

## A Study of the Phosphorus-Containing Proteins of Cells

### I. The Isolation of Phosphoserine from the Liver Proteins of Calf and Rat

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Within recent years numerous studies have been made of the incorporation of  $P^{32}$  into the cells of various body organs (*e. g.*<sup>1-3</sup>). These studies have commonly employed either the method of Schmidt and Thannhauser<sup>4</sup> or that of Schneider<sup>5</sup> for the chemical fractionation of cells. Both methods have proved useful and have disclosed several interesting features of cell metabolism. One of these is the high activity of the "phosphoprotein phosphorus", a property which lends especial interest to this fraction. Nevertheless, before much more can be said about the metabolic significance of cellular phosphoproteins, it is necessary that their chemical nature be more closely defined. In this sense, the work of Hess, Yasnoff, and Lagg, who prepared a protein fraction from lymphocytes containing 0.65 % phosphorus<sup>6</sup>, is a step in the right direction.

In this laboratory we have also been interested in the chemical characterization of phosphoproteins, and have hitherto concentrated our efforts toward the development of methods for the isolation and purification of phosphorus-containing amino acids and peptides, using enzymatic and acid partial hydrolysates of bovine casein as model substances<sup>7,8</sup>. The present paper describes the extension of these methods to the partially hydrolysed Schneider "protein" fraction from calf and rat liver, whereby phosphoserine has been prepared in crystalline form, and several other phosphorus-containing fractions have been obtained.

#### METHODS

*Calf liver* \* was obtained at the slaughter house and frozen in liquid air within 30—40 minutes after the killing of the animal. After thawing the liver was homogenized in ice-cold acetone in a Waring blender and subsequently extracted for 20 minutes with cold 10 % TCA. Lipides were extracted with the same series of solvents used by Davidson<sup>1</sup>, and nucleic acids were extracted according to Schneider<sup>5</sup> for 15 minutes at 90° C with

\* The livers of pasture calves only, were used.

5 % TCA. The residue from this treatment was washed twice with warm 5 % TCA, then with alcohol-ether (3 : 1) and ether, and finally taken as the Schneider protein fraction.

In order to obtain phosphorus-containing amino acids and peptides the protein fraction was partially hydrolysed according to Lipmann<sup>9</sup> with 10 ml 2 N HCl per gram protein under reflux on a water bath for 20 hours. Subsequently, after most of the HCl had been removed by repeated evaporation *in vacuo*, the hydrolysate was run through a short column of charcoal, similar to those described previously<sup>8</sup> except that it had been washed with 1 N HCl before use. The charcoal removed most of the humin which had been formed during hydrolysis, without adsorbing small phosphorus-containing peptides and amino acids. Thus, the latter were readily washed from the column with water. Finally, the wash water was concentrated to a few ml, applied to a 120 × 4 cm column of Dowex 50 (8 % cross linking, 250–500 mesh), and the phosphorus-containing compounds were eluted with 0.01 N HCl.

The fractionation was followed with the aid of an automatic conductivity recording device<sup>10</sup>, and the fractions obtained were further studied using two dimensional paper chromatography. The solvent systems found to be the most suitable were *isobutyric acid-ammonia*<sup>11</sup> and *benzene-propionic acid*<sup>8</sup>. The paper used was Munktell No. OB; and separation was indicated using the ninhydrin and phosphate spot test reagents as in a previous paper<sup>8</sup>. These two reagents could be applied successively to the same chromatogram, and in this way not only ninhydrin positive, but also phosphorus-containing compounds could be detected.

Crystallizing fractions were studied using powder and single crystal diagrams.

*Rat livers* were obtained from 6 adult male animals of the Wistar strain from the departmental colony. The rats were injected subcutaneously between the shoulder blades with 1  $\mu$ c P<sup>32</sup>-phosphate (carrier free) per g body weight. 4 hours after injection the animals were killed by cervical dislocation and the livers were rapidly removed and frozen in liquid air. After pulverizing the livers in a mortar with dry ice, they were extracted and washed with 10 % TCA and then subjected to the same extraction and hydrolysis procedures described for the preparation of the calf liver protein hydrolysate. After hydrolysis and removal of the HCl, the concentrated hydrolysate was applied directly to a 6 × 40 cm Dowex 50 column and eluted with 0.01 N HCl.

Fractionation was followed this time with a device for the continuous registration of radioactivity of the effluent<sup>12</sup>. For a more sensitive indication of the fractionation, 1 ml aliquots from each of the test tubes in the fraction collector were evaporated on aluminum discs, and the number of impulses per minute counted with the aid of Tracerlab's SC-18 Superscaler. For further study of the fractions two dimensional paper chromatography again proved useful. Moreover, the P<sup>32</sup>-containing spots could be indicated by autoradiography.

The phosphorus of the various fractions was determined as in a previous paper<sup>8</sup>.

## RESULTS

*Calf liver.* Fig. 1 is a photograph of the essential sections of the conductivity curve obtained when the calf liver protein hydrolysate was eluted from a Dowex 50 column, compared with a similar curve of a casein hydrolysate. Most of the phosphoserine of the latter had previously been removed in order to facilitate recording of the peaks due to other compounds. The peaks corresponding to inorganic phosphate have been omitted from both curves, so that the first peak shown in each case is that corresponding to a mixture of pyroglutamic acid (as indicated by a crystal diagram) and several ninhydrin-positive substances. The second peak of the liver curve has no obvious counterpart on the casein hydrolysate curve. A two dimensional chromatogram of this material has shown the presence of a substance which contains phosphorus as well as several which react with ninhydrin. On the other hand the third peak of the liver hydrolysate may possibly be similar to the second peak of the

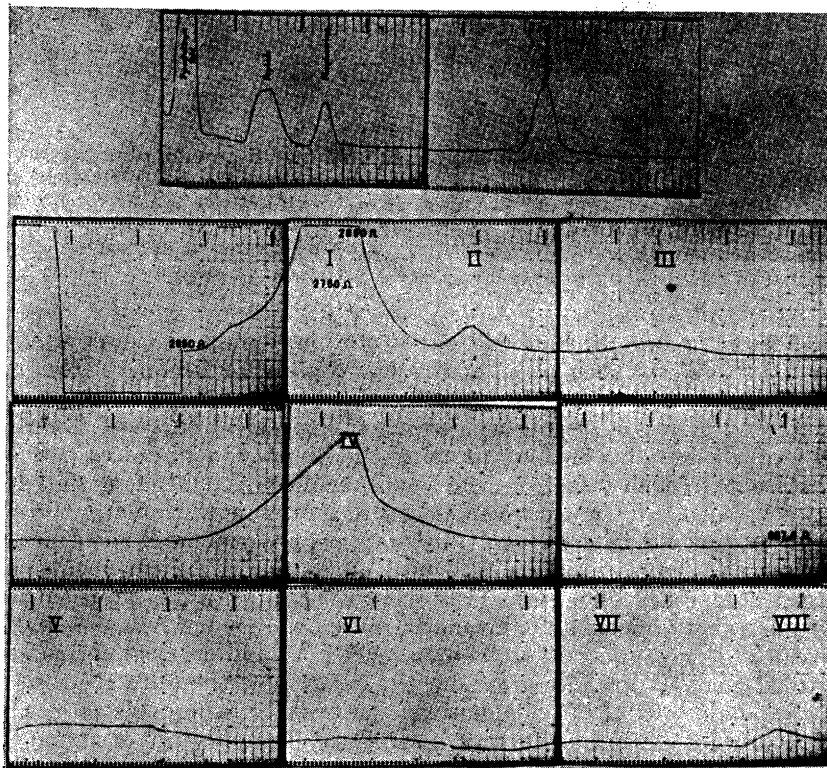


Fig. 1. Conductivity curve from a casein hydrolysate (above), compared with a similar curve from the hydrolysate from the calf liver protein fraction.

casein hydrolysate, which has been found to contain peptides containing phosphoserine and glutamic acid. From peaks IV and III of the liver and casein hydrolysate, respectively, phosphoserine can be obtained in crystalline form. It must be observed that the mother liquor in both instances contains several ninhydrin positive substances. Fractions V and VI from liver contain both ninhydrin positive substances and substances which give a positive phosphate reaction. Fraction VII probably contains phosphothreonine (for reasons to be discussed later with the results from the rat liver protein hydrolysate). Fraction VIII contains several ninhydrin positive substances, two of which give a positive phosphate reaction. One of the latter appears to be similar to the phosphorus-containing spot of fraction VII. Fraction VII and VIII thus probably correspond to fraction IV of the casein hydrolysate.

Fig. 2 is a photograph of the left half of a rotating crystal diagram of the crystals from fraction IV of the calf liver hydrolysate juxtaposed, to facilitate comparison, with the right half of a similar diagram of phosphoserine crystals from casein. The identity of the two crystal preparations is evident.



Fig. 2. Rotating crystal photographs of phosphoserine from casein (right) and crystals from peak IV (left). (*b*-axis rotation, taken with a Weissenberg-goniometer equipped with a cylinder camera, diameter 57.3 mm.)

Fig. 3 shows a one-dimensional paper chromatogram of the crystals from fraction IV compared with phosphoserine and phosphothreonine from casein, using *isobutyric acid-ammonia* as solvent.

*Rat liver.* Fig. 4 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. Only four well defined peaks can be seen, the first corresponding to inorganic phosphate, the second to peptide-bound phosphorus, the third to phosphoserine, and the fourth very possibly to phosphothreonine plus small quantities of another phosphorus-containing compound. Preliminary work with the  $P^{32}$ -containing substances eluted from a two dimensional chromatogram of the second peak has indicated the presence of glutamic acid as well as serine. When material corresponding to the third peak is chromatographed, the position of the only  $P^{32}$ -containing spot corresponds to that of phosphoserine. Moreover, when this spot is eluted from the chromatogram and further hydrolysed, the presence of serine can be shown by conventional amino acid chromatography<sup>8</sup>. The grounds for supposing that the major phosphorus-containing component of the fourth peak is

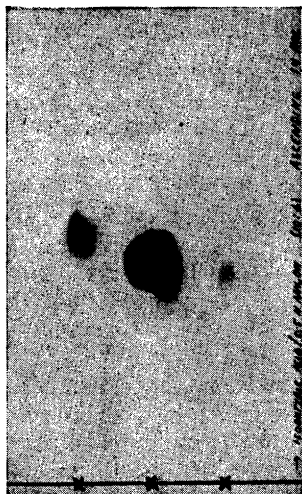


Fig. 3. One dimensional chromatogram of crystals from peak IV, developed with *isobutyric acid-ammonia*. For purposes of comparison, phosphoserine (right) and phosphothreonine (left) from casein have been run on the same chromatogram.

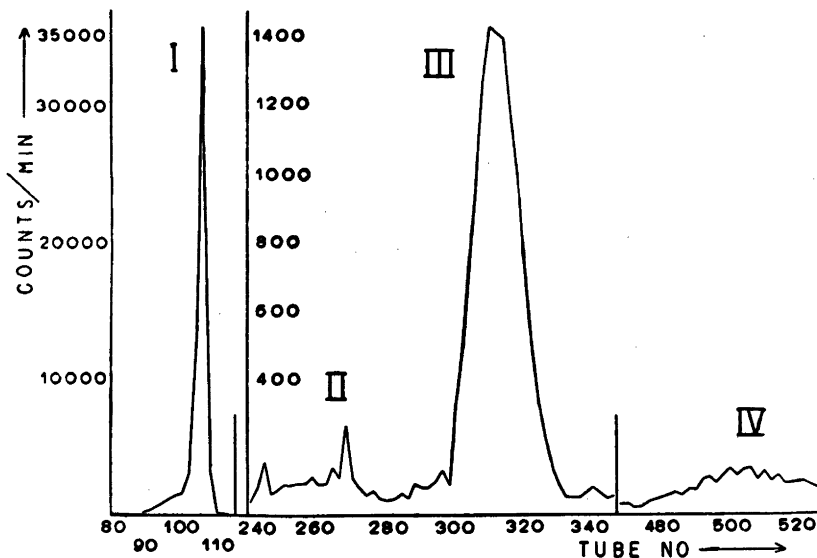
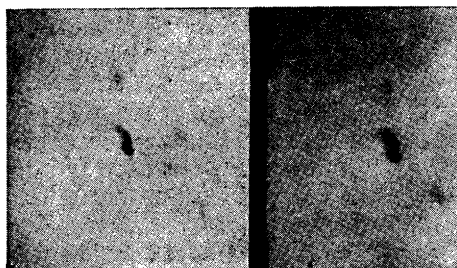


Fig. 4. Radioactivity curve from the hydrolysate of the rat liver protein fraction.

actually phosphothreonine are the following. The fraction is eluted from the Dowex 50 column at about the same position as phosphothreonine. Secondly, the principal phosphorus-containing spot of a two dimensional chromatogram of this fraction also corresponds to the position of phosphothreonine. Fig. 5 is a photograph of a chromatogram of material from the fourth peak which has been sprayed with ninhydrin. In the same figure, for comparison, is an autoradiogram of the same chromatogram.

The test tubes corresponding to the inorganic phosphate and phosphoserine peaks, respectively, were combined and the radioactivity evaluated as the number of counts/min./100  $\mu\text{g}$  P. Whereas the total amount of phosphorus in the fractions was 13.9 mg P and 0.42 mg P, respectively, the specific activities of the two fractions were 9 700 and 52 400 counts/min./100  $\mu\text{g}$  P, respectively. Thus, while the total amount of phosphoserine phosphorus was only

Fig. 5. Two dimensional chromatogram of material from the fourth peak of the rat liver protein hydrolysate, developed with benzene-propionic acid, and isobutyric acid-ammonia (right). To the left in the figure is an autoradiogram of the same chromatogram. (Gevaert Curix X-ray film, 10 day exposure).



1/33rd as great as the total amount of inorganic phosphorus, the specific activity of the phosphoserine phosphorus was 5.4 times greater than that of the inorganic phosphorus.

#### DISCUSSION

Davidson and co-workers<sup>1,13,14</sup> have amply demonstrated the need for a more rigorous definition of the "RNA" phosphorus obtained by the method of Schmidt and Thannhauser. That the "phosphoprotein" phosphorus of this method, as well as that obtained by the Schneider method, is also in need of a closer characterisation is evident from the varying and divergent results obtained by the several investigators who have used the two procedures<sup>1-3,15,16</sup>. While the Schneider method has consistently yielded higher values for the total amount of phosphorus, the method of Schmidt and Thannhauser has yielded the higher specific activity values. At the same time in neither of the two methods has it been shown that the phosphorus measured is actually phosphoprotein phosphorus.

To date protein-bound phosphorus is generally thought of in terms of phosphoserine or phosphothreonine<sup>7</sup>. Therefore, until other types of phosphorus-protein linkages are demonstrated, the present method for the isolation of phosphoserine (and possibly phosphothreonine) from cell protein preparations would seem to be of value, especially in studies of protein phosphorus metabolism. Something should be said from the outset, however, about the scope and limitations of the method.

The method is based on the work of Lipmann<sup>9</sup>, who isolated phosphoserine from a partial acid hydrolysate of casein. During the course of the hydrolysis, however, a good deal of the phosphoserine itself is hydrolyzed, presumably along with other, more acid labile phosphorus compounds. This constitutes the major limitation of the method.

Another point which should be mentioned is that a part of the phosphoserine isolated from the "Schneider protein residue" may possibly have originated from serine phospholipides bound to the protein residue. That this possibility should not be overlooked is suggested by the experiments of Folch<sup>17</sup>, who has shown that some inositol phosphatides are not extracted by the usual lipid solvents. Although Folch has also shown that these phosphatides are split off from the protein residue by treatment with hot TCA, it may be that some additional phosphatides — and among them serine phosphatides — still remain bound to the protein. Should this be the case it is possible that some phosphoserine might be liberated on acid hydrolysis, although this is rendered improbable by the earlier observations of Folch<sup>18</sup> that phosphoglycerine and not phosphoserine is the major product of the partial acid hydrolysis of serine phosphatides.

Preliminary experiments where the activity of the phosphoserine phosphorus of rat liver protein was determined in animals killed 1/2, 4, and 24 hours after injection of radioactive inorganic phosphate indicate that the present method is of value in spite of the limitations noted above. In these experiments the phosphorus bound to serine has exhibited a higher turnover rate than the inorganic phosphorus liberated by the acid hydrolysis. In this respect

at least it would appear to resemble the Schmidt and Thannhauser protein phosphorus. It is expected that enzymatic and milder acid hydrolysis techniques will make it possible to find out more about the source of the inorganic phosphorus.

Other experiments now in progress have demonstrated the presence of phosphoserine in the proteins of all the cell fractions obtained by the Schneider and Hogeboom techniques<sup>19,20</sup>.

#### SUMMARY

A method has been described for the isolation of phosphoserine and some other phosphorus-containing compounds from a partial hydrolysate of the Schneider protein residue of cells.

With the aid of this method crystalline phosphoserine has been obtained from a partial hydrolysate of calf liver protein.

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