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A Study of the Phosphorus-Containing Proteins of Cells

II. The Turnover Rate of P^{32}-labelled Phosphoserine of the Schneider Protein Residues of Several Rat Organs

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The method for the study of acid-stable protein phosphorus of cells described previously [1] has been extended, and used in a study of protein phosphorus turnover. Of the several organs studied, liver and kidney were found to have the highest specific activity values for protein phosphoserine. The maximum incorporation for all organs investigated occurred at approximately three hours after injection of radioactive phosphate. The acid hydrolysate of the Schneider protein residue has been subdivided in about 50 separate fractions all containing phosphopeptides. The methods described have been of great aid in this procedure.

In the first paper of this series [1] a method was presented for the study of the more acid-stable phosphorus of cellular proteins. Using this method it was possible to isolate crystalline phosphoserine from calf liver protein. Preliminary experiments where the activity of the phosphoserine phosphorus of the rat liver protein was determined in animals killed ½, 4, and 24 hours after injection of radioactive inorganic phosphorus also indicated that the method was of use for tracer studies. The phosphorus bound to serine exhibited a high turnover rate. In a short communication [2] we have described the further extension of the method and its application to a tracer study of the rat liver protein phosphorus *.

METHODS

Groups of 10 male rats from the departmental colony averaging 300 g weight were used for each experiment. These rats are inbred and free of infection. Their care has been described by Hallgren [4]. They were fasted for approximately 18 hours before injection with P^{32}-phosphate (1 μC subcutaneously per g body weight corresponding to $3.05 \cdot 10^5$

* After these two articles had been submitted for publication a paper appeared by Kennedy and Smith describing a similar method and its use in demonstrating the presence of phosphoserine in mouse ascites tumor cells and in liver mitochondria.

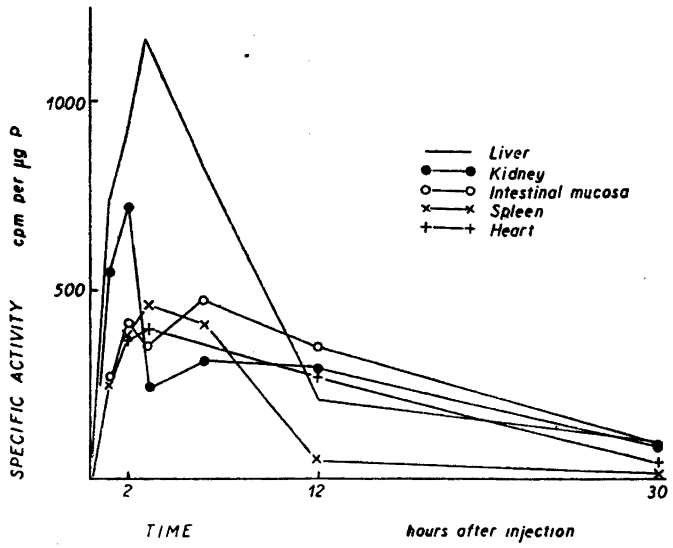

Fig. 1. Specific activities of phosphoserine fractions obtained from the Dowex 50 column after injection of 1 µC $H_3P^{32}O_4$ per g body weight.

cpm with the scaler used). After varying intervals of time the animals were killed and the Schneider protein fractions of various organs prepared and treated as described previously [1]. Subsequently, the Dowex 50 fractions were further resolved using the procedure of Busch, Hurlbert, and Potter [5] on Dowex 1 formate, 2 % DVB.

Paper chromatography of the phosphorus-containing fractions was carried out as before, except that when relatively large amounts were to be run Whatman No. 3 paper, washed according to Hanes, Hird, and Isherwood [6], was used. Scanning of the chromatograms was done according to the following procedure: the chromatogram is taped to an aluminium or brass strip, which is then mechanically moved under an end window Geiger tube (20th Century Electronics, type EW3H). The tube is enclosed in a lead chamber and is shielded from the chromatogram by an exchangeable slotted mask, generally having a slot width of 5 mm. The Geiger tube is connected to a counting rate meter and recorder as described in another paper [7] except that a Leeds and Northrup Speedomax Recorder 0–6 mV is used instead of a Micromax. The roller feeding the strip is connected to the roller in the chart recorder by a synchronous link, both having the same diameter to facilitate evaluation of the chromatogram.

For analysis of the small amounts of substances isolated, it was necessary to use a more sensitive method for phosphorus determination than that used previously. For digestion prior to phosphorus determination it was convenient to use the sealed tube method of Grunbaum, Schaffer and Kirk [8]. Subsequently, inorganic phosphorus was determined using a procedure based on that of Martin and Doty [9], but modified in so far as smaller volumes were used with micropipettes and the colorimetric determinations made in 5 cm cuvettes in a Beckman model B spectrophotometer. This method was not as sensitive as that described by Schaffer, Fong, and Kirk [10], but it allowed the determination of less than 0.1 µg phosphorus and it did not require special equipment.

Radioactivity measurements were made in duplicate on stainless steel planchettes; at least 1000 impulses were counted.

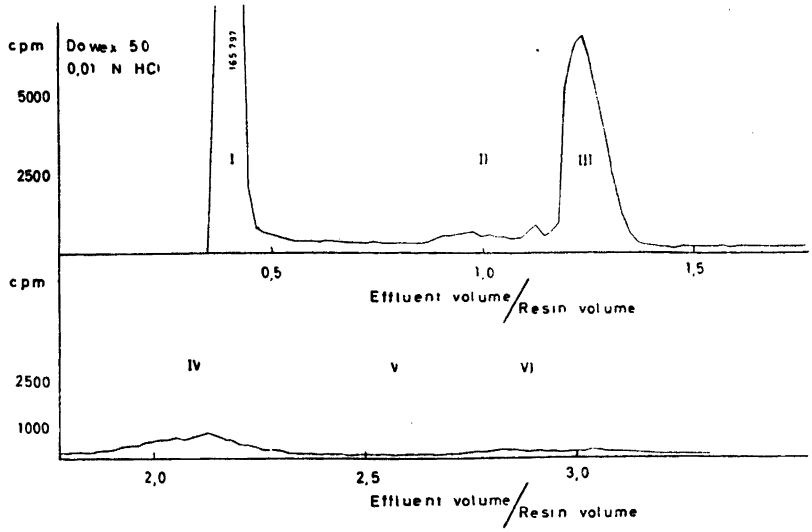

Fig. 2. Radioactivity curve from a hydrolysate of the rat liver protein fraction. The animals killed 3 hr after injection of 5 μC P^{32} per g body weight.

RESULTS

Preliminary data [2] showed that the phosphoserine fraction of a rat liver protein hydrolysate as isolated on a Dowex 50 column had a high specific activity with a maximum at approximately three hours after injection of radioactive phosphate. As is evident from Fig. 1, where the specific activity values of rat liver, kidney, spleen, intestinal mucosa, and heart phosphoserine fractions are compared, the only other organ having a comparatively high activity is the kidney.

Subsequent experiments indicated that each of the phosphorus-containing fractions obtained from Dowex 50 columns could be further subdivided on Dowex 1 columns. When this was done, it became apparent that while inorganic phosphate and phosphoserine dominate the respective Dowex 50 fractions, the peptide fractions are decidedly complex. Moreover, most of the peptide subfractions could be further subdivided using paper chromatography with benzene-propionic acid or *iso*butyric acid-ammonia.

In order to determine whether the phosphorus of the many fractions which could be obtained using the two types of ion exchange resin differed in specific activity, 10 rats were injected with 5 μc P^{32}-phosphate per g body weight. The animals were killed after three hours and the liver and kidney phosphopeptides fractionated. Fig. 2 is a photograph of the radioactivity curve of the rat liver protein hydrolysate obtained by plotting the number of impulses per minute in 1 ml aliquots of each tube from the fraction collector. To facilitate a comparison between different runs the ordinata has been expressed as parts of the volume of the resin bed. Only six comparatively well defined peaks can be

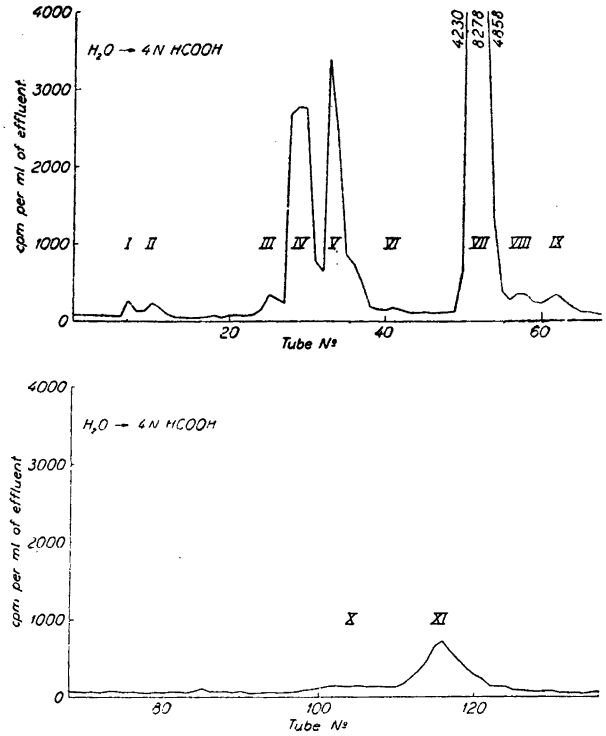

Fig. 3. Peak IV from the Dowex 50 column (Fig. 2) separated on a Dowex 1 column. Column dimensions: 11 × 185 mm.

seen, the first containing inorganic phosphorus, the second peptide bound phosphorus as well as peaks Nos. IV, V, and VI. The third peak mainly consists of phosphoserine. About 92 % of the radioactivity can be eluted from the column with 0.01 *N* HCl.

A qualitative amino acid analysis was also run on each of the fractions isolated in continuation of the study of the peptides present. Whereas the specific activity value of the pure phosphoserine was about 4 500 cpm per μg P the value of the other fractions ranged from 350 to 13 000. Fraction II from the Dowex 50 column (Fig. 2) yielded 15 fractions when Dowex 1 was used in conjunction with paper chromatography. Serine and glutamic acid were found to dominate most of these. About 89 % of the activity of fraction III from Dowex 50 was found to be located in one Dowex 1 peak. The material from this peak moved as one spot on chromatograms run in benzene-propionic acid Moreover, the only amino acid present after a further hydrolysis with 2 *N* HCl for 20 hours at 120° C was serine. This subfraction will henceforward be designated "chromatographically pure phosphoserine".

Like fraction II, fraction IV from the Dowex 50 column was also a complex mixture of peptides. Fig. 3 shows the Dowex 1 elution curve of this frac-

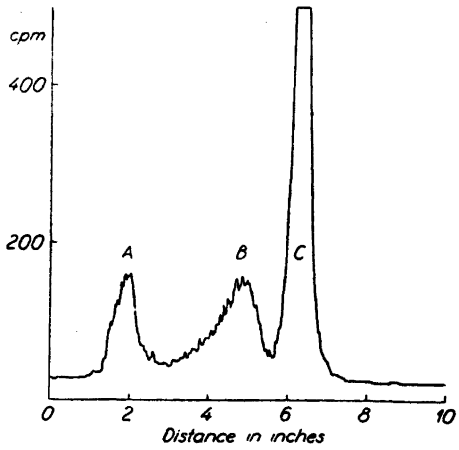

Fig. 4. A paper chromatogram from peak V (Fig. 3) scanned through an automatic device described in the text. Solvent system: benzene-propionic acid.

tion. Of special interest is peak No. VII which on further subdivision using paper chromatography could be shown to contain a fraction which after hydrolysis gave only threonine and traces of glycine (spec. activity 6 160).* Fig. 4 is a photograph of the radioactivity curve obtained when a one-dimensional (benzene-propionic acid) chromatogram of peak No. V was run through the scanning device. When the sections of the chromatograms corresponding to peaks A, B, and C were cut out and eluted, and the phosphopeptides hydrolysed, several amino acids were found to be present in each: alanine, glutamic acid, serine, and valine. In addition A contained glycine and leucine; B contained glycine; C contained threonine and tyrosine, with relatively larger quantities of glutamic acid than the two other.

The experiment where larger amounts of radioactive phosphate were injected also made it possible to detect two additional peaks, V and VI, emerging from the Dowex 50 column. Of these, VI was the strongest, and further fractionation indicated that it contained greater amounts of a peptide of serine, glutamic acid, and leucine. It was of interest that the specific activity of this peptide, 4 500, approximated that of chromatographically pure phosphoserine.

In this experiment it has also been possible to separate the first peak from the Dowex 50 column into seven fractions by the Dowex 1 procedure. About 84 % of the activity from the column moves as orthophosphoric acid. It has a specific activity of 955, the other fractions range from 782 to 2 395. These have not been hydrolysed and analysed for amino acids.

For comparison the TCA-soluble phosphorus-containing compounds from the same experiment have been fractionated according to the system described by Busch *et al.*[5] A part of the curve obtained is shown in Fig. 5. 26 fractions were collected and the specific activities of these were determined. They

* Since this paper was submitted for publication it has been possible to isolate crystalline phosphothreonine from a partial hydrolysate of Schneider protein from sheep liver.

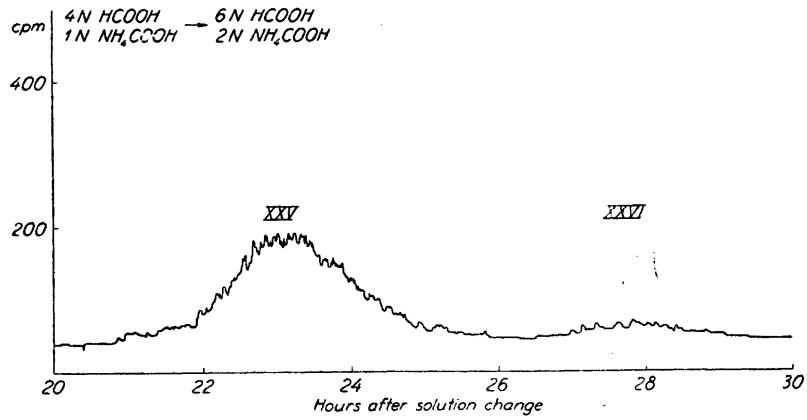

Fig. 5. The last part of the radioactivity curve obtained from the TCA-soluble substances from rat liver. The animals killed 3 hr after injection of 5 μC P^{32} per g body weight. Separation through a Dowex 1 (2 % DVB) column. Column dimensions: 26.7 × 280 mm. Automatic registration according to [7].

ranged from 62.1 to 148 000, most values lying between 3 000—8 000. So far it has not been possible to show the presence of free phosphoserine in this extract.

Having shown that the "phosphoserine fraction" from the Dowex 50 column was not completely homogeneous, it was felt that the determination of the specific activity values of chromatographically pure phosphoserine at different periods after injection was necessitated. In Table 1 some of these values are given. The Table also contains the specific activity values of the inorganic phosphate fraction (peak I in Fig. 2) and in some cases the phosphate purified through Dowex 1. Calculated from the peaks emerging from the Dowex 50 column, the total amount of phosphoserine phosphorus is only about 1/20th as great as the total amount of inorganic phosphorus. If the phosphate present in peptides is included this figure increases to about 1/10th. At the time for maximal incorporation, the specific activity of the phosphoserine values is 2.7 to 10 times greater than that of the inorganic phosphorus. The former value refers to the kidney, the latter to the spleen.

DISCUSSION

Using the present methods it is possible to isolate pure phosphoserine from the cell protein residue. Nevertheless, the limitations of the methods for the study of protein phosphorus turnover discussed previously should be kept in mind. In addition there is the possibility that not all the phosphoserine isolated was originally present as such before hydrolysis. Although it is unlikely that inorganic phosphate becomes esterified during the course of hydrolysis (this does not happen when casein is partially hydrolysed in the presence of P^{32} and we have not been able to observe it in experiments with organ protein)

Table 1. Specific Activities cpm/μg P.

	Phosphoric acid	Phosphoric acid purified on Dowex 1	Phosphoserine	Phosphoserine purified on Dowex 1
Liver				
1 hr	164		836	
1	220	90	643	715
2	156		929	1 078
3 *	299	191	1 152	911
3	400	104	1 204	669
6	143		827	
12	111	349	210	306
30	54		84	
Kidney				
1 hr	256		691	556
1	359	269	398	
2	271		725	441
3*	195	182	328	795
3	164	220	153	
6	191		316	351
12	81	72	297	371
30	44		89	50
Spleen				
1 hr	32		253	
2	73		387	
3 *	55		479	
3	44		439	
6	90		415	
12	54		44	
30	32		7	
Intestinal mucosa				
1 hr	61		255	
1	46		297	
2	71		417	
3 *	22		350	
3	118		401	
6	125		478	
12	70		348	
30	81		96	
Heart				
1 hr	45		259	
2	90		373	
3 *	99		232	
3	90		568	
12	61		270	
30	22		42	
Brain				
6 hr	10		201	

* The animals in this group were injected with 5 μC per g body weight. Values divided by 5 for comparison.

it may be that some phosphorus may have migrated from a phosphoamide linkage in serine to the hydroxyl group. Plapinger and Wagner-Jauregg [11] have shown this to occur when N-di*iso*propyl phosphorylserine is hydrolysed with aqueous hydrochloric acid. Little can be said at the moment of this possibility.

Even if the phosphoserine isolated does not come exclusively from protein-bound phosphoserine, it goes without saying that a measurement of the P^{32} turnover such as we have made can only give an average value for presumably different kinds of phosphoproteins at different loci within the cell. It is clear that work with protein preparations of the various cell organelles is called for. Moreover, it is possible that even if a single phosphoprotein could be isolated from a given cell component, the phosphorus bound to different serine residues, *e. g.* diester phosphorus contra monoester phosphorus, might differ in specific acitivity.

The phosphoric acid in the first peak from the Dowex 50 column must have different origins. Partly it comes from the phosphoproteins but most probably also from not completely extracted nucleic acids and phospholipids. This may explain the differences in values between different organs.

It must also be kept in mind that some of the subfractions isolated contain substances adsorbing ultraviolet light at 260 mμ pointing to the fact, that some nucleotides have withstood the strong acid hydrolysis.

With all these reservations in mind and the future experiments they suggest, the results of the present investigation would seem to be of value in narrowing the search to two organs, kidney and liver. It is of interest that our results tend to confirm those of Johnson and Albert [12] who, using a modified Schneider procedure for the determination of the "phosphoprotein phosphorus", found higher values for maximum specific activity in kidney and liver.

Since we have employed rats in these studies it has not been feasible to study the P^{32} turnover of phosphoserine in pancreas. Such a study would be of interest, however, in view of the high protein synthesis activity of this organ [13]. Further study of the brain, preferably using intracisternal radioactive phosphate injection [14], should prove interesting since the specific activity of brain phosphoserine is relatively high even with subcutaneous injections.

Another point of fundamental importance concerns the source and destination of the phosphoserine phosphorus. But before this problem can be elucidated many more studies of the turnover rates of purified phosphorus compounds of the cell are needed. It would be of interest to compare the specific activities of some of the lower energy compounds of the TCA-soluble fraction, especially those that are not directly involved in glycolysis. Also it would be interesting to compare the activities of phosphoserine with the phosphoinositol which Dawson [15] has shown to have a much greater specific activity than the other phosphorus-containing compounds of the lipid fraction.

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