.) and water (25 ml.) for 40 min. Then 25 ml. were distilled off. The residue required 6.96 ml. of 0.1N-sodium hydroxide on titration; *i.e.*, the total quantity of alcohol liberated was 1.96×10^{-4} mole. To the distillate were added 0.3472N-potassium dichromate (5 ml.) and concentrated sulphuric acid (5 ml.), and the solution was heated for 15 minutes on a steam-bath. After addition of water (250 ml.) and potassium iodide (3 g.) the liberated iodine was titrated with 0.1N-sodium thiosulphate. 16.95 ml. were required in the above experiment and 17.35 ml. were required in a blank run. The ethanol liberated thus amounted to 0.10×10^{-4} mole, and the ratio, mole of ethanol liberated/mole of hydroxyethylamine liberated, is 0.054. Other determinations gave values of 0.045 and 0.062 for this ratio.

(b) *Alkaline hydrolysis.* The ester (6×10^{-4} mole) was hydrolysed by heating it under reflux with 0.1N-sodium hydroxide (25 ml.) for 3 hours and the extent of the hydrolysis was determined by titration with acid. The ethanol liberated was determined by distillation and oxidation as already described. In this case the ratio, mole of ethanol liberated/mole of hydroxyethylamine liberated, was 0.25, 0.24, 0.18, and 0.21 in four determinations.

These results may be summarised: in the acid hydrolysis of the triester the ethyl ester linkage is attacked at about one-twentieth of the rate, and in the alkaline hydrolysis at one-quarter of the rate, of the aminoethyl ester linkage.

Direction of Fission during the Hydrolysis of the Bis(diphenyl phosphate) of Di-2'-hydroxyethyl-2-naphthylamine.—(a) *Acid hydrolysis.* The ester (200 mg.) was refluxed for 40 minutes with 0.1N-sulphuric acid (10 ml.) in water (40 ml.), and then the mixture was steam-distilled for 20 minutes. The total extent of hydrolysis was determined by titration of the residual solution with alkali, and the liberated phenol was determined in the distillate by bromination (Scott, *Ind. Eng. Chem., Anal. Ed.*, 1931, 3, 67). In a typical run: ester taken = 2.9×10^{-4} mole; total alcohol liberated = 1.7×10^{-4} mole; phenol liberated = 0.067×10^{-4} mole. It is not possible to define exactly the amount of triester which is converted into diester in the acid hydrolyses since, in contrast to alkaline hydrolyses, where the amount of diester hydrolysed is negligible, some diester is then always attacked. The amount of monoester formed cannot be determined by a phenolphthalein-methyl-orange titration since di-2'-hydroxyethyl-2-naphthylamine interferes with the end-points but the amount clearly cannot exceed 0.067/1.7 (or about

4%) of the total ester hydrolysed since phenol must be formed in the production of monoester. Colorimetric tests show that no inorganic phosphate is formed in these runs. Thus the amount of triester hydrolysed to diester must be equal to, or greater than, $1.7 - 2 \times 0.067 = 1.566$ moles in the above experiment and the ratio, mole of phenol liberated/mole of hydroxyethylamine liberated in the conversion of triester into diester, cannot exceed $0.067/1.566 = 0.043$ and may be much less. In two other runs this ratio was found to be 0.02 and 0.025.

(b) *Alkaline hydrolysis.* The bis(diphenyl phosphate) of di-2'-hydroxyethyl-2-naphthylamine (100 mg.) was heated under reflux with *N*-sodium hydroxide (2 ml.), water (6 ml.), and ethanol (8 ml.) for 1 hour. This resulted in the complete hydrolysis of triester to diester, as shown by the titration of the residue after steam-distillation, to methyl-orange and phenolphthalein. In each of three determinations the amount of phenol produced corresponded exactly with the amount of hydrolysis occurring, that is, one mole of triester produced one mole of phenol.

These results may be summarised: during the hydrolysis with alkali the diphenyl phosphate is attacked exclusively at the phenyl ester linkage whereas during acid hydrolysis it is the 2-aminoethyl linkage which preferentially undergoes fission.

DISCUSSION

Table 2 shows that anions of phosphoric monoesters have competition factors (*F*) comparable with those of carboxylic acids (compare Part III where *F* for the acetate ion was shown to be 100). The values for the glycerophosphates are somewhat lower than for the other monoesters; α - and β -glycerophosphate clearly have similar *F* values. The competition factors of the anions of phosphodiester on the other hand are very much lower. In view of the reputed instability of triesters of phosphoric acid it was at first

TABLE 2. Competition factors of phosphoric acid derivatives.

Anion	Competition factor (<i>F</i>): Concentration			pH at end of reaction: * Concentration	
	0.01N	0.02N	0.05N	0.01N	0.02N
Orthophosphate	—	108	—	—	8.7
Pyrophosphate	53	45	—	9.3	7.7
Ethyl phosphate	167	152	112	—	8.8
Hydroxyethyl phosphate	117	100	66	—	8.6
2-Hydroxypropyl phosphate	126	134	—	—	8.8
Glycerophosphate (β)	75	66	45	—	—
„ (65% α)	—	68	—	—	—
Diethyl phosphate	—	5.3	3.9	—	3.3
Di-2-hydroxypropyl phosphate	1.2	3	—	—	—
Diphenyl phosphate	—	—	4.4	—	—

* This was the pH of the solution after 24 hours and before titration.

thought that the low *F* value of the primary diester phosphoryl group might be due to the ready hydrolysis of the triester first formed under the conditions of the determination. It will be seen from Table 2 that owing to the lack of buffering action of the diesters the pH of the solution falls to a greater extent than for the monoesters. However, it was later shown that the diethyl and diphenyl phosphates of di-2'-hydroxyethyl-2-naphthylamine were not appreciably hydrolysed at pH 3 in 50% acetone during 24 hours. This is also confirmed by the results in Table 1 which indicate that these phosphates are more resistant to hydrolysis than the corresponding acetate.

The low *F* value of the primary phosphoryl group in these simple diesters was rather surprising in view of the high degree of reaction between "sulphur mustard gas" and the primary phosphoryl groups of thymus nucleic acid reported by Elmore, Gulland, Jordan, and Taylor (*Biochem. J.*, 1948, **42**, 308). An investigation carried out in collaboration with Dr. P. Alexander has shown that the competition factor of the carboxyl group in polymethylacrylic acid towards di-2'-chloroethyl-2-naphthylamine is considerably higher than that in a simple carboxylic acid, values of up to 3000 having been obtained. It has been suggested that this high value might be due to the adsorption of the "mustard" on to the polymer, thus facilitating reaction with the nucleophilic groups thereon as opposed to reaction with water. Some similar explanation may apply to the reaction of di-2'-chloroethyl sulphide with the primary phosphoryl groups in thymus nucleic acid.

The stability of the phosphoric triesters (Table 1) relative to the acetate was also rather unexpected. This suggests that more stable linkages would be formed by the reaction of chloroethylamines with primary phosphoryl groups in nucleic acids than with carboxylic groups in proteins, though it is realised that structural effects may vitiate this argument. The structures dealt with in this work are much simpler than those likely to be concerned in biological systems. An attempt to prepare a di-2-hydroxyalkyl phosphate of di-2'-hydroxyethyl-2-naphthylamine (which would be a triester more closely related to the product obtained when di-2'-chloroethyl-2-naphthylamine reacts with a pentose nucleic acid—it has a hydroxy-group β to the phosphate linkage) was unsuccessful.

It has been suggested (Butler *et al.*, *loc. cit.*) that the degradation of nucleic acid structures after treatment with chloroethylamine might be due to hydrolysis of triesters formed by the reaction of the halide with primary phosphoryl groups. Should such triesters undergo fission at one of the sugar-phosphate linkages then the nucleic acid chain will be broken. This suggestion prompted the present study of the direction of fission of triesters derived from the biologically active di-2'-chloroethyl-2-naphthylamine. Under alkaline conditions fission occurs exclusively at the "non-mustard" linkage in the case of the diphenyl phosphate and quite appreciably at this linkage in the case of the ethyl derivative. Acid hydrolysis in each case results in preferential hydrolysis of the aminoethyl ester linkage. It is realised that these results are incomplete but there is some support for the hypothesis of Butler *et al.* since it has been shown that esters derived from the reaction of a "mustard" with a primary diester phosphoryl group can hydrolyse with fission at the "non-mustard" linkage.

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