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Further Synthetic Studies with Phosphorylated Peptides*

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Synthetic carbobenzoxy-β-benzyl-L-aspartyl-L-serylglycine benzyl ester and carbobenzoxy-β-benzyl-L-aspartyl-L-seryl-L-glutamic dibenzyl ester were phosphorylated by means of di-*p*-nitrobenzylphosphorochloridate in the presence of imidazole in 90% and 50% yield, respectively. The crude phosphate triesters, carbobenzoxy-β-benzyl-L-aspartyl-*O*-(*O,O*-di-*p*-nitrobenzylphospho)-L-serylglycine benzyl ester and carbobenzoxy-β-benzyl-L-aspartyl-*O*-(*O,O*-di-*p*-nitrobenzylphospho)-L-seryl-L-glutamic dibenzyl ester could not be purified by fractional precipitation from usual solvents; however, countercurrent distribution has been found to be a suitable technique for isolating these and related substances in analytically pure form. Preliminary experiments on the synthesis of phosphodiesters of serine are also described. In this connection, *O*-butylphospho-D,L-serine was synthesized and its isolation from by-products, *e.g.*, phosphoserine and serine, was effected by countercurrent distribution.

The study of phosphoproteins, with special reference to the modes of linkage of the phosphoryl groups, has been hampered by the experimental difficulties in the available methods for the synthesis of phosphopeptides needed as model compounds.

In our laboratory we are investigating the problem of synthetic methods leading to the desired model phosphopeptides containing phosphomonoester, phosphodiester, mixed-amide ester, and pyrophosphate bonds. Diphenyl- or dibenzylphosphorochloridate has been used for the phosphorylation of the hydroxyl function of *N*-carbobenzoxy peptide esters of serine (Riley *et al.*, 1957; Fölsch, 1959), but the limitations of these reagents are particularly evident. Phosphorylation by these reagents does not proceed to completion and, since no method is available for the purification of the oily phosphotriesters usually obtained, the homogeneity of the products remains in doubt. In fact, hydrogenolysis of oily diphenylphosphate esters proceeds very slowly and the end products are contaminated with monophenyl derivatives, unphosphorylated products, and, in certain cases, with traces of the respective amino acids (Fölsch, 1959; Theodoropoulos *et al.*, 1960a). Isolation of the desired phosphopeptides from these by-products requires a laborious fractionation on ion-exchange columns (Strid, 1959).

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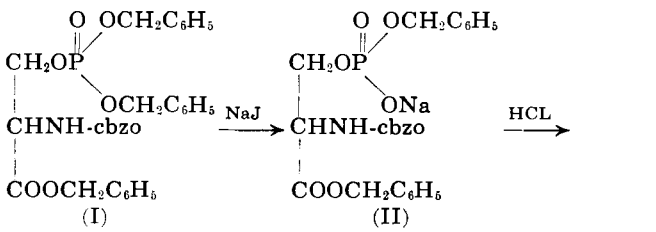
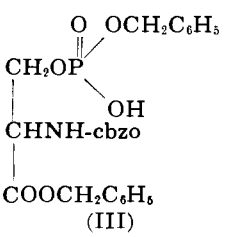
Recently we reported a new procedure for the synthesis of phosphopeptides containing a phosphomonoester bond (Theodoropoulos *et al.*, 1960b). This procedure involving phosphorylation with di-*p*-nitrobenzylphosphorochloridate in the presence of imidazole, was successfully applied to the synthesis of phosphoserine and certain simple phosphorylated peptides. This paper will give evidence for the suitability of our procedure in the synthesis of complex phosphopeptide derivatives and the required intermediates for the synthesis of phosphodiesters of serine. In this connection it should be emphasized that di-*p*-nitrobenzylphosphorochloridate, under the conditions used, usually gives solid products, but in spite of the difference in polarity between di-*p*-nitrobenzylphosphate esters and their unphosphorylated analogs, no satisfactory separation can always be effected by fractionation from usual solvents. Where classical fractionation failed, countercurrent distribution was found to be a powerful technique for separating such related synthetic substances.

Results and Discussion

Phosphorylation of protected peptides of serine with DNBPCl,[1] in the presence of imidazole, proceeds under the most mild conditions in comparison to other methods (Riley *et al.*, 1957; Fölsch, 1959). In preliminary experiments we have also treated cbzo-D,L-serine benzyl ester with tetra-*p*-nitrobenzyl- and tetrabenzylpyrophosphate (Zervas and Dilaris, 1956), respectively, in the presence of imidazole. The corresponding triesters were isolated in 40% and 37% yield. The latter, cbzo-*O*-(*O*,*O*-dibenzylphospho)-D,L-serine benzyl ester (I) does not appear to have been reported in literature. Using DBPCl in pyridine we have failed to obtain crystalline compound I so far.

Compound I, as expected, upon treatment with sodium iodide in acetone, afforded the sodium salt of cbzo-*O*-(*O*,*O*-benzylphospho)-D,L-serine benzyl ester (II), which on acidification gave the corresponding acid-ester III.





In a similar manner the acid-esters,[2] cbzo-glycyl-*O*-(*O*,*O*-*p*-nitrobenzylphospho)-D,L-serine benzyl ester (IV) and cbzo-*O*-(*O*,*O*-*p*-nitrobenzylphospho)-L-seryl-L-alanine benzyl ester (V) were prepared from the respective crude triesters (Theodoropoulos *et al.*, 1960b). Catalytic hydrogenation of compounds IV and V afforded pure glycyl-*O*-phospho-D,L-serine and *O*-phospho-L-seryl-L-alanine. We thought, therefore, that partial debenzylation could be an alternative in isolating protected phosphopeptides from by-products, when recrystallization was not effective. This however has not proved always to be the case, especially with longer peptides such as cbzo-β-benzyl-L-aspartyl-*O*-(*O*,*O*-DNBP)-L-serylglycine benzyl ester and cbzo-β-benzyl-L-aspartyl-*O*-(*O*,*O*-DNBP)-L-seryl-L-glutamic dibenzyl ester. An increase in the hydrophobic part of the molecule renders the respective sodium salts sparingly soluble in water.

Phosphopeptide units containing the sequence asp·serP·gly and asp·serP·glu are of special interest. It is well known that several DFP-inhibited enzymes afford, after partial hydrolysis, the common amino acid sequence asp·serP·gly; however, synthetic proof of this and related structures is lacking.

The other sequence, asp·serP·glu, occurs in ovalbumin. It is the phosphorserine residue of this particular amino acid sequence, which resists dephosphorylation by alkaline phosphatase according to Flavin (1954). Interesting enough, the phosphoserine residue is surrounded by acidic amino acids. Whether this particular structure hinders the phosphatase action might not be clarified unless synthetic asp·serP·glu is tested.

The syntheses of cbzo-β-benzyl-L-aspartyl-L-serylglycine benzyl ester (VI) and cbzo-β-benzyl-L-aspartyl-L-seryl-L-glutamic dibenzyl ester (VII) were first investigated. Coupling of cbzo-β-benzyl-L-aspartate with L-serylglycine benzyl ester by the DCC method (Sheehan and Hess, 1955) gave the expected cbzo-tripeptide ester in good yield. Similarly, cbzo-β-benzyl-L-aspartate with L-seryl-L-glutamic dibenzyl ester gave the expected cbzo-tripeptide ester. Catalytic hydrogenation of compounds VI and VII afforded L-aspartyl-L-serylglycine and L-aspartyl-L-seryl-L-glutamic acid, both isolated as *dihydrates*. Some difficulty was encountered however, in the isolation of analytically pure samples. Prolonged exposure to 90% ethanol, used for recrystallization, leads to the formation of an extra distinct spot, postulated as a β- or γ-ethyl derivative, according to paper chromatography.

Phosphorylation of the protected tripeptide VI with DNBPCl, in the presence of imidazole, produced an amorphous product; its phosphorus content indicated that compound VI was converted into cbzo-β-benzyl-L-aspartyl-L-*O*-(*O*,*O*-DNBP)-L-serylglycine benzyl ester (VIII) in about 90% yield. On the contrary, phosphorylation of the glutamic analog VII, under the same conditions, did not proceed as well as with the glycine derivative; phosphorus analysis of the obtained crude product indicated it to be a mixture of unphosphorylated cbzo-tripeptide VII and cbzo-β-benzyl-L-aspartyl-*O*-(*O*,*O*-DNBP)-L-seryl-L-glutamic dibenzyl ester (IX) in about equal amounts. This was confirmed in a series of experiments. We have no explanation for the striking difference in phosphorylation between the cbzo-tripeptide esters VI and VII, though the limited steric hindrance in the case of glycine, as postulated by Naughton *et al.* (1960), was brought to mind.

Several attempts to purify the above phosphorylated peptides VIII and IX by fractional precipitation have failed regardless of the solvents used. For this reason, the application of countercurrent distribution

[1] In the text and the experimental part, the standard amino acid abbreviations are used. Other abbreviations used are: cbzo, carbobenzoxy-; DNBPCl, di-*p*-nitrobenzylphosphorochloridate; DBPCl, dibenzylphosphorochloridate; DBP, dibenzylphospho-, DNBP, di-*p*-nitrobenzylphospho-; DCC, dicyclohexylcarbodiimide; DFP, diisopropylphosphofluoridate.

[2] Intermediates of this nature are of importance in connection with the synthesis of phosphopeptides containing pyrophosphate bonds (Theodoropoulos, D. [1960], *Chimia* 14, 377).

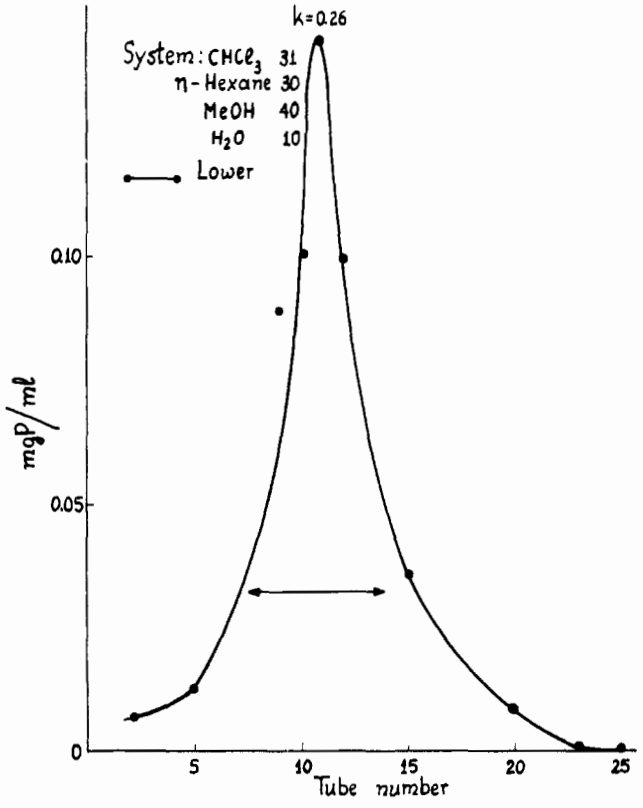


FIG. 1.—Countercurrent distribution pattern of crude cbzo-β-benzyl-L-aspartyl-*O*-(*O*,*O*-DNBP)-L-serylglycine benzyl ester (VIII) after fifty transfers.

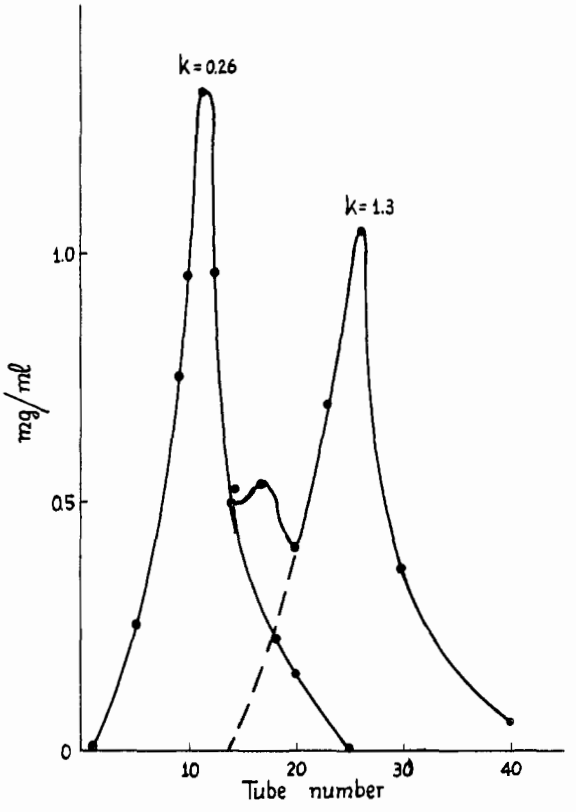


FIG. 2.—Countercurrent distribution of a mixture of equal amounts (0.4 g) of compound VIII and cbzo-β-benzyl-L-aspartyl-L-serylglycine benzyl ester (VI). Curve with K = 0.26 was obtained by phosphorus analysis, the values of which were converted to mg/ml of substance VIII. Curve with K = 1.3 (overlapping) was based on weight analysis.

was attempted. Simple systems like 2-butanol–aqueous acetic or hydrochloric acid were avoided because of the known sensitivity of the aspartyl bond. Systems containing either inorganic or organic bases were avoided because of the danger of β-elimination. A modest beginning has been made along this line with the system chloroform - *n* - hexane - methanol-water (31:30:40:10). A 50-stage distribution with twenty-five 25-ml volumes, starting with 1 g of cbzo-β - benzyl - L - aspartyl - *O* - (*O*,*O* - DNBP) - L - seryl-glycine benzyl ester (crude product), gave the pattern shown in Figure 1. Analysis was done by quantitative phosphorus determination. After recovery of the material in tubes 7–14, elementary analysis gave the expected values for C, H, N, and P. In order to confirm the presence of two main components and learn the extent of the influence of the concentration effects, the experiment was repeated using a mixture of equal amounts of phosphopeptide VIII and its unphosphorylated analog, cbzo-β-benzyl-L-aspartyl-L-serylglycine benzyl ester, scattered in two tubes. After applying fifty transfers, the pattern in Figure 2 was obtained. Analysis at this point was done by phosphorus determination and weight analysis, according to the technique described by Craig *et al.* (1951). Two major partly overlapping components are indicated. A third experiment using 1.2 g of compound VIII and 400 mg of compound VI, scattered in three tubes, gave a clear-cut separation after seventy-five transfers (Fig. 3). Clear-cut separation was achieved also with the glutamic analog after fifty transfers in the same system.

The purified phosphopeptide derivatives VIII and IX gave absorption spectra that were identical with respect to the positions of maxima and minima; the maximum was at 270 mμ (ϵ_{mol} = 19,000 for compound VIII and ϵ_{mol} = 19,900 for compound IX), and the minimum at 235 mμ (ϵ_{mol} = 5,900 for compound VIII and ϵ_{mol} = 5,700 for compound IX). Upon the action of 0.01 N of aqueous-ethanolic alkali the absorption spectrum differed in having much higher apparent molecular extinction coefficient at 235 mμ (ϵ_{mol} = 9,300 for compound VIII and ϵ_{mol} = 11,000 for compound IX), a fact indicating a β-elimination reaction (Riley *et al.*, 1957; Theodoropoulos *et al.*, 1960b). Typical absorption spectra are given in Figure 4. The conversion of compounds VIII and IX into their free phosphopeptides will be discussed in a forthcoming paper, when more experimental data of their hydrolytic behavior are at hand.

Concerning the synthesis of phosphodiesters of serine and related peptides, some preliminary experiments will be discussed in this paper. The sodium salt of cbzo-*O*-(*O*,*O*-*p*-nitrobenzylphospho)-D,L-serine benzyl ester was converted into the corresponding silver salt (X), which reacts readily with alkyl halides to produce the respective phosphotriesters. Thus compound X treated with *n*-butyl bromide produced oily triester, cbzo-*O*-(*O*-*p*-nitrobenzyl *O*-*n*-butylphospho)-D,L-serine benzyl ester (XI).

The absorption spectrum of compound XI indicated a maximum at 270 mμ (ϵ_{mol} = 11,500) and a minimum at 240 mμ (ϵ_{mol} = 4,500), assuming a molecular weight of 600. These values were higher than those expected in comparison to cbzo-*O*-(*O*,*O*-*p*-nitrobenzylphospho)-D,L-serine benzyl ester.[3] The latter acid-ester gave maximum at 270 mμ (ϵ_{mol} = 8,500) and a minimum at 235 mμ (ϵ_{mol} = 2,800). The observed higher values for compound XI were attributed to contami-

[3] Recrystallized from ethyl acetate–ether–petroleum ether (1:2:2) had mp 101–103°, reported mp 83–85° (Theodoropoulos *et al.*, 1960b).

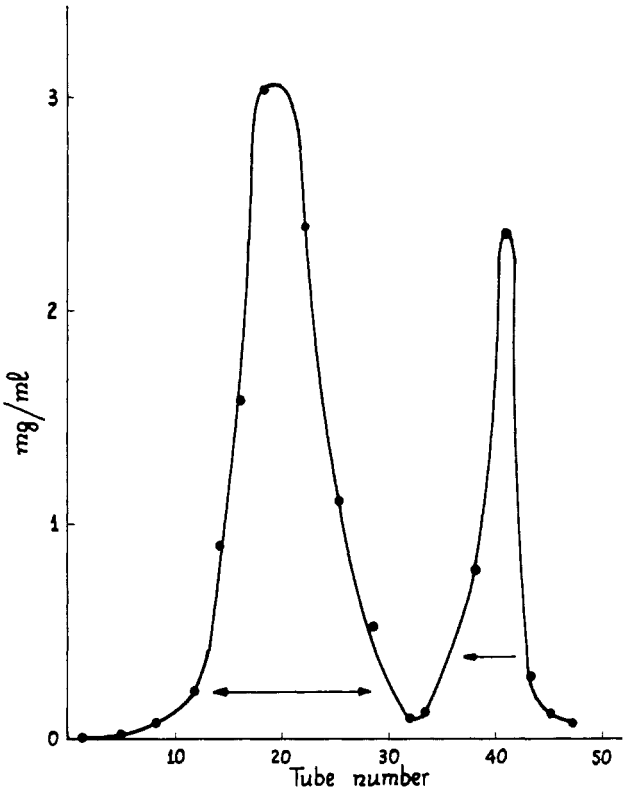
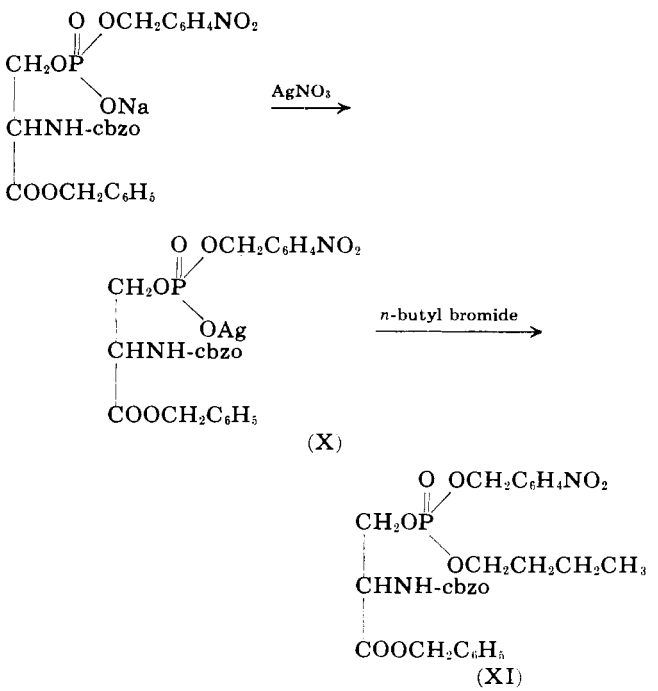


FIG. 3.—Clear-cut separation of Compounds VIII and VI by countercurrent distribution after seventy-five transfers.



nating acid-ester, resulting from the silver salt X. In repeated trials we failed to remove the acid-ester by extraction with 10% pyridine solution. Stronger bases were avoided because of the danger of β-elimination. Finally the problem was solved by countercurrent distribution; the solvent system used was methanol-water-ether (2:3:5). After fifty transfers the triester XI was collected in tubes 42–49, while the acid-ester remained behind in tubes 20–30. The recovered triester XI, which solidified after countercurrent distribution, gave an absorption spectrum with a maximum at 270 mμ (ϵ_{mol} = 9,300) and a minimum

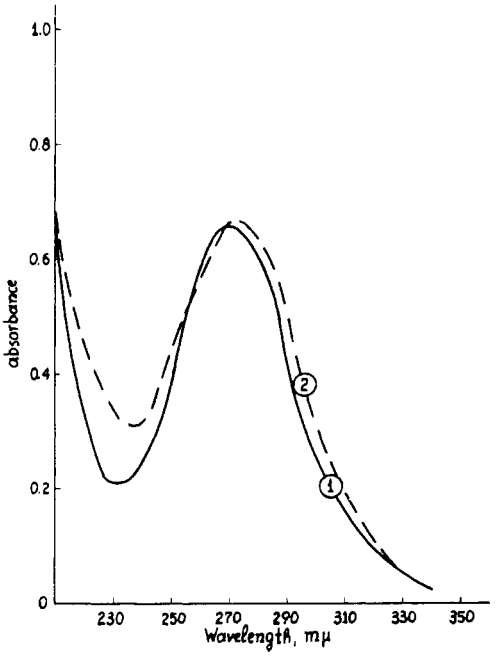


FIG. 4.—Absorption spectra of solutions of cbzo-β-benzyl-L-aspartyl-*O*-(*O*,*O*-DNBP)-L-serylglycine benzyl ester (3.5×10^{-5} M) in (1) 50% ethanol; (2) 0.01 N aqueous-ethanolic alkali.

at 235 mμ (ϵ_{mol} = 3,100); these values were close enough to those expected.

Surprisingly, the triester XI, after hydrogenolysis, gave a mixture of three components, as indicated by paper chromatography (Fig. 5). The slower-moving component corresponded to the position of phosphoserine and the major and faster-moving component was in fact the desired *O*-butylphospho-D,L-serine. The third spot (weak), moved to the position of serine and checked by paper electrophoresis, was neutral.[4]

Isolation of the desired *O*-butylphospho-D,L-serine from the above mixture was achieved by countercurrent distribution (Fig. 6). The system used was 1-butanol-ethanol-water (3:1:4). After 100 transfers the material in tubes 20–30 was recovered and finally the diester, *O*-butylphospho-D,L-serine (Fig. 7), was isolated as its mercury salt. It is noteworthy that the corresponding lead salt is water soluble. Other metals like barium, calcium, and iron form water-soluble salts also.

It is hoped that the information gained with the relatively simple phosphodiester, *O*-butylphospho-D,L-serine, will be valuable for the more difficult problems of synthesis and isolation of complex phosphodiesters of serine and threonine peptides.

EXPERIMENTAL

Cbzo-O-(O,O-dibenzylphospho)-D,L-serine Benzyl Ester (I).—To a suspension of dibenzylphosphate (5.6 g) in dry chloroform (10 ml), cooled to 0°, was added 2 g of DCC. Dicyclohexylurea was precipitated immediately, a fact indicating the formation of tetrabenzylpyrophosphate. After 15 minutes imidazole (1.3 g) and cbzo-D,L-serine benzyl ester (3.3 g), prepared according to Riley *et al.* (1957), were added. The reaction mixture was kept overnight at room tempera-

[4] The presence of phosphoserine and serine after the hydrogenolysis of triester XI may be due to a nucleophilic attack on the phosphorus by the amino group, provided that there is a difference in the hydrogenolysis rate between *N*-cbzo and *O*-benzyl group.

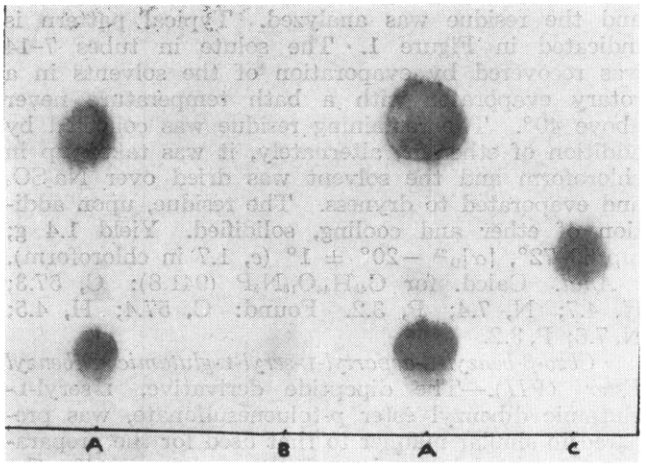


FIG. 5.—Paper chromatography of the hydrogenolysis product of cbzo-*O*-(*O*-*p*-nitrobenzyl *O*-butylphospho)-D,L-serine benzyl ester (A); B = phosphoserine and C = serine (standard).

ture and then dicyclohexylurea was removed by filtration. The filtrate was diluted with chloroform (50 ml) and washed twice with 10% pyridine solution (100 ml), 10% hydrochloric acid and water. After drying over exsiccated Na_2SO_4, the solvent was evaporated *in vacuo* and the remaining residue was dissolved in acetone. Traces of dicyclohexylurea were removed by filtration and the solvent was evaporated to dryness. The residue, upon addition of ether and cooling, solidified; yield 2.4 g (40.7%), mp 59–62° (softens at 57°). It was recrystallized from the minimal amount of absolute ethanol. Yield 2.2 g (37%); mp 62–64°. Its absorption spectrum indicated a maximum at 260 mμ (ϵ_{mol} = 650) and a minimum at 240 mμ (ϵ_{mol} = 250). The action of aqueous-ethanolic alkali indicated high apparent molecular extinction coefficient at 240 mμ (ϵ_{mol} = 5.135).

Anal. Calcd. for $C_{32}H_{32}O_8NP$ (589.5): C, 65.1; H, 5.4; N, 2.3; P, 5.2. Found: C, 65.3; H, 5.4; N, 2.4; P, 5.4.

In a similar manner, cbzo-*O*-(*O,O*-DNBP)-D,L-serine benzyl ester (Theodoropoulos *et al.*, 1960b) was obtained in 40% yield; mp 109–110°. In ethanol, the substance showed an absorption maximum at 265 mμ (ϵ_{mol} = 19,500), and a minimum at 230 mμ (ϵ_{mol} = 6,800). In 0.01 N ethanolic alkali showed maximum at 270 mμ (ϵ_{mol} = 19,200) and minimum at 230 mμ (ϵ_{mol} = 9,800).

Cbzo-O-(O,O-benzylphospho)-D,L-serine Benzyl Ester Sodium Salt (II).—This compound was prepared from triester I in similar manner to that used in the preparation of cbzo-*O*-(*O,O-p*-nitrobenzylphospho)-D,L-serine benzyl ester sodium salt (Theodoropoulos *et al.*, 1960b). Starting with 2.95 g of compound I, 1.95 g of product was obtained. Washed with a mixture of dry ether-acetone (1:1), it had mp 257–260° (Kofler block).

Anal. Calcd. for $C_{25}H_{25}O_8NP$ (521.4): N, 2.6; P, 5.9. Found: N, 2.4; P, 5.6.

Cbzo-O-(O,O-benzylphospho)-D,L-serine Benzyl Ester (III).—A solution of compound II in water (10 ml) was acidified to Congo red with hydrochloric acid. The resulting oil soldified upon standing in the refrigerator. Yield 1.8 g (75%); mp 98–102°. It was recrystallized from ethyl acetate–ether. Yield 1.75 g; mp 105–106° (decomp).

Anal. Calcd. for $C_{25}H_{26}O_8NP$ (468.4): N, 2.9; P, 6.6. Found: N, 2.5; P, 6.3.

Cbzo-glycyl-O-(O,O-p-nitrobenzylphospho)-D,L-serine Benzyl Ester (IV).—To a solution of cbzo-glycyl-*O*-

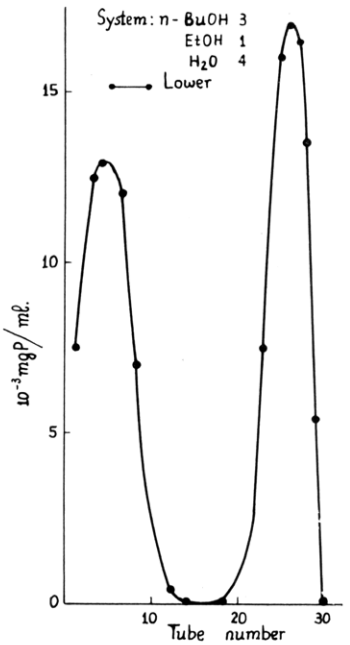


FIG. 6.—Countercurrent distribution pattern of the hydrogenolysis product of compound XI after 100 transfers.

(*O,O*-DNBP)-D,L-serine benzyl ester (3.67 g) in dry acetone (5 ml), was added sodium iodide (0.8 g), preheated for 18 hours to 120°. The mixture was heated under reflux for 1 hour and then the solvent was evaporated *in vacuo*. The remaining residue was almost dissolved in slightly hot water and this solution was washed three times with a mixture of ethyl acetate–ether (1:1). The water layer was acidified to Congo red with hydrochloric acid and the resulting oil solidified upon cooling. Yield 2.5 g (84%); mp 134–136°. After recrystallization from ethanol-ether (equal volumes), it melted at 136° (decomp). Before analysis it was dried at 100° for 2 hours in high vacuum (P_2O_5).

Anal. Calcd. for $C_{27}H_{28}O_{10}N_3P$ (577.4): C, 58.2; H, 4.8; N, 7.2; P, 5.3. Found: C, 57.8; H, 4.9; N, 7.0; P, 5.4.

Cbzo-O-(O,O-p-nitrobenzylphospho)-L-seryl-L-alanine Benzyl Ester (V).—This compound was prepared from cbzo-*O*-(*O,O*-DNBP)-L-seryl-L-alanine benzyl ester (3.77 g) in a similar manner to that described for compound IV. Yield 2.4 g; mp 157–160°. After recrystallization from ethanol–ethyl acetate–petroleum ether (1:6:6), it melted at 159–160°. Yield 1.95 g (61%).

Anal. Calcd. for $C_{28}H_{30}O_{10}N_3P$ (599.5): C, 56.0; H, 5.0; N, 7.0; P, 5.1. Found: C, 55.8; H, 5.2; N, 6.8; P, 5.0.

Cbzo-β-benzyl-L-aspartyl-L-serylglycine Benzyl Ester (VI).—A mixture of cbzo-β-benzyl-L-aspartate (1.79 g), prepared according to Bryant *et al.* (1959), 2.0 g of L-serylglycine benzyl ester *p*-toluenesulfonate (Theodoropoulos and Gazopoulos, 1962), triethylamine (0.7 ml), and DCC (1 g) in methylene chloride (50 ml), was stirred for 12 hours. Dicyclohexylurea was removed by filtration and the filtrate was washed quickly with 10% aqueous sodium bicarbonate and water. After drying over exsiccated Na_2SO_4, the solvent was evaporated to dryness. The residue was dissolved in acetone and traces of dicyclohexylurea were removed by filtration. The solvent was evaporated *in vacuo* at 35° and the remaining residue crystallized upon addition of ethyl ether. It was recrystallized from ethyl acetate–petroleum ether. Yield 1.75 g (60%);

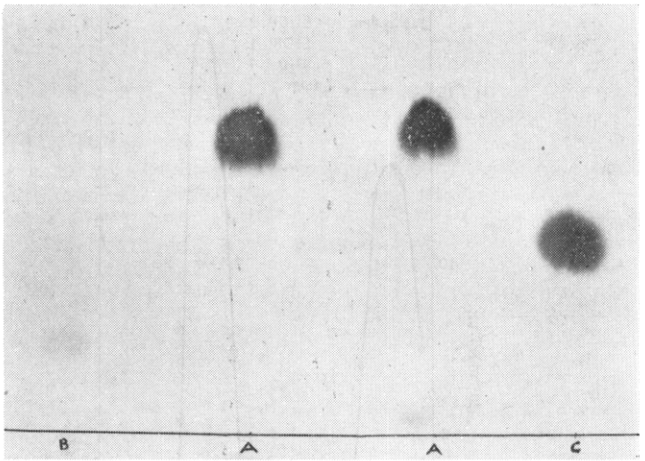


FIG. 7.—Paper chromatography of butylphospho-D,L-serine (A) after countercurrent distribution; B = phosphoserine; C = serine.

mp 112–116°, $[\alpha]_D^{25}$ − 24.5° ± 1° (*c*, 1 in acetic acid).

Anal. Calcd. for $C_{31}H_{33}O_9N_3$ (591.5): C, 62.9; H, 5.6; N, 7.1. Found: C, 62.4; H, 5.8; N, 7.0.

L-*Aspartyl*-L-*serylglycine.*—The above ester VI (1.4 g) was subjected to catalytic hydrogenation in 70% ethanol in the presence of 0.3 g of palladium black. When evolution of CO_2 ceased, the catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo* at 40°. The peptide was collected by addition of absolute ethanol. Yield 0.62 g (80%); mp 160–161°. Paper chromatography (Whatman No. 1) with 1-butanol–pyridine–acetic acid–water (15:10:3:12) gave a single spot with an R_F of 0.11 (ninhydrin-positive). It was recrystallized by dissolving in water (10 ml) and adding ethanol (100 ml). Yield 0.55 g, mp 170–171°, $[\alpha]_D^{25}$ − 8.7° ± 1° (*c*, 3.2 in water). Paper chromatography, after the purification process, revealed an extra faint spot with an R_F value of 0.35.

Anal. Calcd. for $C_9H_{15}O_7N_3 \cdot 2H_2O$ (313.2): N, 13.4; Found: N, 13.4.

Cbzo-β-benzyl-L-*aspartyl-O-*(*O,O-DNBP*)-L-*serylgycine Benzyl Ester* (*VIII*).—To a suspension of di-*p*-nitrobenzylphosphorochloridate (3.86 g) in dry chloroform (5 ml), cooled to 0°, was added imidazole (1.2 g) with shaking. After 2 minutes a solution of cbzo-β-benzyl-L-aspartyl-L-serylglycine benzyl ester (VI), dissolved in chloroform (5 ml), was added and the reaction mixture was cooled for 15 minutes. Subsequently it was permitted to remain at room temperature for 24 hours and was then diluted with chloroform (100 ml). The solution was washed with 2 × 50 ml of 10% aqueous pyridine solution, 10% hydrochloric acid (100 ml), and water. It was dried over Na_2SO_4 and the solvent was evaporated to dryness under reduced pressure at 35°; the remaining residue, upon addition of ether and cooling, solidified. Yield 3.8 g (80%); mp 65–69° (softens at 58°). Several analyses of phosphorus gave values never above 2.9.

Anal. Calcd. for $C_{45}H_{44}O_{16}N_5P$ (941.8); P, 3.2. Found: P, 2.9.

Purification of the above crude product was effected by a 50-transfer countercurrent distribution in the system chloroform-*n*-hexane-methanol-water (31:30:40:10). For preparative purposes 1.9 g of crude product, scattered in two tubes, was used in each experiment. Analysis was done by quantitative phosphorus determination (Fiske and Subbarow, 1925). Aliquots were withdrawn from the lower phase in test tubes, the solvent was evaporated carefully in a water bath, and the residue was analyzed. Typical pattern is indicated in Figure 1. The solute in tubes 7–14 was recovered by evaporation of the solvents in a rotary evaporator with a bath temperature never above 40°. The remaining residue was collected by addition of ether or, alternately, it was taken up in chloroform and the solvent was dried over Na_2SO_4 and evaporated to dryness. The residue, upon addition of ether and cooling, solidified. Yield 1.4 g; mp 69–72°, $[\alpha]_D^{25}$ −20° ± 1° (*c*, 1.7 in chloroform).

Anal. Calcd. for $C_{45}H_{44}O_{16}N_5P$ (941.8): C, 57.3; H, 4.7; N, 7.4; P, 3.2. Found: C, 57.4; H, 4.5; N, 7.6; P, 3.2.

Cbzo-β-benzyl-L-*aspartyl*-L-*seryl*-L-*glutamic Dibenzyl Ester* (*VII*).—The dipeptide derivative, L-seryl-L-glutamic dibenzyl ester *p*-toluenesulfonate, was prepared in similar manner to that used for the preparation of L-serylglycine benzyl ester *p*-toluenesulfonate. It failed to cystallize, but it was successfully used in the subsequent experiment.

In methylene chloride (50 ml) were mixed cbzo-β-benzyl-L-aspartate (1.79 g), L-seryl-L-glutamic dibenzyl ester *p*-toluenesulfonate (2.92 g), triethylamine (0.7 ml), and 1 g of DCC. After 12 hours dicyclohexylurea was removed by filtration and the filtrate was treated as in the case of the glycine analog VII. Yield 2.9 g (78%); mp 64–70°. Recrystallization from ethanol-water gave 1.56 g (68%) of the desired product VII, mp 87–90°, $[\alpha]_D^{25}$ − 45.4° ± 1° (*c*, 1.1 in acetic acid).

Anal. Calcd. for $C_{41}H_{43}O_{11}N_3$ (753.7): C, 65.3; H, 5.7; N, 5.5. Found: C, 65.4; H, 5.6; N, 5.6.

L-*Aspartyl*-L-*seryl*-L-*glutamic Acid.*—Hydrogenolysis of cbzo-β-benzyl-L-aspartyl-L-seryl-L-glutamic dibenzyl ester (1.5 g) in the presence of palladium black afforded 0.6 g (83%) of tripeptide, mp 109–111° (decomp.), $[\alpha]_D^{25}$ − 10° ± 1° (*c*, 3.5 in water). Paper chromatography in 1-butanol–pyridine–acetic acid–water (15:10:3:12) gave one spot with an R_F value of 0.12.

Anal. Calcd. for $C_{12}H_{23}O_{11}N_3 \cdot 2H_2O$ (385.3): N, 10.9. Found: N, 10.9.

Cbzo-β-benzyl-L-*aspartyl-O-*(*O,O-DNBP*)-L-*seryl*-L-*glutamic Dibenzyl Ester* (*IX*).—Following exactly the directions given for compound VIII, cbzo-β-benzyl-L-aspartyl-L-seryl-L-glutamic dibenzyl ester (3.76 g), afforded, after phosphorylation, 3.5 g (63%) of crude product, mp 60–63°.

Anal. Calcd. for $C_{55}H_{54}O_{18}N_5P$ (1103.9): P, 2.8; Found: P, 1.4.

Purification of 1.8 g of crude product by countercurrent distribution in the system previously used gave, after fifty transfers and recovering of the solute in tubes 3–10, 0.85 g of pure compound IX. It melted at 62–63° and had an $[\alpha]_D^{25}$ value of − 41° ± 1° as 1% solution in acetic acid.

Anal. Calcd. for $C_{55}H_{54}O_{18}N_5P$ (1103.9): C, 59.8; H, 5.0; N, 6.3; P, 2.8. Found: C, 60.1; H, 5.0; N, 6.4; P, 2.7.

Cbzo-O-(*O,O-p-nitrobenzylphospho*)-D,L-*serine Benzyl Ester Silver Salt* (*X*).—To a solution of 2.83 g of cbzo-*O-*(*O,O-p*-nitrobenzylphospho)-D,L-serine benzyl ester sodium salt (Theodoropoulos *et al.*, 1960b) in water (10 ml) was added, with stirring, silver nitrate (0.85 g), dissolved in 2 ml of water. The precipitated waxy product was solidified upon cooling and scratching with a glass rod. It was filtered, washed well with water, and dried *in vacuo* over P_2O_5, in the absence of light. Yield 2.6 g (80%); mp 134–137° (softens at 128°).

Anal. Calcd. for $C_{25}H_{24}O_{10}N_2PAg$ (651.3): N, 4.3; P, 4.7. Found: N, 4.2; P, 4.5.

Cbzo-O-(*O-p-nitrobenzyl O-n-butylphospho*)-D,L-*serine Benzyl Ester* (*XI*).—To a suspension of the above silver

salt X (4.1 g) in dry chloroform (30 ml) was added *n*-butyl bromide (0.7 ml). The mixture was heated under reflux for 6 hours with exclusion of moisture and sunlight. The precipitated silver bromide was removed by filtration and the filtrate was washed with 10% pyridine solution, dilute hydrochloric acid, and water. It was dried over Na_2SO_4 and the solvent was evaporated to dryness at 35° *in vacuo*. The remaining residue was further purified by countercurrent distribution. The system used was methanol-water-ether (2:3:5). After fifty transfers (twenty-five 25-ml volumes) the solute in tubes 40–49 was recovered by evaporation of the solvents at 35° *in vacuo*. Yield 2.4 g. The waxy product, upon standing in the refrigerator for several days, solidified, but its melting point, being lower than 40°, did not permit accurate determination.

In the same system used for countercurrent distribution, the acid-ester, cbzo-*O*-(*O*,*O*-*p*-nitrobenzylphospho)-D,L-serine benzyl ester, had K = 1.3 and was located in tubes 20–30.

Anal. Calcd. for $C_{29}H_{33}O_{10}N_2P$ (600.5): N, 4.6; P, 5.1. Found: N, 4.4; P, 4.9.

O-Butylphospho-D,L-serine Mercury Salt.—The triester XI (2.3 g) was dissolved in 80% ethanol and hydrogenated in the presence of palladium black. When evolution of CO_2 had ceased, the catalyst was removed by filtration and washed well with water and ethanol. The combined filtrate was evaporated to dryness at 40° and the remaining residue was checked by paper chromatography (Fig. 5). With the system 1-butanol–pyridine–acetic acid–water (15:10:3:12) the slower-moving component corresponded to the position of phosphoserine (R_F = 0.1), while the faint spot, in front of phosphoserine, appeared to be serine (R_F = 0.2). The latter, after elution and paper electrophoresis at *p*H 5.5, was found to be neutral. The faster-moving component (R_F = 0.35), which was expected to be the desired phosphodiester, was isolated from the above by-products by countercurrent distribution. The system used was 1-butanol-ethanol-water (3:1:4). Emulsion was destroyed by addition of few drops of ethanol. After fifty transfers (twenty-five 25-ml volumes) phosphorus analysis gave the pattern shown in Figure 6. The solute in tubes 22–41 was recovered by evaporation of the solvents at 40° *in vacuo*. The oily diester thus obtained was found to be homogeneous according to paper chromatography (Fig. 7) and paper electrophoresis (acidic spot). No attempt was made to crystallize the oily diester, but it was converted into its mercury salt as follows. The diester (0.5 g) was dissolved in water (2 ml) and mixed with mercury acetate (0.8 g) dissolved in 10 ml of water. The precipitate was filtered and washed with 7 ml of cold water. Yield 0.4 g; mp 180–182° (decomp). From the filtrate, by addition of 10 ml of ethanol, was obtained an additional amount of 0.2 g, with identical mp 180–182°. It was dried to 100° *in vacuo* over P_2O_5 before analysis.

Anal. Calcd. for $C_7H_{14}O_6NPHg$ (439.7): N, 3.1; P, 7.0. Found: N, 3.0; P, 6.9.

A sample was treated with Dowex 50 in H^+ form in water and the water solution was checked by paper chromatography. Again there was detected one spot with an R_F value of 0.35 in the system used before.

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