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DETERMINATION OF O-PHOSPHOTHREONINE, O-PHOSPHOSERINE, O-PHOSPHOTYROSINE AND PHOSPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure has been developed to measure O-phosphoamino acids (O-phospho-L-serine, O-phospho-L-threonine, O-phospho-L-tyrosine) and phosphate. A strong basic anion-exchange column (Partisil 10 SAX Whatman) enables their separation within 20 min. Detection is made by ultraviolet absorption at 210 nm or measurement of ^{32}P radioactivity. The best separation is obtained with a 0.04 *M* phosphate aqueous mobile phase at pH 4. The performance of the procedure has been tested by analysis of hydrolysed phosphoamino acids and hydrolysed phosphorylated histones; all hydrolysates are purified on Sephadex G-25 before chromatographic analysis.

INTRODUCTION

Protein phosphorylation by protein kinase is a major regulatory mechanism for hormonal regulation of cell function [1]. This phosphorylation is usually studied by measuring the incorporation of [^{32}P]phosphate into amino acids. Among all possible phosphate esters of the natural amino acids, only three, i.e. O-phospho-L-serine (P-Ser), O-phospho-L-threonine (P-Thr) and O-phospho-L-tyrosine (P-Tyr), have reasonable chemical stability over a large pH range [2]. Characterization of phosphoamino acids after protein acid hydrolysis has therefore been restricted to these.

The separation of ^{32}P-labelled amino acids and [^{32}P]phosphate has been studied since at least 1962 [3], and paper [3—7] or thin-layer cellulose plate electrophoresis [8—16] were the most widely used methods. Alternatively, some authors have used high-performance liquid chromatography (HPLC) [17—19] or amino acid analysis [20—22].

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This paper is devoted to the setting up of a rapid analysis involving HPLC on an anion-exchange resin (Partisil 10 SAX Whatman). The performance of the procedure has been tested by measuring phosphate bond hydrolysis rate in P-Thr, P-Ser and P-Tyr and peptidic plus phosphate bond hydrolyses of ^{32}P-phosphorylated histones.

EXPERIMENTAL

Chemical reagents

Potassium dihydrogen phosphate, trichloroacetic acid (TCA), phosphoric acid, hydrochloric acid, diphosphorus pentoxide, barium hydroxide, absolute ethanol, and L-tyrosine (Prolabo, Paris, France) were analytical grade; magnesium chloride, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), cyclic AMP, histones from calf thymus type II S, protein kinase, O-phospho-L-serine and O-phospho-DL-threonine were obtained from Sigma (Osi, France); [γ-^{32}P]ATP and [^{32}P]phosphate were obtained from New England Nuclear (U.K.).

Analysis of O-phosphothreonine, O-phosphoserine, O-phosphotyrosine and phosphate by HPLC

The liquid chromatography apparatus consisted of a Model 848 pump module (Dupont, Orsay, France) with a Rheodyne 7125 injection valve, a 100-μl loop (Touzart et Matignon, Vitry sur Seine, France), and an absorbance monitor ISCO Model 1840 (Roucaire, Velizy, France) equipped with an 8-μl flow-cell unit. The chromatographic effluent was collected on an LKB 2212 Redirac fraction collector with a 24-sec delay. The system was operated at room temperature.

Chromatography was performed on a Partisil 10 SAX Whatman column, 25 × 0.4 cm I.D., particle size 10 μm, with a mobile phase of 0.04 *M* potassium phosphate aqueous solution at pH 4. The flow-rate was 1.5 ml/min (80 bars). The O-phosphoamino acids were detected at 210 nm; ^{32}P-radioactive compounds were counted using Aquassure (New England Nuclear) as scintillation fluid in a spectrometric counter SL 4000 (Intertechnique, Montigny Le Brotonneux, France).

Synthesis of O-phosphotyrosine

O-Phosphotyrosine was synthesized as described by Mitchell and Lunan [23]. It was purified by the method of Plimmer [2] by forming a soluble alkaline barium salt, which was separated by filtration from insoluble barium phosphate. O-Phosphotyrosine barium salt was then precipitated by adding an equal volume of absolute ethanol. The isolated solid was homogeneous on cellulose and silica thin-layer chromatography [8, 24]; its UV spectrum showed an absorption maximum at 265 nm as previously described [18, 23].

Synthesis of ^{32}P-phosphorylated histones

Phosphorylated histones were prepared as described [25]. The incubation medium contained: 10 mg/ml histones, 10 m*M* magnesium chloride, 0.05 m*M* cyclic AMP, [γ-^{32}P]ATP (4 μCi/ml), 1.5 mg/ml protein kinase, 50 m*M* HEPES pH 6.8. The reaction was run for 10 min and stopped with TCA (5% final

concentration). After 1 min of centrifugation in a Beckman microfuge, the supernatant was discarded and the precipitated phosphohistones were stored at 4°C.

At 5 min, 40% of the γ-^{32}P of ATP was incorporated into histones and the maximum of the phosphorylation step was reached.

Analysis of unlabelled phosphoamino acids and ^{32}P-phosphorylated histones

Hydrolysis. P-Thr (40 mg) with $^{32}PO_4$ as internal standard (0.25 μCi) was dissolved in 20 ml of 5.6 *M* hydrochloric acid; 2-ml fractions were poured into glass tubes which were sealed under vacuum and heated at 105°C for periods of 0.5, 1, 2, 4, 7, 16 and 24 h. In the same way, P-Ser (40 mg) and P-Tyr (100 μg) together with $^{32}PO_4$ (0.25 μCi) as internal standard were hydrolysed for 0.5, 1, 2, 4, 7 and 16 h. [^{32}P]Phosphohistones (3 mg) were dissolved in 1.5 ml of 5.6 *M* hydrochloric acid; 0.25-ml fractions were hydrolysed for periods of 0.5, 1, 2, 4 and 7 h.

All hydrolysates were dried by evaporation, and the residues dissolved in 150 μl of water and purified on Sephadex G-25 (Pharmacia, Bois d'Arcy, France) before HPLC analysis.

Purification on Sephadex G-25. The hydrolysates were purified on Sephadex G-25 before analysis by HPLC. Sephadex G-25 was equilibrated with 0.04 *M* potassium phosphate aqueous solution (pH 4) and 2 ml of gel were poured into an Econo-Column (Bio-Rad, Touzart et Matignon). The void volume (V_0) of the column was determined using dextran blue. A 100-μl volume of each hydrolysate was deposited on the column; fractions of 180 μl were collected and 45 μl of each were counted. All the fractions containing radioactivity were pooled and lyophilized. The dry residue was dissolved in 200 μl of water.

Chromatography on SAX column (HPLC)

A 50-μl volume of unlabelled phosphoamino acid hydrolysate was injected into the loop to measure the phosphate bond cleavage; 50 μl of hydrolysed [^{32}P]phosphohistones together with 20 μl of non-radioactive O-phosphoamino acids (P-Thr and P-Ser $5 \cdot 10^{-2}$ *M*, and P-Tyr 10^{-4} *M*) as markers were injected into the loop.

Standardization

HPLC assay. The reproducibility and the recovery of the HPLC procedure were tested by making five successive injections of a standard solution consisting of $5 \cdot 10^{-2}$ *M* P-Thr, $5 \cdot 10^{-2}$ *M* P-Ser, 10^{-4} *M* P-Tyr, and [^{32}P]-phosphate (50 000 cpm/ml); 20 μl were injected into the loop at 0.2 a.u.f.s. (absorbance units full scale).

The capacity ratio, k', was defined as $k' = (t - t_0)/t_0$, where t is the retention time of a given compound and t_0 the retention time of the void volume of the column.

RESULTS

HPLC analysis of O-phosphoamino acids and [^{32}P]phosphate

Injection of a mixture of P-Thr, P-Ser, P-Tyr and [^{32}P]phosphate on the

SAX column enabled their clear separation within 20 min using 0.04 *M* potassium phosphate aqueous phase at pH 4 (Fig. 1).

The plot of capacity ratio (k') of O-phosphoamino acids and phosphate as a function of potassium phosphate concentrations at pH 4.5 (Fig. 2) shows a good separation between P-Tyr, P-Ser and P-Thr; phosphate overlapped P-Tyr or P-Ser except at 0.03 *M* and 0.04 *M* phosphate.

The relationship between k' values and the pH of 0.04 *M* potassium phosphate aqueous phase (Fig. 3) indicates that, in the range studied, the best separation is obtained at pH 4 with $k'_{PO_4} > k'_{P\text{-}Tyr} > k'_{P\text{-}Ser} > k'_{P\text{-}Thr}$.

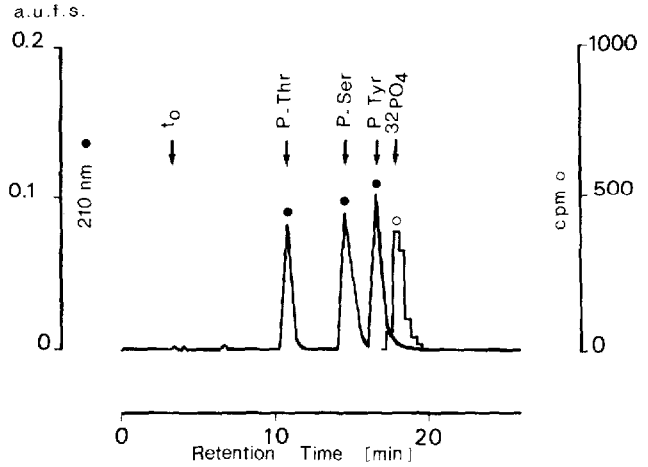

Fig. 1. Chromatogram of standards on Partisil 10 SAX Whatman. Peaks: P-Thr = O-phosphothreonine, 1 μmol; P-Ser = O-phosphoserine, 1 μmol; P-Tyr = O-phosphotyrosine, 2 nmol; $^{32}PO_4$ = phosphate labelled with ^{32}P, 1000 cpm. Injection volume = 20 μl. The mobile phase consisted of 0.04 *M* potassium phosphate aqueous solution, pH 4. Flow-rate = 1.5 ml/min. t_0 = retention time corresponding to the void volume of the column. a.u.f.s. = absorbance unit full scale. cpm = counts per min.

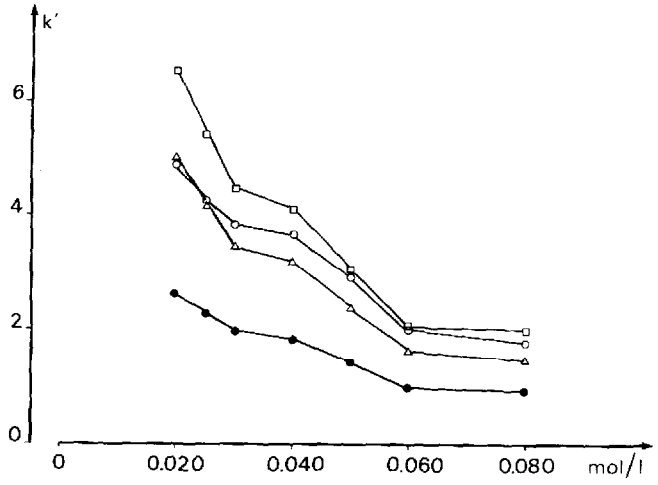

Fig. 2. k' Values of P-Thr, P-Ser, P-Tyr and $^{32}PO_4$ plotted as a function of potassium phosphate concentration at pH 4.5. (▫) O-Phosphotyrosine; (○) phosphate labelled with ^{32}P; (△) O-phosphoserine; (●) O-phosphothreonine.

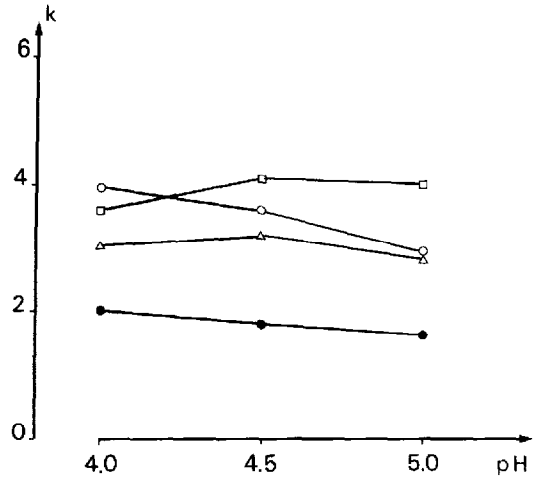

Fig. 3. k' Values of P-Thr, P-Ser, P-Tyr and $^{32}PO_4$ plotted as a function of pH at a phosphate concentration of 0.04 *M*. (□) O-Phosphotyrosine; (○) phosphate labelled with ^{32}P; (△) O-phosphoserine; (●) O-phosphothreonine.

Five repeated injections of the markers (1 μmol P-Thr, 1 μmol P-Ser and 2 nmol P-Tyr) gave relative standard deviations of 1.1%, 1.1% and 1.5%, respectively (n = 5). The recovery of [^{32}P]phosphate was 94% with a relative standard deviation of 2% (n = 5). The limit of detection of [^{32}P]phosphate was 450 cpm per injection.

Analysis of unlabelled phosphoamino acids and [^{32}P]phosphate on Sephadex G25

The recovery of [^{32}P]phosphate from Sephadex G-25 was 94% with a relative standard deviation of 3% (n = 3).

The chromatographic profiles of phosphate and phosphoamino acids overlapped. This was assessed by comparing two HPLC profiles (three experiments); (1) a 5-μl volume of the standard solution was injected into the loop at 0.05 a.u.f.s.; (2) a 100-μl volume of the same standard solution was chromatographed on Sephadex G-25, all radioactive fractions were pooled, adjusted to 1 ml and 50 μl were injected into the HPLC loop at 0.05 a.u.f.s.

The recoveries of phosphoamino acids were: P-Thr 100%, P-Ser 100%, and P-Tyr 97%, with relative standard deviations of 2%, 1.5% and 2%, respectively.

Cleavage of phosphate bonds of P-Thr, P-Ser and P-Tyr

After phosphoamino acid hydrolysis in acid, each sample was purified on a calibrated Sephadex G-25 and analysed by HPLC.

The rates of hydrolysis of P-Thr, P-Ser and P-Tyr are shown in Fig. 4. P-Thr is the most resistant to hydrolysis and P-Tyr the least. After 4 h, 70% of P-Thr, 40% of P-Ser and 20% of P-Tyr remained unhydrolysed.

The recoveries of [^{32}P]phosphate as internal standard were 99 ± 6% after G-25 and 97 ± 7% after HPLC assay (mean ± S.D. of fifteen experiments).

Analysis of hydrolysed phosphorylated histones

The radioactive composition of hydrolysed phosphohistones was studied

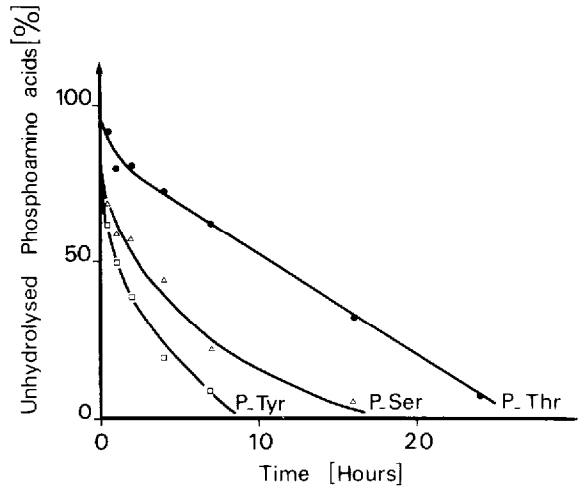

Fig. 4. Measurement of percentage unhydrolysed phosphoamino acid after hydrolysis of O-phosphotyrosine (□), O-phosphoserine (△) and O-phosphothreonine (•) in 5.6 *M* hydrochloric acid at 105° C.

after hydrolysis for 0.5, 1, 2, 4 and 7 h. Each sample was purified on a calibrated Sephadex G-25 prior to its analysis by HPLC. The determinations were made in triplicate.

Analysis on Sephadex G-25. Unhydrolysed phosphohistones eluted at V_0 (Fig. 5a). After 0.5 h of hydrolysis, the broad radioactive peak eluted between V_0 and the PO_4 elution volume (Fig. 5b). After 2 h hydrolysis, the radioactive peak was sharper (Fig. 5c) and eluted as PO_4.

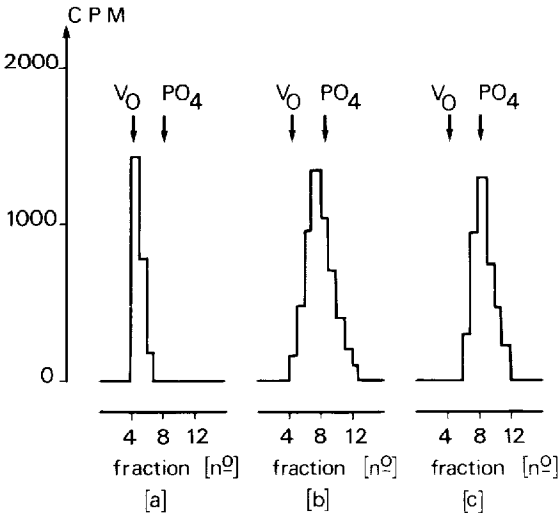

Fig. 5. Chromatographic profiles of Sephadex G-25 (2-ml volume): (a) unhydrolysed [^{32}P]-phosphohistones; (b) [^{32}P]phosphohistone hydrolysis for 0.5 h in 5.6 *M* hydrochloric acid at 105° C; (c) [^{32}P]phosphohistone hydrolysis for 2 h in 5.6 *M* hydrochloric acid at 105° C. V_0 is the void volume of the column. PO_4 indicates the elution volume of [^{32}P]phosphate. Each fraction represents a volume of 180 µl.

These results indicate that unhydrolysed phosphohistones do not contain free [^{32}P]phosphate. At 0.5 h, the elution profile would correspond to an incomplete cleavage of the phosphohistone peptide bonds while these are completely hydrolysed after 2 h hydrolysis.

HPLC analysis. Fig. 6 shows a chromatogram obtained after 1 h of [^{32}P]phosphohistone hydrolysis.

The radioactivity profiles suggest that several products are detected. These can be divided into two groups: (1) two unknown compounds, x and y, with

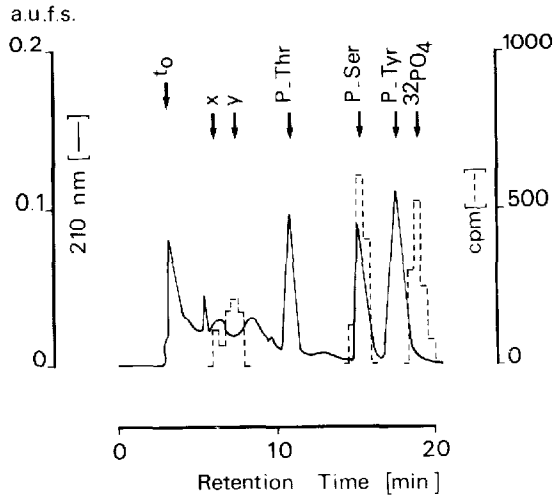

Fig. 6. Chromatogram of hydrolysed [^{32}P]phosphohistones on Partisil 10 SAX Whatman. [^{32}P]Phosphohistones were hydrolysed for 1 h in 5.6 *M* hydrochloric acid at 105°C. The injection volume was 20 µl of the standard solution of unlabelled O-phosphoamino acids and 50 µl of the [^{32}P]phosphohistone hydrolysate (2000 cpm). Peaks: P-Thr = O-phosphothreonine, 1 µmol; P-Ser = O-phosphoserine, 1 µmol; P-Tyr = O-phosphotyrosine, 2 nmol; x and y are undetermined radioactive compounds. The mobile phase consisted of 0.04 *M* potassium phosphate aqueous solution, pH 4. Flow-rate: 1.5 ml/min. t_0 = retention time corresponding to the void volume of the column. a.u.f.s. = absorbance unit full scale. cpm = counts per min.

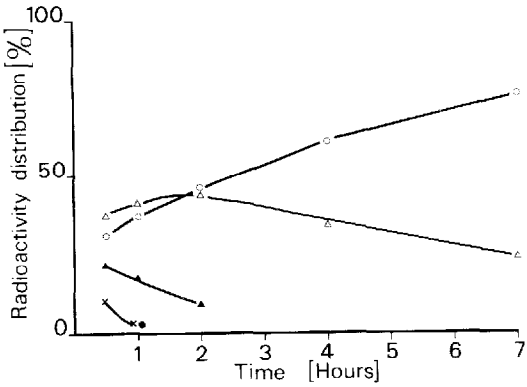

Fig. 7. Radioactivity distribution (%) of hydrolysed [^{32}P]phosphohistones for periods of 0.5, 1, 2, 4, and 7 h in 5.6 *M* hydrochloric acid at 105°C. (○) Phosphate labelled with ^{32}P; (△) O-phosphoserine; (▲) compound y; (×) compound x; (●) O-phosphothreonine.

capacity ratios smaller than those of phosphoamino acids or phosphate (k'_x = 0.95, k'_y = 1.4); (2) three known products, namely P-Thr, P-Ser and PO_4.

The evolution of the HPLC radioactivity profiles as a function of hydrolysis time is shown in Fig. 7; x and y decrease from 0.5 to 1 h and from 0.5 to 2 h, respectively. P-Thr is almost absent while P-Ser increases during the first 2 h and then decreases. PO_4 increases regularly. The recovery of the injected radioactivity is 88 ± 10% (mean ± S.D. of fifteen experiments).

DISCUSSION

The characterization of [^{32}P]phosphoamino acids and phosphate using paper and thin-layer plates has already been described. Thus, Eckhart et al. [8] and Hunter [9] developed methods on thin-layer plates to fractionate phosphoamino acids by electrophoresis in combination with chromatography or by two-dimensional electrophoresis. These methods are indeed adequate but they are time-consuming (some 10 h being required).

Swarup et al. [17] were the first to determine O-phosphoamino acids and phosphate using an anionic resin. However, with this procedure, the time required for complete analysis was 80 min and the separation between P-Ser and P-Tyr was relatively poor (only 2 min apart). In 1983, Swarup et al. [26] reported the advantage of high-voltage electrophoresis (40 min to perform the analysis) over their previous HPLC method.

Alewood et al. [18] described a preparation of O-phosphotyrosine. These authors studied the conversion of tyrosine to phosphotyrosine by HPLC with a C_{18} column, and its optical purity by a modification of the Manning-Moore procedure [27]. However, they did not study the chromatographic profile of other phosphoamino acids.

In 1982, using an anionic resin, Yang et al. [19] were able to reduce the time required for analysis down to 60 min with a good separation. However, other high-voltage electrophoresis techniques appeared [28, 29] which seemed to leave all HPLC procedures aside.

Our previous work on the determination of histamine [30] and 3-methylhistidine [31] in biological samples using a cationic HPLC resin, led us to consider the putative capacities of such techniques when appropriate mobile phases are used.

Our study shows a good resolution between phosphoamino acids. We paid particular attention to the retention time of phosphate because it is always released during phosphoprotein hydrolysis. It was possible to make the phosphate retention time longer than those of O-phosphoamino acids using a 0.04 *M* potassium phosphate aqueous phase at pH 4. The good separation of O-phosphoamino acids and phosphate within 20 min compares favourably with the time reported for high-voltage electrophoresis and other HPLC procedures.

Although P-Thr and P-Ser have a relatively low absorption at 210 nm, unlabelled phosphoamino acids are used as markers because they can be introduced in detectable amounts (1 μmol of each) without affecting the resolving power of the column.

The relative sequence of phosphoamino acid hydrolysis rates is P-Tyr > P-Ser > P-Thr. Nevertheless, in our experiments, the absolute hydrolysis rates of

P-Thr and P-Ser were faster than those described by Bitte and Kabat [4], who reported less than 15% cleavage of the phosphate bond after 2 h heating.

Cooper et al. [28] hydrolysed P-Tyr for 1 h in 1 *M* hydrochloric acid at 100°C or for 5 h in 5 *M* sodium hydroxide at 150°C. We agree that P-Tyr is not very stable in 5.6 *M* hydrochloric acid; it almost disappeared after 7 h of hydrolysis. In fact, Eckhart et al. [8] and Hunter [9] hydrolysed phosphotyrosylproteins and phosphotyrosylpeptides in 5 *M* hydrochloric acid at 110°C for 2 and 1 h, respectively.

After 0.5 or 1 h of phosphohistone hydrolysis, the broad radioactive peak obtained from Sephadex G-25 contained phopshorylated peptides. They were detected by HPLC as the unknown compounds x and y and represented 30% and 20% of the radioactivity distribution, respectively. After 4 or 7 h of hydrolysis, only phosphate and P-Ser appeared. The time course of the appearance of P-Ser from phosphohistone hydrolysis is biphasic. We suggest that it represents the sum of two phenomena: (1) hydrolysis of peptide bonds, which leads to the release of P-Ser, and (2) the cleavage of the P-Ser phosphate bond leading to phosphate release. According to this suggestion, we can use the rate of phosphate bond cleavage of P-Ser (Fig. 4) to calculate the theoretical levels of P-Ser liberated from phosphohistone hydrolysis: after 2 h of hydrolysis, the appearance of 44% of P-Ser would represent 83% of the ^{32}P liberated from the histones as P-Ser; after 7 h of hydrolysis, it would be 96% of the ^{32}P liberated.

Finally, we find that 2 h of phosphohistone hydrolysis corresponded to the best experimental conditions because 83% of the protein bonds were hydrolysed and a high percentage of unhydrolysed P-Ser was seen.

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