ACTA CHEMICA SCANDINAVICA 16 (1962) 2434–2451

Calcium, Magnesium, and Manganese(II) Complexes of Some O-Phosphorylated Peptides *

RAGNAR ÖSTERBERG

Department of Medical Biochemistry, University of Göteborg, Sweden

Complex formation between calcium, magnesium, and manganese(II) ions and O-phosphorylated ethanolamine, seryllysine, serylglycine, glycylserine, glycylserylglycine and serylglutamic acid has been measured by pH titration at 25° in an ionic medium, whose anion concentration, [Cl^-], was kept constant by potassium chloride, and whose initial ionic strength was 0.15 M. The experimental data could be explained by assuming that complexes of the type M_pH_qA are formed, where $(p;q)$ = (1;1), (1;0) for the first two ligands, $(p;q)$ = (1;1) (1;0), (2;0) for the next three ligands ($(p;q)$ = (2;0) was not measurable, when M = Ca^{2+}), and $(p;q)$ = (1;2), (1;1), (1;0), (2;1), (2;0) for the last ligand. The stability constants of the complexes have been computed, and the coordination sites are discussed.

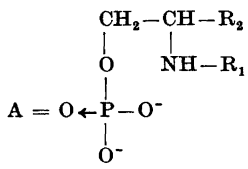
The observed differences in complexity between the calcium and magnesium ions support Williams' conclusion [1] that their complexing ability can be correlated to ionic size. The definitely greater tendency of magnesium and manganese(II) to dinuclear complex formation as compared to calcium is discussed in relation to the specificity of enzymes for these metal ions.

Most enzyme reactions, which involve phosphorylated substrates, have an absolute requirement for divalent metal ions, in general, magnesium, calcium or manganese (see Refs.[1–3], for review). In some such systems the metal ion is assumed to activate by forming a ternary complex, coordinating both the substrate phosphoryl and some enzyme protein groups [1]. The relative catalytic activity of these metal ions varies, however, from enzyme to enzyme, and in some cases one of them can even completely inhibit the action of one other [1–3]. In respect to magnesium and calcium this contrary action does not agree with their similarity in chemical reactions and electron configuration of the outer shell.

Williams recently explained the enzymic calcium/magnesium specificity on the basis of ionic size [1]. He suggested that the larger calcium ion forms com-

* Presented in part at the Vth International Congress of Biochemistry, Moscow, August 10–16, 1961.

Table 1. Ligands.



Ligand	Abbreviation	R_1	R_2
O-Phosphorylethanolamine	P-ethanolamine	$-H$	$-H$
O-Phosphoryl-D,L-serylglycine	SerP-Gly	$-H$	$-CO-NH-CH_2-COO^-$
O-Phosphoryl-L-seryl-L-glutamic acid	SerP-Glu	$-H$	$-CO-NH-CH(CH_2-CH_2-COO^-)-COO^-$
O-Phosphoryl-L-seryl-L-lysine	SerP-Lys	$-H$	$-CO-NH-CH((CH_2)_4-NH_3^+)-COO^-$
Glycyl-O-phosphoryl-D,L--serylglycine	Gly-SerP-Gly	$-CO-CH_2-NH_2$	$-CO-NH-CH_2-COO^-$
Glycyl-O-phosphoryl-D,L-serine	Gly-SerP	$-CO-CH_2-NH_2$	$-COO^-$

plexes different from those of the magnesium ion by the coordination of more dentate groups. On the other hand, no systematic study appears to have been undertaken on the complex formation between these metal ions or between Mn(II) and such phosphorylated ligands that include different types of metal binding groups present in proteins. Therefore, calcium, magnesium, and manganese(II) complexes of some O-phosphorylated peptides have been investigated and the results interpreted quantitatively in the form of stability constants. The ligands studied are shown in Table 1.

SYMBOLS *

A	ligand, see Table 1
a	molar concentration of free A
h	molar concentration of free hydrogen ions
M	complex forming metal ion: Ca^{2+}, Mg^{2+} or Mn^{2+}
m	molar concentration of free M
p,q	number of metal and hydrogen ions bound in M_pH_qA
Q,S,T	maximum number of q in H_qA, MH_qA and M_2H_qA, see eqn. 1
r	number of hydrogen ions bound in M_pH_rA; $p \neq 0$
C_A	total molar concentration of ligand $= \sum_{p=0} \sum_{q=0} [MpHqA]$
C_M	total molar concentration of metal ions $= m + \sum_{p=1} \sum_{q=0} p[M_pH_qA] + [MOH]$

* All charges are omitted for the sake of convenience.

C_S total molar concentration of bound hydrogen ions =
$= \sum_{p=0} \sum_{q=1} q[M_pH_qA] - [MOH] = jC_A - [KOH] - h + [OH]$
where j is the number of dissociable hydrogen ions bound to the ligand A form in solide state, and [KOH] the molar concentration of KOH added.

Z ligand number $= \sum_{p=1} \sum_{q=0} [M_pH_qA](m + \sum_{p=1} \sum_{q=0} p[M_pH_qA] + [MOH])^{-1}$, see eqn. 6

$b_{M_pH_qA}$ stoichiometric stability constant of the complex
$M_pH_qA = [M_pH_qA]m^{-1}([M_{p-1}H_qA])^{-1} = \varkappa_{pq_1}(\varkappa_{(p-1)q_1})^{-1}$; $p \neq 0$

$K_{M_pH_qA}$ stoichiometric acid ionization constant of M_pH_qA
$= h[M_pH_{q-1}A]([M_pH_qA])^{-1} = \varkappa_{p(q-1)_1}(\varkappa_{pq_1})^{-1}$; $q \neq 0$

$*K_1$ stoichiometric hydrolysis constant $= [MOH]hm^{-1}$

$*K_{MA}$ stoichiometric hydrolysis constant $= [MAOH]h([MA])^{-1}$

$pK_{M_pH_qA}$ $= -\log K_{M_pH_qA}$

$\varkappa_{pq_1}$ stoichiometric equilibrium constant, defined by eqn. 3, $\varkappa_{100} = \varkappa_{010} = \varkappa_{001} = 1$

φ_r defined by eqn. 4a

METHOD

pH titrations [4] were carried out for the values of C_A and C_M, as shown in Table 2. In each titration these quantities were held practically constant, and h and C_S were varied by additions of potassium hydroxide. Activity coefficients were assumed to be constant by keeping the concentration of the negatively charged counter-ion (Cl^-) constant [5] with potassium chloride at a value, which corresponded to 0.15 M initial ionic strength.

If mononuclear and dinuclear complexes only are formed, the following equilibria (*cf.* discussion on pp. 2445–2449) may be successively present during the titration process:

$$\left\{H_QA\right\} \rightleftharpoons \ldots \rightleftharpoons \left\{\begin{matrix} H_SA \\ MH_SA \end{matrix}\right\} \rightleftharpoons \ldots \rightleftharpoons \left\{\begin{matrix} H_TA \\ MH_TA \\ M_2H_TA \end{matrix}\right\} \rightleftharpoons \ldots \rightleftharpoons \left\{\begin{matrix} A \\ MA \\ M_2A \end{matrix}\right\} \quad (1)$$

where $Q > S > T$. The complex formation is summarized by

$$pM + qH + A \rightleftharpoons M_pH_qA \quad (2)$$

and is defined by the equilibrium constants

$$\varkappa_{pq_1} = \frac{[M_pH_qA]}{m^p h^q a} \quad (3)$$

p and q may attain values, which conform to eqn. 1, *i.e.* ($p = 0$; $q = 0, 1, \ldots, Q$), ($p = 1$; $q = 0, 1, \ldots, S$), ($p = 2$; $q = 0, 1, \ldots, T$).

In accord with eqn. 1, pH regions are selected, where M_pH_rA ($r = S, S-1, \ldots, 0$) species successively dominate and metal complexes with $q < r$ can be neglected [6]. In each region $\varkappa_{1r1}$ and $\varkappa_{2r1}$ are solved by (*cf.* eqn. 7a in Ref.[6])

$$\frac{Z(1 + *K_1h^{-1})}{(1-Z)ah^r} - \sum_{q=r+1}^{S} \varkappa_{1q_1}h^{q-r} - \sum_{q=r+1}^{T} \frac{(1-2Z)}{(1-Z)} \varkappa_{2q_1}mh^{q-r} = \frac{m(1-2Z)}{(1-Z)} \varkappa_{2r1} + \varkappa_{1r1} \quad (4)\ *$$

using data of two or more C_M. In general $\varkappa_{1r1}$ is first computed from measurements with $C_M \sim C_A$, and then $\varkappa_{2r1}$ from titrations with $C_M \geq 5C_A$, where $m \approx C_M$. Alternatively,

* In the following all equations assume occurrence of MOH species, and that $*K_1$ is known from the literature.

$\varkappa_{1r1}$ and $\varkappa_{2r1}$ are obtained from two series of measurements with $C_M \geqslant 5C_A$. The left hand side is easily determined, as a and Z can be computed from known, (C_A, C_M, $\varkappa_{pq1}$; $q > r$, if $p \neq 0$), and measured, (h, C_S), quantities.

When $C_M \geqslant 5C_A$, the following equations are used [6]

$$a = \frac{C_S - rC_A + {}^*K_1 mh^{-1}}{\sum_{p=0} \sum_{q=0} (q-r)\varkappa_{pq1} m^p h^q} \quad (q > r, \text{ when } p = 1,2) \tag{5}$$

$$Z = \frac{C_A - a \sum_{q=0} \varkappa_{0q1} h^q}{C_M} \tag{6}$$

The $\varkappa_{0q1}$ are obtained from pK values, computed from a titration in the absence of complex forming metal ions. $\varkappa_{pq1}$ ($q > r$) are known from calculations performed on data in lower pH regions. The quantity $m \approx C_M$ may be better approximated by the relation

$$m = C_M - Z'C_M - am^2 \sum_{q=r} \varkappa_{2q1} h^q - {}^*K_1 mh^{-1} \tag{7}$$

where Z' is an approximate value of Z. The m value obtained is refined by successive approximation *.

When $C_M \sim C_A$, dinuclear species may be neglected. For $r < S$, a and Z are solved for each measuring point from the intersection of the $Z(a)_{C_S,h}$ curves of eqn. 6 and the following equation [6]:

$$Z = 1 - \frac{\left[C_S - rC_A - a \sum_{q=0}^{Q} (q-r)\varkappa_{0q1} h^q\right]\left[1 + {}^*K_1 h^{-1}\right]}{C_M\left[a \sum_{q=r+1}^{S} (q-r)\varkappa_{1q1} h^q - {}^*K_1 h^{-1}\right]} \tag{8}$$ **

For $r = S$ eqns. 5 and 6 are used.

EXPERIMENTAL

Material. The phosphorylated compounds were those synthesized by Fölsch [7-10]. Analytical data for O-phosphorylated glycylserine and glycylserylglycine have been given in Ref.[7], and for O-phosphorylated ethanolamine in Ref.[9] O-Phosphorylated serylglutamic acid and seryllysine were freed from minor impurities before titration and converted to free and hydrochloride forms, respectively, by means of cation exchange chromatography [10]; analytical data were reported in Ref.[10]

A new preparation of O-phosphorylserylglycine, which had been run through a cation exchange column (Dowex 50-X2, H^+-form, 1.6 × 40 cm, eluted with water) moved as one spot (0.5 mg applied) at paper chromatography, exactly as a sample of a previous preparation [7], in the solvents: phenol/water, 4:1 (v/v), R_F 0.10, and butanol:acetic acid: water, 4:1:5 (v/v/v), upper phase), R_F 0.10, as developed by ninhydrin and phosphate (ammonium molybdate) reagents. (Found: N 11.5; P 12.6. Calc. for $C_5H_{11}O_7N_2P$ (242.1): N 11.6; P 12.8). Acid-base titration gave pK values: 3.13, 5.41, and 8.00, which agree with those reported [9]: 3.13, 5.41 and 8.01.

Aqueous stock solutions of all phosphoryl compounds in free form and in one case, O-phosphorylseryllysine, in the dihydrochloride form, were standardized by potentiometric titration. When not used, the solutions were kept in a refrigerator at −20°. Aqueous stock solutions of complex forming metal ions were prepared from Merck's *p. a.* $CaCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$ and $MnCl_2 \cdot 4H_2O$. For standardization, samples of these

* The first approximation does not consider the last two terms. A roughly estimated value of $\varkappa_{2r1}$ was used, as the figure corresponding to $q = r$ in the sum was usually less than 1 % of m.

** The equation is identical with eqn. 9 of Ref.[6], when used for MA ($r = 0$) in the presence of MHA.

Table 2. Stability constants * (in M^{-1}) of the metal complexes.

Ligand	Metal ion	$C_M^o \times 10^3$	$C_A^o \times 10^3$	b_{MHA}	b_{MA}	b_{M_2A}
P-ethanolamine	Ca	5.19	4.97	14 ± 2	37 ± 1	—
P-ethanolamine	Ca	19.9	2.32	12 ± 2	36 ± 3	—**
P-ethanolamine	Mg	4.99	4.97	18 ± 2	50 ± 3	—
P-ethanolamine	Mg	9.80	1.90	16 ± 2	49 ± 4	—**
P-ethanolamine	Mg	19.8	1.90	17 ± 2	53 ± 5	—**
P-ethanolamine	Mn	4.99	4.97	53 ± 2	359 ± 24	—
P-ethanolamine	Mn	20.0	2.32	53 ± 2	354 ± 41	—**
SerP-Lys	Ca	19.9	1.86	14 ± 1	34 ± 5	
SerP-Lys	Mg	9.90	1.86	16 ± 1	43 ± 7	—**
SerP-Lys	Mg	19.9	1.86	18 ± 1	43 ± 5	—**
SerP-Lys	Mn	19.9	1.86	60 ± 2	212 ± 20	
Gly-SerP-Gly	Ca	19.9	2.00	28 ± 2	65 ± 3	
Gly-SerP-Gly	Mg	9.80	1.74	29 ± 3	61 ± 3	9 ± 6
Gly-SerP-Gly	Mg	19.8	1.74	30 ± 2		
Gly-SerP-Gly	Mn	20.0	2.00	119 ± 2		
Gly-SerP	Ca	19.9	1.48	30 ± 2	59 ± 2	
Gly-SerP	Mg	9.80	2.33	45 ± 2	72 ± 3	24 ± 8
Gly-SerP	Mg	19.8	2.33	43 ± 2		

Ligand	Metal ion	$C_M^o \times 10^3$	$C_A^o \times 10^3$	b_{MH_2A}	b_{MHA}	b_{M_2HA}	b_{MA}	b_{M_2A}
SerP-Gly	Ca	5.19	5.21		21 ± 1		71 ± 3	—
SerP-Gly	Ca	19.9	2.00		19 ± 1		71 ± 1	—**
SerP-Gly	Mg	4.99	5.21		28 ± 2		87 ± 5	—
SerP-Gly	Mg	9.81	2.03		23 ± 3		***	17 ± 3
SerP-Gly	Mg	14.8	2.03		23 ± 3		86 ± 2	18 ± 4
SerP-Gly	Mg	19.8	2.03		28 ± 3			18 ± 2
SerP-Gly	Mn	4.99	5.21		75 ± 3		422 ± 11	—
SerP-Gly	Mn	20.0	2.00		80 ± 2		—	35 ± 4
SerP-Glu	Ca	5.19	4.74	12 ± 1	44 ± 2	—	138 ± 3	—
SerP-Glu	Ca	19.9	1.98	12 ± 2	—	11 ± 3	—	29 ± 2
SerP-Glu	Mg	4.99	4.74	10 ± 2	43 ± 2	—	122 ± 3	—
SerP-Glu	Mg	9.91	1.98	10 ± 2	—	36 ± 5	—	66 ± 4
SerP-Glu	Mg	19.9	1.98	11 ± 1	—	28 ± 4	—	62 ± 4
SerP-Glu	Mn	4.99	4.74	26 ± 3	173 ± 4	—	962 ± 4	—
SerP-Glu	Mn	19.9	1.98	27 ± 4	—	21 ± 3	—	89 ± 3

* Uncertainties given refer to greatest deviation from the mean observed for individual calculations.

** No true evidence for existence of M_2A species, see the text.

*** Value obtained by extrapolation of the line: $(\varphi_0)_{h/K_{HA}=0} = \frac{(1-2Z)m}{(1-Z)} b_{MA}b_{M_2A} + b_{MA}$; to $\frac{(1-2Z)m}{(1-Z)} = 0$ for these three C_{Mg}; the slope $b_{MA}b_{M_2A}$ gave $b_{M_2A} = 19$; ordinates were obtained by extrapolation of eqn. 4a ($r = 0$) to $h/K_{HA} = 0$ (Fig. 3).

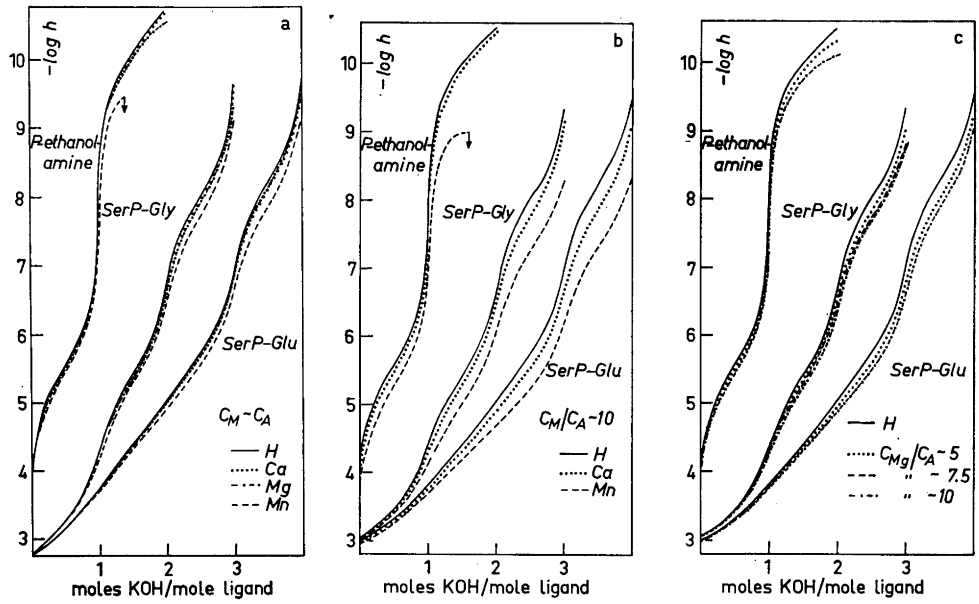


Fig. 1. Titration curves of O-phosphorylated ethanolamine, serylglycine and serylglutamic acid in the absence of complex forming metal ions, H, and in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+}.

a. $C_M \sim C_A \sim 0.005$. Apparent confluence of Ca and Mg curves is shown as Mg curve. The H curves gave pK_{H_4A}; pK_{H_3A}; pK_{H_2A}; pK_{HA} (P-ethanolamine): —; —; 5.57; 10.16; (SerP-Gly): —; 3.13; 5.41_5; 8.02; (SerP-Glu): 3.02_5; 4.38_5; 5.68; 8.25.

b. $C_A \sim 0.002$. The H curves gave pK_{H_4A}; pK_{H_3A}; pK_{H_2A}; pK_{HA} (P-ethanolamine): —; —; 5.57; 10.12; (SerP-Gly): —; 3.13_5; 5.41_5; 8.00; (SerP-Glu): 3.02_5; 4.38_5; 5.69; 8.25.

c. $C_A \sim 0.002$. The H curves gave pK_{H_3A}; pK_{H_2A}; pK_{HA}; (P-ethanolamine): —; 5.57; 10.12_5; (SerP-Gly): 3.13_5; 5.41_5; 8.00; (SerP-Glu): see *a*.

solutions were passed through a hydrogen ion saturated cation exchanger, and then titrated with standard potassium hydroxide. The manganese solutions were never used later than 6 h after preparation.

Carbonate-free 0.2979 M KOH, containing 0.15 M KCl, was prepared according to Powell and Hiller [11], and standardized against potassium hydrogen phthalate. Merck's *p.a.* bromide-free KCl was used as supporting electrolyte, without further purification. All stock solutions were prepared from freshly triply quartz-distilled water.

Measurements. Titration solutions were prepared by mixing stock solutions of phosphate ester, complex forming metal(II) chloride, and potassium chloride to obtain the concentrations, C_A^o, C_M^o (Table 2), the volume, 10 ml, and the ionic strength, 0.15 M, originally present. pH was measured with a Radiometer glass electrode (G202B), in combination with a saturated calomel electrode (K100), connected *via* a saturated KCl bridge. Standardization of the pH meter (Radiometer PHM 4) and routine calibration of the measuring system (reproducibility: 0.01 pH unit) were performed as described [9]. The readings were converted to values of $-\log h$ by a calculated apparent activity coefficient [9] of 0.79 ± 0.02. The titration equipment was that previously described [9]. All titrations were carried out at 25.00 ± 0.03° in a thermostatically controlled water bath in a room temperature of 23 ± 1°. Nitrogen, which had previously bubbled through 10 % H_2SO_4, 10 % NaOH and 0.15 M KCl, was bubbled through the solution. After

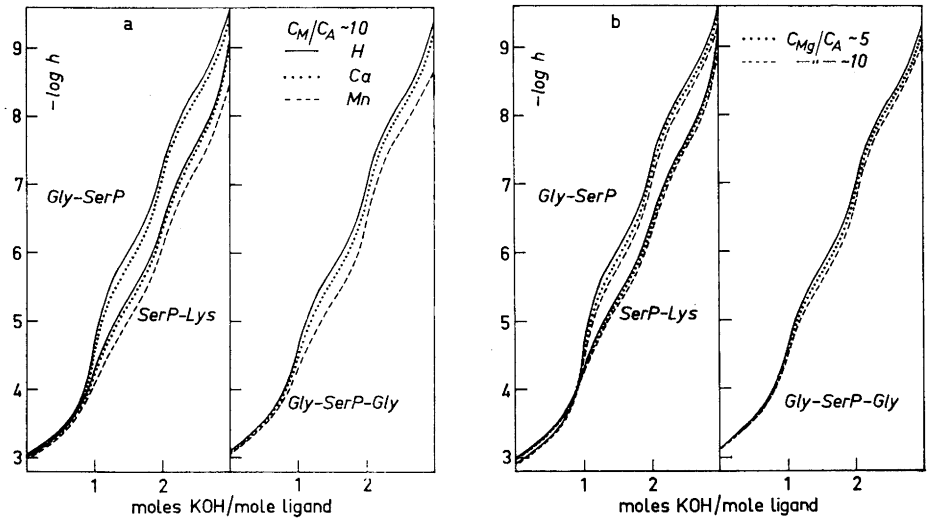


Fig. 2. Titration curves of O-phosphorylated glycylserine, seryllysine and glycylserylglycine in the absence of complex forming metal ions, H, and in the presence of Ca^{2+}, Mn^{2+} and Mg^{2+}. At zero abscissa one equivalent of phosphorylseryllysine dihydrochloride has been titrated.

a. $C_A \sim 0.002$. The H curves gave pK_{H_3A}; pK_{H_2A}; pK_{HA}(Gly-SerP): 2.91_5; 6.03_5; 8.42; (SerP-Lys): 2.98_5; 5.33_5; 7.58_5; (Gly-SerP-Gly): 3.29_5; 5.77_5; 8.21_5.
b. $C_A \sim 0.002$. The H curves gave pK_{H_3A}; pK_{H_2A}; pK_{HA} (Gly-SerP): 2.89_5; 6.03_5; 8.41_5; (SerP-Lys): see *a.*; (Gly-SerP-Gly): 3.29; 5.77_5; 8.21_5.

titration, peptide solutions with $C_M \sim 10C_A$ were subjected to paper chromatography in the aforementioned solvents (p. 2437). Each sample produced a single ninhydrin- and phosphate-positive spot within an R_F-region as expected.

The results, represented as titration curves, are shown in Figs. 1 and 2. These curves are based on mean values of data recorded from two or more identical experiments and measured at an interval of about 0.1 pH unit. The reproducibility was within 0.01 pH unit (0.005 pH for $C_A \sim 0.005$) in the pH ranges used for calculations of the constants. These calculations utilized the mean values.

Titrations for each ligand stock solution were performed in the absence of complex forming metal ions (Figs. 1 and 2). Then pK values could be computed, which conformed as exactly as possible to the experimental conditions. The results (see the legends of Figs. 1 and 2) agree, within the limits of experimental uncertainty, with those previously reported [9,10].

EVALUATION OF CONSTANTS

The ionization steps, shown in Figs. 1 and 2, apparently involve the successive ionization of the second phosphate and amino groups of phosphorylethanolamine (Fig. 1); the α-carboxyl, second phosphate, and α-amino groups of phosphorylserylglycine, phosphorylseryllysine, glycylphosphorylserylglycine,

Table 3. Computation of $b_{M_pH_qA}$ (in M^{-1}) of the O-phosphorylserylglutamic acid-calcium(II) system *. The numbers within parentheses refer to equation numbers **.

$C_A^o = 4.74 \times 10^{-3}$; $C_M^o = 5.19 \times 10^{-3}$; $[Cl^-] = 0.145$					$C_A^o = 1.98 \times 10^{-3}$; $C_M^o = 19.9 \times 10^{-3}$; $[Cl^-] = 0.130$					
V	$-\log h$	$a \times 10^{10}$ (5)		b_{MH_2A} (9)	V	$-\log h$	$a \times 10^{10}$ (5)			b_{MH_2A} (9)
0.1620	3.764	3.049		13.0	0.0740	3.878	2.573			14.0
0.1760	3.875	6.406		12.3	0.0820	4.012	6.071			13.0
0.1900	3.987	13.27		11.4	0.0900	4.146	13.86			12.5
0.2040	4.097	26.56		11.2	0.0980	4.280	30.51			12.3
0.2180	4.205	51.34	$b_{MH_2A} =$	11.7	0.1040	4.382	54.35	$b_{MH_2A} =$		12.3
0.2320	4.313	97.30	$= 12 \pm 1$	11.3				$= 12 \pm 2$		
V	$-\log h$	$a \times 10^7$ (6,8)	Z (6,8)	b_{MHA} (4)	V	$-\log h$	$a \times 10^7$ (5)	Z (6)	X_1*** (4)	b_{M_2HA} (4)
0.3120	4.948	2.716	0.0552	42.2	0.1280	4.802	0.4809	0.0191	51.1	8.4
0.3240	5.048	4.303	0.0606	42.4	0.1330	4.895	0.7431	0.0208	51.0	8.4
0.3360	5.148	6.692	0.0670	43.8	0.1380	4.987	1.124	0.0228	51.8	9.4
0.3480	5.249	10.28	0.0734	44.3	0.1430	5.079	1.662	0.0250	54.2	12.4
0.3600	5.350	15.51	0.0802	44.9	0.14805	5.173	2.458	0.0271	54.7	13.0
0.3720	5.453	23.22	0.0864	44.7	0.1530	5.267	3.589	0.0289	53.9	12.1
0.3840	5.558	34.41	0.0920	43.9	0.1580	5.364	5.184	0.0313	55.2	13.8
0.3960	5.666	50.74	0.0952	41.6	0.1630	5.464	7.478	0.0335	55.2	13.9
		$b_{MHA} = 44 \pm 2$					$b_{M_2HA} = 11 \pm 3$			
V	$-\log h$	$a \times 10^4$ (6,8)	Z (6,8)	b_{MA} (4)	V	$-\log h$	$a \times 10^4$ (5)	Z (6)	X_0*** (4)	b_{M_2A} (4)
0.4920	7.242	3.247	0.161	137.0	0.2100	7.176	6.764	0.0537	212.9	31.0
0.4960	7.323	3.835	0.164	135.0	0.2130	7.293	8.467	0.0550	212.0	31.0
0.5020	7.436	4.810	0.170	135.9	0.2160	7.396	10.20	0.0563	208.5	29.7
0.5080	7.534	5.800	0.177	139.5	0.2190	7.492	12.03	0.0577	209.7	30.3
0.5140	7.624	6.835	0.182	138.7	0.2220	7.581	13.89	0.0588	206.5	29.1
0.5220	7.731	8.220	0.190	138.5	0.2250	7.663	15.70	0.0601	206.2	29.1
0.5320	7.855	10.03	0.199	138.0	0.2290	7.770	18.21	0.0618	204.4	28.5
0.5420	7.969	11.84	0.211	140.8	0.2330	7.873	20.68	0.0636	204.3	28.6
0.5520	8.079	13.70	0.220	140.0	0.2370	7.976	23.13	0.0653	204.1	28.7
		$b_{MA} = 138 \pm 3$			0.2410	8.081	25.50	0.0671	205.6	29.6
							$b_{M_2A} = 29 \pm 2$			

* pK values of Fig. 1a and 1b were used.
** V = ml 0.2979 M KOH added; $C_A = C_A^o\ (1 + 0.1\ V)^{-1}$; $C_M = C_M^o\ (1 + 0.1\ V)^{-1}$.
*** $X_1 K_{HA}$ and X_0 are equal to the left hand side of eqn. 4 for $r = 1$ and $r = 0$, respectively.

glycylphosphorylserine (Figs. 1 and 2); and the α-carboxyl, γ-carboxyl, second phosphate, and amino groups of phosphorylserylglutamic acid (Fig. 1) *.

* The first phosphate group in all ligands and the ε-amino group of the lysine peptide were not titrated. The ionization of these groups did not require any consideration, as the pH values used in the calculations were more than 3 pH units away from their pK values [9,10].

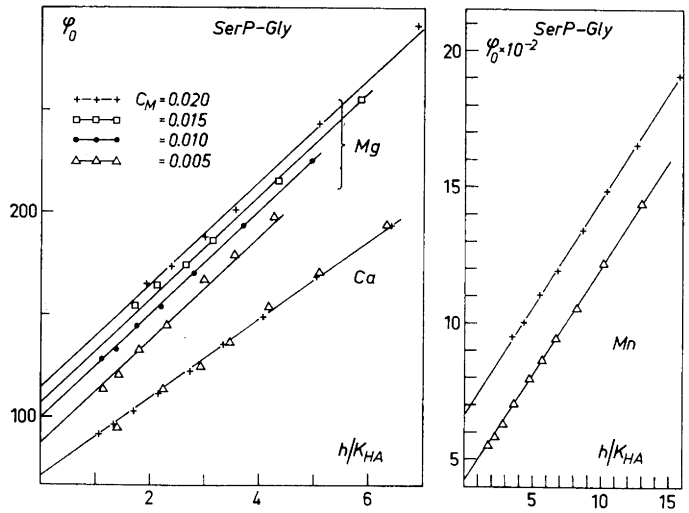


Fig. 3. Survey of the computation of b_{MHA}, b_{MA} and b_{M_2A} in the O-phosphorylseryl-glycine systems, *cf.* the text. Experimental data plotted by eqn. 4a for $r = 0$, assuming $S = 1$ and $T = 0$. The curves are calculated from the constants of Table 4 and those of the legend of Fig. 1, using eqn. 4a.

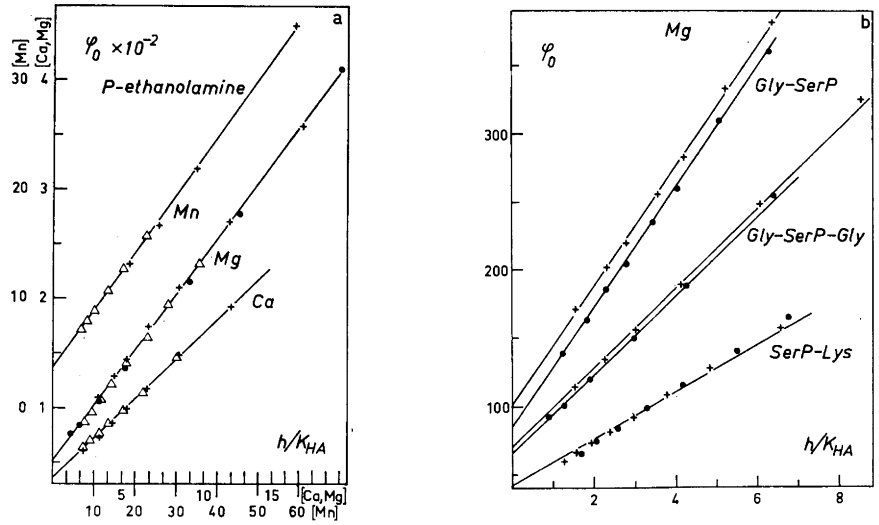


Fig. 4. Survey of the computation of b_{MHA}, b_{MA} in the systems of O-phosphorylated ethanolamine (*a.*) and seryllysine (*b.*), and b_{MHA}, b_{MA}, b_{M_2A} in those of O-phosphorylated glycylserine and glycylserylglycine (*b.*). Experimental data plotted by eqn. 4a for $r = 0$, assuming $S = 1$ and $T = 0$ and using the symbols of Fig. 3. The curves are calculated from the constants of Table 4 and those of the legends of Figs. 1 and 2, as well as b_{MHA}; b_{MA}; b_{M_2A} (Gly-SerP): **44**; 72; 24; (Gly-SerP-Gly): 29; 61; 9 M^{-1}, using eqn. 4a.

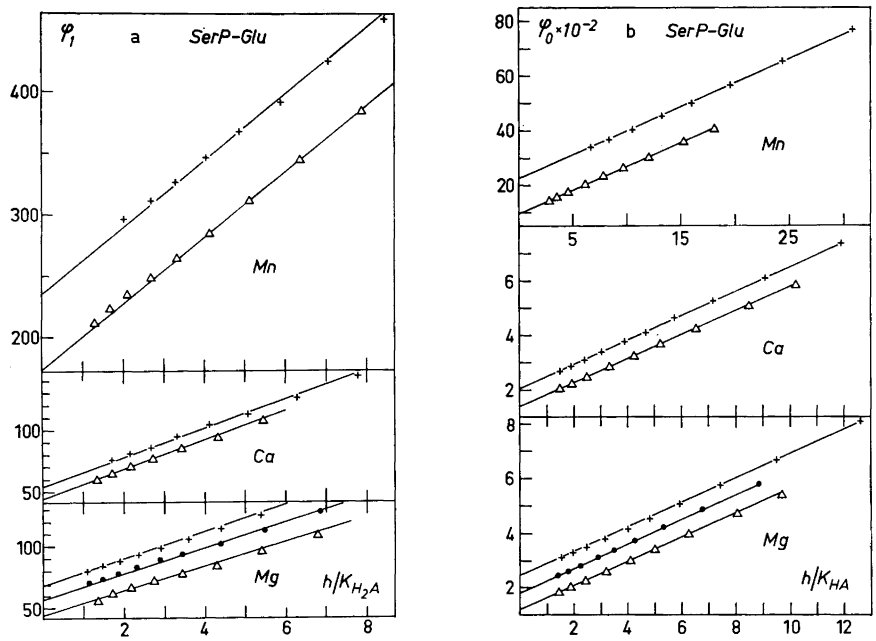


Fig. 5. Survey of the computation of b_{MH_2A}, b_{MHA}, b_{M_2HA} (*a.*) and b_{MHA}, b_{MA}, b_{M_2A} (*b.*) in the O-phosphorylserylglutamic acid systems. Experimental data plotted by eqn. 4a for $r = 1$ (*a.*) and $r = 0$ (*b.*), assuming $S = 2$ and $T = 1$ and using the symbols of Fig. 3. The curves are calculated from the constants of Table 2 and those of the legend of Fig. 1, using eqn. 4a.

Figs. 1 and 2 also indicate that the complex formation begins in the buffer region of the second phosphate group, however, for phosphorylserylglutamic acid systems in that of the γ-carboxyl group. Then complexes corresponding to $S = T = 2$ (phosphorylserylglutamic acid systems) and $S = T = 1$ (other systems) had to be considered at the computation of the $\varkappa_{pq1}$ constants. An example of the computations comprises Table 3, where the Ca(II)-phosphorylserylglutamic acid system is presented. The $\varkappa_{pq1}$ constants have been converted to $b_{M_pH_qA}$.

The computation of $\varkappa_{121}$ could not entirely neglect MHA + M_2HA species. Expressions for $q = 1$ ($p = 1, 2$) were therefore introduced in eqn. 5, using approximate values for $\varkappa_{111}$ and $\varkappa_{211}$ *. The constant was obtained from

$$\varkappa_{121} = \frac{C_S - C_A - a \sum_{q=0}^{q=4} (q-1)\varkappa_{0q1}h^q}{h^2a\left[C_M - C_A + a \sum_{q=0}^{q=4} \varkappa_{0q1}h^q\right]} \qquad (9)$$

and the value obtained was refined by successive approximation.

* The contributions of $-\varkappa_{111}\, mh$, ($C_A \sim C_M$), and $-mh(\varkappa_{111} + m\varkappa_{211})$, ($C_M \sim 10C_A$), to the denominator of eqn. 5 were 0.05–1 % and 0.4–5 %, respectively, in the calculations compiled in Table 3.

Species of the type MOH were considered in all of the Mn(II) systems and in the Mg(II)-phosphorylethanolamine system (for $r = 0$); $*K_1 = 10^{-10.7}$ and 10^{-12} were used [12].

The S values assumed gave approximately constant values for $\varkappa_{121}$ (phosphorylserylglutamic acid systems, $r = 2$) and the left hand side of eqn. 4 (other systems, $r = 1$) in the selected parts of the titration curves (*cf.* Table 3). Other S values (1 and 0, respectively) gave a definitely trend in the left hand side of eqn. 4 ($r = 1$ and $r = 0$) with pH.

In the former calculations $\varkappa_{121}$ and the left hand side of eqn. 4 were also almost constant for different C_M, and consequently the species were mononuclear [13]. For $r = 1$, 0 in the phosphorylserylglutamic acid systems and $r = 0$ in some of the other systems, the computations indicated dinuclear complex formation. T will then be 1 and 0, respectively, which is apparent from Figs. 3—5. Individual calculations of systems studied at different C_M are surveyed graphically [6] for these r values by the plot φ_r against $h(K_{H_{r+1}A})^{-1}$, where

$$\varphi_r = \frac{Z(1 + *K_1 h^{-1})}{(1-Z)\varkappa_{0r1} a h^r} - \sum_{q=r+2}^{S} \varkappa_{1q1}\varkappa_{0r1}^{-1} h^{q-r} - \sum_{q=r+1}^{T} \frac{1-2Z}{1-Z} \varkappa_{2q1}\varkappa_{0r1}^{-1} h^{q-r} m =$$

$$= h b_{MH_{r+1}A}(K_{H_{r+1}A})^{-1} + b_{MH_rA} + \frac{(1-2Z)}{(1-Z)} b_{MH_rA} b_{M_2H_rA} m \qquad (4a)$$

Due to the last expression in the right hand side, the presence of dinuclear species will increase φ_r with C_M (Figs. 3, 4b, 5). When the equilibria are mononuclear, this expression cancels, and the plotted data are independant of C_M (Figs. 3 and 4).

Phosphorylated seryllysine, glycylserine * and glycylserylglycine were titrated at one (Ca^{2+} and Mn^{2+}) and two (Mg^{2+}) C_M only (Table 2), due to a lack of material. All of these Ca^{2+} systems as well as the Mn^{2+}system of phosphorylseryllysine were assumed to be mononuclear; *cf.* the plots of the Ca(II)-phosphorylserylglycine and Mg(II)-phosphorylseryllysine systems in Figs. 3 and 4b. With regard to other glycine peptides (Figs. 3 and 4b) glycylphosphorylserylglycine was assumed to form Mg_2A species, though there is only weak experimental evidence (Fig. 4b). As this ligand might also form Mn_2A complexes, no calculations were carried out for $r = 0$ in its Mn(II) system.

Values computed for $\varkappa_{pq1}$ were converted to corresponding stability constants, $b_{M_pH_qA}$ which are found in Table 2.

DISCUSSION

Data of the phosphorylserylglycine systems indicate the presence of dinuclear magnesium and manganese(II) complexes, while the existence of such a calcium complex could not be demonstrated (Fig. 3). The same type of complex formation seems to be valid for the other glycine peptide systems. This follows from Fig. 4b and from their "b_{CaA}", which are smaller than the b_{CaA} of the phosphorylserylglycine system (Table 2). Occurrence of a greater amount

* No Mn^{2+} titration could therefore be carried out with this ligand.

of Ca_2A should have appreciably increased the size of these "b_{CaA}" (in this case taken as equal to the left hand side of eqn. 4 at $r = 0$), as $C_M \sim 10C_A$.

The increase in φ_r (eqn. 4a) with C_M is used to show the presence of dinuclear species (Figs. 3, 4b, 5). As this increase is not generally observed (in all systems at all r; *cf.* Figs. 3 and 4 and the previous section), it cannot be due to systematic errors, arising from the variations in C_M. Such errors would be, *e.g.*, changed activity factors and liquid junction potentials, caused by the increasing contribution of complex forming metal ions to the ionic strength.

At the present C_A, weak MH_3A (phosphorylserylglutamic acid systems) and MH_2A (other systems) complexes (stability constant < 3 M^{-1}) cannot be detected. Existence of such species would, however, neither invalidate those reported, or appreciably change the relations between their constants. There is no support for the occurrence of complexes with two or more ligands per central metal ion, as no upward trend in φ_r is seen for decreasing h (Figs. 3—5). We can also eliminate complexes of the type MA(OH), which most probably should be formed in the phosphorylethanolamine-Mn(II) system. If we assume $*K_{MA}$ to be equal to $*K_1$ ($= 10^{-10.7}$, Ref.[12]) in this system, [MA(OH)] will constitute 2 % of [MA] and b_{MA} will be changed from 342 to 332 M^{-1} at the most alkaline point of calculation (0.1000 ml 0.2979 M KOH; $-\log h = 8.850$) at $C_M \sim 10C_A$ *. Phosphorylethanolamine-Mn(II) was the only system, where MOH species had a marked influence on b_{MA}. Neglecting these species, "b_{MA}" will be 460 ± 20 ($C_M \sim C_A$) and 690 ± 40 M^{-1} ($C_M \sim 10C_A$).

Previous works on complexes of related phosphate esters. At 0.1—0.2 M ionic strength and 20—25° stability constants of the following 1:1 complexes have been reported: **

HPO_4^{2-}—Ca^{2+}; —Mg^{2+}; —Mn^{2+} (Ref.[12]): 32; —; —; (Ref.[14]): 50; 76; 382; AMP^{2-}—Ca^{2+}; —Mg^{2+}; —Mn^{2+} (Ref.[14]): 27; 49; 156; (Refs.[15,16]): 26; 49; —; (Ref.[17]): 58; 89; 204; (Ref.[18]): —; 100; —; CrP^{3-}—Ca^{2+}; —Mg^{2+}; —Mn^{2+} (Ref.[14]): 14; 20; 110; $FructoseP^{2-}$—Ca^{2+}; —Mg^{2+} (Ref.[19]): 30; 39; $GlycerolP^{2-}$—Ca^{2+}; —Mg^{2+} (Ref.[19]): 46; 63; $GA2P^{3-}$ —Mg^{2+}; —Mn^{2+} (Ref.[20]): 280; 1225; PAP^{3-} —Mg^{2+}; —Mn^{2+} (Ref.[20]): 180; 560; $SerP^{2-}$ —Ca^{2+}; —Mg^{2+}; —Mn^{2+} (Ref.[6]): 27; 40; 81; $SerP^{3-}$ —Ca^{2+}; —Mg^{2+}; —Mn^{2+} (Ref.[6]):138; 245; 6600 M^{-1}.

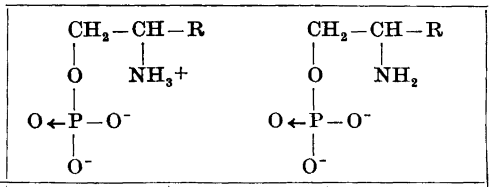
In all ligands the phosphate group has the charge —2. With the exception of the $GA2P^{3-}$, PAP^{3-}, and $SerP^{3-}$ constants, all corresponding constants, including the present (Table 2), are similar in magnitude. The phosphate group may therefore be the main dentate group in these systems [19].

The $GA2P^{3-}$ and PAP^{3-} constants can be explained by the presence of the carboxyl group; therefore these ligands may also require consideration of M_2A species for accurate interpretation of data $C_M > C_A$. Wold *et al.*[20] apparently used such concentrations (no C_M is given), though they did not consider any dinuclear species. The aforementioned $SerP^{3-}$ constants [6] have

* As $r = 0$, the expressions $-b_{MA}*K_{MA}h^{-1}$ and $-b_{MA}*K_{MA}h^{-1}m$ related to [MA(OH)] were introduced in eqn. 4 (left hand side) and eqn. 5 (denominator), respectively, using $b_{MA} = 356$ M^{-1}.

** The abbreviations signify: AMP: adenosine-5′-monophosphate, CrP^{3-}: creatinephosphate; $FructoseP^{2-}$: fructose monophosphate; $GlycerolP^{2-}$: glycerol monophosphate; $GA2P^{3-}$: glyceric acid 2-phosphate; PAP^{3-}: enolpuruvic acid phosphate; SerP: O-phosphorylserine.

Table 4. Effect of different substituents, R, on the complex formation of compounds indicated.



Compound	R	b_{MHA}			b_{MA}			b_{M_2A}		
		Ca	Mg	Mn	Ca	Mg	Mn	Ca	Mg	Mn
P-ethanolamine	$-H$	13	17	53	36	50	356	—*	—*	—*
SerP **	$-COO^-$	27	40	81	138	245	6600	—*	14	17
SerP-Gly	$-CO-NH-CH_2-COO^-$	20	25	78	71	86	422	—*	18	35
SerP-Glu	$-CO-NH-CH(-(CH_2)_2-COO^-)-COO^-$	44	43	173	138	122	962	29	64	89
SerP-Lys	$-CO-NH-CH(-(CH_2)_4-NH_3^+)-COO^-$	14	17	60	34	43	212	—*	—*	—*

* No true evidence for existence of M_2A species, see the text.
** Ref.[6]

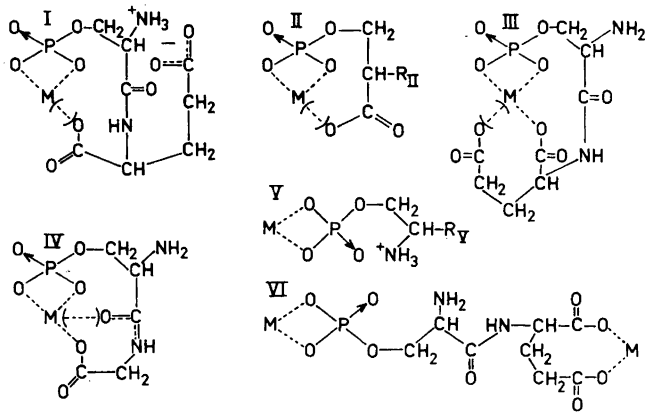


Fig. 6. Schematic structures of metal complexes. Coordination positions of the metal ions may be filled by water molecules.
I, III, VI: MHA, MA and M_2A complexes of phosphorylserylglutamic acid.
II: MHA complex; phosphorylserine: $R_{II} = -NH_3^+$; glycylphosphorylserine: $R_{II} = -NH-CO-CH_2-NH_3^+$.
IV: MA complex of phosphorylserylglycine.
V: MHA complex; phosphorylethanolamine: $R_V = -H$; phosphorylseryllysine: $R_V = -CO-NH-CH(-(CH_2)_4-NH_3^+)-COO^-$.

been computed from data $C_M \sim C_A/2$. A reconsideration of the findings of that work [6], when compared with the present results, suggests that magnesium and manganese(II) may form M_2A complexes, whereas calcium might not (Figs. 4b, c in Ref.[6], where $Z/(1-Z)A$ equals φ_0 of eqn. 4a).

Coordination sites. Table 4 shows influence of substituents (R) on the coordination of compounds with phosphorylethanolamine structure. The carboxyl group substituents increase the complex forming tendency; the phosphorylserine complexes are more stable than those of phosphorylethanolamine and the glutamic acid peptide complexes are more stable than those of the glycine peptide.

The α-carboxyl group may, thus, partially contribute to the MHA stability of phosphorylserylglutamic acid and probably to that of phosphorylated serine, glycylserine, and glycylserylglycine (*cf.* Table 2). This is shown by I and II in Fig. 6. The γ-carboxyl group of phosphorylserylglutamic acid may be intramolecularly neutralized by the amino group (I). Such "neutralization" may occur also in the MHA species of phosphorylated serylglycine (amino and carboxyl groups) and seryllysine [10] (ε-amino and carboxyl groups).

Decharging of the amino groups of the glutamic acid and glycine peptides may liberate the carboxyl groups mentioned, which are then free to interact with the central metal ions (III and IV in Fig. 6). These groups rather than the amino groups may engage the metal ions, as the present metal ions bind to oxygen in preference to nitrogen [21-24]. Contribution through the peptide bond oxygen [25], favoured due to resonance, is possible in the phosphorylserylglycine MA complexes, IV in Fig. 6 (more unlikely due to steric reasons in the reversed dipeptide and the tripeptide complexes, as judged from Stuart-Briegleb molecular models). The relatively high b_{MA} of the phosphorylserine complexes (Table 4) may be explained by the capacity of this ligand to bind both through the phosphate group and a five-membered chelate ring, formed by the amino and carboxyl groups *.

The MHA complex formation of phosphorylated ethanolamine and seryllysine may be influenced by the α-amino groups (V in Fig. 6); *cf.* the low b_{MHA} in Table 4. These groups may also be involved in the MA complex formations (Mn^{2+} complexes, specially [21,22]), but they are then deprotonized and increase the stability. — The general weak complexity of phosphorylseryllysine and inability to form M_2A species (Table 4) indicate some impairment of its donor groups; probably due to interaction of the charged ε-amino group [10].

Complex formation of dinuclear type may straighten the ligands, and one metal ion may be bound at each end. Phosphate and carboxyl groups may be the main dentate groups (*cf.* VI in Fig. 6). Apart from the carboxyl group the peptide bond carbonyl group [25] may contribute in the glycine peptide complexes and the amino group in the phosphorylserine complexes. Their b_{M_2A} (Table 2) compare better with b_{MA} (Ref.[4]) of glycollate, lactate ($-Mg^{2+}$: 8—23), glycylglycine ($-Mg^{2+}$: 11), succinate ($-Mg^{2+}$: 16), and glutarate ($-Mg^{2+}$: 12) complexes than b_{MA} (Ref.[4]) of acetate, propionate, and butyrate

* Dividing the b_{MA} (Table 4) with approximate b_{MA} of the corresponding complexes of an esterbound phosphate group (see compilation on page 2445 and Table 2), (Ca^{2+}): 25; (Mg^{2+}): 35; (Mn^{2+}): 100, results in: 6; 7; 66, which may be compared to b_{MA} of alanine complexes [26] (multiplied with an activity factor [27], 0.56, and a statistical factor [28], 0.17): 2; 8; 97 M^{-1}.

(—Mg^{2+}: ~3) complexes [23]. The lower stability of the dinuclear tripeptide complex relative to those of the other glycine peptides (Table 2) may be attributed to an error in b_{M_2A}, accumulated from errors in $\varkappa_{0q1}$ and $\varkappa_{1q1}$, or to the more rigid nature of the tripeptide.

The b_{M_2A} of the Ca and Mg(II) complexes of phosphorylserylglutamic acid (Table 4) are very similar to b_{CaA} and b_{MgA} reported for glutamic acid complexes [29]: 27 and 79 M^{-1} in 0.1 M KCl at 25°. A structure (VI, Fig. 6) involving the two carboxyl groups as dentate sites thus seems reasonable [30].

Conclusions. pK values of the acid complexes (Table 5) indicate that all present complexes, except the MH_2A of the phosphorylserylglutamic acid systems and the MA of those of phosphorylethanolamine, exist in the physiological pH range (pH 6—8). The pK_{MHA} values are appreciably lower than their corresponding pK_{HA} (the legends of Figs. 1 and 2). The amino groups will, thus, become more acidic, when the ligands are complexed. These groups will also increase the complex stability 2—4 times by their deprotonizing processes (*cf.* b_{MHA} and b_{MA} in Tables 2 and 4), though the may not act as donor groups themselves (Fig. 6).

Table 2 shows that the stability order $Ca^{2+} < Mg^{2+} < Mn^{2+}$ in general is valid. An increasing number of dentate groups, however, will successively increase the calcium complex stability relative that of magnesium [1] (Table 4). As a consequence the larger calcium ion forms mononuclear phosphorylserylglutamic acid complexes with equal or greater strength as compared to magnesium. The smaller magnesium ion may then fail to engage as many donor atoms as the calcium ion [1,23].

A further consequence of ionic size may be that magnesium and manganese ions form dinuclear complexes more easily than calcium ions (Tables 2 and 4). The dinuclear species may have a conformation other than the mononuclear species (*cf.* Fig. 6 and p. 2447), and Mg^{2+} or Mn^{2+} may induce a stretched conformation, when Ca^{2+} induces a compact conformation. The findings therefore offer a possibility beside that proposed by Williams [1] to explain the action of Mg^{2+} (Mn^{2+}) as opposed to Ca^{2+} in certain enzyme systems. The enzymic meaning may be that the activating ions by complex formation give the enzyme active site (alternatively the substrate or both the enzyme

Table 5. pK values of acid complexes.

Ligand	Constant	Ca	Mg	Mn
P-ethanolamine	pK_{MHA}	9.70	9.65	9.29
SerP-Gly		7.45	7.46	7.27
SerP-Lys		7.18	7.18	7.04
Gly-SerP-Gly		7.85	7.89	—
Gly-SerP		8.12	8.19	—
		7.76	7.80	7.50
SerP-Glu	pK_{MH_2A}	5.12	5.05	4.87
	pK_{M_2HA}	7.34	7.47	6.88

active site and the substrate) a suitable conformation for catalysis. When an extended shape is catalytically favoured, magnesium (manganese) ions may activate and calcium ions inhibit, while these effects may be reversed, if a compact form is preferred.

APPENDIX

The results obtained for titrations with $C_M \sim C_A$ rest on the assumption that the species formed are mononuclear in M and A. We therefore tried to check some of these results by calculations performed by another procedure. Values of a were found directly by integration of a $\bar{q}$ (log h) curve, where $\bar{q} = C_S/C_A$. The equation used was derived in analogy with the principles given by Sillén [31], and we will only give the main result.

We define the function

$$S = \sum_p \sum_q \sum_v [M_pH_qA_v] = \sum_p \sum_q \sum_v m^p h^q a^v \varkappa_{pqv} \quad \begin{matrix}(p,q,v = 0, 1, 2, \ldots; \\ v \neq 0, \text{ if } (p+q) > 1)\end{matrix} \qquad (10)\ *$$

and write the differential of this function $S(m,h,a)$

$$dS = (C_A - a)\ d\ \ln\ a + C_S\ d \ln h + (C_M - m)\ d \ln m \qquad (11)$$

By introducing $\bar{q}C_A = C_S$ and then transform to

$$d(C_A \ln\ a + C_M \ln\ m - S - a - m) = \ln\ a\ dC_A - \bar{q}C_A\ d\ \ln\ h + \ln\ m\ dC_M \qquad (12)$$

we get

$$\left(\frac{\partial \ln a}{\partial \ln h}\right)_{C_A,C_M} = -\left(\frac{\partial \bar{q}C_A}{\partial C_A}\right)_{h,C_M} = -\left[q + \left(\frac{\partial \bar{q}}{\partial \ln C_A}\right)_{h,C_M}\right] \qquad (13)$$

We solve this equation for log a in the range log h_o to log h and add $-\log C_A$ to both sides

$$\log\ a/C_A = \log\ a_0/C_A - \left[\int_{\log h_o}^{\log h} \left[\bar{q} + \log e \left(\frac{\partial \bar{q}}{\partial \log C_A}\right)_{h,C_M}\right] d\ \log h\right]_{C_A,C_M} \qquad (14)$$

For $p = 0,1$ we can write the differential of the function C_M (m,h,a)

$$dC_M = C_M\ d\ \ln\ m + (C_A - a\sum_{q=0} h^q \varkappa_{0q1}) d\ \ln\ a + (C_S - a\sum_{q=1} q h^q \varkappa_{0q1}) d\ \ln\ h = \\ = C_M\ d\ \ln\ m \qquad (15)$$

Transformation and substitution, $(\bar{q}C_A = C_S)$, gives

$$d(C_A \ln\ a - a\sum_{q=0} h^q \varkappa_{0q1}) = \ln\ a\ dC_A - \bar{q}C_A\ d\ \ln\ h \qquad (16)$$

whence we obtain eqns. 13 and 14, which are independent of constancy in C_M. For $v = 0, 1$ the partial derivative $(\partial \bar{q}/\partial\ \log\ C_A)_{h,C_M}$ in eqn. 14 becomes zero. Consequently, the condition $p,v = 0, 1$ will reduce eqn. 14 to

* We thus first assume that v may attain higher values than 0 and 1, and that species corresponding to these v are included in C_A, C_M and C_S (*cf.* eqns. 2, 3 and the symbol section).

Table 6. Comparison of a,Z values for the Ca(II)—O-phosphorylserylglutamic acid system ($C_M \sim C_A$, for details see Table 3), as computed by eqns. 5, 6, 8, and eqns. 14a *, 17, 18*.

** $-\log h$	$a \times 10^{10}$ (14a)	$a \times 10^{10}$ (5)	** $-\log h$	$a \times 10^{7}$ (14a)	$a \times 10^{7}$ (6,8)	** Z (17,18)	Z (6,8)	** $-\log h$	$a \times 10^{4}$ (14a)	$a \times 10^{4}$ (6,8)
			4.948	2.716	2.716	0.0547	0.0552	7.242	3.251	3.247
3.764	3.051	3.049	5.048	4.303	4.303	0.0607	0.0606	7.323	3.842	3.835
3.875	6.406	6.406	5.148	6.690	6.692	0.0666	0.0670	7.436	4.811	4.810
3.987	13.26	13.27	5.249	10.28	10.28	0.0729	0.0734	7.534	5.796	5.800
4.097	26.54	26.56	5.350	15.52	15.51	0.0794	0.0802	7.624	6.827	6.835
4.205	51.32	51.34	5.453	23.20	23.22	0.0860	0.0864	7.731	8.200	8.220
4.313	97.23	97.30	5.558	34.32	34.41	0.0936	0.0920	7.855	9.975	10.03
			5.666	50.40	50.74	0.1011	0.0952	7.969	11.75	11.84
								8.079	13.55	13.70

* The trapezoid formula was used in the plot $\bar{q}$ or $\bar{q}_h$ *versus* log h. Smoth curves were drawn through the experimental points, and the integration was done with intervals of 0.05 log h units.

** $\log h_0 = -3.750$, where $\log a_0/C_{A_0} \approx \log [(\bar{q}-2)/\sum_{q=0}^{q=4} (q-2)h^q \varkappa_{0q_1}]$ and $\log C_{A_h}/C_{A_0} =$ $= \log \left(a_0 C_{A_0}^{-1} \sum_{q=0}^{q=4} h^q \varkappa_{0q_1}\right)$; the $\varkappa_{0q_1}$ were obtained from the pK of the legend of Fig. 1a.

$$\log a/C_A = \log a_0/C_{A_0} - \int_{\log h_0}^{\log h} \bar{q} \, \mathrm{d} \log h \qquad (14a)$$

Table 6 shows values of a computed by eqn. 14a for the Ca(II)-O-phosphorylserylglutamic acid system ($C_M \sim C_A$), as compared to those computed by eqns. 5, 6, and 8 (*cf.* Table 3).

In the pH region, where the acid ionizations of this ligand overlap, some Z values were checked by

$$Z = \frac{1 - C_{A_h}/C_A}{C_M/C_A} \qquad (17)$$

Here C_{A_h} equals $a \sum_{q=0}^{q=4} h^q \varkappa_{0q_1}$ and C_{A_h}/C_A was obtained from (*cf.* eqn. 25 in Ref.[31])

$$\log C_{A_h}/C_A = \log C_{A_h}/C_{A_0} + \int_{\log h_0}^{\log h} \bar{q}_h \, \mathrm{d} \log h - \int_{\log h_0}^{\log h} \bar{q} \, \mathrm{d} \log h \qquad (18)$$

where

$$\bar{q}_h = \left(a \sum_{q=1}^{q=4} q h^q \varkappa_{0q_1}\right) C_{A_h}^{-1} \qquad (19)$$

The first integral in eqn. 18 was determined by a $\bar{q}_h(\log h)$ curve, based on an acid-base titration of the ligand (H in Fig. 1a). The Z values calculated are listed in Table 6, and for comparison those calculated previously (Table 3) by eqns. 6, 8, are included.

The stability constants ($b_{MH_2A} = 12 \pm 1$; $b_{MHA} = 44 \pm 2$; $b_{MA} = 140 \pm 9$), which finally were computed, agree with those of Table 3 within the uncertainties given.

Acknowledgements. I wish to express my sincere gratitude to Dr. G. Fölsch for the gift of the phosphorylated compounds, to Laborator Ido Leden for valuable comments on the manuscript, to Drs. S. Aleby and K. Larsson for valuable discussion, and to Mrs. Clara Wadsworth for correcting the English. The investigation was aided by grants from the *Medical Faculty, University of Göteborg* and from *Lennanders' fund.*

REFERENCES

1. Williams, R. J. P. in Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes*, 2nd ed., Academic Press, New York **1** (1959) 391.
2. Koshland, D. E., Jr. *Ibid.* **1** (1959) 305.
3. Vallee, B. L. *Ibid.* **3** (1960) 225.
4. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability Constants, Part I: Organic Ligands*, The Chemical Society Spec. Publ. No. 6, London 1957.
5. Biedermann, G. and Sillén, L. G. *Arkiv Kemi* **5** (1953) 425.
6. Österberg, R. *Arkiv Kemi* **13** (1959) 393.
7. Fölsch, G. *Acta Chem. Scand.* **12** (1958) 561.
8. Fölsch, G. *Acta Chem. Scand.* **13** (1959) 1407.
9. Fölsch, G. and Österberg, R. *J. Biol. Chem.* **234** (1959) 2298.
10. Fölsch, G. and Österberg, R. *Acta Chem. Scand.* **15** (1961) 1963.
11. Powell, J. E. and Hiller, M. A. *J. Chem. Educ.* **34** (1957) 330.
12. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability Constants, Part II: Inorganic Ligands*, The Chemical Society Spec. Publ. No. 7, London 1958.
13. Leden, I. *Dissertation*, Lund 1943, pp. 22–23.
14. Smith, R. M. and Alberty, R. A. *J. Am. Chem. Soc.* **78** (1956) 2376.
15. Martell, A. E. and Schwarzenbach, G. *Helv. Chim. Acta* **39** (1956) 653.
16. Schwarzenbach, G. and Martell, A. E. *J. Phys. Chem.* **62** (1958) 886.
17. Walaas, E. *Acta Chem. Scand.* **12** (1958) 528.
18. Nanninga, L. B. *J. Phys. Chem.* **61** (1957) 1144.
19. Schwarzenbach, G. and Anderegg, G. *Helv. Chim. Acta* **40** (1957) 1229.
20. Wold, F. and Ballou, C. E. *J. Biol. Chem.* **227** (1957) 301.
21. Irving, H. and Williams, R. J. P. *J. Chem. Soc.* **1953** 3192.
22. Schwarzenbach, G. *Anal. Chem.* **32** (1960) 6.
23. Williams, R. J. P. *J. Chem. Soc.* **1952** 3770.
24. Basolo, F. and Pearson, R. G. *Mechanisms of Inorganic Reactions*, John Wiley & Sons, London 1958, pp. 60–61.
25. Rabin, B. R. *Trans. Faraday Soc.* **52** (1956) 1130.
26. Monk, C. B. *Trans. Faraday Soc.* **47** (1951) 297.
27. Davies, C. W. *J. Chem. Soc.* **1938** 2093.
28. Bjerrum, J. *Dissertation*, Copenhagen 1941 p. 40.
29. Lumb, R. F. and Martell, A. E. *J. Phys. Chem.* **57** (1953) 690.
30. Schubert, J. *J. Am. Chem. Soc.* **76** (1954) 3442.
31. Sillén. L. G. *Acta Chem. Scand.* **15** (1961) 1981.

Received May 22, 1962.