Phosphorylation Products of Poly-DL(L)-serine

By

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With 2 Figures

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Two phosphorylation products of polyserine are described. The first product was obtained by coupling poly-DL-serine with benzyl phosphoric acid, in the presence of dicyclohexyldiimide, and subsequent debenzylation with anhydrous HBr. The second product was obtained by the phosphorylation of poly-DL-(or L)-serine with chlorophosphoric acid.

The peptide derivative (IV), obtained via a benzyl phosphate intermediate, was shown to consist of approximately equimolar amounts of seryl and O-phosphoseryl residues. All of the phosphorous in this polyserine derivative is bound to the hydroxyl groups of the seryl residues and appears in the form of phosphoric acid monoester.

The phosphorylation of poly-DL-(or L)-serine with chlorophosphoric acid yielded a derivate (VI) containing one phosphate group per each serine residue. An extensive rearrangement of the polypeptide chain occured during the phosphorylation reaction with chlororophosphoric acid as a result of an N to O acyl shift. Approximately 80 percent of the phosphate groups of VI are linked to the α-amino groups of the polyester-polyamide VI, the remaining 20% of the phosphates are linked to the β-hydroxyl groups of the modified peptide.

Phosphorous containing proteins are found in many tissues, and are particularly abundant in embryonic and rapidly growing tissues[1]. The two phosphoproteins which have so far been isolated in pure form and

* Dedicated to Prof. Dr. *F. Wessely* on the occasion of his 70th birthday.

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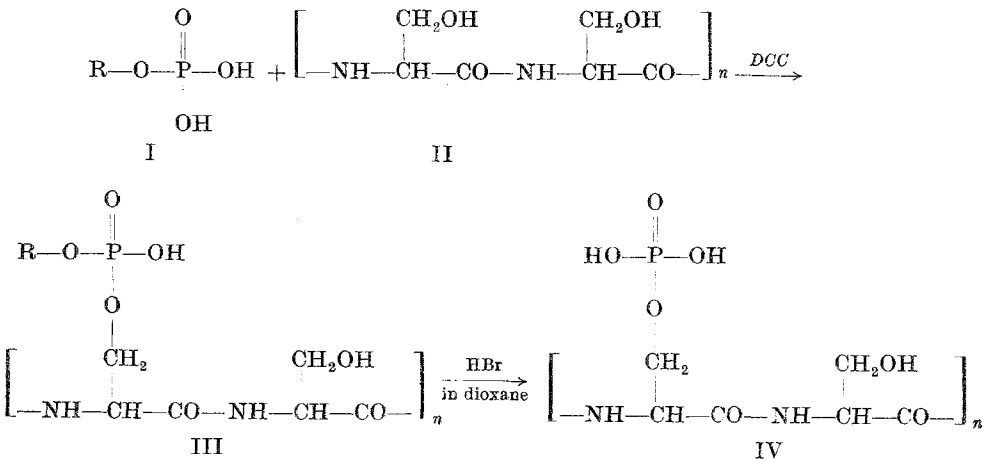
[1] *E. Perlmann*, Adv. Prot. Chem. **10**, 1 (1955).

studied extensively are casein from milk and phosvitin which is the major phosphoprotein component of vertebrate egg yolk and fish roe. In a study of the properties of phosvitin it has been shown by *Rabinowitz* and *Lipmann*[2] that phosphate groups from phosvitin can be transferred enzymatically to ADP to form ATP. Chemical analysis revealed that all the phosphorus in phosvitin is present as a monoester of phosphoric acid and is linked predominantly to the hydroxyl groups of serine[3]. Furthermore, it has been shown that blocks containing up to 6 phosphoseryl residues are present in the peptide chain of both phosvitin and casein[4]. Phosphorylated polyserine may thus be regarded as a synthetic model of phosphoproteins and might be used for the study of their chemical and biological properties.

In this communication we wish to describe the products obtained on phosphorylating poly-DL(L)-serine with chlorophosphoric acid and with monobenzyl phosphate activated with *DCC*.

The preparation of phosphorylated polyserine derivatives using benzyl phosphoric acid is summarized in the following scheme:



$R = C_6H_5CH_2$
$DCC = C_6H_{11}—N=C=N—C_6H_{11}$

The pyridinium salt of monobenzyl phosphate (I) was reacted with polyserine (II) in the presence of *DCC* under conditions similar to those used by *Gilham* and *Tener* for the phosphorylation of alcohols[5]. The poly-benzyl phosphoserine obtained was isolated by gel filtration and the

[2] *M. Rabinowitz* and *F. Lipmann*, J. Biol. Chem. **235**, 1043 (1960).
[3] *S. E. Allerton* and *G. E. Perlmann*, J. Biol. Chem. **240**, 3892 (1965).
[4] *J. Williams* and *F. Sanger*, Biochim. Biophys. Acta **33**, 294 (1959).
[5] *P. T. Gilham* and *G. M. Tener*, Chem. Ind. 542 (1959).

Table I. Analysis of Poly-O-phospho-DL-serine (Compound IV)

Sample	P/N	Amino Acid Found After 3 h Hydrolysis		After 22 h Hydrolysis
		Phospho-serine	Serine + Phospho-serine	Serine
	moles/mole	moles/mole P	moles/mole N	moles/mole N
I	0.65	0.46	0.77	0.64
II	0.47	0.51	0.74	0.75
III	0.44	0.53	0.87	0.76
Phospho-serine		0.52	0.82	0.85

benzyl groups were then removed with HBr in dioxane. The phosphorylated polyserine was finally purified by gel filtration.

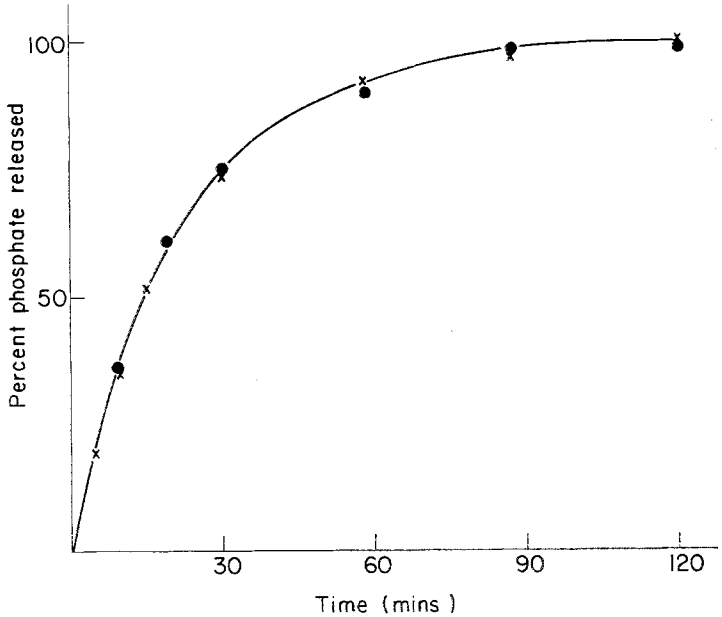


Fig. 1. Release of phosphate from phosphorylated poly-DL-serine (Compound IV) and from phosvitin in 0.5*N*-NaOH at 50° (for details see Experimental section). —●— Phosphorylated poly-DL-serine; —×— phosvitin

The final phosphorylation product contains about one phosphate group per two nitrogens (Table I) indicating that the procedure adopted leads to the phosphorylation of about half of the seryl residues. The polymer contains no acid labile phosphate groups indicating the absence of pyrophosphate and N—P bonds. All of the phosphate groups could be released with alkali, at a rate similar to the dephosphorylation of phosvitin under the same conditions (Fig. 1). The only amino acids obtained on

short acid hydrolysis are serine and phosphoserine (Table I). The amount of phosphoserine recovered after hydrolysis corresponds to about half of the phosphorus present in the sample. Acid hydrolysis thus leads to the

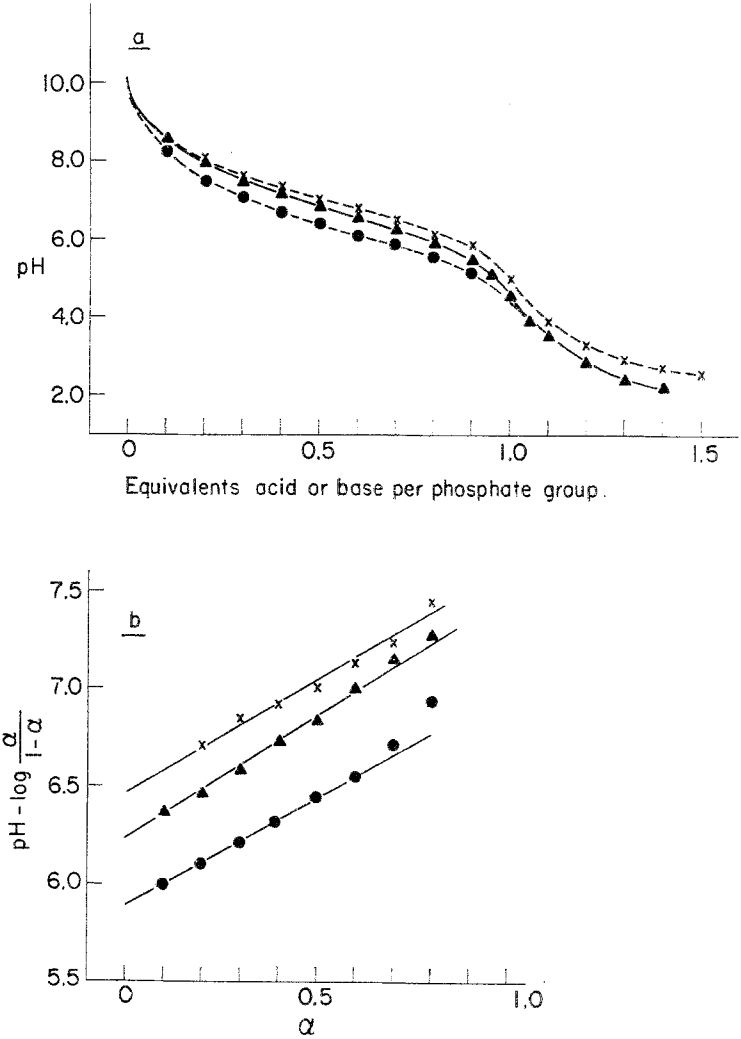


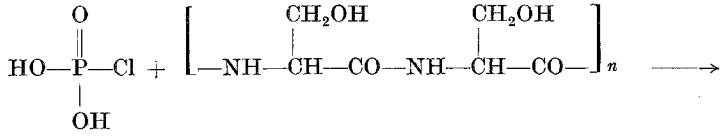
Fig. 2. Potentiometric titrations of phosphorylated poly-DL-serine (for details see Experimental section). —▲— in 0.02*M*-KCl; —●— in 0.2*M*-KCl; —×— in dioxane—water (1:1 v/v)

destruction of approximately half of the phosphoseryl residues of the peptide. A similar recovery of phosphoserine was reported by *Allerton* and *Perlmann*[3] for the hydrolysates of phosvitin.

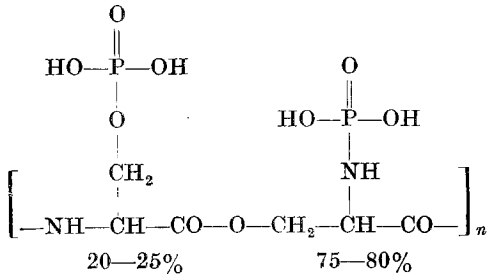
The potentiometric titration of phosphorylated poly-DL-serine is shown in Fig. 2 a. The first ionizable group of the bound phosphate is

only partly titrated at pH 2 and therefore has a $pK_1 < 2$. The second ionizable group is titrated between pH 5 and 9 and its titration curve is shifted to higher pH values by the addition of dioxane, and to lower pH values by increasing the ionic strength. The plot of $\text{pH} - \log \frac{\alpha}{1-\alpha}$ vs. α (Fig. 2 b) has a marked positive slope, characteristic for the titration of polyanions. Extrapolation to zero charge (i.e. to the point $\alpha = -1.0$ on the Figure) yields a value of $pK_2 \cong 5.0$ for the second intrinsic dissociation constant.

Phosphorylation of poly-DL-serine (II) with chlorophosphoric acid (V)



V II



VI

led to 100% phosphorylation (nitrogen-phosphorus ratio of 1:1). A characteristic absorption in the infra red spectrum (KBr pellet) at 1750 cm^{-1} indicated, however, the presence of ester linkages. The amount of ester bonds in the phosphorylation product (75%) was assayed by the hydroxamic acid test[6]. It is thus plausible to assume that an N → O acyl shift to the extent of 75% occurred during the phosphorylation of polyserine with chlorophosphoric acid. Acyl derivatives of hydroxy amino acids are known to undergo an N → O shift in the presence of strong acids and an O → N shift in alkaline media[7]. *Elliott*[8], for example, demonstrated that treatment of silk fibroin or lysozyme with concentrated sulphuric acid leads to an N → O shift at the serine and

[6] *S. Hestrin*, J. Biol. Chem. **180**, 249 (1949).

[7] *M. Bergmann, E. Brand,* and *F. Weinmann*, Z. Physiol. Chem. **131**, 1 (1923).

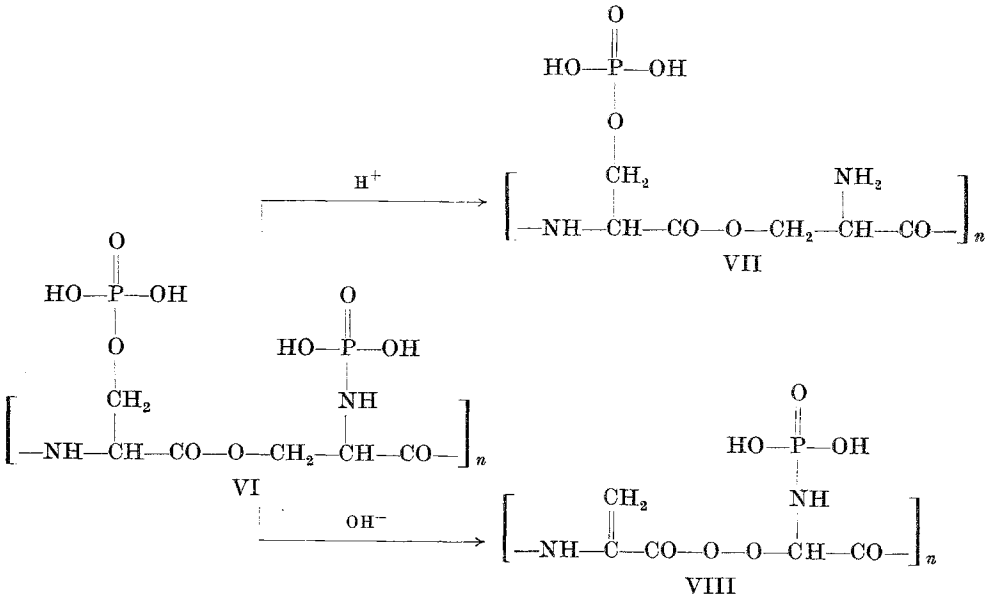
[8] *D. F. Elliott*, Biochem. J. **50**, 542 (1952).

threonine residues. *Fasman*[9] and *Rabinowitz*[10] found that concentrated sulphuric acid causes an N → O shift in polyserine to an extent of 70%.

Analysis of the stability of the covalently bound phosphate groups yielded supporting evidence for the occurrence of an N → O shift during the phosphorylation of polyserine with chlorophosphoric acid. Thus when the phosphorylation product VI was treated with 1 *N*-HCl at 45°, up to 24 hours, dephosphorylation to an extent of 72% occurred (Table II).

Table II. Dephosphorylation of Compound VI in 1*N*-HCl at 45° C

Time hrs.	% Dephosphorylation
2	32
4	50
6	64
12	67
16	72
20	72
24	72



Dephosphorylation (Table III) to an extent of 20% occurred, on the other hand, on treatment of IV with 0.5 *N*-alkali at 37° up to 24 hours. No ^{18}O-labelled inorganic phosphate was formed on alkaline dephosphorylation

[9] *A. Lapidot* and *M. Halmann*, J. Chem. Soc. 1713 (1958).
[10] *L. Anderson* and *J. J. Kelley*, J. Amer. Chem. Soc. **81**, 2275 (1959).

of VI in $H_2{}^{18}O$. Dephosphorylation in the presence of alkali thus occurs by a β-elimination mechanism[10]. Since N—P bonds are known to be acid labile and alkali stable[9] whereas the phosphates attached to the hydroxyl group of the serine residues in phosphoproteins are acid stable and alkali labile[10] it might be concluded that about 20% of the phosphate groups in the product formed on phosphorylation of poly-DL-serine with chlorophosphoric acid are O-phosphates and the rest are N-phosphates.

Table III.
Dephosphorylation of Compound VI in 0.5*N*-NaOH at 37° C

Time hrs.	% Dephosphorylation
0.5	8
1	11
2	15
6	20
24	20

Complete dephosphorylation of compound VI occurred on incubation with acid monophosphatase[11] at pH 5.3. Compound III, on the other hand, yielded only minute amounts of inorganic phosphate when incubated with several acid and alkaline phosphatases.

Experimental

Organic phosphate was determined by the method of *King*[12]. Nitrogen was determined by the micro Kjeldahl method.

Alkali labile phosphate was determined by adding 1 vol. of 2 *N*-NaOH to 3 vols of a test solution containing 0.8—1.0 μmoles organic phosphate per ml. Aliquots of 1.0 ml were withdrawn at the times indicated, added to 1.5 ml of a solution 10% in *TCA* and 0.5 N in H_2SO_4, and the mixture was centrifuged for 15 min. at 10,000 g. Inorganic phosphate was determined in 1.0 ml of the supernatant.

For the potentiometric titrations solutions containing 30—40 μmoles organic phosphate in 2.0 ml of the desired solvent were employed. The solutions were titrated automatically (Autotitrator TTT 1 c, Radiometer, Copenhagen) to pH 10 with 0.2 *N*-KOH, then back titrated with 0.2 *N*-HCl.

*Poly-O-benzylphospho-*DL*-serine*

Poly-DL-serine[13] (150 mg) was dissolved in water (1 ml) and pyridine (5 ml) was added, whereupon the polymer precipitated as a gel. The solvent was removed under reduced pressure on a rotary evaporator at room tempe-

[11] *T. P. Singer*, J. Biol. Chem. **174**, 11 (1948).

[12] *E. J. King*, Biochem. J. **26**, 292 (1932), modified by J. Biol. Chem. **209**, 197 (1954).

[13] *Z. Bohak* and *E. Katchalski*, Biochemistry **2**, 228 (1963).

rature until a thick slurry remained. Pyridine (5 ml) was added and evaporation continued to complete dryness. Dry pyridine (5 ml) and mono-benzyl phosphoric acid[14] (1.5 g) were then added and the solvent evaporated. The addition of pyridine (5 ml) and evaporation was repeated twice to yield a homogenous pasty residue. The latter was then suspended in pyridine (7 ml) and *DCC* (1.5 g) was added. The mixture was stirred at 4° for 24 hours. The solvent was then removed under reduced pressure, water (10 ml) was added to the residue, and the pH of the suspension was adjusted to 7.5—8 by the addition of solid ammonium bicarbonate. The suspension was stirred for 15 min., filtered and the solid washed again with water (10 ml) until free of phosphorus. The filtrate and washings were combined and lyophilized to yield a light brown powder. The powder was dissolved in water (3 ml) and the pH of the solution adjusted to 7.5 with ammonium bicarbonate, and chromatographed on a column of Sephadex G-25 (1.8 × 134 cm) using 0.04 *M*-ammonium bicarbonate pH 7.5 as eluent (20 ml/hr). The peak emerging at the breakthrough volume of the column was collected and lyophilized. Yield 305 mg of a light yellow powder. The product obtained contained acid labile phosphate corresponding to 20 percent of total phosphate. It was used, however, without further purification for the preparation of poly-O-phospho-DL-serine.

*Poly-O-phospho-*DL*-serine*

The benzylphosphorylation product of poly-DL-serine (900 mg) was suspended in anhydrous dioxane saturated with HBr at 0° (60 ml, 40% HBr w/w), and the suspension stirred at 4° for 48 hours. The mixture was allowed to settle, excess solvent was decanted and the residue washed with several portions (about 100 ml each) of anhydrous ether. The final precipitate was suspended in water (5 ml) and brought into solution after raising the pH to 7.2 with solid sodium acetate. The solution was applied to a column of Sephadex G-25 (2.0 × 120 cm) which was developed with 0.01 *M*-NaCl (20 ml/hr). The eluent was monitored by light absorption at 220 mμ (Automatic UV Analyzer 1056 A, Vanguard Instrument Corp., Lagrange, Illinois, U.S.A.) and the peak emerging at the breakthrough volume was collected and lyophilized. Yield 400 mg of a colorless powder.

Analysis: Found N 6.33, NaCl 28.7.

A small amount (about 10 mg) of phosphorylated polyserine was freed of NaCl by dialysis (Visking 23/32 tube) against water, and used for amino acid analysis (see Table I).

The molecular weight of the polymer (M. W. = 7800) was determined by ultracentrifugation in 0.3 M NaCl ($S_{20,w} = 1.31 \times 10^{-13}$ sec, $D_{20} = 16.4 \times 10^{-7}$ cm^2 sec^{-1}. v assumed 0.75).

Incubation of the phosphorylated poly-DL-serine with wheat-germ and prostate acid phosphatases (in 0.15 *M*-acetate buffer, pH 5.3), or with intestinal alkaline phosphatase (in 0.1 *M*-veronal buffer, pH 9.0, 0.01 *M* in $MgCl_2$) and *E. coli* alkaline phosphatase (in 0.15 *M*-veronal buffer, pH 9.0, 1.0 *M* in NaCl), caused only slight dephosphorylation. The incubation mixtures contained 2 μmoles organic phosphorus per ml, and 0.1 to 0.2 units per ml of enzyme (one unit being the amount of enzyme which hydrolyses 1 μmole p-nitrophenylphosphate in one minute). After incubation for 18 hours at 23°, 5 to 10% of the total phosphorus was liberated as inorganic phosphorus.

[14] *F. Cramer* and *G. Weimann*, Chem. Ber. **94**, 996 (1961).

Phosphorylation of Polyserine with Chlorophosphoric Acid

Chlorophosphoric acid[15] (3 g) was added to poly-L-(or DL)-serine (200 mg). The reaction mixture was stirred for 20 hours at 40° and protected from moisture with a $CaCl_2$ tube. After cooling to room temperature, ethanol (20 ml) was added and the polymer was precipitated with ether (100 ml). The precipitate was isolated by centrifugation, washed several times with ethanol (5 ml) and ether (10 ml) and dried in a vacuum desiccator. The dry polymer was dissolved in water (5 ml) and the solution mixed with absolute ethanol (200 ml). The precipitate (VI) was isolated by centrifugation and dried in a vacuum desicator. Yield 220 mg.

Analysis: Found P 18.3, N 8.3. Extent of phosphorylation $\frac{14 \cdot 18.3 \cdot 100}{31 \cdot 8.3} =$ $= 99.5\%$.

Enzymic Dephosphorylation of Compound VI

A solution of acid phosphatase (0.5 ml, 2 mg/ml purchased from Nutritional Biochemical Corp.) in acetate buffer pH 5.3, was added to a solution of compound VI (0.5 ml, 22 μmoles P/ml). The total volume was brought to 3 ml by adding acetate buffer pH 5.3 and the reaction mixture was incubated at 37° for 3 hours. The protein was precipitated by adding 20% *TCA* (1 ml), and the precipitate was removed by centrifugation. Analysis of inorganic phosphate in the supernatant showed quantitative dephosphorylation. In a blank experiment (without enzyme) there was no release of inorganic phosphate.

Acknowledgment

The authors wish to thank Mr. *M. A. Weissenberg* for repeating and checking some of the preparative procedures, and Mrs. *S. Ehrlich Rogozinski* for the performance of the analyses and potentiometric titrations.

2—3 mg Samples were hydrolyzed with 6*N*-HCl in sealed evacuated tubes at 110°. Analyses were carried out on a Beckman 120 amino acid analyzer. Determinations of total nitrogen and phosphorus were carried out on samples weighed out together with the samples for amino acid analysis.

[15] *F. C. Neuhaus* and *S. Kookes*, Biochem. Preparations **6**, 75 (1958).