It is not possible at present to assign unequivocally an order of electrophilicity on halogen or nucleophilicity on carbon to all the halogens and interhalogen compounds. It can be concluded from the relative stability of active *sec*-octyl iodide in the presence of iodine, as well as from runs made with iodine monochloride in the presence of iodine that iodine is much less nucleophilic on carbon than iodine monochloride which is more nucleophilic than molecular chlorine. The optical stability of active *sec*-octyl chloride in the presence of chlorine is probably due to the lack of complex formation between the chloride and chlorine.

Experimental

Materials.—*d*,1-2-Octanol was resolved by the customary procedure[17] and purified by fractional distillation, b.p. 88–89° (23 mm.), $n^{21.5}D$ 1.4250 (lit.[18] $n^{20}D$ 1.4244), $[\alpha]^{25}D$ ±8.99 to ± 9.9° (ethanol) (lit.[16] $[\alpha]^{20}D$ 9.9°).

sec-Octyl iodide was prepared by the method of Berlack and Gerrard,[15] $n^{20}D$ 1.4880, $[\alpha]^{30}D$ ±23.7 to ±52.7° (carbon tetrachloride), b.p. 49–50° (1.4 mm.) [lit.[15,19] $[\alpha]^{18}D$ ±56.8°, b.p. 98–100° (18 mm.), $n^{25}D$ 1.4263].

sec-Octyl chloride was made according to the procedure of McKenzie and Tudhope,[20] b.p. 62° (14 mm.), $n^{19.3}D$ 1.4270, $[\alpha]^{25}D$ ±24.2° (ether) [lit. b.p. 79 (~16 mm.),[21] $[\alpha]^{20}D$ (max.) ±36.14°[14]]. The chloride exhibited characteristic absorption in the infrared at 690 cm.$^{-1}$ (strong, sharp, C–Cl stretching).

Methyl *sec*-octyl ether was prepared by the reaction of methyl iodide with potassium *sec*-octoxide in refluxing ether. It was purified by filtration through a column of activated alumina (in pentane) and subsequent distillation, b.p. 57.5° (13 mm.), $n^{20}D$ 1.4083 [lit.[21] b.p. 76–77° (44 mm.), $n^{25}D$ 1.4212]. The ether showed characteristic C–O stretching absorption in the infrared at 1090–1100 cm.$^{-1}$ and no trace of alcoholic hydroxyl.

(−)*sec*-Octyl Thiocyanate.—A solution of one gram (0.00415 mole) of (+)*sec*-octyl iodide ($[\alpha]^{34}D$ +52.7°) and 20 g. of potassium thiocyanate in 150 ml. of methanol was heated to reflux overnight. The solvent was removed under reduced pressure and the residue was treated with water and extracted with pentane–methylene chloride (40:1). The pentane solution was concentrated and the residue was fractionally distilled through a semi-micro column to give 42% (−)*sec*-octyl thiocyanate, b.p. 119–120° (18 mm.), $n^{22}D$ 1.4630, $[\alpha]^{34}D$ −60.8° (ethanol) [lit.[16] b.p. 119–120° (20 mm.), $n^{17}D$ 1.4651, $[\alpha]^{20}_{5893}$ −64.68° (ethanol) for a sample obtained *via* the tosylate from *sec*-octyl alcohol, $[\alpha]^{20}_{5893}$ +9.48°].

General Procedure for Exchange Reactions.—To a solution of active *sec*-octyl iodide in the solvent or solvent mixture, chilled under anhydrous conditions in a Dry Ice–acetone bath, was added rapidly a cold solution of excess chlorine (or iodine monochloride) in the particular solvent (final concn. ~10 *M*). After storage in the dark at cooling-bath temperature for 5–15 minutes, the reaction mixture was allowed to warm to about 0° and then washed successively with 10% aqueous sodium bisulfite and water. The solution of the chloride was then diluted with pentane, washed successively with concentrated sulfuric acid and water, dried and distilled fractionally to obtain a sample of pure chloride for the determination of optical rotation. In some cases the product was first isolated without washing with sulfuric acid by total distillation for infrared analysis. The yield of chloride, b.p. 60–62° (14 mm.), $n^{20}D$ 1.4280–1.4310, varied from 30 to 50% depending on the efficiency of the isolation. The crude chloride obtained from the exchange runs using methanol as solvent without a sulfuric acid washing contained methyl *sec*-octyl ether as an impurity as determined from the infrared spectrum. The pure chloride could be obtained by removal of the ether using a sulfuric acid wash.

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[CONTRIBUTION FROM THE POLYMER DEPARTMENT, THE WEIZMANN INSTITUTE OF SCIENCE]

Phosphate Anhydrides of Amino Acids

BY A. KATCHALSKY AND M. PAECHT

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A method for the synthesis of free phosphate anhydrides of leucine, aspartic and glutamic acid is described. The silver salt of carbobenzyloxy amino acid is condensed with dibenzyl chlorophosphate and the carbobenzyloxy and benzyl groups are split off by a stream of dry hydrogen bromide. The free phosphate anhydrides of these amino acids are obtained as heavy oils of 70–90% purity. The substances are extremely hygroscopic and reactive. They react spontaneously with alcohols to give esters: with amines, to give amides; and with hydroxylamine, to give hydroxamic acids suitable for analytical determination. In aqueous solution at room temperature, the phosphate anhydride of leucine polymerizes spontaneously to produce polypeptides of 3–20 amino acids.

Introduction

For the past twenty years reactive intermediates in the biosynthesis of proteins have been sought[1] which would condense, under conditions found in the living cell, to form polypeptides and proteins.

Lipmann has suggested[2] that these intermediates are the mixed anhydrides of phosphoric and amino acids. Several attempts have therefore been made to synthesize such compounds, which could serve as models for the reactive substances that enable the peptide bond formation. Hitherto, however, the compounds have proved very unstable and could not, therefore, be isolated. Some of the more stable derivatives of the phosphate anhydrides have been prepared by Chantrenne.[3]

The phosphate anhydrides of glycine and alanine were synthesized in this laboratory[4] as follows.

The silver salt of N-carbobenzyloxy amino acid was coupled with dibenzyl chlorophosphate[5] and the product treated with anhydrous hydrogen bromide, analogous to the method of Ben-Ishai and

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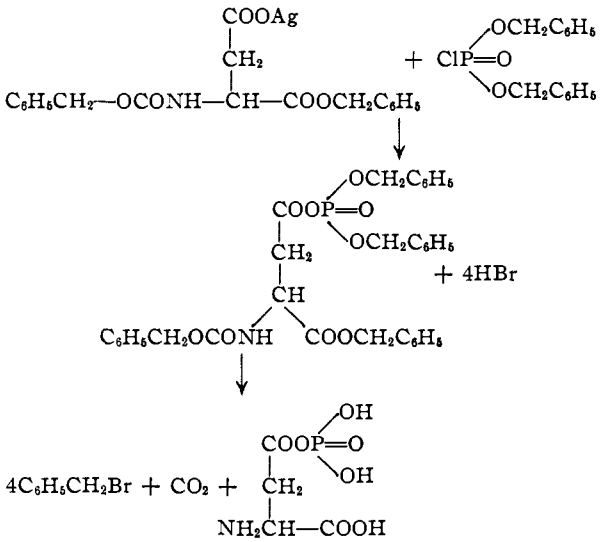
Berger,[6] to remove the carbobenzyloxy and benzyl groups.

The synthesis of the amino acid phosphates, starting from the azides of the corresponding acids has recently been reported by Bentler and Netter.[7] However, no properties of the products are described.

This paper describes the preparation, according to our method, of the free phosphate anhydride of leucine, and of the β- and γ-phosphates of aspartic and glutamic acid, respectively. Properties of the five phosphate anhydrides thus far prepared are described as well.

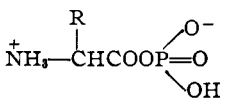
Synthetic Procedure.—The starting material for the synthesis of the phosphate of the dicarboxylic amino acids was the silver salt of the α-benzyl ester of the carbobenzyloxy amino acid.[8] The synthesis followed the same procedure as that used for the neutral amino acids



The free phosphate anhydrides are obtained as clear, heavy and highly hygroscopic oils. Attempts to remove all the impurities (benzyl bromide), result in decomposition.

These phosphates react with hydroxylamine to form hydroxamic acids, and with aqueous ammonia to give amides—glutamic and aspartic phosphate giving glutamine and asparagine, respectively, in over 90% yield.

Properties of the Phospho-anhydrides of Amino Acids.—All five amino acid phosphates are free of bromide ion, proving that no bromohydrate is formed at the amino group. Considering the strong acidity of the phosphate group, it is probable that the substances are dipolar ions as



Heat of hydrolysis, calorimetrically measured, is about 20 kcal./mole; exact measurements, however, could not be made owing to technical difficulties. These phosphate anhydrides phosphorylate adenylic acid in aqueous solution to adenosine-diphosphate; special studies have been carried out with leucine phosphate.[9] All the phosphate anhydrides, and especially those of glycine and alanine, give ammonia. In aqueous solution at room temperature and neutral *p*H, the neutral amino acid phosphates are not only hydrolyzed but react readily with themselves to give polypeptides. Hydroxylamine and other organic bases react with the amino acid of the phosphate anhydrides. In addition, however, they also act as strong polymerization catalysts.

Polypeptides of 5–10 amino acids are formed spontaneously from the monoamino-monocarboxylic acids investigated. The dibasic amino acids fail to polymerize readily.

Experimental

A. Leucine Phosphate. (1) Preparation of the Silver Salt of Carbobenzyloxy-N-leucine.—To a 2 *M* aqueous solution of the sodium salt of carbobenzyloxyleucine[10] (no excess of sodium hydroxide), an equivalent amount of silver nitrate solution was slowly added. The precipitated silver salt of the carbobenzyloxyleucine was washed with distilled water, extracted with ether, and then dried to constant weight in the dark.

(2) Coupling of the Silver Salt of Carbobenzyloxyleucine with Dibenzyl Chlorophosphate.—To a water-free carbon tetrachloride solution of dibenzyl chlorophosphate prepared, according to Todd,[5] from 8.6 g. of dibenzylphosphite, was added 12 g. of the silver salt of carbobenzyloxyleucine. The reaction mixture was agitated for two hours at room temperature by a stream of dry nitrogen. After the mixture had stood overnight, the silver chloride and the excess of silver salt of carbobenzyloxyleucine were removed by filtration, and the carbon tetrachloride was removed by distillation under reduced pressure. The carbobenzyloxyleucine dibenzylphosphate remained as a viscous oil.

Anal. Calcd. for $C_{28}H_{32}O_7NP$: C, 63.8; H, 6.1; N, 2.7; P, 5.9. Found: C, 64.4; H, 6.5; N, 2.5; P, 6.2.

(3) Preparation of Free Phosphate Anhydride of Leucine.—To prepare the free leucine phosphate, a stream of dry hydrogen bromide[11] was passed through a 10% solution of carbobenzyloxyleucine dibenzylphosphate in water-free carbon tetrachloride.[6] The temperature of the solution rose during this reaction. After about 20 minutes, when the solution had become saturated with the gas, the leucine phosphate separated as a heavy oil. The oil was washed several times with absolute ether, and the solvents removed *in vacuo* with a diffusion pump at room temperature. This procedure did not remove all the entrapped benzyl bromide, but more drastic procedures resulted in decomposition of the phosphate.

Quantitative Determination of the Anhydride.—In all cases, the anhydride content was determined colorimetrically by converting the phosphates to the corresponding hydroxamic acids. The phosphate anhydrides were transformed into the amino acid ethyl esters by interaction with absolute ethyl alcohol. After standing for a few minutes, the phosphate salt of the ester precipitated. The esters were then treated with aqueous hydroxylamine, and the color developed according to the method of Hestrin.[12] The corresponding ethyl esters of the amino acids were used as standards.

Analysis of the leucine phosphate by this method shows a yield of 87%, calculated on the weight of the carbobenzyloxyleucine dibenzylphosphate used.

(4) Polymerization.—Leucine phosphate polymerizes readily in aqueous solution, the degree of polymerization depending on the *p*H of the solution. The polyleucines obtained

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(9) Results to be published subsequently.

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in aqueous solution gave, at various *p*H, the following values for the ratio total N (Dumas)/amino N (Van Slyke), which corresponds to the degree of polymerization (buffer concentration *M*/30, concentration of substance, 100 mg. in 5 cc. of buffer solution).

*p*H	9	6	5	4
Dumas N/Van Slyke N	1.5	3	4	20 (insol. product)

Chromatographic analysis showed that all the polyleucines were mixtures of at least 3 substances. Each of them gave pure leucine on hydrolysis.

B. **Aspartyl Phosphate.** (1) **Carbobenzyloxyaspartic Acid-α-benzyl Ester β-Dibenzylphosphate.**—Carbobenzyloxy-*l*-aspartic-α-benzyl ester was prepared according to Bergmann, *et al.*[10,8] 5.4 g. of carbobenzyloxy-l-aspartic acid was boiled quickly with 16 ml. of acetic anhydride, immediately cooled, and absolute ether and petrol ether were added. Four grams of carbobenzyloxy-l-aspartic acid anhydride precipitated as white crystals, which had, after washing with ether, a melting point of 124°.

The carbobenzyloxyaspartic acid-α-benzyl ester was prepared by heating 3 g. of the above anhydride with 1.9 g. of freshly distilled benzyl alcohol for 3.5 hours in a closed tube kept in boiling water. The oil formed was extracted with ether, and the etheric solution washed twice with a solution of bicarbonate. The bicarbonate solution was then washed with ether; upon the addition of diluted hydrochloric acid, the benzyl ester precipitated as an oil which quickly crystallized. Recrystallization from ether–petrol ether gave a melting point of 84°.

Five equivalents of the silver salt of the benzyl ester to one of dibenzyl chlorophosphate was used for the preparation of the carbobenzyloxyaspartic acid-α-benzyl ester β-dibenzylphosphate. The method used to prepare carbobenzyloxyleucine dibenzylphosphate was followed, yielding a heavy, yellowish oil. Calculated on the amount of dibenzyl chlorophosphate, a 100% yield was obtained.

Anal. Calcd. for $C_{33}H_{31}O_9NP$: C, 64.1; H, 5.0; N, 2.3; P, 5.2. Found: C, 63.7; H, 5.4; N, 2.3; P, 4.9.

(2) **β-Aspartyl Phosphate.**—As with leucine phosphate, the β-aspartyl phosphate was prepared by passing a stream of dry hydrogen bromide through a water-free carbon tetrachloride solution of carbobenzyloxyaspartic acid-α-benzyl ester β-dibenzylphosphate. In this case the carbobenzyloxy and three benzyl groups were removed, producing an oil which was 92% pure, according to the colorimetric analysis described above. The yield, calculated on the weight of carbobenzyloxyaspartic acid benzyl ester dibenzylphosphate, was 80%.

(3) **Asparagine Formation.**—One hundred mg. of β-aspartyl phosphate was added to 5 ml. of 1 *M* aqueous ammonia. After standing for about 5 minutes, the solution was concentrated by distillation to 1 ml. The phosphate salt of *asparagine* precipitated as a white crystalline substance; m.p. 168°, mixed melting point with the phosphate salt of natural asparagine 168°.

Anal. Calcd. for $C_4H_{11}O_7N_2P$: C, 20.5; H, 4.8; N, 12.1; P, 13.5. Found: C, 19.8; H, 5.5; N, 12.1; P, 13.6.

C. **Glutamyl Phosphate.** (1) **Carbobenzyloxy-*l*-glutamyl-α-benzyl Ester γ-Dibenzylphosphate.**—This substance was prepared according to the procedure used to obtain the aspartic acid derivative, yielding the carbobenzyloxy-*l*-glutamic acid-α-benzyl ester as a colorless oil. Only a slight excess of its silver salt was required to obtain a good yield of carbobenzyloxy-*l*-glutamyl-α-benzyl ester γ-dibenzylphosphate in the form of a heavy oil.

Anal. Calcd. for $C_{34}H_{32}O_9NP$: C, 64.6; H, 5.1; N, 2.2; P, 4.9. Found: C, 64.2; H, 5.9; N, 2.3; P, 5.2.

(2) **γ-Glutamyl Phosphate.**—The γ-glutamyl phosphate was prepared in the same way as the β-aspartyl phosphate. The yield was 85%. The reaction with hydroxylamine showed that the product was 92% pure.

(3) **Glutamine Formation.**—One hundred mg. of γ-glutamyl phosphate was dissolved in 1 ml. of 1 *M* aqueous ammonia. *Glutamine* immediately precipitated; m.p. 185°. On heating, the glutamine was converted into the ammonium salt of pyrrolidone carboxylic acid, which has the same melting point as glutamine.[13] The amino nitrogen of the latter was 6.3%, while its total nitrogen was 19.0% (theoretical 19.2%). The amino nitrogen of the glutamine, air-dried, was 18.9%.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Streaming Orientation Studies on Denatured Proteins. V. Bovine Serum Albumin[1]

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The denaturation of bovine serum albumin under various conditions has been investigated by flow birefringence. No common pattern of alteration, under the various denaturing conditions, is evident. Heat denaturation in either acid or alkaline aqueous solution leads to moderately highly birefringent solutions, probably largely through aggregation as previously concluded by Joly and Barbu. Samples heat denatured in the presence of 80% glycerol possess relatively large rotary diffusion constants suggesting neither appreciable aggregation nor drastic unfolding of the molecule. The rotary diffusion behavior following heat denaturation is not appreciably altered by inclusion of anionic detergent ions (in alkaline solutions) or cationic detergent ions (in acid solutions). Denaturation by guanidine hydrochloride or thiocyanate at 37° leads to measurable, though relatively weak, birefringence of flow. Guanidine thiocyanate (3.0 *M*) in alkaline solution and in absence of buffer ions appears to be particularly effective in minimizing aggregation. The ability of caprylate ions to repress or modify denaturation is not significant unless prolonged interaction with the protein is permitted prior to subjection of the protein to the denaturing conditions. It is tentatively suggested that the primary denaturation process in the case of this protein is essentially an expansion of the molecule, rather than an unfolding such as has been suggested for ovalbumin in previous studies of this series.

Previous contributions in this series[3] have been concerned with an attempt to clarify, through studies of streaming birefringence behavior, the character of the configurational changes involved in the denaturation of ovalbumin under a variety of conditions. It has been shown that any interpretation of such results on a molecular level is complicated by the tendency of denatured ovalbumin to aggregate. Nevertheless, evidence has been presented to show that under conditions which minimize intermolecular effects similar rotary diffusion constants are obtained under a variety of denaturing conditions including heating aqueous

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