[CONTRIBUTION FROM THE BIOCHEMISTRY BRANCH, CHEMICAL CORPS MEDICAL LABORATORIES]

The Reaction of Phosphorus-containing Enzyme Inhibitors with Amines and Amino Acid Derivatives[1]

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To obtain a better understanding of the chemical reaction between certain enzymes (chymotrypsin, trypsin, cholinesterase) and the powerful inactivators diisopropylfluophosphate (DFP) and tetraethylpyrophosphate (TEPP), model reactions have been carried out between these latter compounds or analogs and low molecular weight compounds similar to those found in the enzyme molecule. The chloro analog of DFP, diisopropylchlorophosphate, (DClP) is more reactive than DFP and should give the same reaction products, since halogen is split off in the reaction. N-Diisopropylphosphorylated derivatives of the following have been obtained by reaction with DClP at room temperature and in non-aqueous media: glycinamide; glycine, threonine and serine esters; cyclohexylamine, benzylamine. Certain of these derivatives can be distilled in high vacuum. All were obtained crystalline except the serine derivative. DFP is much less reactive toward amines than is DClP under similar conditions. In aqueous alkaline solution, preliminary results indicate that phenolic hydroxyl groups react with DFP. TEPP and cyclohexylamine react with good yield in water, quantitatively in a non-polar organic solvent, to give N-diethylphosphorylcyclohexylamine and the diethylphosphoric acid salt of cyclohexylamine. Glycine ethyl ester and TEPP react to form a phosphorylation product whose structure is discussed. The hydrolysis products of DFP (diisopropylphosphoric acid and HF) form salts with guanidine, benzylamine and cyclohexylamine, some of which are suitable for identification purposes.

Relatively little is known concerning the mechanism of reaction between certain enzymes (chymotrypsin, trypsin, cholinesterase) and the powerful inactivators diisopropylfluophosphate (DFP) and tetraethylpyrophosphate (TEPP). In the case of cholinesterase, it has been established in this Laboratory[2] that at least the phosphorus atom of DFP becomes firmly bound to the enzyme, in direct proportion to the degree of enzyme inactivation. For chymotrypsin, it has been shown that the crystalline reaction product with DFP contains one atom of phosphorus and two isopropoxy groups per molecule of protein, and that it is free of fluorine.[3] Some type of phosphorylation is apparently involved, but the portion of the protein molecule concerned is not yet known. Presumably reaction is taking place with an amino acid residue in the protein, although the possibility of an as yet unknown non-amino acid moiety being concerned has not been ruled out.

The phosphorylation of amino acids with $POCl_3$ in aqueous solution in the presence of excess MgO was described by Neuberg and Oertel,[4] more than 35 years ago. The reaction was reinvestigated later by Winnick and Scott.[5] The P–N linkage of the resulting magnesium salts of N-phosphorylated amino acids is hydrolyzed somewhat more rapidly by dilute acid than is the corresponding linkage in phosphocreatine.

Our attempts to phosphorylate amino acids in a slightly alkaline aqueous medium under various conditions with DFP were without success. The DFP molecule is not only slowly hydrolyzed by water but also its reactivity toward ordinary amino or hydroxyl groups in organic molecules seems to be exceedingly low.

The chloro analog of DFP, diisopropylchlorophosphate (DClP), is much more reactive *in vitro* than DFP; for example, DClP reacts with aniline, whereas DFP does not.[6] In order to get some information on the chemical properties of diisopropylphosphorylated amino acid derivatives we studied the reaction

$$2RCH_2NH_2COOR' + ClPO(OC_3H_7\text{-}i)_2 = RCH_2NH[PO(OC_3H_7\text{-}i)_2]COOR' + RCH_2NH_2COOR\cdot HCl$$

which readily took place in chloroform, carbon tetrachloride, benzene or ethyl acetate.

The following glycine ester, glycineamide and *d,l*-threonine methyl ester derivatives have been synthesized by this method and isolated in pure form

I	$i\text{-}(C_3H_7O)_2PO\cdot NHCH_2COOC_2H_5$	m.p. 28–29°
II	$i\text{-}(C_3H_7)_2PO\cdot NHCH_2CONH_2$	m.p. 81–84°
III	$CH_3\text{-}CHOH\text{-}CHNH\cdot PO(OC_3H_7\text{-}i)_2\text{-}COOCH_3$ (*d,l*)	m.p. 54–56°

These compounds are soluble in water and organic solvents. The analogous N-diphenylphosphoryl amino acid esters, synthesized by Sciarini and Fruton,[7] using diphenylphosphoryl chloride as a phosphorylating agent, ethyl acetate as a solvent and $NaHCO_3$ for the neutralization of the HCl formed during the reaction, are insoluble in water. N-Diphenylphosphoryl glycine ester $(C_6H_5O)_2PONHCH_2COOC_2H_5$ melts at 77–78°.

The reaction product of DClP with serine methyl ester is liquid at room temperature and could not be distilled in a high vacuum without decomposition.

Evidence that the phosphoryl group is bound to nitrogen in the phosphorylated threonine derivative III is given by the fact that the substance does not react alkaline, and does not give the nitrous acid reaction for free amino groups until after acid or alkaline hydrolysis.

Even when 2 moles of DFP in the presence of 2 moles of triethylamine were allowed to react with one mole of threonine methyl ester only phosphorylation of the amino group occurred. It has been shown that 1-fluoro-2,4-dinitrobenzene also reacts only with the amino group of threonine and serine,

(1) This paper was presented at the 119th A. C. S. Meeting in Boston, Mass., April, 1951.

(2) Michel and Krop, *J. Biol. Chem.*, **190**, 119 (1951).

(3) Jansen, Nutting and Balls, *ibid.*, **179**, 201 (1949); Jansen, Nutting, Jang and Balls, *ibid.*, **185**, 209 (1950).

(4) Neuberg and Oertel, *Biochem. Z.*, **60**, 491 (1914).

(5) Winnick and Scott, *Arch. Biochem.*, **12**, 201 (1947).

(6) Saunders, *et al.*, *J. Chem. Soc.*, 695 (1948).

(7) Sciarini and Fruton, THIS JOURNAL, **71**, 2940 (1949).

in the presence of sodium bicarbonate.[8] On the other hand, dibenzylchlorophosphate is more reactive than DClP because it phosphorylates alcohols rapidly at low temperature in the presence of tertiary bases, in addition to the phosphorylation of amines.[9]

N-Phosphorylated amino acids are dephosphorylated easily by acids.[5] Concerning the stability of the diisopropylphosphorylated amino acid derivatives described in this paper we only know at the present time that *d,l*-N-diisopropylphosphoryl-threoneine methyl ester can be dissolved in water and recovered undecomposed by evaporating the aqueous solution to dryness at room temperature. In acid or alkaline solution the phosphorus-containing residue is split off from the nitrogen.

It is to be expected that it will not be easy to saponify the carboxylic ester group without splitting the P–N linkage. Sciarini and Fruton[7] found that in the diphenylphosphorylated amino acid esters the phosphorylation of the α-amino group greatly increased the stability of the carboxylic ester linkage. A similar behavior has been described by Posternak, *et al.*,[10] in the case of tyrosine- and serine-containing peptides; the phosphorylation of the OH group in these compounds protected the peptide linkage against enzymatic hydrolysis. The preparation of the free N-alkyl phosphorylated amino acids probably would be possible by catalytic reduction of the corresponding benzyl esters.

As expected, the reaction of DClP is not limited to amino acid esters but occurs also with other amines of higher basicity. By this method we prepared N-diisopropylphosphoryl-benzylamine (m.p. 49–50°) and N-diisopropylphosphorylcyclohexylamine (m.p. 54–55°). The water insolubility of these substances is in direct contrast with the corresponding amino acid ester derivatives, which are easily water-soluble.

As an example of a secondary cyclic amine, ethylenimine reacts with DClP to yield N-diisopropylphosphoryl-ethylenimine, an oil with a boiling point of 76–78° (1–2 mm.). Bestian[11] has shown that in $POCl_3$ all three chlorine atoms react with ethylenimine.

When diisopropylfluophosphate (DFP) is substituted for diisopropylchlorophosphate (DClP) little or no reaction was observed with amino acid esters at room temperature. Benzylamine reacts to a small degree, cyclohexylamine to a more considerable extent, and dodecylamine almost quantitatively with DFP.

According to Sizer[12] the phenolic OH group of tyrosine has an essential function for the enzymatic activity of chymotrypsin; primary amino, sulfhydryl or disulfide groups are not required for chymotryptic activity. In preliminary experiments with tyrosine ethyl ester we found that both the α-amino group and the hydroxyl group are reactive with DClP. Furthermore, we have been able to phosphorylate phenol at room temperature, in the presence of triethylamine, by this reagent. Phenol reacts even with DFP, in aqueous potassium carbonate, to give diisopropylphosphoryl-phenol.

The insecticide tetraethylpyrophosphate (TEPP) is similar in many ways to DFP. For example, it owes its effectiveness to a powerful anticholinesterase activity[13] and it reacts with and inactivates chymotrypsin to give a phosphorylated protein containing one atom of phosphorus and two alkoxy groups per molecule of protein.[14] Comparison of the reactivity of TEPP under conditions similar to those described above for DFP and DClP should therefore be of interest.

As an example of the reaction between primary amines and TEPP, we treated this substance with cyclohexylamine in benzene solution. The reaction takes place very fast and almost quantitatively according to the equation

$$2C_6H_{11}NH_2 + (C_2H_5O)_2\cdot OPOPO\cdot(OC_2H_5)_2 = \underset{(a)}{C_6H_{11}NH\cdot PO\cdot(OC_2H_5)_2} + \underset{(b)}{C_6H_{11}NH_2\cdot HOPO(OC_2H_5)_2}$$

Both reaction products have been isolated. N-Diethylphosphorylcyclohexylamine (a) has a melting point 71–72°, which is 17° higher than that of the corresponding diisopropyl derivative. The properties of the cyclohexylamine salt of diethylphosphoric acid (b) will be described later.

When the reaction between cyclohexylamine and TEPP was carried out in a water–ether mixture with stirring the same reaction took place as indicated above; however, on account of the hydrolysis of TEPP the relative amount of N-diethylphosphorylcyclohexylamine formed was less than in a non-aqueous medium.

When we heated glycine ethyl ester, TEPP and triethylamine in equimolecular amounts in boiling benzene we isolated from the reaction mixture an oil, which, according to its analytical data seems to be the N-(bis-diethylphosphoryl)-glycine ethyl ester. The formation of this substance could be characterized by the equation

$$H_2NCH_2COOC_2H_5 + (C_2H_5O)_2\cdot OPOPO\cdot(OC_2H_5)_2 = (C_2H_5O)_2PO{-}\underset{\displaystyle CH_2COOC_2H_5}{\overset{\displaystyle |}{N}}{-}PO(OC_2H_5)_2 + H_2O$$

It is to be noted that an alternate isomeric formula for the reaction product, $(C_2H_5O)_2OPOP(OC_2H_5)_2$ with $\overset{\displaystyle \|}{N}CH_2COOC_2H_5$, cannot as yet be excluded. Further work will be necessary to establish definitely the purity and structure of this substance.

The toxicity of the reaction product of glycine ethyl ester and TEPP for rabbits was about equal to that of DFP and about $^1/_{10}$ that of TEPP. The aqueous solution is hydrolyzed on standing at room temperature for several hours.

An attempt to introduce a second diethylphosphoryl group into N-diethylphosphorylcyclohexyl-

(8) Sanger, *Biochem. J.*, **39**, 507 (1945); *Nature*, **160**, 295 (1947). However, the reaction between 1-fluoro-2,4-dinitrobenzene and alcohols in the presence of triethylamine readily furnishes the corresponding 2,4-dinitrophenyl ethers (Whalley, *J. Chem. Soc.*, 2241 (1950)).

(9) A. R. Todd, *Bull. soc. chim. France*, 933 (1948).

(10) Posternak, *et al.*, *Helv. Chim. Acta*, **24**, 921 (1941); **28**, 1258 (1945).

(11) Bestian, *Ann.*, **566**, 210 (1950).

(12) Sizer, *J. Biol. Chem.*, **160**, 547 (1945).

(13) Mangun and DuBois, *Federation Proc.*, **6**, 353 (1947).

(14) Fleisher, Jandorf, Summerson and Norton, *ibid.*, **9**, 171 (1950).

amine by boiling this substance with 1 mole of TEPP and 1.5 moles of triethylamine in benzene was unsuccessful.

The characterization of organic phosphoric acids has been carried out successfully by the formation of salts with nitrogen-containing bases.[15] For the identification of DFP, amine salts of its products of hydrolysis should be useful. For example, diisopropylphosphoric acid readily forms a guanidine salt, which has a melting point of 155–157° and can be precipitated from aqueous solution by acetone. We have been able to prepare this compound starting with the Ba-salt of diisopropylphosphoric acid or directly from DFP, hydrolyzed with water. Another crystalline derivative is the cyclohexylamine salt of diisopropylphosphoric acid (m.p. 193°) prepared from cyclohexylamine and DFP in the presence of an aqueous suspension of $Ca(OH)_2$. It is remarkable that the next lower homolog, the cyclohexylamine salt of diethylphosphoric acid, has a much lower melting point (78°), and is very hygroscopic.

No solid benzylamine salt of diisopropylphosphoric acid could be obtained. However, from a concentrated mixture of benzylamine and hydrolyzed DFP, benzylamine hydrofluoride (m.p. 149–150°) can be precipitated by acetone. This salt might also be useful for the identification of DFP after hydrolysis.

The reactions described here demonstrate that diisopropylhalogenophosphates and tetraethylpyrophosphate phosphorylate amines in the usual manner. The same is true for the interaction of diisopropylhalogenophosphates and amino acid esters. However, TEPP and glycine ester obviously react in a different manner; the chemical structure of the reaction product is not entirely established as yet.

Whereas the velocity of the reaction between amines and TEPP is such that this reaction might possibly be considered as a model for the reaction between enzymes and TEPP, this is not true for DFP. Preliminary experiments with phenols appear to be more promising in the latter case. Further work along these lines should yield more information concerning the nature of the chemical grouping in enzymes which is involved in the phosphorylation of these proteins by such substances as DFP and TEPP.

Experimental[16]

Reaction of Diisopropylphosphorylchloride (DClP) with Amino Acid Esters.—An excess of the free amino acid ester was mixed with DClP in a small amount of dry chloroform. Heat was evolved in the case of glycine ethyl ester and the formation of the ethyl ester hydrochloride occurred immediately. The reaction mixture with threonine methyl ester was left standing for 24 hours at room temperature. The ester hydrochlorides were filtered off on a buchner funnel and washed with dry ether. The solvents of the filtrate were removed by vacuum-distillation and the remaining sirup was extracted with a small amount of petroleum ether (30–60°) plus some drops of absolute ether. The solution was kept for crystallization at −20° or, in the case of the glycine ester, the product was distilled in a high vacuum. The products were recrystallized from low boiling petroleum ether at low temperature.

Diisopropylphosphorylglycine Ethyl Ester (I).—From 1.94 g. of glycine ethyl ester, prepared according to the method of E. Fischer,[17] and 1.6 ml. of DClP in 8 ml. of $CHCl_3$ there was obtained about 0.5 g. of the reaction product; b.p. 115–128° (~0.5 mm.); m.p. 28–29°; soluble in water with *p*H 4.7–5.0.

Anal. Calcd. for $C_{10}H_{22}O_5NP$: P, 11.60. Found: P, 11.53.

N-Diisopropylphosphorylglycinamide (II).—Three hundred and sixty mg. of glycinamide was suspended in 36 ml. of dry ethyl acetate, 0.42 ml. of DClP was added, and the mixture stirred for 3 hours. The next day the glycinamide hydrochloride was filtered off and the filtrate was evaporated in a vacuum. The remaining crystals were dissolved in a very small amount of chloroform and precipitated from this solution by addition of petroleum ether. After repetition of this procedure 350 mg. of fine needles, m.p. 81–84°, were obtained. The compound was easily soluble in water or $CHCl_3$, only slightly soluble in petroleum ether or ether.

Anal. Calcd. for $C_8H_{19}N_2O_4P$: P, 13.06. Found: P, 13.2.

Diisopropylphosphoryl-*d,l*-threonine Methyl Ester (III).—*d,l*-Threonine methyl ester hydrochloride was prepared according to the method of E. Fischer and Suzuki[18]; m.p. 112–116° (from methanol + ether).[19] The free amino acid ester, prepared from 1 g. of the hydrochloride by the method of G. Hillmann,[20] plus 1.25 ml. of DClP in 3 ml. of dry $CHCl_3$ yielded about 0.5 g. of the reaction product; m.p. 54–56°, with a wax-like touch (crystallized from petroleum ether). The compound is soluble in methanol, methylene dichloride and ether. The aqueous solution is very slightly acid. The substance is stable toward HIO_4 under conditions where threonine gives one mole of acetaldehyde. The presence of three alkoxy groups was shown analytically.

Anal. Calcd. for $C_{11}H_{24}NO_6P$: N, 4.71; P, 10.45. Found: N, 4.34; P, 10.6.

N-Diisopropylphosphorylbenzylamine.—2.01 g. (1 equivalent) of DClP was added to a solution containing 2.14 g. (2 equivalents) of benzylamine dissolved in 8 ml. of benzene previously dried over sodium. Heat was evolved and an immediate precipitate formed while the solution was being stirred. The precipitate of benzylamine hydrochloride was removed by filtration after 24 hours standing; yield 1.50 g., m.p. 246° (literature value 248°).

From the filtrate the benzene was evaporated under vacuum and a small amount of petroleum ether was added. A small amount of precipitate formed which was removed by filtration. The filtrate was placed at −20° and fine white crystals formed. After twice recrystallizing from petroleum ether, the crystals melted at 48.5–50°. Properties: The compound is soluble in petroleum ether at room temperature, insoluble at low temperature; insoluble in water; m.p. 48.5–50°, b.p. 238°.

Anal. Calcd. for $C_{13}H_{22}O_3NP$: P, 11.4; N, 5.2. Found: P, 11.3; N, 5.1.

N-Diisopropylphosphorylcyclohexylamine.—1.96 g. of redistilled cyclohexylamine (b.p. 133.5–134°) was dissolved in 8 ml. of carbon tetrachloride previously dried over calcium chloride. To this was added 2.01 g. of DClP. The reaction was vigorous and completed in a matter of minutes. A large amount of heat was liberated with the immediate formation of a white precipitate, which gave a positive silver nitrate test and had a m.p. of 204° (corrected) which corresponds to the melting point of cyclohexylamine hydrochloride. The carbon tetrachloride was removed from the filtrate and petroleum ether was added. More of the hydrochloride precipitated almost immediately; the reaction mixture was placed at −20° overnight, and then filtered. The petroleum ether was evaporated off to yield a compound soluble at room temperature in petroleum ether.

The compound was recrystallized from petroleum ether and washed with water. It is soluble in warm petroleum ether or in acetone at room temperature. It can be crys-

(15) T. Wagner-Jauregg, *et al.*, *Z. physiol. Chem.*, **239**, 188 (1936); *Ber.*, **70**, 1, 8, 1458, 1459 (1937); **77**, 481 (1944); Barrenscheen, *ibid.*, **281**, 98 (1944); Todd, *et al.*, *J. Chem. Soc.*, 582, 2476 (1949).

(16) Microanalyses were made by the Analytical Branch, Chemical Corps Chemical and Radiological Laboratories, Army Chemical Center, Maryland. All melting points are uncorrected.

(17) Fischer, *Ber.*, **34**, 433 (1901).

(18) Fischer and Suzuki, *ibid.*, **38**, 4173 (1905).

(19) Carter, Norris and Rockwell, *J. Biol. Chem.*, **170**, 296 (1947).

(20) Hillmann, *Z. Naturforsch*, **1**, 682 (1946).

tallized from acetone by adding water until an oil forms, which, placed in the cold overnight yields well formed crystals, m.p. 53.5–55°.

Anal. Calcd. for $C_{12}H_{26}O_3NP$: N, 5.3; P, 11.8. Found: N, 5.2; P, 11.8.

N-Diisopropylphosphoryl Ethylene Imine (a).—1.3 ml. of ethylene imine was dissolved in a mixture of 12 ml. of dry benzene and 3.5 ml. of triethylamine. The solution was stirred and 2.1 ml. of DClP was added dropwise. There was an immediate evolution of heat and a precipitate formed which prevented further stirring. After standing at room temperature for 24 hours triethylamine hydrochloride was filtered off and the solvent removed from the filtrate by vacuum distillation. The remaining oil yielded three fractions on distillation at a pressure of 1–2 mm.; I (67–70°), 0.5 ml.; II (75°), 1.0 ml.; III (76–78°), 0.6 ml. The products were water soluble. Fraction III, $n^{23.5}D$ 1.430, was analyzed.

Anal. Calcd. for $C_8H_{18}O_3NP$: N, 6.78; P, 14.95. Found: N, 6.9; P, 14.8.

(b).—2 ml. of ethylene imine and 5 ml. of DFP were mixed and kept at room temperature for 5 days. The liquid was poured off from some solid polymerization product and distilled at about 1 mm. pressure. Three fractions: I (45–50°), 2 ml.; II (50–65°), 0.5 ml.; III (75–80°), 0.6 ml. Fraction III was analyzed for phosphorus.

Anal. Calcd. for $C_8H_{18}O_3NP$: P, 14.95. Found: P, 14.3.

Interaction of Cyclohexylamine and Tetraethylpyrophosphate (TEPP).—Five ml. of pure cyclohexylamine, 7.5 ml. of benzene and 5 ml. of TEPP were mixed in a stoppered erlenmeyer flask. Heat was evolved and crystals appeared immediately. After 12 hours the stiff cake of crystals was filtered on a buchner funnel and washed with a very small amount of ice-cold ether. After several recrystallizations from hot benzene the cyclohexylamine salt of diethylphosphoric acid so obtained softens in a capillary tube between 65 and 70° to give an opaque mass which melts to a clear liquid at 78°. The crystals are easily soluble in chloroform or acetone, less soluble in ether or petroleum ether at room temperature, and soluble in boiling benzene.

Anal. Calcd. for $C_6H_{11}NH_2 \cdot HOPO(OC_2H_5)_2 + 1H_2O$: P, 11.44. Found: P, 11.5.

From the analytically determined phosphorus content, the crystals appear to contain one molecule of water of crystallization, probably obtained either from the atmosphere or from the washing ether, which was found on test to contain appreciable moisture. In order to remove any water the substance was melted at 100° in a high vacuum until no more gas bubbles escaped from the molten mass. The waxy mass which resulted at room temperature gave, after recrystallization from hot benzene, prismatic crystals of the same melting point as given above.

Anal. Calcd. for $C_6H_{11}NH_2 \cdot HOPO(OC_2H_5)_2$: C, 47.5; H, 9.55; N, 5.54. Found: C, 48.3; H, 9.3; N, 5.35 (kjeldahl).

The benzene–ether filtrate from the cyclohexylamine salt was washed with water in a separatory funnel and dried over anhydrous sodium sulfate. After evaporation of the solvent the solid residue was recrystallized from petroleum ether. The resulting compound, N-diethylphosphorylcyclohexylamine, had a melting point of 71–72°; the yield was 2.7 g. of purified product. This material was non-toxic to rabbits.

Anal. Calcd. for $C_6H_{11}NH \cdot PO(OC_2H_5)_2$: N, 5.96; P, 13.2. Found: N, 5.9; P, 13.1.

Interaction of Glycine Ethyl Ester with TEPP (a).—3.35 ml. of glycine ethyl ester, 9.65 g. of TEPP, 4.7 ml. of triethylamine and 10 ml. of benzene were refluxed for 4 hours. By vacuum distillation a fraction was obtained boiling at 110–148° (12 mm.), which on distillation in a high vacuum yielded the fractions

No.	B.p. (1–2 mm.), °C.	Yield, ml.	Found
I	58– 65	~1.5	
II	125–139	~4	N, 3.3; P, 15.5
III	140–143	~3	P, 16.6

In order to remove acid impurities, fraction II was dissolved in benzene and washed at 15° with a suspension of $NaHCO_3$ in an aqueous saturated NaCl solution.[21] Redistillation in a high vacuum gave the two fractions

No.	B.p. (1–2 mm.), °C.	Yield, ml.	$n^{25}D$	Found
IIa	135–140	~1.5	1.4275	N, 3.5
IIb	140–142	~2	1.4290	N, 3.7; P, 16.2

Anal. Calcd. for $C_{12}H_{27}O_8NP_2$: N, 3.7; P, 16.5.

The substance is hygroscopic; the following analytical figures obtained with an older sample correspond to the uptake of one mole of water.

Anal. Calcd. for $C_{12}H_{27}O_8NP_2 \cdot H_2O$: C, 36.65; H, 7.4; OC_2H_5, 57.4. Found: C, 36.72, 36.62; H, 7.30, 7.31; OC_2H_5, 58.2.

(b).—5.5 ml. of glycine ethyl ester, dissolved in 10 ml. of absolute benzene, was mixed with 6.7 ml. of TEPP. The mixture was kept for 12 days at room temperature and was then washed with 5 ml. of the Toy $NaHCO_3$ suspension in saturated NaCl solution. For the separation of the two layers anhydrous sodium sulfate was added. The benzene layer was dried on anhydrous $MgSO_4$. After evaporation of the solvent in a vacuum a portion of the residue solidified crystalline. By distillation in a high vacuum (~ 1–2 mm.), two fractions were obtained: I, b.p. 100–120°; II, b.p. 120–160°. Fraction I partly crystallized. Fraction II on redistillation yielded several drops of a first fraction and a main fraction, boiling at 145–150° (1–2 mm.); $n^{25.5}D$ 1.4306. This portion crystallized at −20° and melted at about 14°. Toxicity: LD_{50}, 1 mg. per kg. in rabbits, intravenously; the symptoms of intoxication in animals were more muscular than central. The toxicity disappeared after keeping an aqueous solution of the substance for one hour at room temperature.[22]

Cyclohexylamine Salt of Diisopropylphosphoric Acid.—To a mixture of 1.8 ml. of cyclohexylamine and 2.6 ml. of DFP, 0.55 g. of calcium hydroxide suspended in 2.5 ml. of water was added with stirring in portions during a period of 1.5 hours. Stirring was continued for 2.5 more hours. The mixture which had a *p*H of about 10 was filtered on a buchner funnel, the precipitate washed with ether and the combined filtrates evaporated at 60° under reduced pressure, finally in high vacuum. The crystals remaining were recrystallized twice from acetone, by cooling the clear solution at −20°. The material crystallized in the form of fine needles, melting at 193° (softening of the crystals about 20° lower; between 110 and 120° shrinkage can be observed, probably due to the loss of water of crystallization). The salt is soluble in water, ether, petroleum ether, chloroform, carbon tetrachloride and hot benzene. The substance was dried for analysis at 100° in a vacuum over $Mg(ClO_4)_2$.

Anal. Calcd. for $C_6H_{11}NH_2 \cdot HOPO(OC_3H_7)_2$: F, 11.03. Found: P, 11.2.

Benzylamine salt of diisopropylphosphoric acid was prepared by the same method as used for the cyclohexylamine salt. The resulting sirup was dissolved in a small amount of acetone and the solution precipitated with petroleum ether. When this procedure was repeated several times, the product was crystalline at −30° but a thick sirup at room temperature. For analysis the substance was dried in a high vacuum at 100°.

Anal. Calcd. for $C_6H_5CH_2NH_2 \cdot HOPO(OC_3H_7)_2$: N, 4.8; P, 10.7. Found: N, 4.8; P, 10.3.

Benzylamine Hydrofluoride.—2.45 g. of benzylamine dissolved in 2.5 ml. of distilled water plus 2.0 ml. of DFP gave a homogeneous solution which was evaporated at room temperature *in vacuo* over phosphorus pentoxide. When crystals appeared on the surface of the solution, acetone was added. On cooling to −20°, nice crystal leaflets were obtained, which weighed 0.7 g. after suction. After redissolving in a small amount of water and precipitation with acetone at −10°, followed by filtration and drying over sulfuric acid in a vacuum the melting point was 149–150°. The material was soluble in hot chloroform, insoluble in carbon tetrachloride. For analysis the crystals were dried at 100° *in vacuo*.

(21) Toy, THIS JOURNAL, **72**, 2065 (1950).

(22) We are indebted for the determination of toxicities to Dr. Bernard McNamara, Pharmacology Section, Medical Division, Army Chemical Center, Maryland.

Anal. Calcd. for $C_6H_5CH_2NH_2 \cdot HF \cdot H_2O$: F, 13.1; N, 9.7. Found: F, 12.7; N, 10.4.

The fact that the fluorine content was found to be too low, the nitrogen too high, can be explained by a certain tendency of the salt to lose HF, benzylamine remaining in the residue. This was shown in the following way. (a) The salt dissolves in water with neutral reaction; but after standing for a while, the solution becomes alkaline, probably by loss of HF. (b) When the dry salt is heated in suspension with benzene in a test-tube, the glass becomes etched and the benzene contains an alkaline substance, probably benzylamine.

Guanidine Salt of Diisopropylphosphoric Acid (a).—Three hundred mg. of the barium salt of diisopropylphosphoric acid was dissolved in a small amount of water and passed through a column of Dowex 50 ion-exchange resin. The column was washed twice with water; the solution and washings were caught in an evaporating dish and concentrated by evaporation. Guanidine carbonate was added and carbon dioxide was liberated. The solution was filtered and the guanidine salt of diisopropylphosphoric acid crystallized from the mixture after adding acetone; m.p. 152–154°.

(b).—2.5 g. of the barium salt of diisopropylphosphoric acid was dissolved in a small amount of water and 2.1 ml. of 5 *N* H_2SO_4 added, the precipitated barium sulfate centrifuged off, and 900 mg. of guanidine carbonate added to the clear solution. After concentration on a boiling water-bath, acetone was added to the solution. The precipitate so obtained, which melted at 155–156°, was redissolved in distilled water and reprecipitated with acetone; beautiful long needles, m.p. 155–157°, were obtained. The material was insoluble in ether or petroleum ether.

(c).—One ml. of DFP, 450 mg. of guanidine carbonate and 2 ml. of distilled water were heated on a boiling water-bath until no more carbon dioxide was evolved. After cooling and addition of acetone, silky needles, m.p. 267–270°, were obtained. The material contained nitrogen and fluorine and was presumably guanidine hydrofluoride. To the filtrate more acetone was added and the crystals obtained were reprecipitated twice from water–acetone; m.p. 153–158°; yield 100 mg. For analysis the material was dried over phosphorus pentoxide in a vacuum.

Anal. Calcd. for $C_7H_{20}N_3O_4P$: C, 34.9; H, 8.35; N, 17.42; P, 12.88. Found: C, 34.9; H,8.3; N, 17.59; P, 13.3.

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A Study of *n*-Octadecenoic Acids. III. X-Ray Diffraction Behavior of 6,7- through 12,13-Dihydroxystearic Acids

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Both the low-melting and the high-melting series of 6,7- through 12,13-dihydroxystearic acids (obtainable, respectively, from the *cis* and *trans* series of octadecenoic acids by performic acid oxidation) show alternation of properties. By m.p.'s and interplanar spacings, it is possible to classify these acids into four sub-groups, namely, the even (6,7-, 8,9-, etc.) and the odd (7,8-, 9,10-, etc.) low-melting and the even and odd high-melting isomers. Within each sub-group the individual members cannot be satisfactorily distinguished by means of m.p. and interplanar spacings. They can be distinguished, however, by the relative intensities of the different orders of their long spacings. These relative intensities fall in line with calculated values. An incomplete correspondence of the 6,7-acids with other even acids was partially accounted for by their polymorphism. (The 9,10-compounds were also found to be polymorphic.)

The characterization of individual *cis* and *trans* 6- through 12-octadecenoic acids is a difficult matter. The first two papers of this series,[1,2] which describe the synthesis of both *cis* and *trans* acids and diffraction patterns of the latter, reveal an alternation in both m.p. and interplanar spacings, such that classification of either a *cis* or a *trans* acid as a member of an "even" (6-, 8-, etc.) or "odd" (7-, 9-, etc.) sub-group is readily made, but positive identification of any individual odd (or even) acid is not easy. It is true that odd (or even) *trans* acids show diffraction differences, but only in fine details revealed by high resolution technique. Mixed m.p. methods can, of course, be used; and Bumpus, *et al.*,[3] have shown that it is possible to characterize the octadecenoic acids according to double bond position by chromatographic examination of their monocarboxylic degradation products.

A simple and reasonably direct means of characterizing individual octadecenoic acids was therefore still to be sought. It seemed that the relative intensities of the long spacings ((001) reflections) of their dihydroxy derivatives should give clear-cut distinction between individual octadecenoic acids because of the differences in relative position along the diffracting unit of the added strongly diffracting hydroxyl groups. In other words, it was to be expected that the relative intensities of a pair of successive long spacing orders, for example, should change with change in hydroxyl positions. On testing this idea, it proved successful, for the most part, as shown by the results reported in this paper.

An excellent method for preparing dihydroxy acids has been described by Swern, *et al.*,[4] and diffraction data for products thus prepared from *cis*- and *trans*-9-octadecenoic acids have been reported by Witnauer, *et al.*[5]

In the first paper of the present series[1] the preparation and m.p.'s of the low-melting and high-melting dihydroxy derivatives (hereafter referred to as LMDH and HMDH, respectively) of the 7- through 12-octadecenoic acids were reported. These samples were used in the present diffraction study. Data are also reported which were obtained by studying the LMDH and HMDH derivatives of 6-octadecenoic acid.

It has been shown by various workers[6,7] that the

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