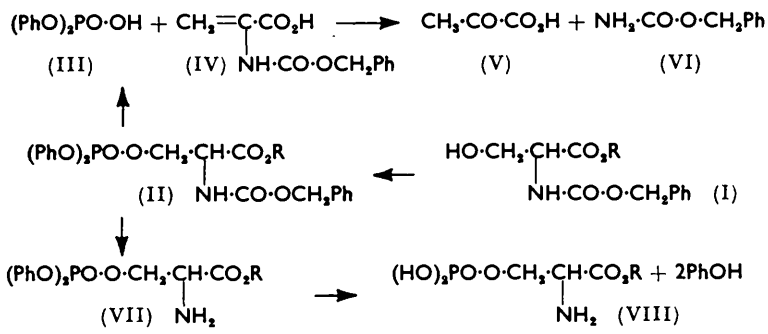
260. *Synthesis of Some Phosphorylated Amino-hydroxy-acids and Derived Peptides related to the Phosphoproteins.*

By G. Riley, J. H. Turnbull, and W. Wilson.

Diphenyl phosphorochloridate has been used to prepare a number of (di-*O*-phenylphospho)serine derivatives. In attempts to remove protecting phenyl groups, the action of alkali on these products has been studied; *N*-acylated compounds readily afford diphenyl hydrogen phosphate and acrylic acid derivatives by a β-elimination process. However, compounds having a free serine amino-group can be hydrolysed by alkali to phenol (2 mols.) and *O*-phosphorylserine derivatives. These reactions have been used in syntheses of phosphoserine, phosphothreonine, phosphoserylglycine, and phosphoserylglutamic acid. Hydrogenolysis also has been successfully used for removing phenyl groups in this series.

Several phosphorylated peptides have been obtained by partial hydrolysis of natural phosphoproteins, and similarly from certain enzymes (*e.g.*, cholinesterases and chymotrypsin) which have been inhibited by di*iso*propyl phosphorofluoridate.[1] By further hydrolysis of these peptides the following simple fragments have been isolated: serine dihydrogen phosphate ester (phosphoserine);[2] threonine dihydrogen phosphate ester (phosphothreonine);[3] phosphoserylglycine;[4] and phosphoserylglutamic acid.[5] If it is assumed[4,6] that no rearrangement occurs during hydrolysis, these phosphate units should exist in the parent phosphoproteins. The present work (for a preliminary account see ref. 7) concerns the development of general methods of synthesising such units. Diphenyl phosphorochloridate has been used throughout as the phosphorylating agent. Earlier workers in this field tended to use more drastic reagents.[8]



The synthesis of DL-phosphoserine was first investigated. *N*-Benzyloxycarbonyl-DL-serine ethyl ester (I; R = Et), with diphenyl phosphorochloridate in pyridine, afforded

[1] Perlmann, *Adv. Protein Chem.*, 1955, **10**, 1; Ågren and Glomset, *Acta Chem. Scand.*, 1953, **7**, 1071; Flavin, *J. Biol. Chem.*, 1954, **210**, 771; Oosterbaan, Kunst, and Cohen, *Biochim. Biophys. Acta*, 1955, **16**, 299.

[2] de Verdier, *Acta Chem. Scand.*, 1954, **8**, 1302; Ågren, de Verdier, and Glomset, *Acta Chem. Scand.* 1951, **5**, 324; 1954, **8**, 1570; Lipmann and Levene, *J. Biol. Chem.*, 1932, **98**, 109; Lipmann, *Biochem. Z.*, 1933, **262**, 3; Schaffer, May, and Summerson, *J. Biol. Chem.*, 1954, **206**, 201; 1953, **202**, 67.

[3] de Verdier, *Acta Chem. Scand.*, 1953, **7**, 196.

[4] Schaffer, Harshman, and Engle, *J. Biol. Chem.*, 1955, **214**, 799.

[5] Levene and Hill, *ibid.*, 1933, **101**, 711; Posternak and Pollaczek, *Helv. Chim. Acta*, 1941, **24**, 921.

[6] Plapinger and Wagner-Jauregg, *J. Amer. Chem. Soc.*, 1953, **75**, 5757.

[7] Riley, Turnbull, and Wilson, 14th Internat. Congr. Pure Appl. Chem., Zurich, 1955, Handbook, p. 133.

[8] Plimmer, *Biochem. J.*, 1941, **35**, 461; Levene and Schormüller, *J. Biol. Chem.*, 1934, **106**, 595; Posternak and Grafi, *Helv. Chim. Acta*, 1945, **28**, 1258.

the diphenyl phosphate ester (II; R = Et). In such reactions it was necessary to protect the carboxyl group by esterification.[9] Attempts to remove the phenyl groups from the ester (II) by alkaline hydrolysis failed: a β-elimination reaction (cf. refs. 10) ensued yielding diphenyl hydrogen phosphate (III) and α-*N*-benzyloxycarbonylaminoacrylic acid (IV). The structure of the latter was established by the existence of intense light absorption at 241 mμ (ε 5300), and by its hydrolysis to benzyl carbamate (VI) and pyruvic acid (V). Alternatively the benzyloxycarbonyl group was removed from the diphenyl phosphate (II) by treatment with hydrogen bromide in acetic acid.[11] The resulting amino-ester (VII; R = Et) (obtained as the crystalline hydrobromide) has little tendency to undergo the elimination; careful treatment with alkali at room temperature rapidly yielded phenol (2 mols.) and DL-phosphoserine (VIII; R = H) isolated by way of the lead or barium salt. At higher temperatures (*ca.* 90°) the yield of phosphoserine decreases, and appreciable amounts of diphenyl hydrogen phosphate and pyruvic acid are formed indicating that β-elimination is again favoured. These reactions are readily followed by measuring the light absorption of the phenol produced;[12] diphenyl hydrogen phosphate is easily recognised by the isolation of the *cyclo*hexylammonium salt. The resistance of the amino-ester (VII) to β-elimination in cold alkaline solution is attributed to the inductive effect of the α-amino-group which would hinder expulsion of the α-proton. Alkaline hydrolysis of other diphenyl phosphates (*e.g.*, cholesteryl diphenyl phosphate) has been shown to yield alkali-stable phenyl hydrogen phosphates.[12] The production of the dihydrogen phosphate in the alkaline hydrolysis of the diphenyl phosphate (VII) and the extreme rapidity with which phenol is formed in both aqueous and aqueous-alcoholic media suggest that the formation of cyclic intermediates involving the amino- or carboxyl group may be facilitating removal of *both* phenyl groups.[13]

Reactions similar to the foregoing have been used for making L-phosphoserine and DL-phosphothreonine. The method has also been successfully extended to the synthesis of certain phosphorylated peptides, notably DL-phosphoserylglycine. Attempts to prepare the latter from the diphenyl phosphate ester of *N*-benzyloxycarbonyl-DL-serine ethyl ester by way of the azide were unsuccessful, since the ester underwent β-elimination on treatment with (strongly basic) hydrazine. It appeared to be necessary to start with a serylglycine derivative as in the following synthesis. *N*-Benzyloxycarbonyl-DL-serylglycine ethyl ester, prepared from the azide, reacted smoothly with diphenyl phosphorochloridate in pyridine, yielding the diphenyl phosphate characterised by its readily undergoing β-elimination in alkaline solution. With hydrogen bromide in glacial acetic acid, however, this diphenyl phosphate afforded the corresponding amino-derivative from which DL-phosphoserylglycine was obtained by careful treatment with alkali, two mols. of phenol being produced concurrently. L-Phosphoseryl-L-glutamic acid, previously isolated from casein hydrolysates,[1, 5] was similarly synthesised from diethyl *N*-benzyloxycarbonyl-L-seryl-L-glutamate.

The use of alkaline hydrolysis to remove phenyl groups limits the foregoing method to the synthesis of peptides in which the phosphoserine or phosphothreonine amino-groups are free, for with *N*-substituted derivatives β-elimination predominates. Removal of phenyl groups by catalytic hydrogenolysis should permit the synthesis of peptides containing phosphoserine or phosphothreonine residues in any position. This alternative has now been used for the synthesis of phosphoserine. In preliminary experiments hydrogenolysis of the diphenyl phosphate ester of *N*-benzyloxycarbonyl-DL-serine ethyl ester (II; R = Et) resulted in the removal of the benzyloxycarbonyl and the phenyl groups, affording

[9] Cf. Friedkin and Lehninger, *J. Biol. Chem.*, 1947, **169**, 183.
[10] Linstead, Owen, and Webb, *J.*, 1953, 1211; Riley, Turnbull, and Wilson, *Chem. and Ind.*, 1953, 1181; Brown, Fried, and Todd, *J.*, 1955, 2206.
[11] Ben-Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[12] Montgomery, Turnbull, and Wilson, *J.*, 1956, 4603.
[13] Cf. Chanley and Feageson, *J. Amer. Chem. Soc.*, 1955, **77**, 4002; Moffatt and Khorana, *ibid.*, 1956, **78**, 883.

DL-phosphoserine ethyl ester (VIII; R = Et). Similarly, hydrogenolysis of the corresponding benzyl ester [14,15] (II; R = CH_2Ph) yielded DL-phosphoserine, the ester benzyl group being removed simultaneously. The hydrogenolysis method is being studied further.

The possibility of O ⟶ N migration [6,14] of phosphate groups was borne in mind but no evidence for this was found. Electrophoretic and potentiometric titrations appear to favour the dihydrogen phosphate ester formulation.

Experimental

The expression ε^* is used for the apparent molecular extinction coefficient of the solute after it has undergone some transformation, *e.g.*, hydrolysis.

N-*Benzyloxycarbonyl*-DL-*serine*.—Benzyl chloroformate (8·8 g.) was added to a solution of DL-serine (4·0 g.) in saturated aqueous sodium hydrogen carbonate (80 c.c.). The mixture was stirred vigorously for 2 hr. at 25°, excess of chloroformate removed by extraction with ether, and the aqueous solution acidified with concentrated hydrochloric acid. The oily product slowly deposited crystals (8·0 g.), m. p. 122—125°, which recrystallised from ethyl acetate in needles, m. p. 125—126° (Found: C, 55·2; H, 5·1; N, 5·7. Calc. for $C_{11}H_{13}O_5N$: C, 55·2; H, 5·4; N, 5·8%). Bergmann and Zervas [16] give m. p. 125°; the use of sodium hydrogen carbonate is a substantial improvement on the procedure of these authors.

N-*Benzyloxycarbonyl*-DL-*threonine*.—The *product* (0·75 g.) obtained from benzyl chloroformate (0·95 g.) and DL-threonine (0·5 g.) according to the foregoing method was isolated by extraction with ethyl acetate and recrystallised therefrom in needles, m. p. 74—75° (Found: C, 56·6; H, 5·8. $C_{12}H_{15}O_5N$ requires C, 56·9; H, 5·9%).

N-*Benzyloxycarbonyl*-DL-*serine Ethyl Ester*.—Benzyl chloroformate (4·5 g.) was added to a vigorously stirred mixture of DL-serine ethyl ester hydrochloride (4·0 g.) and saturated sodium hydrogen carbonate solution (70 c.c.) at 20°. After 2 hr. extraction with ether afforded the *ester* (4·5 g., 64%), b. p. 150—156°/0·01 mm. (Found: C, 58·0; H, 6·1; N, 5·2. $C_{13}H_{17}O_5N$ requires C, 58·4; H, 6·4; N, 5·2%).

N-*Benzyloxycarbonyl*-DL-(*di*-O-*phenylphospho*)*serine Ethyl Ester*.—*N*-Benzyloxycarbonyl-DL-serine ethyl ester (8·0 g.), dry pyridine (100 c.c.), and diphenyl phosphorochloridate (9·0 g.) were mixed. After being kept at 0° for 12 hr., the mixture was diluted with chloroform (100 c.c.) and washed with dilute hydrochloric acid and with water. Evaporation and recrystallisation from ether–light petroleum gave the *diphenyl phosphate* as needles (13·7 g., 85%), m. p. 40—41°, $\lambda_{max.}$ 261 mμ (ε 787 in EtOH) (Found: N, 2·7; P, 6·1. $C_{25}H_{26}O_8NP$ requires N, 2·8; P, 6·2%). The compound, on treatment with aqueous-ethanolic alkali, rapidly developed an absorption band at 244 mμ (ε^* 5000) which remained after acidification. This band is due to the formation of *N*-α-benzyloxycarbonylaminoacrylic acid (see following experiment) by β-elimination; diphenyl hydrogen phosphate which is also formed absorbs only weakly ($\lambda_{max.}$ 261 mμ; ε 1000).

α-N-*Benzyloxycarbonylaminoacrylic Acid*.—The foregoing diphenyl phosphate (0·2 g.) was dissolved in acetone (5 c.c.), and 2N-sodium hydroxide (2 c.c.) added. After 10 min., the acetone was removed in a vacuum and the residue carefully acidified to Congo-red by concentrated hydrochloric acid. Recrystallisation of the solid precipitate (50 mg.; m. p. 93—96°) from water gave α-N-*benzyloxycarbonylaminoacrylic acid*, m. p. 106—108°, $\lambda_{max.}$ 241 mμ (ε 5300 in H_2O) (Found: C, 59·5; H, 4·9; N, 6·3. $C_{11}H_{11}O_4N$ requires C, 59·7; H, 5·0; N, 6·3%). Addition of *cyclo*hexylamine to the original mother-liquors afforded *cyclo*hexylammonium diphenyl phosphate, m. p. and mixed m. p. 198°. The above acrylic acid derivative was stable to 2N-sodium hydroxide; 5N-sodium hydroxide converted it into benzyl carbamate, m. p. 88—89°. Refluxing it with dilute hydrochloric acid gave benzyl carbamate and pyruvic acid (2 : 4-dinitrophenylhydrazone, m. p. 217°).

DL-(*Di*-O-*phenylphospho*)*serine Ethyl Ester Hydrobromide*.—*N*-Benzyloxycarbonyl-DL-(di-*O*-phenylphospho)serine ethyl ester (6·0 g.) and a saturated solution of hydrogen bromide in glacial acetic acid (15 c.c.) were mixed at 0° and dry ether (500 c.c.) was added. After several

[14] Jones and Lipkin, *J. Amer. Chem. Soc.*, 1956, **78**, 2408.

[15] Si-Oh Li and Eakin, *ibid.*, 1955, **77**, 1866; Miller and Waelsch, *ibid.*, 1952, **74**, 1092; Baer and Maurukas, *J. Biol. Chem.*, 1955, **212**, 25.

[16] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192; Doub and Vandenbelt, *J. Amer. Chem. Soc.*, 1947, **69**, 2714.

hours at 0°, the ether layer was decanted, the residual syrup triturated with aqueous methanol, and the dry solid product (5 g.; m. p. 46—52°) recrystallised from ethyl acetate–ether. The *hydrobromide* (4·8 g.) formed needles, m. p. 63—64° (Found: C, 45·9; H, 4·9; N, 3·1. $C_{17}H_{21}O_6NPBr$ requires C, 45·8; H, 4·7; N, 3·1%). Light absorption max. at 260 mμ (ε 510 in H_2O). In a similar experiment with hydrogen chloride in glacial acetic acid, the corresponding *hydrochloride* (10%), m. p. 99—100°, was obtained (Found: N, 3·2. $C_{17}H_{21}O_6NPCl$ requires N, 3·5%).

Action of Alkali on DL-(*Di-O-phenylphospho*)*serine Ethyl Ester Hydrobromide.*—(*a*) The hydrobromide was dissolved in aqueous alkali at a suitable temperature and concentration; the amount of phenol liberated was measured at appropriate times by measuring the ultra-violet light absorption of aliquot parts of the solution at 234 mμ (phenoxide ion absorption) or, after acidification, at 270 mμ (undissociated phenol absorption).[16] Optimum conditions for the hydrolysis were with N-sodium hydroxide at 22° for 5 min.; 2 mols. of phenol were liberated.

(*b*) The hydrobromide (0·2 g.) was mixed with 2N-sodium hydroxide (5 c.c.); the oily free base rapidly dissolved on shaking. After 30 min. at 20°, acidification and addition of bromine water gave 2 : 4 : 6-tribromophenol (0·2 g.; m. p. 94—95°). Addition of *cyclo*hexylamine to the mother-liquors gave no precipitate, indicating the absence of significant amounts of diphenyl hydrogen phosphate.

(*c*) The hydrobromide (0·2 g.) was heated at 90° with N-sodium hydroxide. After 5 min., the solution was acidified and bromine water added to precipitate 2 : 4 : 6-tribromophenol (0·1 g.; m. p. 93—94°). Addition of *cyclo*hexylamine to the filtrate gave *cyclo*hexylammonium diphenyl phosphate (70 mg.; m. p. 198—199°). Treatment of the acidified mother-liquors with 2 : 4-dinitrophenylhydrazine gave pyruvic acid 2 : 4-dinitrophenylhydrazone, m. p. 211—213°.

DL-*Phosphoserine.*—(*a*) DL-(Di-*O*-phenylphospho)serine ethyl ester hydrobromide (1·0 g.) was shaken vigorously with N-sodium hydroxide (10 c.c.) until a clear solution was obtained. After a further 30 min. acetic acid was added to pH 4. Phenol was removed by ether-extraction, and the aqueous solution concentrated to a small volume; saturated lead acetate solution was added, and the lead salt of phosphoserine was collected, suspended in water, and decomposed with hydrogen sulphide. The solution was evaporated and ethanolic ether added. The precipitate [100 mg.; m. p. 145—150° (decomp.)] was dissolved in water and reprecipitated with ethanol–ether, to give DL-phosphoserine (75 mg., 18%) as small prisms, m. p. 163—164° (decomp.) (Found: C, 19·4; H, 4·6; N, 7·1. Calc. for $C_3H_8O_6NP$: C, 19·5; H, 4·4; N, 7·6%). Plimmer [8] gives m. p. 165—166° (decomp.), and Plapinger and Wagner-Jauregg [6] m. p. 166—167° (decomp.)

(*b*) DL-(Di-*O*-phenylphospho)serine ethyl ester hydrobromide (0·5 g.) and N-sodium hydroxide (5 c.c.) were set aside for 30 min., then percolated through Amberlite IR-120 resin (H^+ form), and the effluent extracted with ether and made alkaline (phenolphthalein) by aqueous barium hydroxide. The filtrate was evaporated, ethanol added, the precipitated barium salt (0·28 g.) dissolved in water, and percolated through Amberlite IR-120 resin (H^+ form), and phosphoserine [70 mg., 33%; m. p. 162—163° (decomp.)] isolated from the effluent by evaporation and precipitation with ethanol–ether.

N-*Benzyloxycarbonyl*-L-(*di-O-phenylphospho*)*serine Ethyl Ester.*—L-Serine {m. p. 219—220° (decomp.), $[\alpha]_D$ −6·2° (*c* 2·3 in H_2O)} with absolute ethanol and hydrogen chloride gave the ethyl ester hydrochloride, m. p. 130—131°, $[\alpha]_D$ −4·8° (*c* 2·1 in H_2O), which was converted into the *N*-benzyloxycarbonyl derivative, then treated with diphenyl phosphorochloridate in pyridine, as in the case of the DL-isomer, affording the *diphenyl phosphate ester*, m. p. 39—40°, $[\alpha]_D^{20}$ −1° (*c* 2·9 in EtOH) (Found: C, 60·0; H, 5·4; N, 2·9. $C_{25}H_{26}O_8NP$ requires C, 60·1; H, 5·2; N, 2·8%), $\lambda_{max.}$ 261 mμ (ε 760 in EtOH); treatment with 0·5N-sodium hydroxide in 50% ethanol at 20° for 30 min. produced a strong absorption band (238 mμ, ε* 5450, unchanged on cautious acidification), indicating the formation of *N*-benzyloxycarbonylaminoacrylic acid.

L-(*Di-O-phenylphospho*)*serine Ethyl Ester Hydrobromide.*—Prepared from the foregoing diphenyl phosphate (1·5 g.) and hydrogen bromide in glacial acetic acid, this *hydrobromide* was obtained as needles (1·1 g.), m. p. 67—68° (Found: N, 3·1; P, 6·5. $C_{17}H_{21}O_6NPBr$ requires N, 3·1; P, 7·0%), $\lambda_{max.}$ 260 mμ (ε 500 in EtOH). Treatment with 0·02N-aqueous sodium hydroxide at 20° for 30 min. liberated two mols. of phenol by hydrolysis.

L-*Phosphoserine.*—The above hydrobromide (0·25 g.) was dissolved in methanol, N-sodium hydroxide (2 c.c.) added, and the mixture left for 30 min. at 20°. A barium salt was isolated,

by the foregoing procedure for DL-phosphoserine. The barium salt (100 mg.), $[\alpha]_D^{19}$ +5·5° (*c* 3·6 in HCl), gave free L-phosphoserine (32% yield), m. p. 175—176° (decomp.), $[\alpha]_D^{19}$ +12° (calc. from Ba salt; *c* 3·6 in N-HCl) (Found: N, 7·5; P, 16·3. Calc. for $C_3H_8O_6NP$: N, 7·6; P, 16·7%). Ågren *et al.*[3] give m. p. 167° (decomp.), $[\alpha]_D^{23}$ +7·2° (*c* 4·2 in H_2O). Levene and Schormüller [8] give $[\alpha]_D^{25}$ +16·3° (calc. from Ba salt; *c* 6·0 in 10% HCl), and Lipmann and Levene [3] $[\alpha]_D^{25}$ +17·3° (calc. from Ba salt; *c* 1·7 in 10% HCl).

N-*Benzyloxycarbonyl*-DL-(*di*-O-*phenylphospho*)*threonine Ethyl Ester*.—DL-Threonine ethyl ester hydrochloride (m. p. 116—117°) was converted into crude *N*-benzyloxycarbonyl-DL-threonine ethyl ester in 82% yield. This (1·6 g.) with diphenyl phosphorochloridate (1·6 g.) in dry pyridine gave the *diphenyl phosphate ester* (1·8 g., 61%), m. p. 56—57° after recrystallisation from ether–light petroleum (Found: N, 3·1; P, 5·8. $C_{26}H_{28}O_8NP$ requires N, 2·7; P, 6·0%), $\lambda_{max.}$ 261 mμ (ε 730 in EtOH). In dilute sodium hydroxide solution it developed characteristic absorption at 231 mμ (ε* 6250).

DL-(*Di*-O-*phenylphospho*)*threonine Ethyl Ester Hydrobromide*.—The foregoing product (1·3 g.) and a saturated solution of hydrogen bromide in glacial acetic acid (3 c.c.) gave, as in the similar reactions already described, the *hydrobromide* (0·4 g., 45%), m. p. 88—89°, $\lambda_{max.}$ 260 mμ (ε 430 in H_2O) (Found: N, 3·0; P, 6·4. $C_{18}H_{23}O_6NPBr$ requires N, 3·0; P, 6·7%). Phenol (2 mols.) was rapidly liberated in 0·1N-sodium hydroxide at 20°.

DL-*Phosphothreonine*.—The above hydrobromide (0·3 g.) with N-sodium hydroxide (3 c.c.) and methanol (3 c.c.) at 20° for 30 min. afforded a crude barium salt (0·15 g.) by the procedure outlined for DL-phosphoserine. The barium salt in water with Amberlite IR-120 resin (H^+ form) yielded DL-phosphothreonine (12 mg., 10%), m. p. 150—152° (decomp.) (Found: N, 6·7. Calc. for $C_4H_{10}O_6NP$: N, 7·0%). Plimmer [8] gives m. p. 169° (decomp.), Plapinger and Wagner-Jauregg [6] give m. p. 184° (decomp.).

N-*Benzyloxycarbonyl*-DL-*serine Hydrazide*.—Crude *N*-benzyloxycarbonyl-DL-serine ethyl ester (4·5 g.), hydrazine hydrate (2·5 c.c.), and absolute ethanol (50 c.c.) were left at 20° for 24 hr. An equal volume of ether was added, and the mixture set aside at 0° for several hr. The precipitate (3·3 g., m. p. 156—157°) was recrystallised from ethanol–ether, yielding the *hydrazide* (3·2 g., 75%) as fine needles, m. p. 162—163° (Found: C, 52·3; H, 6·1; N, 16·0. $C_{11}H_{15}O_4N_3$ requires C, 52·5; H, 6·0; N, 16·5%).

N-*Benzyloxycarbonyl*-L-*serine Hydrazide*.—Prepared from *N*-benzyloxycarbonyl-L-serine ethyl ester (1·9 g.) by the foregoing method, this was obtained as needles (1·4 g.), m. p. 180—181°, from ethanol. Fruton [17] gives m. p. 181°.

N-*Benzyloxycarbonyl*-DL-*threonine Hydrazide*.—This *derivative* was obtained from *N*-benzyloxycarbonyl-DL-threonine ethyl ester as needles, m. p. 171—172° (Found: C, 53·4; H, 6·5; N, 16·0. $C_{12}H_{17}O_4N_3$ requires C, 53·9; H, 6·4; N, 15·7%).

N-*Benzyloxycarbonyl*-DL-*serylglycine Ethyl Ester*.—*N*-Benzyloxycarbonyl-DL-serine hydrazide (3·0 g.) was suspended in water (30 c.c.) and glacial acetic acid (3 c.c.), and concentrated hydrochloric acid (1·0 c.c.) was added. Sodium nitrite (0·5 g.) in water (3 c.c.) was added dropwise at 0°. The azide was extracted into ethyl acetate, washed with water, and dried. Meanwhile, glycine ethyl ester hydrochloride (5·0 g.) was treated with cold saturated potassium carbonate, the free glycine ester isolated with ether, and the ether solution dried, then mixed with the above azide solution. After 24 hr. at 20°, the mixture was washed with dilute hydrochloric acid, sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and evaporated. Light petroleum (b. p. 40—60°) precipitated a solid which was recrystallised from ethyl acetate–light petroleum (b. p. 40—60°), affording the *peptide* (0·4 g., 57%), m. p. 86—87° (Found: C, 55·6; H, 6·3; N, 9·0. $C_{15}H_{20}O_6N_2$ requires C, 55·5; H, 6·2; N, 8·6%).

DL-(*Di*-O-*phenylphospho*)*serylglycine Ethyl Ester Hydrobromide*.—*N*-Benzyloxycarbonyl-DL-serylglycine ethyl ester (1·0 g.) with diphenyl phosphorochloridate in pyridine gave the crude diphenyl phosphate ester (1·6 g.) as an oil ($\lambda_{max.}$ 260 mμ, ε 537). This developed a characteristic absorption band at 236 mμ (ε* 7000) in dilute aqueous-alcoholic sodium hydroxide.

The crude diphenyl phosphate (1·7 g.) with saturated hydrogen bromide in glacial acetic acid gave a salt which, recrystallised from ethanol–ethyl acetate, afforded the *hydrobromide* (0·6 g., 45%), m. p. 129—130° (Found: C, 45·1; H, 4·5; N, 5·5. $C_{19}H_{24}O_7N_2PBr$ requires C, 45·5; H, 4·8; N, 5·6%). The substance, with N-sodium hydroxide at 20°, liberated 2 mols. of phenol in less than 4 min.

[17] Fruton, *J. Biol. Chem.*, 1942, **146**, 463.

DL-*Phosphoserylglycine*.—The above hydrobromide (0·5 g.), methanol (2 c.c.), and N-sodium hydroxide (5 c.c.) were left at 20° for 30 min. The solution was percolated through Amberlite IR-120 resin (H^+ form), extracted with ether, neutralised with barium hydroxide solution, and evaporated below 30°; ethanol was added, the barium salt isolated, dissolved in water, and treated with Amberlite IR-120 (H^+ form), and the solution evaporated and diluted with ethanol. The crystalline product (45 mg., 18%), m. p. 150—154° (decomp.) was DL-phosphoserylglycine (Found: N, 11·5. Calc. for $C_5H_{11}O_7N_2P$: N, 11·6%). Fölsch [18] records m. p. 178°.

Diethyl N-*Benzyloxycarbonyl*-L-(*di*-O-*phenylphospho*)*seryl*-L-*glutamate*.—*N*-Benzyloxycarbonyl-L-serine hydrazide was converted into the azide, which was dissolved in anhydrous ethyl acetate and mixed with ethereal diethyl L-glutamate. After 20 hr., the product was recrystallised from ethyl acetate–light petroleum (b. p. 40—60°), yielding diethyl *N*-benzyloxycarbonyl-L-seryl-L-glutamate (58%; m. p. 81—82°) (Fruton [17] gives m. p. 85—86°). This ester (1·0 g.) with diphenyl phosphorochloridate–pyridine gave the crude diphenyl phosphate ester (1·45 g.) as an oil, $\lambda_{max.}$ 261 mμ (ε 800 in EtOH). With 0·04N-sodium hydroxide in aqueous ethanol at 20° the solution developed a characteristic absorption band at 234 mμ (ε* 8000 after 30 min.).

L-*Phosphoseryl*-L-*glutamic Acid*.—The foregoing crude diphenyl phosphate with hydrogen bromide gave diethyl L-(di-*O*-phenylphospho)seryl-L-glutamate hydrobromide as a syrup, $\lambda_{max.}$ 260 mμ (ε 430). The substance rapidly liberated 2 mols. of phenol in cold 0·1N-aqueous ethanolic sodium hydroxide. The crude hydrobromide with aqueous-methanolic sodium hydroxide gave, after appropriate processing, a barium salt {0·6 g.; $[\alpha]_D^{22}$ −1·3° (*c* 3·12 in N-HCl)} which was converted into L-*phosphoseryl*-L-*glutamic acid* (35 mg.), m. p. 145—147° (Found: N, 8·8. $C_8H_{15}O_9N_2P$ requires N, 8·9%). The brucine salt was obtained as small plates, m. p. 160—162° (Levene and Hill [5] record m. p. 171—172° for the salt).

DL-*Phosphoserine Ethyl Ester*.—*N*-Benzyloxycarbonyl-DL-(di-*O*-phenylphospho)serine ethyl ester (0·585 g.) was shaken in glacial acetic acid (20 c.c.) with platinum oxide (45 mg.) in hydrogen. After 16 hr., the solution gelled; a little water was added and hydrogenation continued for 3 hr. The solution was evaporated to small bulk below 40°; addition of ethanol precipitated the *ester* (0·17 g.), m. p. 170—171° (Found: N, 6·5; OEt, 20·05. $C_5H_{12}O_6NP$ requires N, 6·6; OEt, 21·1%).

DL-*Serine Benzyl Ester Benzenesulphonate*.—Serine (3·6 g.) and commercial hydrated benzenesulphonic acid (5·5 g.) were dissolved in benzyl alcohol (30 c.c.) and dry benzene (20 c.c.). The benzene was slowly distilled off, whilst more benzene (40 c.c.) was dropped in. The solution was cooled, and dry ether added: the solid (11 g.; m. p. 96—100°) was recrystallised three times from acetone–ether, to yield the *benzyl ester benzenesulphonate* (5 g.), m. p. 115—116° (Found: N, 3·9. $C_{16}H_{19}O_6NS$ requires N, 4·0%). A portion of the salt was treated with triethylamine in chloroform, triethylammonium benzenesulphonate filtered off, and the filtrate evaporated and treated with hydrogen chloride in ether. This yielded the benzyl ester hydrochloride, m. p. 154°. The foregoing procedure is a modification of that of Miller and Waelsch.[15]

N-*Benzyloxycarbonyl*-DL-*serine Benzyl Ester*.—The foregoing benzenesulphonate (3·5 g.) was treated with benzyl chloroformate (2·0 g.) and saturated sodium hydrogen carbonate solution (75 c.c.). After vigorous stirring during 1 hr., the product (2·8 g.; m. p. 70—72°) was filtered off. Recrystallisation from ether–light petroleum (b. p. 40—60°) gave the benzyloxycarbonyl derivative (2·3 g.), m. p. 74—75° (cf. Miller *et al.*[15]) (Found: N, 4·5. Calc. for $C_{18}H_{19}O_5N$: N, 4·25%).

N-*Benzyloxycarbonyl*-DL-(*di*-O-*phenylphospho*)*serine Benzyl Ester*.—*N*-Benzyloxycarbonyl-DL-serine benzyl ester (1·6 g.) was treated in pyridine (3 c.c.) with diphenyl phosphorochloridate (1·5 g.) in pyridine (2 c.c.). After 2 hr., ether and water were added, and the ether layer washed with dilute hydrochloric acid. Evaporation of the ether and trituration with light petroleum (b. p. 40—60°) gave a wax. Recrystallisation from ether–light petroleum (b. p. 40—60°) gave the *diphenyl phosphate ester* (2·4 g.), m. p. 49—50° (Found: N, 2·85. $C_{30}H_{28}O_9NP$ requires N, 2·5%), $\lambda_{max.}$ 260 mμ (ε 900 in EtOH). Treatment with 0·01N-aqueous-ethanolic sodium hydroxide at 20° developed a characteristic band at 239 mμ (ε* 5500).

DL-*Phosphoserine*.—The foregoing diphenyl phosphate (0·5 g.) in glacial acetic acid (10 c.c.) with platinum oxide (55 mg.) was shaken in hydrogen for about 7 hr.; a little water was added

[18] Fölsch, *Acta Chem. Scand.*, 1955, **9**, 1039.

during the reaction to dissolve a gel which formed. The solution was filtered, then concentrated in a vacuum, and absolute ethanol added. The crystalline product (0·15 g.) had m. p. 160—161° (decomp.) (Found : N, 7·4. Calc. for $C_3H_8O_6NP$: N, 7·6%).

Potentiometric Titrations.—DL-Phosphoserine (11 mg.) was dissolved in water (5 c.c.) and titrated with 0·029N-sodium hydroxide. A Marconi pH meter in combination with a glass electrode was employed. The titration curve showed typical inflexions at pH 4·25, 7·75, and 10·4. DL-Phosphoserine ethyl ester, titrated similarly, gave a curve with inflexions at pH 3·8, 6·8, and 9·5.

Paper Electrophoresis.—Electrophoresis was carried out on Whatman No. 3 paper in 0·1M-acetate buffer at pH 4·15 with a current of 30 mA and a gradient of 8 v cm.$^{-1}$. Ninhydrin was used as developer. The following migrations were observed :

Distance (cm.) moved towards :	cathode	anode
Serine	3·4	—
DL-Phosphoserine	—	11·5
DL-Phosphoserine ethyl ester	1·4	—

Grateful acknowledgment is made to Professor M. Stacey, F.R.S., for constant encouragement. We are much indebted to Mr. D. Sutcliffe for the potentiometric and electrophoretic data. One of us (G. R.) thanks the Department of Scientific and Industrial Research for a maintenance grant.

CHEMISTRY DEPARTMENT, THE UNIVERSITY,
EDGBASTON, BIRMINGHAM, 15.

[*Received, October 23rd,* 1956.]