DNA-like and Phospholipid-like Phosphorylated Polystyrenes: Characterization, Distribution of Functional Groups, and Calcium Complexation Properties

E. IMBERT, A. SOUIRTI, V. MENARD, F. PFLUGER, M. JOZEFOWICZ, and V. MIGONNEY*

Laboratoire de Recherches sur les Macromolécules, Institut Galilée, Université Paris XIII, Avenue J.B. Clément, 93430 Villetaneuse, France

SYNOPSIS

The phosphorylation of cross-linked polystyrene resins has been previously described. As seen by their interaction with lupus antibodies, these polymers were found to mimic either DNA or phospholipid antigens, depending on the degree of substitution by phosphate groups. When an alkyl spacer was included between phosphate groups and macromolecular chains, difficulties in synthesis and desorption of the biological species from the resins were seen. In this article, kinetic aspects of the synthesis of phosphorylated polymers, possessing sulfamide spacers, are discussed. The characterization of these phosphorylated polymers by acidimetric titration is extensively described. This technique differentiates and quantifies the grafted phosphate groups, which can be either phosphomonoester or phosphodiester in nature. We demonstrate that these polymers form complexes with calcium ions, and that the affinity of the ions is closely associated with the type of phosphate groups. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

The random substitution of macromolecular chains with chemical functional groups leads to the development of new polymeric, biospecific surfaces. The nature, number, and distribution of the functional groups along the macromolecular chains are the keys, which control the final biologic properties of the materials. By this process, the required biospecific sites for specific protein interactions are created on the surface.[1,2] Heparin-like biomaterials were the first examples[3-5] of biospecific polymers using this principle, which principle has since been extensively verified.

Based on this principle, phosphorylated polystyrene resins have been synthesized[6]; it was demonstrated that they mimic natural antigens, whose epitopes include phosphate groups, such as DNA or phospholipids, and it was shown they interact specifically with anti-DNA and antiphospholipid antibodies from the sera of systemic lupus erythmatosis patients. The specificity of the interactions depends on the amount of substitution of phosphate groups: resins with approximately 25% substitution are DNA-like, while resins containing approximately 60% phosphate groups behave in a phospholipid-like manner.[7] The difference in the recognition of the resins by two antibodies (anti-DNA or antiphospholipid) was attributed to a different distribution of phosphate groups at the surface. These results indicate two distinct idiotypic sites, which are composed of arrays of phosphate groups. Also, these findings indicate that the antibodies adsorb via their Fab fragments.

The primary characterization of these resins was heretofore performed by elemental analysis and IR spectroscopy. These methods were limited by an inability to discriminate the types of phosphate groups present. These polymers, derived from poly(hydroxyethylstyrene) by a reaction with oxyphosphorous chloride, have a variety of active sites, probably composed of phosphomonoester, phosphodiester, or pyrophosphate groups. In order to determine more

* To whom correspondence should be addressed.

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precisely the nature of the phosphate groups, acidimetric titration was performed, taking advantage of the differences in pK_As of the different acidic groups on the resins. When complexed by calcium, phospholipids can develop highly specific interactions with vitamin K-dependent factors, which lead to the formation of ternary complexes: phospholipid/Ca^{2+}/coagulation factor.

We have shown that the same phenomenon could occur using phospholipid-like resins instead of phospholipids[8]; furthermore, the differences in the type of phosphate groups present on the resins may induce differences in the complexation of calcium ions. In this study, the complexation of Ca^{2+} by various phosphorylated resins has been performed in order to investigate this hypothesis. As a result, the biological properties of these biospecific polymers may be of great interest.

EXPERIMENTAL

Materials

Crosslinked polystyrene resins, BioBeads S-X2 200 (Bio-Rad Laboratories, Richmond, CA, USA), were washed successively with 1 M NaOH and 1 M HCl. The beads were then thoroughly washed with distilled water and were dried at 60°C under vacuum. All solvents, tetrahydrofuran (THF), dioxane, acetone, dichloromethane, trimethylphosphate, and dimethylsulphoxide (DMSO), were supplied by Carlo Erba (Rueil–Malmaison, France) and were reagent grade or better. Chlorosulfonic acid, phosphoryl chloride, ethanolamine, calcium chloride, and sodium chloride were purchased from Prolabo (Paris, France). Elemental analyses were performed by the Service Central d'Analyses CNRS (Vernaison, France).

Acidimetric titration was performed with an automatic titrator TT100/TT300 from Tacussel Solea (Villeurbanne, France). The process involved a potentiometric titration in the heterogeneous phase.

Calcium complexation is measured by coagulation assays, using a method that involves essentially activated partial thromboplastin time (APTT) technique.

Methods

Preparation of Phosphorylated Polystyrene

The preparation of the phosphorylated polystyrene resins was performed in three steps:

1. Chlorosulfonation of the phenyl groups of poly(styrene) (PS) was performed by reaction with chorosulphonic acid $HClSO_3$ at room temperature. A large excess of $HClSO_3$ is used (× 10/PS) in order to reach approximately 90% substitution of the chlorosulphonyl groups in 30 min. The subsequent poly(chorosulphonyl styrene) (PCSS) obtained is quickly washed with dichloromethane, and rinsed with acetone and dichloromethane.
2. Condensation of ethanolamine with the PCSS, obtained in the first step, is achieved by the addition of an excess of ethanolamine (× 5/SO_2Cl) in dichloromethane. The suspension is refluxed for 18 h and extensively washed with the following solvents: dichloromethane, mixtures of water/dioxane (20/80, 50/50, 80/20), dioxane, mixtures of water/ethanol (20/80, 50/50, 80/20), and then dried under vacuum at 60°C.
3. Phosphorylation is performed on the poly(ethanolamine sulfamide styrene) (PEASS) in dried trimethyl phosphate by adding phosphorous oxychloride. The suspension is refluxed for 8, 18, or 40 h, after which the reaction is stopped by the addition of water. The total hydrolysis of the phosphorous chloride intermediate is performed by extensive washings in large amounts of water and 1 M sodium hydroxide. The resin is then dried at 50°C under vacuum.

When the sulfonated polystyrene (SPS) derivative is desired, a 24 h hydrolysis of PCSS (from step 1), with a 2 M sodium hydroxide aqueous solution, is performed.

Acidimetric Titration

Acidimetric titrations were performed as follows: 50 to 100 mg of phosphorylated resin were suspended in 5 mL of a mixture of DMSO/water (75/25). In order to increase the conductivity of the solution, tetrabutylammonium perchlorate was added to obtain a final concentration of 0.25 M. A combined pH reference microelectrode was introduced into the microcell in order to measure accurately the pH of the solution. An automatic titrator was responsible for the addition of small amounts (1 μL) of the titrating solution (sodium hydroxyde 0.283 M), which could be performed every 100 sec. The time between additions could be increased if the exchange equilibrium between the ionic polymer and the solution

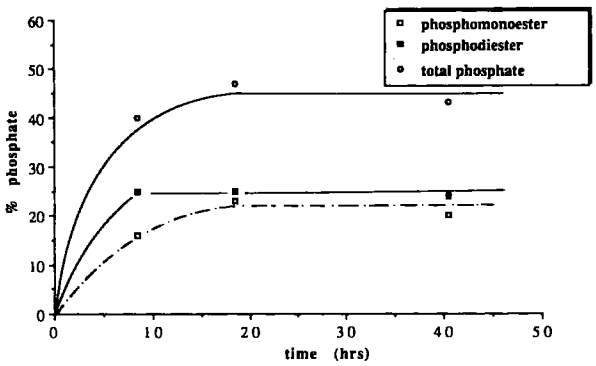

Figure 1 Influence of the reaction time on the phosphate contents of the phosphorylated derivative.

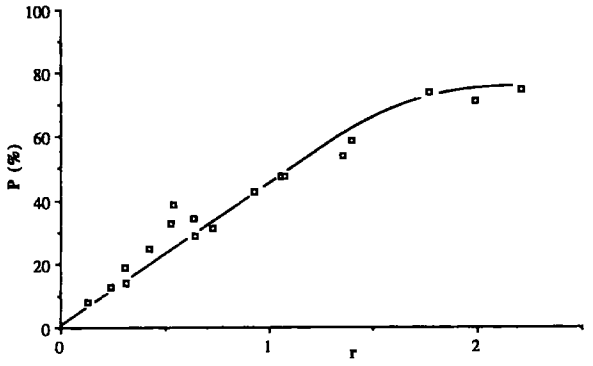

Figure 2 The dependence of the phosphate content of the resin on the molar ratio r ($POCl_3$/OH).

was not reached. Under these conditions, the titration curves obtained were reproducible.

Complexation of Calcium Ions by the Resins

Resins were first passivated by bovine serum albumin (BSA) as follows: 25 to 30 mg of phosphorylated resin were incubated for 30 min with a 0.4 g/L BSA solution in Michaelis Buffer (pH 7.3). The passivated resins were rinsed five times with Michaelis Buffer and were centrifugated at 13,000 rpm; the supernatent was then removed.

One mL of 0.01 M to 0.1 M calcium solution in Michaelis Buffer was incubated with the BSA-passivated resin for 1 h. The calcium concentration of the supernatant was measured by a coagulation assay, using the activated partial thromboplastin time (APTT) method.

The APTT assay was performed as follows: 100 μL of platelet poor plasma are incubated at 37°C for 3 min with 100 μL of cephalin–kaolin; then 100 μL of $CaCl_2$ solution in Michaelis Buffer (standard or supernatant) are added at the start of the chronometer. The APTT, corresponding to the apparition of the clot, is read. A calibration curve of APTT time vs. calcium chloride is established, by varying

Table I Chemical Composition of the Phosphorylated Polystyrene[a]

	%					
Resins	PS	$-SO_3$	—OH	Pmono	Pdi	PS—P
PS—P_6	6.5	4.5	76.5	0.3	6	6.3
PS—P_{11}	10.5	2.5	65.5	0.6	10.5	11
PS—P_{12}	15	0.1	60	0.6	12	12.5
PS—P_{17}	8	1	58	1	16	17
PS—P_{23}	10	4	41.5	2	21	23
PS—P_{27}	16.5	1	32.5	5	22	27
PS—P_{29}	20	3.5	25	7.5	22	29.5
PS—P_{31}	15	0	31	8	23	31
PS—P_{32}	15	3	26	10	22.5	32.5
PS—P_{37}	7	0	32	12.5	24.5	37
PS—P_{41}	13.5	3	18	17	24	41
PS—P_{46}	10	3	16.5	21.5	24.5	46
PS—P_{46}	14.5	8	7	22.5	23.5	46
PS—P_{52}	11.5	10	0	28	24	52
PS—P_{57}	3.5	0	13	31	26	57
PS—P_{70}	0	4.5	0	44	25.5	69.5
PS—P_{72}	0	1	0	45	27	72
PS—P_{73}	0	0	0	46	27	73

[a] Results from elemental analysis and acidimetric titration.

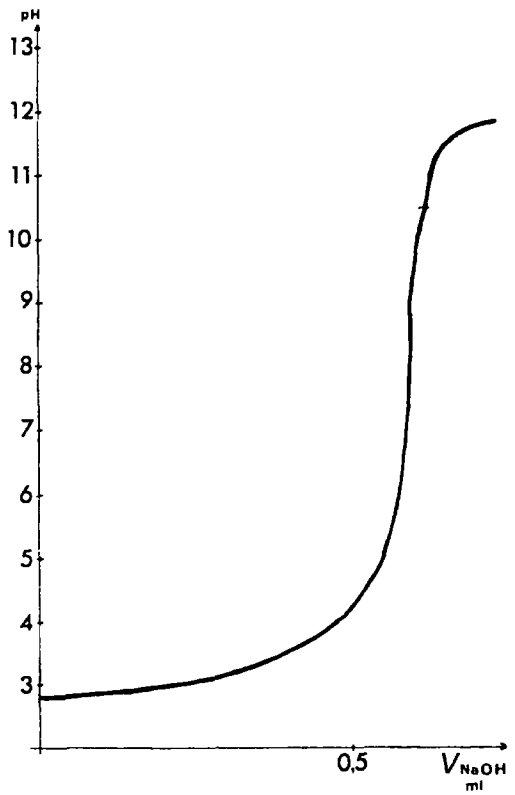

Figure 3 Typical acidimetric titration curve of a low phosphorylated polystyrene derivative.

the concentration of the calcium chloride solutions in the coagulation assay from 0.01 M to 0.05 M.

The calibration curve was used to calculate the amount of complexed calcium in mol per gram of resin through the APTT of the supernatant.

RESULTS AND DISCUSSION

Effect of the Phosphorylation Time

PEASS was allowed to react with $POCl_3$ in the ratio of 1 $POCl_3$ for 1 hydroxyl group, with three reaction times: 8, 18, and 40 h. The total phosphorous amount was determined by elemental analysis and the amounts of phophodiester and phosphomonoester were determined by acidimetric titration (Table III).

The results show (Fig. 1) that an 18 h reaction time is enough to reach a final state. Subsequent phosphorylation experiments were thus performed with this reaction time.

Effect of the Concentration of $POCl_3$

The effect of the molar ratio $POCl_3$/OH, r, on the phosphate content, was also studied for the 18 h reaction time. The variation of the ratio (% of fixed P)/OH, vs. r, is plotted in Figure 2. This relationship was used to choose r in order to obtain a predetermined composition of phosphorylated resin. Numerous phosphorylated resins were synthesized and were characterized with elemental analysis and acidimetric titration (Table I).

Acidimetric Titration

In order to obtain a pH-scale that encompasses the pK_As exhibited by the different phosphate groups, a variety of solvent mixtures were tried. The optimal titration curves were obtained using 75% DMSO, 25% H_2O as solvent, with tetrabutylammonium perchlorate as electrolyte. The resins were systematically washed in hydrochloric acid to acidify the functional groups, and the resins were then thoroughly rinsed. Titration was performed with sodium hydroxyde.

The shape of the titration curves depends on the phosphorous content: resins with the lower phosphorous contents (less than about 1 mmol/g) give evidence to only one strong acidity (Fig. 3); resins with the higher phosphorous contents (more than

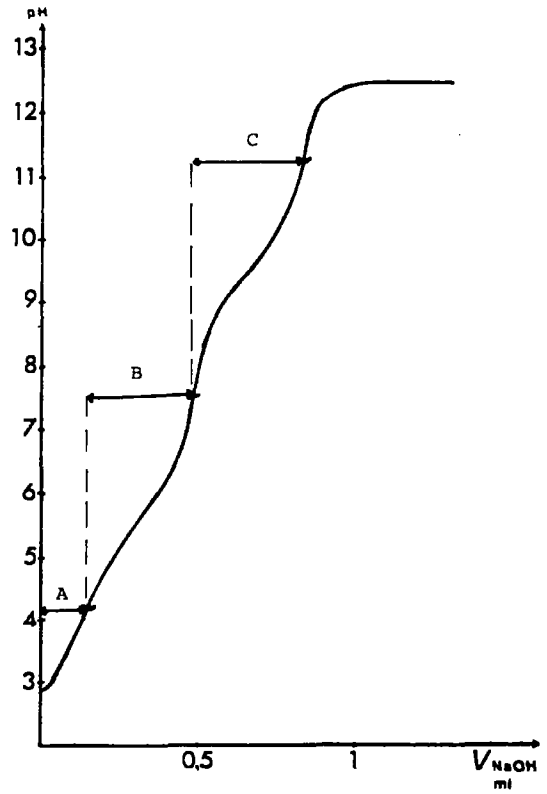

Figure 4 Typical acidimetric titration curve of a high phosphorylated polystyrene derivative.

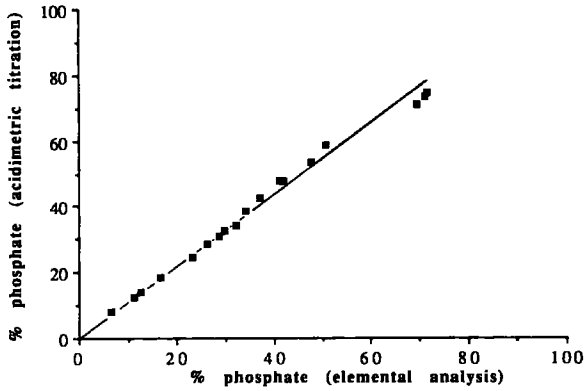

Figure 5 Correlation between the phosphate content, determined by elemental analysis and acidimetric titration.

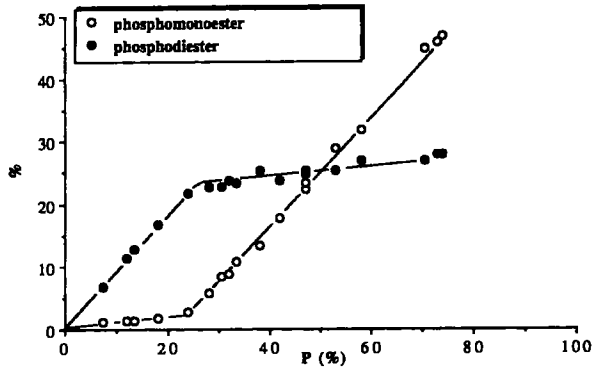

Figure 6 Distribution of the phosphate groups: relationship between the total phosphate content of the resin and (a) the phosphodiester content or (b) the phosphomonoester content.

about 2 mmol/g) have titration curves with three steps (Fig. 4), showing one strong acidity, *A*, and two weak acidities, *B* and *C*, whose pK_As are about 5.5 and 10. Acidities *B* and *C* correspond to the same concentration, thus being assigned to a weak diacid.

We assumed that acidity *A* corresponds to the monoacidic phosphodiester groups, and acidities *B* and *C* to the diacidic phosphomonoester groups. The equivalence point, between regions *A* and *B*, is not always well-defined (for intermediate phosphorous contents), but we could determine the total phosphorous concentration as *A* + *B*, the phosphomonoester concentration as *C*, and finally the phosphodiester concentration by substraction. Figure 5 shows the good correlation of acidimetrically determined total phosphorous content with the results of elemental analysis.

Distribution of the Phosphate Groups

Figure 6 shows the percentage of phosphodiester or phosphomonoester groups plotted against the molar ratio of phosphorous over the total hydroxy-units. These results suggest that phosphorous oxychloride reacts first to produce almost exclusively phosphodiester, until about half of the hydroxy functions are substituted, and that phosphomonoester is only produced later. These results are presently being investigated by comparison with computed models.

Complexation of Calcium Ions by Phosphorylated Resins

Complexation of calcium ions was performed with several phosphorylated polystyrene derivatives, but

Table II Complexation of Calcium Ions with Polystyrene Derivatives: Dissociation Constants and Binding Sites of High (Kd_1, B_1) and of Low (B_2, Kd_2) Affinity

Polymer	Phosphate (%)	B_1 (mmol/g)	B_2 (mmol/g)	Kd_1 (10^{-3} M)	Kd_2 (10^{-1} M)
PSOH	0		1.8		
PSP15	15	0.8	2.4	7.0	2.2
PSP21	21	1.0	2.2	6.2	1.0
PSP22	22	0.8	1.3	10	1.4
PSP34	34	1.1	2.3	2.4	1.1
PSP43	43	0.8	3.9	3	3.2
PSP45	45	1.3	3.1	5	1.9
PSP56	56	1.1	4	8.9	1.0
PSP62	62	1.37	4.7	7.0	
$PSSO_3$	0		4.0		

Table III Time Dependence of the Phosphorylation

Reaction Time (h)	% Phosphomonoester	% Phosphodiester	% Total Phosphate
8	15	24	39
18	22	24	46
40	19	23	42

also with the PEASS and a sulfonated polystyrene (SPS); the results are reported Table II.

For the phosphorylated derivatives, a typical complexation curve and its corresponding Scatchard's plot are presented Figure 7(a) and (b). This figure shows there were two kinds of calcium interaction sites (of high B_1 and of low B_2 affinity) on the phosphorylated resins, regardless of the degree of substitution by phosphate groups. However, this is not the case for the SPS or PEASS derivatives, which contain only one type of interaction site. For the hydroxylated resin PEASS, the observed weak complexation could be explained by its higher swelling, which induces an equilibrium of the distribution of the calcium ions between the aqueous buffer solution and the swelled polymer.

When the amount of complexed calcium, B_1 (mmol/g of resin), which corresponds to the sites of higher affinity, are plotted against the total phosphorous content of the resin (% P), a linear variation is observed up to a total phosphorous content of about 25%, as seen in Figure 8; above this value, B_1 was constant, even if total phosphorous content increased. In parallel, the analysis of the acidimetric titration curves showed that until 25% of substitution of phosphate, the grafted groups were only phosphodiester in nature and, furthermore, when the total phosphorous content of the resin was increased above 25%, the PDE group content did not vary. When B_1 and PDE are plotted together against $\%P$, good correlation was observed (Fig. 8); this demonstrates that the high affinity sites for calcium ions corresponded to the PDE concentration and

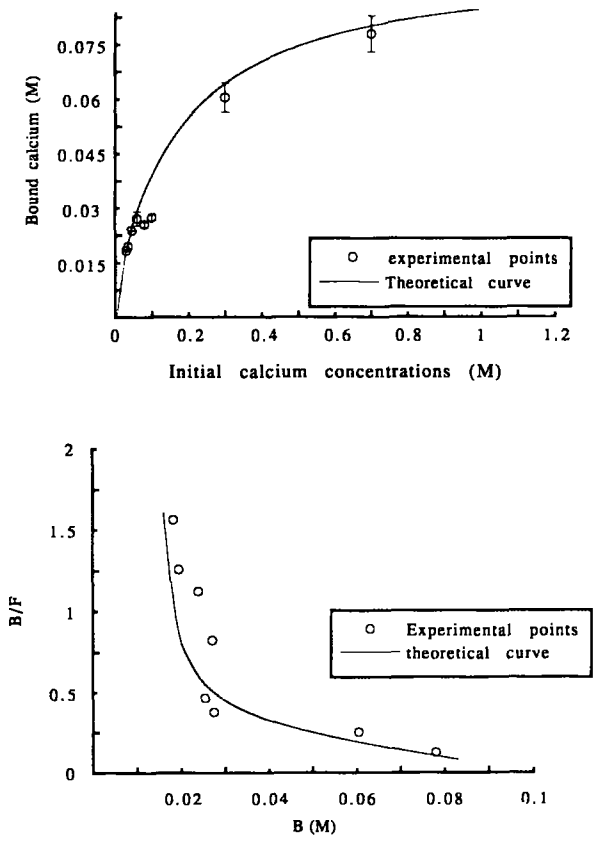

Figure 7 Complexation of calcium ions with phosphorylated polystyrene derivative: typical complexation curve (a) and its corresponding Scatchard's plot (b).

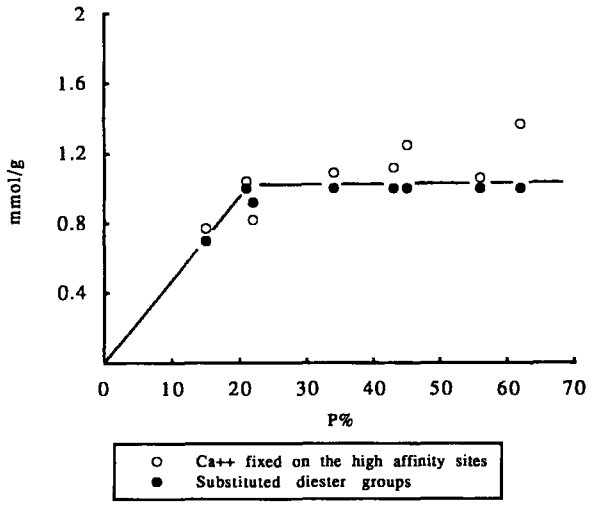

Figure 8 Correlation between the high affinity binding sites for calcium ions and the PDE content of the phosphorylated resins.

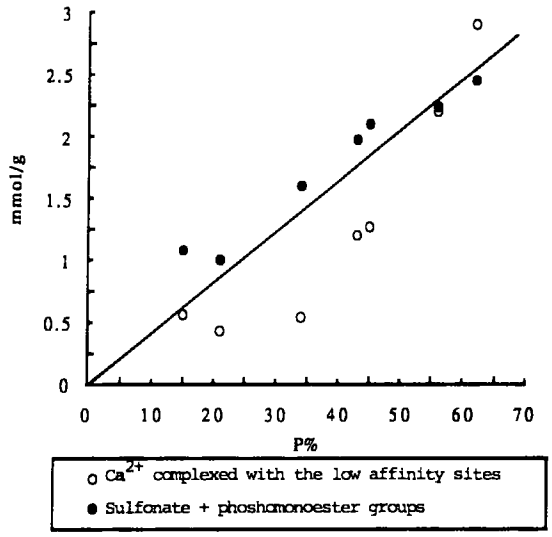

Figure 9 Correlation between the low affinity binding sites for calcium ions and the PME + sulfonate content of the resins.

that the complexation was one Ca^{2+} complexed ion per PDE group.

At later time points, the phosphate groups grafted to the resin were found to be PME, increasing linearly with the total phosphorous content of the resin, and the PDE content remained constant at 1 mmol/g. When the amount of calcium ions complexed to the low affinity sites, B_2 was plotted against the PME + residual sulfonate groups of the resin, a linear relation is again observed (Fig. 9). This demonstrates that the weaker affinity sites of the resin for calcium ions could be correlated with the PME and sulfonate groups and that the fixation of calcium ions corresponds to one calcium per PME or sulfonate group.

CONCLUSIONS

Described in this article is a kinetically controlled method of phosphorylation of polystyrene resins. This method permits the synthesis of the precise, predetermined chemical composition of phosphorylated resins. The nature of the phosphate groups, grafted on these resins, has been thoroughly characterized. Phosphorous, bound to the macromolecular chain through the hydroxyl groups, can lead to the formation of either PDE or PME groups. The respective amounts of each type of phosphate group were determined by acidimetric titration and were found to depend on the total phosphorous content of the resin.

The complexation of calcium ions with these resins depends on the nature of the phosphate groups, PME or PDE, and it yielded two dissociation constants, as evidenced by the Scatchard's plots. The analysis of the affinity of calcium, in relation to the chemical composition, shows the high affinity sites could be correlated with the PDE groups, while the low affinity sites could be attributed to the PME groups.

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