

## The Apparent Acid Ionization Constants of L-Seryl-L-Lysine, L-Seryl-L-Glutamic Acid and their O-Phosphorylated Analogues. Proposed Mechanism for the Absence of Tryptic Action on O-Phosphorylated Lysine Peptides \*

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1. The peptides L-seryl-L-lysine and O-phosphoryl-L-seryl-L-lysine have been synthesized.

2. Apparent acid ionization constants have been determined at 25° in 0.15 M KCl for L-seryl-L-lysine, L-seryl-L-glutamic acid and their corresponding O-phosphorylated analogues.

3. The effect of the phosphate group on the ionization of the other acid groups in these peptides is discussed in relation to previous studies on synthetic phosphopeptides.

4. Some of the p*K* values obtained are discussed in relation to p*K* values and possible amino acid sequences of phosphopeptides prepared from whole-casein and  $\alpha$ -casein.

5. Electrostatic interaction between the phosphate and  $\epsilon$ -amino groups in O-phosphorylseryllysine is shown to be present. It is proposed that this interaction is the reason for the observed inability of trypsin to hydrolyse peptide bonds involving a lysine carboxyl group, when O-phosphorylserine is attached to the lysine residue.

As a part of a study of phosphorus linkages in casein and its degradation products <sup>2,3</sup>, a phosphopeptide, containing seven phosphate groups was isolated in two different ways from  $\alpha$ -casein <sup>4,5</sup>. The type of phosphate bonds in this peptide has been studied by means of acid-base titration <sup>6</sup>. The analysis of the titration data in the form of p*K*-values for different acid groups assumed to be present, required information about acid ionization constants of these groups in similar peptides of known structure. A preceding paper <sup>3</sup> reported acid-base properties of the  $\alpha$ -amino,  $\alpha$ -carboxyl-, and O-phosphoryl groups in selected synthetic peptides. In the present work peptides containing  $\gamma$ -carboxyl- and  $\epsilon$ -amino groups have been prepared and the ionization con-

\* Synthesis of phosphopeptides, Part IV. For Parts II and III see Ref.<sup>1</sup>

stants of these groups determined. The peptides investigated are listed in Fig. 1. One of these peptides, O-phosphoryl-L-seryl-L-lysine, was of special interest with regard to hydrolysis of peptide bonds in phosphopeptides, as it is known that trypsin is unable to hydrolyse lysyl peptide bonds, when the peptide contains negatively charged groups<sup>7</sup> such as O-phosphoryl<sup>5,8</sup> or carboxyl groups<sup>9-11</sup> near the lysine residue.

The synthesis of L-seryl-L-glutamic acid<sup>1,12</sup> and O-phosphoryl-L-seryl-L-glutamic acid<sup>1,13</sup> have been reported previously. Free and O-phosphorylated L-seryl-L-lysine were obtained as follows (reaction scheme, Fig. 2): N<sup>α</sup>-(N-carbobenzoxy)-L-seryl-N<sup>ε</sup>-carbobenzoxy-L-lysine benzyl ester (I) was prepared by condensation of the appropriate derivatives with N,N'-dicyclohexylcarbodiimide. Hydrogenolysis of (I) in *tert*-butanol-water-hydrochloric acid gave L-seryl-L-lysine as the *mono*-hydrochloride. Phosphorylation of (I) in pyridine with dibenzylphosphoryl chloride gave N<sup>α</sup>-(O-dibenzylphosphoryl-N-carbobenzoxy)-L-seryl-N<sup>ε</sup>-carbobenzoxy-L-lysine benzyl ester (III). Unchanged (I) could be removed from (III) by means of fractional crystallization, but the main portion of crude (III) was hydrogenolyzed directly to a mixture of the two peptides (II) and (IV), and these were separated easily by means of ion exchange chromatography.

Before titration, all peptides (except II, hydrochloride) were freed from acetate and formate ions by means of chromatography on a cation exchange resin, Dowex 50-X2.

Peptide	Abbreviation	Formula
L-Seryl-L-lysine	(a) Ser-Lys	$\begin{array}{c} \text{NH}_2 \\   \\ (\text{CH}_2)_4 \\   \\ \text{CH}_2\text{O}-\text{R} \\   \\ \text{H}_2\text{N}\cdot\text{CH}\cdot\text{CO}-\text{NH}\cdot\text{CH}\cdot\text{CO}\cdot\text{OH} \end{array}$
O-Phosphoryl-L-seryl-L-lysine	(b) SerP-Lys	$\begin{array}{c} \text{COOH} \\   \\ (\text{CH}_2)_2 \\   \\ \text{CH}_2\text{O}-\text{R} \\   \\ \text{H}_2\text{N}\cdot\text{CH}\cdot\text{CO}-\text{NH}\cdot\text{CH}\cdot\text{CO}\cdot\text{OH} \end{array}$
L-Seryl-L-glutamic acid	(c) Ser-Glu	
O-Phosphoryl-L-seryl-L-glutamic acid	(d) SerP-Glu	

Compounds: a,c : R = -H

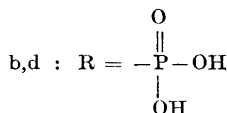


Fig. 1. Structure and symbols of peptides investigated.

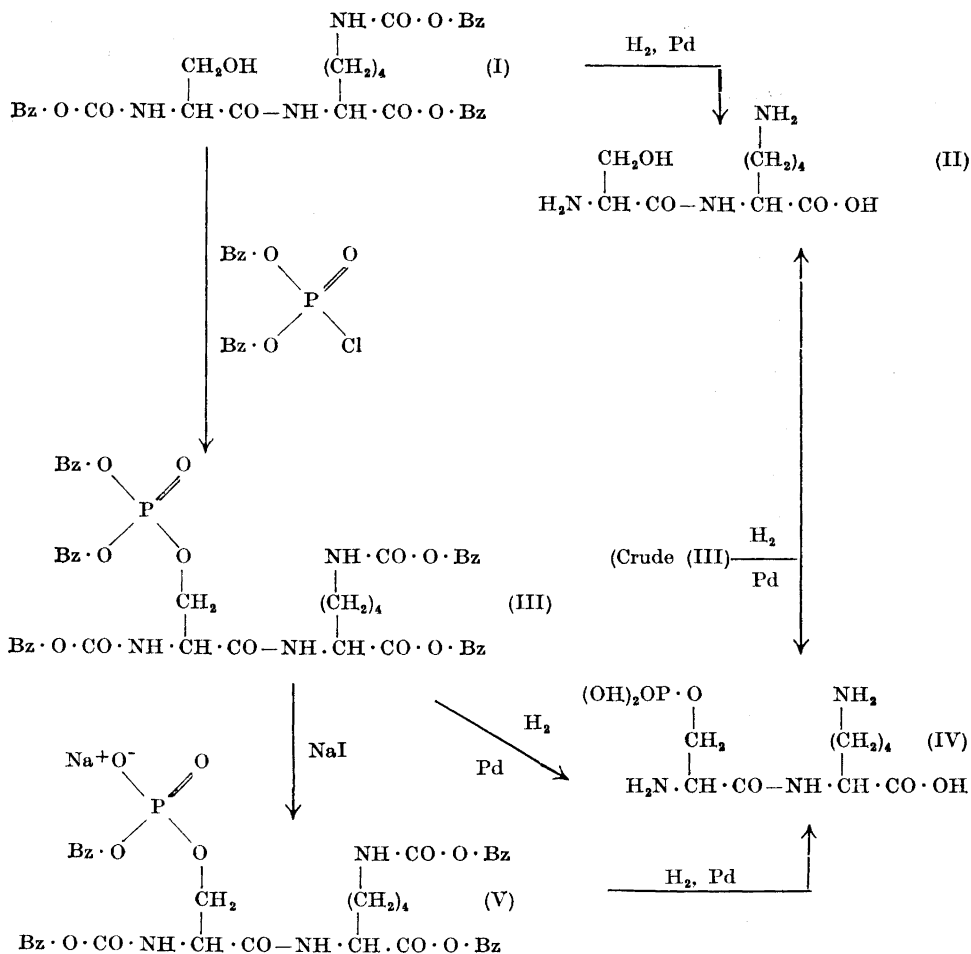


Fig. 2. Reaction scheme. Bz =  $-\text{CH}_2 \cdot \text{C}_6\text{H}_5$ .

### EXPERIMENTAL \*

#### Peptide synthesis

*Na*-(*N*-carbobenzoxy)-*L*-seryl-*N*<sup>ε</sup>-carbobenzoxy-*L*-lysine benzyl ester (Compound I, Fig. 2)

*N,N'*-Dicyclohexylcarbodiimide (6.3 g = 30 mmoles) was added to a solution of 7.2 g (30 mmoles) *N*-carbobenzoxy-*L*-serine<sup>12</sup>, 12.2 g (30 mmoles) *N*<sup>ε</sup>-carbobenzoxy-*L*-lysine benzyl ester hydrochloride<sup>14</sup> and 4.2 ml (30 mmoles) triethylamine in 150 ml of tetrahydrofuran-acetonitrile (2:1). The next day the precipitated dicyclohexylurea was removed

\* Solvents systems used in the paper (Whatman No. 1) chromatography: (a) phenol-water (80:20, v/v), ascending, 6 h; and (b) *n*-butanol-acetic acid-water (40:10:50, v/v/v), descending, 18 h. All papers were sprayed first with ninhydrin and then with phosphate (ammonium molybdate) reagents.

by filtration, and the solvent distilled off *in vacuo*. The residue was dissolved in ethyl acetate and the solution washed successively with water, 2 M  $H_2SO_4$ ,  $KHCO_3$  solution and water. After drying ( $Na_2SO_4$ ), the solution was concentrated to a small volume. Light petroleum was added causing crystallization. The product was recrystallized from ethyl acetate-light petroleum, yielding 15.5 g (87 %) of (I). M.p. 112–113° (Found: C 64.7; H 6.3; N 7.1; Calc. for  $C_{22}H_{27}N_3O_8$  (591.6): C 65.0; H 6.3; N 7.1).

*L-Seryl-L-lysine (Compound II)*

a) *From (I)*. To a solution of 3.0 g (5 mmoles) of (I) in 50 ml *tert*-butanol, water was added to slight turbidity. After adding 5 ml of 1 M HCl, the solution was shaken in an atmosphere of pure hydrogen together with 0.5 g 10 % palladium on charcoal catalyst. In 2 h, 340 ml hydrogen was consumed. The catalyst was filtered off and the solution evaporated to dryness *in vacuo*. The solid residue was dissolved in 5 ml water and precipitated with acetone, yielding (II) as the *mono*-hydrochloride (1.25 g = 93 % after drying over  $P_2O_5$  at room temperature).

$[\alpha]_D^{25}$  –5.9 (1 M HCl,  $c$  3.9);  $R_F$  0.45 (solvent a);  $R_F$  0.20 (solvent b). (Found: C 40.1; H 7.8; N 14.9; Calc. for  $C_9H_{11}N_3O_4 \cdot HCl$  (269.4) : C 40.1; H 7.5; N 15.6). The hydrochloride was strongly hygroscopic.

b) *From crude (III)*. After hydrogenolysis and chromatographic separation (see below, compound IV), 2.5 g of (II) was isolated as the diformate salt. (Found: C 40.5; H 7.1; N 13.2; Calc. for  $C_9H_{11}N_3O_4 \cdot 2CH_2O_2$  (325.3): C 40.6; H 7.1; N 12.9). The compound moved on paper chromatography exactly as the above-mentioned hydrochloride. The formate salt was less hygroscopic than the latter.

*Na-(O-Dibenzylphosphoryl-N-carbobenzoxy)-L-seryl-N $\epsilon$ -carbobenzoxy-L-lysine benzyl ester (Compound III)*

A solution of 11.8 g (20 mmoles) of (I) in 20 ml dry pyridine was cooled to just above the freezing point. Dibenzylphosphoryl chloride, freshly prepared from 10.5 g (40 mmoles) dibenzylphosphite was added. After shaking 15 min at this temperature the flask was left at 4° over night. The reaction product, which had turned pale yellow, was poured into a cold mixture of 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was washed successively with 5 M  $H_2SO_4$ , water,  $KHCO_3$  solution and water. After drying ( $Na_2SO_4$ ), evaporation of the solvent yielded 15.5 g of a slowly solidifying mass. Analysis (Found: N 6.9; P 2.8) indicated that the product contained large amounts of (I)\*. A sample of 1 g was recrystallized four times from ethyl acetate-light petroleum, yielding 0.30 g of fairly pure (III) with m.p. 92–93°. (Found: C 63.5; H 6.0; N 5.3; P 3.4; Calc. for  $C_{44}H_{50}O_{11}N_3P$  (851.9): C 64.8; H 5.9; N 4.9; P 3.6). Most of the material was hydrogenolyzed directly (see below, compound IV).

*O-Phosphoryl-L-seryl-L-lysine (Compound IV)*

a) *From crude (III)* by direct hydrogenolysis. To a solution of 12.0 g of crude (III) in 100 ml *tert*-butanol, water was added to slight turbidity. The solution was shaken with 2.0 g 10 % Pd/C in an atmosphere of pure hydrogen. In 4 h, 930 ml was consumed. The filtered solution was worked up as under (II a), yielding 4.8 g of a solid product. This was found by paper chromatography to be a mixture of (II) and (IV). The product was dissolved in a small amount of water and applied to a column (2.7 × 14 cm) of Dowex 1-X2, 200–400 mesh (formate form) (*cf.* Ref.<sup>14</sup>). Fractions were collected as given in Table 1 and evaporated immediately to dryness *in vacuo*.

\* The N and P analyses as well as the hydrogenolysis below (compound IV) indicated a yield of about 50 % of (III). Careful low temperature phosphorylations of serine derivatives with freshly prepared dibenzyl phosphoryl chloride may yield 60–75 % of phosphate triester<sup>1</sup>. Higher yields are obtained with diphenylphosphoryl chloride<sup>1,13</sup> but the hydrogenolytic elimination of phenyl groups is usually slow. Theodoropoulos *et al.*<sup>15</sup> obtained very low yields with any phosphorylation method tried except when imidazole was added to the phosphorylation mixture.

Table 1. Separation of O-phosphoryl-L-seryl-L-lysine and L-seryl-L-lysine on Dowex 1-X2, 100–200 mesh.

Fraction No.	H <sub>2</sub> O ml	Grams	Fraction No.	1 M HCOOH ml	Grams
1	20	0.1	5	20	0
2	20	2.2	6	20	0.1
3	20	0.2	7	20	0.8
4	30	0.1	8	20	1.5
			9	50	0.1
			10	50	0

The fractions were analysed by paper chromatography in solvent II. Fractions 2, 3 and 4 ( $R_F$  0.20), contained compound (II), whereas fractions 6, 7, and 8 ( $R_F$  0.03), contained the expected phosphopeptide (IV). After drying *in vacuo* at room temperature over P<sub>2</sub>O<sub>5</sub>, the latter had  $[\alpha]_D^{25} - 4.7$  (1 M HCl,  $c$  3.4). Analyses indicated it to be *mono*-formate salt. (Found: C 34.7; H 6.7; N 12.5; P 8.5; Calc. for *mono*-formate salt (C<sub>9</sub>H<sub>20</sub>O<sub>7</sub>N<sub>3</sub>P·CH<sub>2</sub>O<sub>2</sub>) (359.3): C 33.4; H 6.2; N 11.7; P 8.7. Calc. for free phosphopeptide (C<sub>9</sub>H<sub>20</sub>O<sub>7</sub>N<sub>3</sub>P) (313.3): C 34.5; H 6.4; N 13.4; P 9.9).

The sample to be titrated was converted to its hydrochloride by means of a cation exchange column (Dowex 50-X2, 200–400 mesh) in H<sup>+</sup>-form. This had the dimensions 1.5 × 40 cm, and 250 mg peptide was applied. The column was eluted first with 2 l of water and then with 0.5 M HCl. After 1.2 l of the latter eluent the peptide came out, as indicated with Moore and Stein's ninhydrin method<sup>17</sup>. After immediate freeze-drying 150 mg of O-phosphoryl-L-seryl-L-lysine dihydrochloride was obtained. The compound moved as a single spot in paper chromatography;  $R_F$  0.08 (solvent a);  $R_F$  0.04 (solvent b). (Found: C 28.0; H 5.8; N 10.0; P 7.8; Calc. for C<sub>9</sub>H<sub>20</sub>O<sub>7</sub>N<sub>3</sub>P (386.2): C 28.0; H 5.7; N 10.8; P 8.0). The hydrochloride was hygroscopic.

b) *From pure (III)*. Hydrogenolysis of 100 mg of (III) as described for compound (IIa) yielded 35 mg (77 %) of compound (IV), paper chromatographically homogeneous in solvents a ( $R_F$  0.08) and b ( $R_F$  0.04).

c) *From (III) via mono-debenzylation*. A solution of 1.5 g of crude (III) in 10 ml dried acetone was refluxed for 15 min with 0.2 g anhydrous NaI. The expected sodium salt (V) did not separate from the acetone solution within 48 h at 4°. The solvent was distilled off and the residue poured into ethyl acetate and KHCO<sub>3</sub> solution. Acidification of the aqueous phase gave an oily acid. This was extracted with ethyl acetate, the solvent distilled off and the residue hydrogenolyzed as above. Thereby 110 mg of compound (IV) was obtained;  $R_F$  = 0.04 (solvent b).

#### L-Seryl-L-glutamic acid.

Palladium-catalyzed hydrogenolysis of N-carbobenzoxy-L-seryl-L-glutamic acid dibenzyl ester<sup>1</sup> in glacial acetic acid solution (to avoid any ester exchange<sup>18</sup>) resulted in a peptide, which contained strongly bound acetic acid\*. The acetic

\* The presence of acetic acid was indicated by titration. After drying *in vacuo* over KOH at room temperature, the ratio between KOH consumed for ionization of carboxyl groups (below pH 6) and that for the amino group (pH 6–10) was 1.6. Drying over KOH at 1 mm Hg, 5 h at 75° lowered this ratio to 1.18. Further 8 h drying at 100°, however, resulted in a large decrease in free amino groups, and the ratio increased to 4.0. Diketopiperazine formation or polymerisation (involving amino- and  $\alpha$ - or  $\gamma$ -carboxyl groups) may have occurred.

acid was removed by applying the original peptide (407 mg) to an  $1.5 \times 19$  cm column of Dowex 50-X2, 200–400, mesh, in  $H^+$ -form. The column was first eluted with water and then with 0.12 M HCl. After 120 ml (in 10 ml fractions) of the latter effluent the peptide appeared as indicated by ninhydrin <sup>17</sup>. After freeze-drying and drying *in vacuo* over  $P_2O_5$  at room temperature 341 mg of peptide hydrochloride was obtained. (Found: C 35.2; H 5.5; N 10.5; Calc. for  $C_8H_{14}O_6N_2 \cdot HCl$  (270.7): C 35.5; H 5.6; N 10.4).

#### *O*-Phosphoryl-L-seryl-L-glutamic acid

This phosphopeptide, prepared as described previously <sup>1</sup>, was finally purified by cation exchange chromatography (Dowex 50-X2, 200–400 mesh,  $1.5 \times 40$  cm column in  $H^+$ -form). After elution with 250 ml of water, the peptide appeared, and the solution was freeze-dried.  $R_F$  0.04 (solvent I) and  $R_F$  0.06 (solvent II). (Found: C 30.6; H 5.0; N 9.0; P 9.9; Calc. for  $C_8H_{15}O_6N_2P$  (314.2): C 30.6; H 4.8; N 8.9; P 9.9).

In attempts to crystallize *O*-phosphoryl-L-seryl-L-glutamic acid from ethanol the product obtained showed at least two new ninhydrin- and phosphate-positive spots in paper chromatography (solvents a and b). These moved appreciably faster ( $R_F$  0.9 and  $R_F$  0.4 in solvent a;  $R_F$  0.3 and  $R_F$  0.2 in solvent b) than the original peptide. Ethyl esters seem to be formed \*. Ester interchange during hydrogenolysis of benzyl esters in ethanolic solvent has been observed by Crofts, Marques and Rydon <sup>18</sup>, and *tert*-butyl alcohol recommended as solvent. Therefore, hydrogenolysis has been performed in either glacial acetic acid or in *tert*-butanol-water, and acetone has been preferred for precipitation of acidic peptides. With these precautions no esterification has been detected.

### T i t r a t i o n

*Potassium hydroxide*, 0.25 M, containing 0.15 M KCl, and *hydrochloric acid*, 0.30 M, were prepared and standardized as described <sup>2</sup>. All reagents in this work were of analytical grade.

*Titration procedure.* Acid-base titrations were carried out for each peptide by successive addition of standard KOH or HCl to the experimental solution (initial volume 10 ml), containing peptide (approximately  $2 \times 10^{-3}$  M) and 0.15 M KCl, and the pH measured after each addition. In respect of the lysine peptides, the compounds IIa and IVa (hydrochlorides) were titrated. Every titration was reproduced at least once. The true concentration of peptide, used in the calculations, was obtained from the titration curve. As measuring instrument a Radiometer PHM 4a valve potentiometer, equipped with a Radiometer glass electrode G202 B and a calomel electrode K 100 was used. The rest of the apparatus was the same as described previously <sup>3</sup>. All titrations were made at  $25.00 \pm 0.03^\circ$ . Activity coefficients were assumed to be constant owing to the presence of 0.15 M KCl. Standardization of the pH-meter, conversion of measured pH to correspond to hydrogen ion concentration, pcH (*cf.* Ref.<sup>20</sup>), and the routine calibration of the measuring system were made as before <sup>3</sup>. The reproducibility of the measurements was within 0.01 pH unit.

*Calculations* \*\*. Apparent ionization constants were computed for the peptides investigated as described <sup>3</sup> with the exception of the overlapping constants of serylglutamic acid and phosphorylserylglutamic acid.

The ionization constants ( $K_{H_2A}$  and  $K_{H_3A}$ ) of the  $\alpha$ - and  $\gamma$ -carboxyl groups in serylglutamic acid were resolved by comparing the experimental data with a family of normalized curves <sup>21</sup>. The constant,  $K_{H_2A}$ , was normalized and determined the position of the experimental function  $\bar{n}_H$  (pcH) on the abscissa, while the ratio  $K_{H_2A}/K_{H_3A}$  determined the shape. The constants obtained thereby were refined by the projection strip method developed by Rossotti *et al.*<sup>22</sup>

\* Contrary to *O*-phosphorylserylglutamic acid, *O*-phosphorylserine is very resistant to esterification and can be crystallized from boiling methanol.

\*\* The same symbols have been used as in the preceding paper <sup>3</sup>. Thus, any constant  $K_{H_2A}$  is defined  $h(H_{1-1}A)/(H_2A)$ ; A = conjugate base of the polybasic acid  $H_3A$  with all dissociable protons removed;  $h$  = molar free hydrogen ion concentration;  $\bar{n}_H$  = average number of protons bound per all different species of A;  $Y(X)_Z$  means Y as a function of X with Z constant.

Table 2. Apparent ionization constants of the peptides at 25° in 0.15 M KCl.

Compound	$\alpha$ -COOH	$\gamma$ -COOH	$-\text{PO}_3\text{H}^-$	$\alpha$ -NH $_3^+$	$\epsilon$ -NH $_3^+$
Ser-Lys	$pK_{\text{H}_2\text{A}} = 2.92 \pm 0.02$			$pK_{\text{H}_2\text{A}} = 7.16 \pm 0.01$	$pK_{\text{HA}} = 10.49 \pm 0.03$
SerP-Lys	$pK_{\text{H}_2\text{A}} = 2.98 \pm 0.01$		$pK_{\text{H}_2\text{A}} = 5.34 \pm 0.01$	$pK_{\text{H}_2\text{A}} = 7.58 \pm 0.02$	$pK_{\text{HA}} = 11.05 \pm 0.03$
Ser-Glu	$pK_{\text{H}_2\text{A}} = 2.94 \pm 0.02$	$pK_{\text{H}_2\text{A}} = 4.34 \pm 0.01$		$pK_{\text{HA}} = 7.42 \pm 0.01$	
SerP-Glu	$pK_{\text{H}_2\text{A}} = 3.02 \pm 0.02$	$pK_{\text{H}_2\text{A}} = 4.39 \pm 0.01$	$pK_{\text{H}_2\text{A}} = 5.69 \pm 0.005$	$pK_{\text{HA}} = 8.25 \pm 0.005$	
Ser-Gly *	$pK_{\text{H}_2\text{A}} = 3.10 \pm 0.01$			$pK_{\text{HA}} = 7.33 \pm 0.02$	
SerP-Gly *	$pK_{\text{H}_2\text{A}} = 3.13 \pm 0.01$		$pK_{\text{H}_2\text{A}} = 5.41 \pm 0.01$	$pK_{\text{HA}} = 8.01 \pm 0.01$	
Ser-Leu *	$pK_{\text{H}_2\text{A}} = 3.08 \pm 0.01$			$pK_{\text{HA}} = 7.45 \pm 0.01$	
SerP-Leu *	$pK_{\text{H}_2\text{A}} = 3.11 \pm 0.02$		$pK_{\text{H}_2\text{A}} = 5.47 \pm 0.005$	$pK_{\text{HA}} = 8.28 \pm 0.01$	

 \* Data compiled from the preceding paper <sup>3</sup>.

The constants ( $K_{H_1A}$ ,  $K_{H_2A}$  and  $K_{H_3A}$ ) related to the  $\alpha$ -carboxyl-,  $\gamma$ -carboxyl- and second phosphate groups of phosphorylserylglutamic acid were computed by one of the elimination methods described by Sillén<sup>21</sup> for two parameter systems. A preliminary value of  $K_{H_1A}$  was, however, first obtained from extrapolation of the plot,  $\log[h(4 - \bar{n}_H)/(\bar{n}_H - 1)]$  as a function of  $(\bar{n}_H - 1)$ , to  $(\bar{n}_H - 1)$  equal to zero (for details see Ref.<sup>2</sup>). Holding  $K_{H_1A}$  constant,  $K_{H_2A}$  and  $K_{H_3A}$  were then determined by plotting  $K_{H_1A}^{-1}$  as a function of  $h$ , for different  $K_{H_1A}$ . With two similar plots the constants were refined:  $K_{H_1A}$  and  $K_{H_2A}$  were computed from  $K_{H_1A}(h)K_{H_2A}, K_{H_3A}$  curves with different  $K_{H_1A}$ , and values of  $K_{H_1A}$  and  $K_{H_2A}$  were computed from  $K_{H_1A}(h)K_{H_2A}, K_{H_3A}$  curves with different  $K_{H_1A}$ . The latter two plots gave only a minor change in the values of the constants (less than 0.01 pK unit).

### RESULTS AND DISCUSSION

In Table 2 are listed the apparent ionization constants, expressed as pK values, obtained in this investigation, as well as those reported<sup>3</sup> for some analogous peptides. The titrations are shown in Fig. 3. The constants in Table 2 are referred to individual groups, even though they are not true intrinsic constants<sup>23</sup>. The pK value of the first ionization of the phosphate group is not included, as it cannot be determined under the experimental conditions used. Due to some possible overlapping of the ionization of this group and the  $\alpha$ -carboxyl group, less weight was given to experimental data below pCH 3 for the phosphorylated peptides.

No acid-base data are available in the literature for any of the present compounds. The ionization constants obtained for serylglutamic acid and seryllysine may, however, be compared with those of similar peptides, if differences in experimental conditions and chemical structure are considered. The following constants have been reported: glutamylglutamic acid<sup>24</sup>: pK ( $\alpha$ -COOH) 3.14, pK ( $\gamma$ -COOH) 4.38; lysylglutamic acid<sup>24</sup>: pK ( $\alpha$ -COOH) 2.93, pK ( $\gamma$ -COOH) 4.47, pK ( $\epsilon$ -NH<sub>3</sub><sup>+</sup>) 10.50; lysyllysine<sup>25</sup>: pK ( $\alpha$ -COOH) 3.01; and alanyllysylalanine<sup>25</sup>: pK ( $\epsilon$ -NH<sub>3</sub><sup>+</sup>) 10.30 (all L-configurations).

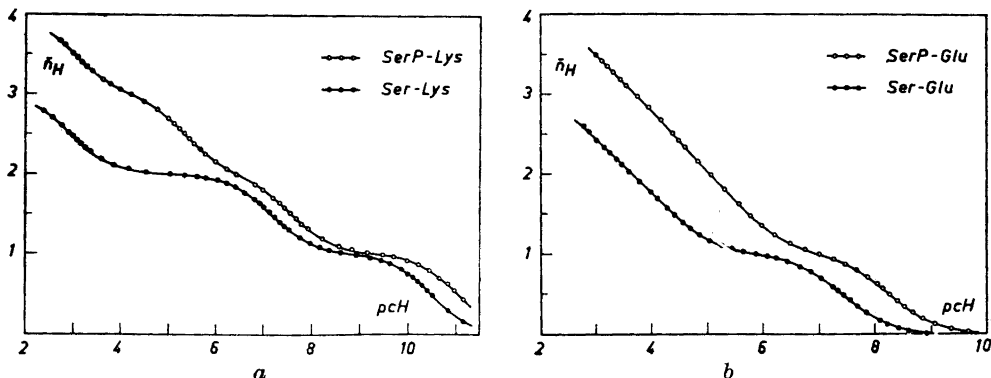


Fig. 3. Potentiometric titration data of serine peptides at 25° in 0.15 M KCl.

a. L-Seryl-L-lysine, ●; O-phosphoryl-L-seryl-L-lysine, ○.

b. L-Seryl-L-glutamic acid, ●; O-phosphoryl-L-seryl-L-glutamic acid, ○.

The drawn curves have been computed from the constants in Table 1.



From Table 2 can be seen that the introduction of an O-phosphoryl group in serylglutamic acid and seryllysine results in a similar slight increase in  $pK$  of the  $\alpha$ -carboxyl group as in the analogues, serylglycine and serylleucine. The increase in  $pK(\alpha-NH_3^+)$  of phosphorylserylglutamic acid relative to serylglutamic acid is also almost as great as that obtained by phosphorylation of serylglycine and serylleucine (0.7–0.8  $pK$  units). This may therefore be due to the same effects in all three peptides, reasonably the combined direct field (+ D) and inductive effects<sup>26</sup> (cf. Fig. 4, conformation a). The  $\gamma$ -carboxyl group seems, thus, to have no measurable effect on the rise in  $pK(\alpha-NH_3^+)$ , caused by the phosphate group.

The other  $pK$  values obtained for phosphorylserylglutamic acid do not allow any further discussion owing to overlap of the ionization of the  $\alpha$ -carboxyl,  $\gamma$ -carboxyl- and second phosphate groups. However, the  $pK$  values of the carboxyl groups may be compared with the corresponding values for the phosphopeptides prepared from  $\alpha$ -casein<sup>6\*</sup> and whole-casein<sup>2\*\*</sup>. The  $pK(\gamma-COOH)$  value obtained in this study agrees roughly with the intrinsic  $pK$  value (= 4.1) for the ten free side-chain carboxyl groups present in the  $\alpha$ -casein peptide<sup>\*</sup>, especially as these groups may constitute both  $\beta$ -aspartic and  $\gamma$ -glutamic carboxyl groups. The titration data of the whole-casein peptide<sup>2</sup> suggest a lower intrinsic  $pK$  value (about 3.8) for its  $\gamma$ -carboxyl groups. This may be properly explained by the fact that one of the glutamyl residues occupies the N-terminal position and one other possibly being neighbour to the basic histidyl residue. The  $pK(\alpha-COOH)$ -values of the synthetic peptides studied (see Ref.<sup>3</sup> and Table 2) further indicate that this histidyl residue may be C-terminal in the whole-casein peptide, due to the apparently low  $pK$  (= 2.7) of its  $\alpha$ -carboxyl group.

In contrast to the other phosphopeptides in Table 2, the increase in  $pK$  of the  $\alpha$ -amino group of phosphorylseryllysine relative to unphosphorylated peptide is much less (0.4  $pK$  units). The  $pK$  of the second phosphate ionization in this compound is only slightly lower than that of phosphorylated serylglycine and serylleucine. Furthermore, the  $pK$  of the  $\epsilon$ -amino group in phosphorylseryllysine is appreciably higher (0.5  $pK$  units) than that of the unphosphorylated peptide and other lysine derivatives<sup>24,25,27</sup>. This suggests that the phosphate group is shared between the two amino groups, when they are charged; schematically by a combination of the forms (a) and (b) of Fig. 4. The approach between the charged side-chains is not sterically hindered according to Stuart-Briegleb molecular models. Owing to the isolated position of the  $\epsilon$ -amino group, differences around this group in inductive, mesomeric or solvation effects between seryllysine and phosphorylseryllysine may be eliminated. Therefore the increase in  $pK$  of the  $\epsilon$ -amino group will be due almost exclusively to the + D effect of the negatively charged phosphate group.

This interaction between the  $\epsilon$ -amino- and phosphate groups may now be discussed in relation to trypsin action. At pH 8.5–10.0 involving an uncharged  $\alpha$ -amino group, conformation of type (b) in Fig. 4 will dominate. Fig. 4 c

\* Asp(Asp<sub>3</sub>, Thr, Ser<sub>8</sub>, Glu<sub>10</sub>, Pro, Gly, Ala<sub>2</sub>, Val<sub>2</sub>, Met<sub>2</sub>, Ileu<sub>4</sub>, Lys)Lys; (NH<sub>3</sub>)<sub>4</sub>; (H<sub>3</sub>PO<sub>4</sub>)<sub>7</sub>.

\*\* Glu(Thr, Ser<sub>3</sub>, Glu<sub>2</sub>, Val, Ileu<sub>2</sub>, His); (H<sub>3</sub>PO<sub>4</sub>)<sub>3</sub>.

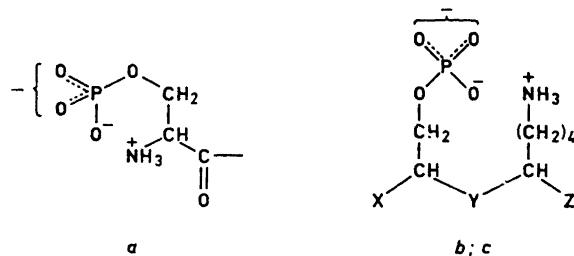


Fig. 4. Probable conformations due to charge interaction in O-phosphorylated serine peptides at pH 6–7 (a), and pH 7–10 (b, c) according to acid-base data.

a. N-terminal O-phosphorylserine residue

b. Amino acid sequence -SerP-Lys-; X = NH; Y = CO-NH; Z = CO

c. " " " -Lys-SerP-; X = CO; Y = NH-CO; Z = NH

as well as molecular models show that the above discussion will apply also to the reversed amino acid sequence, lysyl-(O-phosphoryl)-serine. As in larger peptides and proteins the  $\alpha$ -amino- and  $\alpha$ -carboxyl groups of these sequences are involved in peptide bonds, the phosphate group interacts with the  $\epsilon$ -amino group in the pH optimum region of trypsin. Trypsin requires, however,

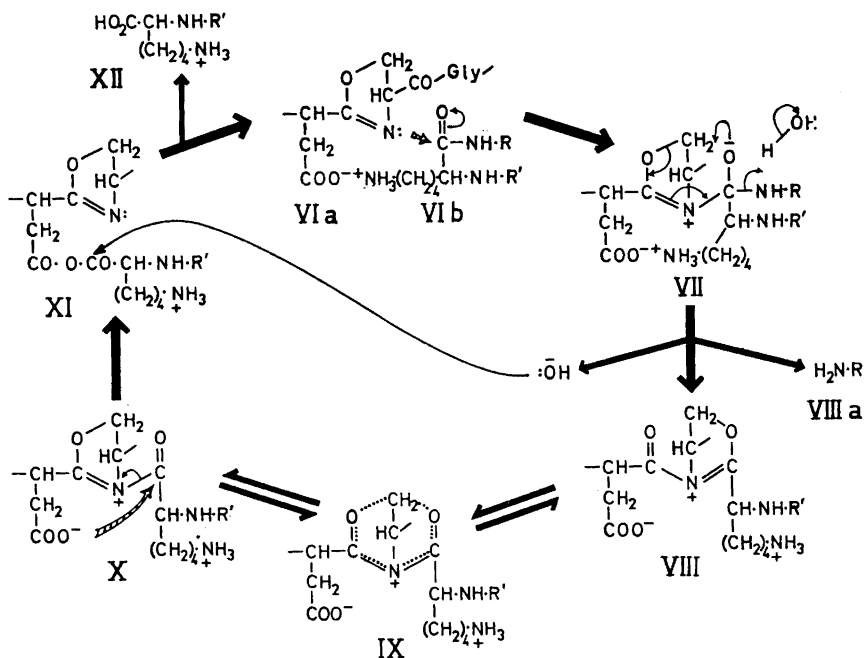


Fig. 5. Tryptic hydrolysis mechanism according to Rydon<sup>34</sup>, adapted for lysine peptides. VI a = active center of enzyme. The serine carbonyl group and the glycine residue are indicated by a bond line only in VII–XI.

a free  $\epsilon$ -amino group for action on a peptide bond involving the lysine carboxyl group<sup>7</sup>. A plausible mechanism taking in account this specificity and the absence of hydrolysis of O-phosphorylated lysine peptides may be formulated as follows:

The amino acid sequence in the "active center" of trypsin<sup>28,29</sup> and several other esterases<sup>28,30-33</sup> has been shown to be ...—Asp—Ser—Gly—.... Rydon<sup>34</sup> has formulated a mechanism for the trypsin catalyzed hydrolysis of esters, assuming a  $\Delta^2$ -oxazoline structure for the active serine residue. The adaptation of his mechanism to peptides and proteins is suggested in Fig. 5. In this figure, (VI a) represents the active center of the enzyme and (VI b) the substrate with a free, positively charged  $\epsilon$ -amino group of lysine. We tentatively propose that the function of this basic group is to bind the substrate to the aspartic acid residue in the active center of the enzyme\*. As shown in Fig. 5, the approach of the  $\epsilon$ -amino group to the aspartic acid  $\beta$ -COO<sup>-</sup> may bring the peptide bond carbonyl group in favourable position for a nucleophilic attack by the oxazoline N atom, forming (VII). Rearrangement of (VII) and proton transfer from a water molecule would liberate the amino acid or peptide chain (H<sub>2</sub>N—R) attached to the lysine carboxyl group and result in (VIII), having a positive charge on the oxazoline N atom. This charge may then repel the  $\epsilon$ -amino group from the aspartic acid carboxyl group and thus enable an attack on the lysine carbonyl group in the tautomeric isomer (X). Finally, the anhydride (XI) so formed would undergo hydrolysis, formally by the uptake of the OH<sup>-</sup> released in stage (VII)→(VIII), and so liberate the lysyl part of the peptide chain (XII) under simultaneous regeneration of the enzyme (VIa).

The observed inability of trypsin to hydrolyse O-phosphorylated peptides\*\* may then be attributed to a hindrance of the enzyme-substrate complex formation involving the enzyme  $\beta$ -carboxyl group and the substrate  $\epsilon$ -amino group. The positive charge on the latter is preferentially taken into account by the phosphate group. Furthermore, the negatively charged enzyme carboxyl- and substrate phosphate groups may repel each other\*\*\*.

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\* On the basis of kinetic data it has been suggested that the combination between enzyme and substrate is essentially electrostatic in character<sup>7,p.108</sup>. The differences in specificity between different esterases, apparently with the same active centers, may be explained by a different mechanism for the enzyme-substrate complex formation; e.g. chymotrypsin may combine with the substrate through hydrogen bonds<sup>7,p.118</sup>. The validity of the electrostatic binding mechanism described below is not dependant on the presence of an oxazoline structure; an unmodified serine residue may undergo similar reactions, when suitably activated, e.g. by a histidine residue.

\*\* In Fig. 5, R = —CH(CH<sub>2</sub>OPO<sub>3</sub><sup>2-</sup>)CO— in a sequence —lysyl-(O-phosphoryl)-serine; R' = —CO·CH(CH<sub>2</sub>OPO<sub>3</sub><sup>2-</sup>)NH— in a sequence —(O-phosphoryl)-seryl-lysine.

\*\*\* A mechanism for the inhibition of trypsin by polyelectrolytes as, e.g., poly- $\alpha$ -L-glutamic acid, has been proposed by Kornguth and Stahmann<sup>35</sup>. In that case the inhibition is entirely independant of the substrate.

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