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Fractionation of the Phosphorus-Containing Proteins of Rat Liver Cell Supernatant

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Rat liver supernatant proteins have been fractionated by ammonium sulfate precipitation, zone electrophoresis and ion exchange chromatography with special reference to phosphoserine and phosphothreonine-containing proteins. Several phosphorus-containing fractions have been obtained which together account for most of the protein-bound phosphoserine and phosphothreonine present.

In earlier publications from this laboratory [1,2] direct evidence was presented concerning the nature of a type of phosphoprotein present in liver. Methods were described by which crystalline phosphoserine and phosphothreonine could be isolated from partial hydrolysates of cell protein. And it was observed that these two phosphorylated amino acids could be prepared from each of the cell fractions obtained by the differential centrifugation method of Hogeboom and Schneider [3]. Moreover, experiments were described which indicated that protein-bound phosphorus might possibly play an active role in the metabolism of the cell. Recently, this possibility has been considerably strengthened by the work of Sutherland [4,5] and of Ågren and Engström [6,7] The former found that liver phosphorylase, a phosphoprotein, is inactivated by dephosphorylation and becomes active again after rephosphorylation. The latter authors presented evidence to show that an exchange of the phosphorus of a muscle phosphorylase and yeast hexokinase preparation occurred during the reactions catalyzed by these enzymes.

In the present paper a separation of the soluble phosphorus-containing proteins of rat liver has been attempted in order to provide a basis for subsequent studies of their structure and function. Both conventional precipitation methods and the more recent methods of ion exchange chromatography and zone electrophoresis have been employed. Several phosphorus-containing peaks have been obtained by zone electrophoresis and phospho-protein fractions corresponding to two of these and containing a considerable part of the protein-bound phosphoserine of the cell sap have been isolated. A preliminary report of some of this work has been published [8]. Some preliminary metabolic experiments have also been performed.

EXPERIMENTAL

Three month old male Wistar rats from the departmental colony were employed as experimental animals. They averaged about 300 g in weight and had not been fasted previously. Generally, they were injected intraperitoneally with 1—2 mC carrier-free radioactive phosphate 1—2 h previous to each experiment. The rats were killed by cervical dislocation and their livers quickly perfused *via* the hepatic portal vein, first with ice-cold isotonic NaCl and then with ice-cold 0.25 M sucrose solution, both solutions having been made up with glass-distilled water and adjusted to a pH of 7.4 with KOH. The perfusion was performed *in situ* using a long plastic tube connected to a syringe. After the plastic cannula had been inserted into the vein and securely tied in place, a few ml of saline were forced into the liver and then the abdominal and thoracic *vena cava* were cut to allow the free flow of blood from the liver. During perfusion the liver was cooled with ice-cold sucrose. The perfused livers were minced with a scissors and homogenized in 9 vol. of 0.25 M sucrose for 2 min in a homogenizer of the Potter & Elvehjem type consisting of a smooth-walled glass tube fitted with a teflon pestle (manufactured by Arthur H. Thomas Co., Philadelphia). The liver supernatant was then prepared essentially according to Hogeboom [9]; the microsomes, however, were spun down at 30 000 R.P.M. for one hour in rotor No. 40 of the Spinco model L preparative centrifuge. All centrifugations and manipulations were carried out at 0°—2°C. Furthermore, all solutions were made up with glass-distilled water using analytical grade reagents and were adjusted to pH 7.4 with KOH unless otherwise noted. All glass-ware and instruments were rinsed with glass-distilled water before use.

Precipitation with ammonium sulfate. Fractions were obtained by adding to the supernatant a saturated aqueous solution of ammonium sulfate, the pH of which had been adjusted so that it was 7.0 after ten-fold dilution. Fraction "AS 50" was obtained by adding 1 vol. of saturated ammonium sulfate. The resulting precipitate was then washed three times with 50 % saturated ammonium sulfate. The first washing having been combined with the original supernatant, fraction "AS 85" was obtained by adding additional saturated ammonium sulfate solution to 85 % saturation. This fraction was also washed three times. The two precipitates were then dissolved in 0.06 M tris(hydroxymethyl)aminomethane buffer, pH 7.8 (unless otherwise noted) and then dialyzed for 2 h before application to the zone electrophoresis columns. In all the ammonium sulfate fractionation experiments, the sucrose used in preparing the cell sap contained 0.01 M versene.

Zone electrophoresis was performed according to Porath [10] on 150 × 3 cm columns. Generally "tris" buffers were employed at a pH of 7—8 and a concentration of 0.03—0.1 M. The electrophoresis required from 36—48 h and was always performed in the cold.

Ion exchange chromatography. Triethylaminoethyl cellulose columns and sulfomethyl cellulose columns prepared according to Porath [11] and ECTEOLA columns prepared at room temperature according to Peterson and Sober [12] (however, using cellulose linters instead of solka floc) were employed for the protein fractionation experiments. The columns were of varying dimensions, but the exchanger was always supported by cellulose discs at the lower end of the column. The chromatography was always carried out in the cold.

The chromatographic and electrophoretic fractionations were followed spectrophotometrically at 260 and 280 mμ. Cold precautions were taken at all possible points. ^{32}P determinations were made on aliquots of the fractions and counted on an automatic scaler. For a more specific determination of the phosphoserine and phosphothreonine-containing proteins, the two amino acids were isolated from partial hydrolysates of the protein fractions as described previously [1,2]. For a crude estimation of the relative amounts of radioactive phosphoserine and phosphothreonine present in the fractions the total activity of the two phosphorus-containing amino acids was determined. For concentration of the protein fractions the ultrafiltration apparatus described by Aronsson [13] was employed. Phosphorus determinations were performed as described previously [2]. The following abbreviations have been used: "tris" = tris(hydroxymethyl)-aminomethane; RNA = ribonucleic acid; PCA = perchloric acid; TEAE = triethylaminoethyl cellulose; ATP = adenosine triphosphate.

RESULTS

The first step of the fractionation procedure was initially planned to comprise a precipitation method in order to concentrate the supernatant (initial volume 140 ml), remove organic phosphorus compounds of low molecular weight and obtain a preliminary fractionation of the proteins.

In preliminary experiments [8] ethanolic fractionation by the method of Cohn *et al.*[14] was attempted. Fractions containing relatively increased amounts of phosphoserine and phosphothreonine proteins could be obtained, but it was difficult to obtain satisfactory reproducibility from experiment to experiment (on the basis of radioactive phosphoserine determinations and the relative number and amount of peaks present when the fractions were subjected to zone electrophoresis) although adequate reproducibility could be obtained with aliquots of the same supernatant preparation. Moreover, similar difficulties were encountered in fractionating solubilized proteins from mitochondria (unpublished results). It was also noted that even when the method was slightly modified so that the first precipitation was performed at a lower ethanol concentration (10 % by volume) some apparent denaturation of a nucleoprotein, phosphoprotein fraction was obtained under such mild conditions as pH 5.8, 10 % ethanol, —3°C, 45 min. Thus, in every experiment a residue remained after the first precipitation step which could not be redissolved although several different solvent systems were tried.

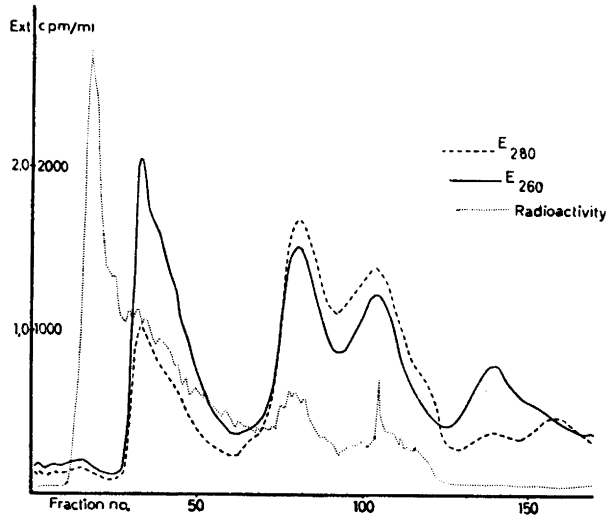

Fig. 1. Electropherogram of liver cell supernatant, fraction "AS 50". 2 rats injected with 1 mC ^{32}P 1 h previous to liver perfusion. Supernatant prepared with 0.25 M sucrose — 0.01 M versene, pH 7.4. 1 vol. saturated ammonium sulfate added: precipitate washed thrice, dialyzed 2 h; insoluble material removed by centrifugation. Clear solution displaced into the zone electrophoresis column with 50 ml buffer (0.06 M tris, pH 7.8). Electrophoresis for 45 h at 40 mA (1 300 V). Fractions of approximately 5 ml collected. Each measured at 260 mμ and 280 mμ; 1 ml aliquots taken for ^{32}P determination.

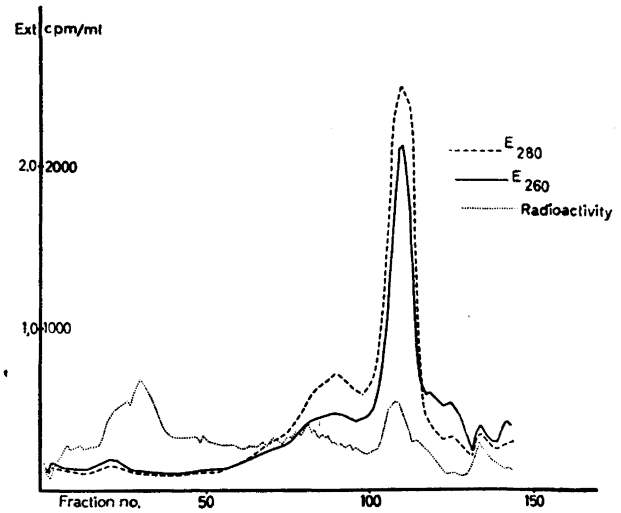

Fig. 2. Electropherogram of liver cell supernatant, fraction "AS 85". Same experiment as shown in Fig. 1. Precipitate obtained with 85 % saturated ammonium sulfate. Treated in same manner as fraction "AS 50".

Ammonium sulfate precipitation. In view of the difficulties encountered in the ethanolic fractionation experiments, a cruder separation into only 2 fractions was attempted, using ammonium sulfate precipitation. Subsequently, the precipitates were further fractionated using a more selective method (zone electrophoresis). In preliminary experiments it was found that approximately 80 % of the phosphoserine protein fraction of the supernatant fraction (and a nearly proportionate amount of the total RNA as estimated by measuring the total quantity of 260 mμ adsorbing material released by warm PCA extraction) could be precipitated by adding 1 vol. saturated ammonium sulfate solution to the cell sap, the resulting precipitate corresponding to 30—40 % of the total cell sap protein by weight. Further, it was found that little or no enzymatic dephosphorylation could be demonstrated after the phosphoserine protein-containing fractions had been allowed to stand in the cold for a period of 48 h (the maximum time usually employed for the zone electrophoresis).

The results of a typical experiment where ammonium sulfate fractionation was followed by a further separation of the fractions by zone electrophoresis are shown in Figs. 1 and 2.

As is apparent, both the ammonium sulfate precipitation and the electrophoresis yield a rather sharp separation of the cell sap proteins. Moreover, this two step separation is reproducible, although it must be mentioned that the ammonium sulfate fractionation at this pH causes the irreversible precipitation of a small amount of protein when the ammonium sulfate concentration approaches 35 % saturation so that this protein does not appear on the zone electrophoresis diagram.

Another point to be mentioned is that some of the protein migrating toward the cathode has not been included in the electrophoresis diagram, it having moved into the unstabilized buffer region.

The fractions which have been of the greatest interest in the present investigation are those which contain radioactive phosphorus. As may be seen there are a number of radioactive peaks. The first radioactive peak of the "AS 50" fraction remained soluble after cold PCA had been added to a final concentration of 0.6 N and was found to consist mainly of inorganic phosphate. The second peak was found to contain RNA. Radioactive phosphoserine and phosphothreonine were isolated in relatively large amounts from the third and fourth radioactive peaks and also from the main peak of fraction "AS 85". The identity of the remaining peaks is under investigation. The adsorption properties of the most slowly moving fractions suggest the presence of relatively large amounts of bound nucleotide. Most of the 260 mμ-adsorbing material of these fractions could be released from the protein by treatment with cold 0.6 N perchloric acid.

Ion exchange chromatography. In a series of experiments acidic and basic cellulose derivatives were substituted for the precipitation with ammonium sulfate as the first step in the procedure. It was found that when the supernatant was passed through the acidic exchanger, sulfomethyl cellulose, at pH 6.0 almost no phosphoprotein was adsorbed and most of the additional cell sap protein also passed through the column. Moreover, when the cation exchanger was used in an attempt to fractionate an already purified phosphoprotein fraction some denaturation apparently occurred.

The basic exchangers used were triethylaminoethyl cellulose and ECTEOLA cellulose. The former is rather similar in its properties to the DEAE cellulose described by Sober and Peterson (see Porath [10]) and affords a similar separation of serum proteins (unpublished experiments). Columns 10 cm in length and 3 cm in diameter were usually employed. The fresh cell sap (usually of a pH of 6.8—7.0) was allowed to pass through the column in the cold room, the column having previously been equilibrated with 0.005 N or 0.01 N tris buffer, pH 7.4. The column was then washed with 0.01 N buffer and subsequent fractions were displaced from the column by successively higher concentrations of Cl^- ion obtained by increasing the concentrations of the buffer solution or by adding NaCl. Fig. 3 shows the type of separation obtained.

Fraction I (not included in the curve) consists of those cell sap components which have not been adsorbed by the ion exchanger under these experimental conditions and comprises about 30 % of the total cell sap protein by weight. This fraction contains only a very small amount of the total radioactive phosphoserine protein and 4 % or less ot the total material yielding 260 mμ adsorbing substances when extracted with warm PCA. In most preparative experiments fractions II—V were combined. The combined fraction then amounted to about 30 % of the total cell sap protein, roughly 20 % of the radioactive phosphoserine protein and about 12 % of the total "RNA". Also, for cruder, initial separations VI—IX were combined corresponding to about 20 % of the total protein but more than 60 % of the total radioactive phosphoserine protein and RNA. Reducing the pH of the displacing buffer (to pH 6 or 5) did not cause the displacement of any additional protein from the column.

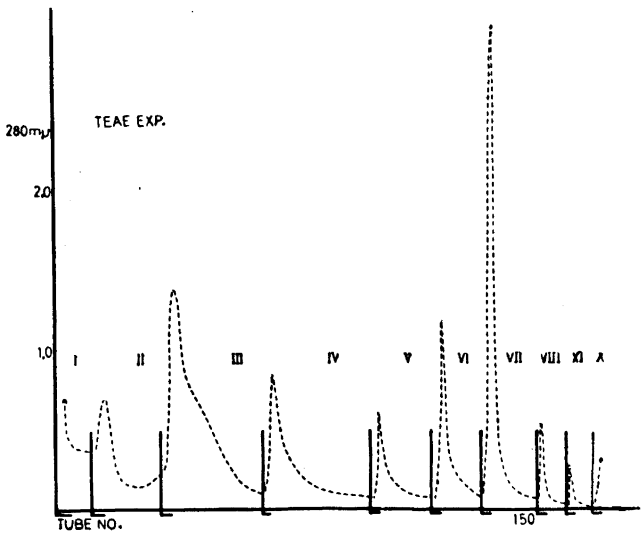

Fig. 3. Fresh supernatant prepared with 0.25 M sucrose from 2 rats, chromatographed on a 3 × 10 cm TEAE column, previously equilibrated with 0.005 M "tris", pH 7.4, subsequently washed with 0.005 M "tris", pH 7.4 to obtain fraction 1. Stepwise elution at same pH with 0.01 M "tris", 0.05 M "tris" + 0.05 M NaCl, 0.05 M "tris" + 0.1 M NaCl, 0.05 M "tris" + 0.2 M NaCl, 0.05 M "tris" + 0.5 M NaCl, 0.1 M "tris" + 0.9 M NaCl, 0.1 M "tris" + 1.5 M NaCl to obtain fractions II—IX, respectively. Remaining adsorbed material eluted with 2 % NaOH. Approximately 15 ml fractions taken, measured at 280 mμ.

However, an additional fraction (not shown in the curve) which corresponded to about 10 % of the total protein was displaced from the column by 2 % NaOH. This apparently corresponds to protein, denatured during the chromatographic procedure (however, see discussion) and in several experiments was found to contain between 20 and 30 % of the total radioactive phosphoserine protein as well as a considerable amount of RNA. Experiments in which the initial pH of the ion exchange column was lower (6.2) (that of the cell sap had also been reduced) yielded no reduction in the amount of "denatured" phosphoprotein. Nor did increasing the initial chloride concentrations of the cell sap and column have a favorable effect.

When TEAE fractions II—V were combined, ultrafiltered, dialyzed and subjected to zone electrophoresis the electropherogram shown in Fig. 4 was obtained. The main peak appears to correspond to the last radioactive peak in Fig. 1. Moreover, when in another experiment, material corresponding to the latter peak was chromatographed on a TEAE column it was found to be eluted with Cl-concentrations corresponding to fractions II—V. TEAE fractions VI—IX yielded the electropherogram shown in Fig. 5. The main peak resembles the third radioactive peak in Fig. 1. The identity of the two peaks was confirmed by chromatography of peak 3, Fig. 1, on a TEAE column. It should be noted that the most slowly moving peaks in Fig. 1 are contained

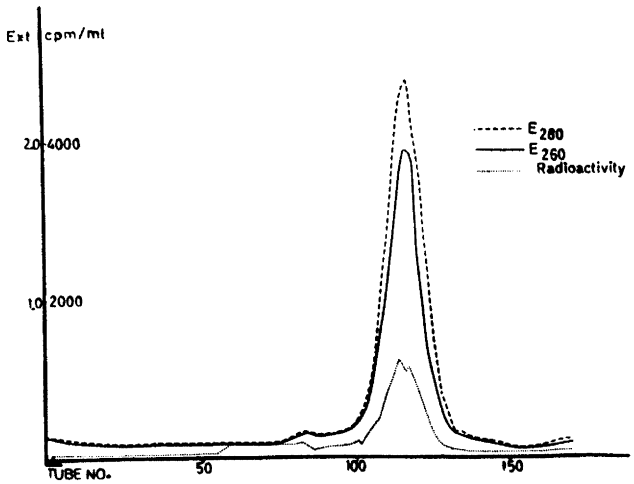

Fig. 4. Same experiment as in Fig. 3. Electropherogram of TEAE fraction corresponding to fractions III—V, Fig. 3. After ultrafiltration and dialysis, electrophoresis and elution as in Fig. 5.

in TEAE fractions VI—IX which otherwise contains only the faster moving electrophoretic components. As may be seen in both Figs. 1 and 5 the slower moving components appear to contain bound nucleotide material, which

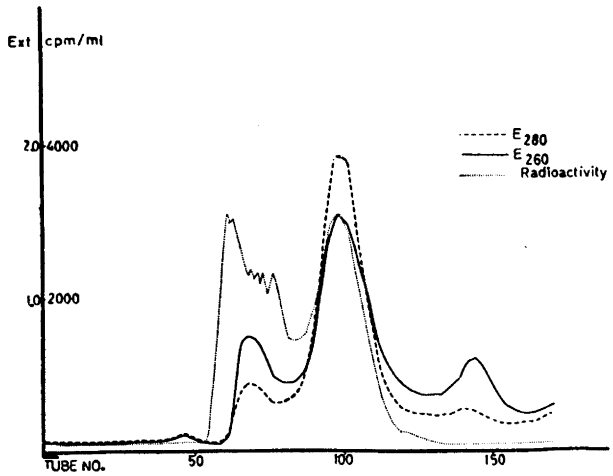

Fig. 5. Electropherogram of TEAE fraction corresponding to fractions VI—VIII, Fig. 3. 4 rats injected with 1.5 mC ^{32}P each, 2 h previous to perfusion. Supernatant prepared with 0.25 M sucrose, pH 7.4, chromatographed on 3 × 10 cm TEAE column. Fraction corresponding to fractions VI—VIII, Fig. 3, ultrafiltered and applied to electrophoresis column. Electrophoresis in 0.1 M "tris", pH 7.8 for 46 h, 45 mA (900 V). Fractions collected and measured as in Fig. 1.

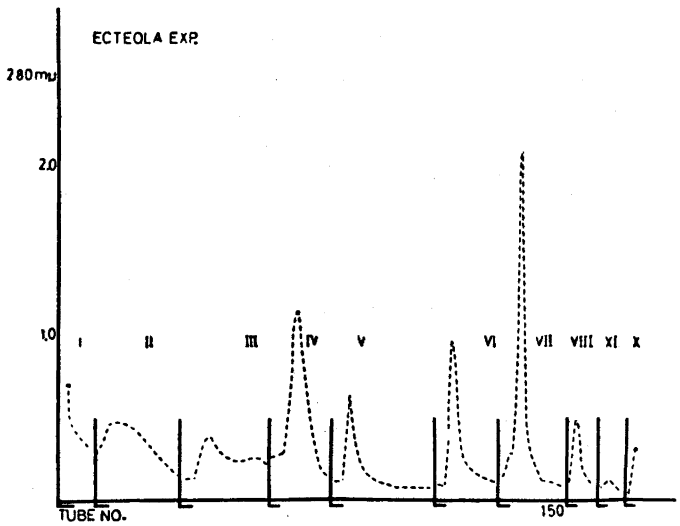

Fig. 6. Fresh supernatant prepared with 0.25 M sucrose from 2 rats, chromatographed on a 3 × 10 cm ECTEOLA column under conditions identical to those in the TEAE experiment shown in Fig. 3.

although released for the most part by cold perchloric acid, does not appear to contain phosphorus which has exchanged with other nucleotide compounds of the cell.

ECTEOLA columns were also found to be useful for the fractionation of the acidic cell sap proteins. While a large part of the total cell sap protein passes through the column with 0.005 N tris, pH 7.4, the phosphoserine and phosphothreonine proteins are retained and may be displaced to yield a fractionation pattern which is quite similar to that of the TEAE columns. Moreover, electropherograms of the ECTEOLA fractions were similar to those obtained with the corresponding TEAE fractions. As is the case with the TEAE columns some of the phosphoprotein is retained by the column in such a way that it can only be displaced by weak NaOH. An elution curve is reproduced in Fig. 6 for purposes of comparison.

Incubation experiments. Some preliminary incubation experiments have been carried out with the whole rat liver supernatant fraction. When a mixture of ^{32}P-containing nucleotides fortified with highly radioactive $AT^{32}P$ was added to fresh non-radioactive supernatant from two rat liver homogenates incorporation of ^{32}P into not only the phosphoserine but also the phosphothreonine of the protein could be demonstrated. Moreover, incorporation of the isotope could also be demonstrated in an experiment in which 1 mC inorganic ^{32}P was added to fresh rat liver supernatant and the mixture shaken at 20—21° C for 15 min. While under these conditions no exchange of RNA phosphorus occurred, the phosphoserine and phosphothreonine isolated from hydrolysates of the protein both contained ^{32}P.

DISCUSSION

Determination of phosphoproteins. Although phosphorus conceivably may be bound to protein in a number of ways, the experiments described in the present paper have mainly concerned those proteins of rat liver cell sap which contain phosphoserine and phosphothreonine. As yet these are the only two phosphorylated amino acids which have been isolated from liver protein. These amino acid derivatives may be qualitatively determined as described previously [1,2], but, unfortunately, there is still no satisfactory method for their quantitative determination in protein linkage. The methods which have hitherto been employed for the determination of protein phosphorus have been based on the old observation by Plimmer [15] that the phosphorus of casein is relatively alkali labile. Thus, Schmidt and Thannhauser [16] and Johnson and Albert [17] among others have subjected organ protein to partial alkaline hydrolysis and designated the liberated inorganic phosphorus as "phosphoprotein phosphorus". The reliability of this type of method for the determination of phosphoserine and phosphothreonine proteins has been questioned earlier [1]. Moreover, since then Crosby *et al.*[18] have reported that the alkaline hydrolysis method used by them to obtain RNA nucleotides from rat liver does not hydrolyze all of the phosphoserine present, and this has been confirmed by the author, under more vigorous alkaline hydrolysis conditions (unpublished experiments). Thus, the alkaline hydrolysis method would definitely seem inadequate when applied to liver phosphoprotein determination.

In the experiments described in this paper the protein separations have been followed by (1) measuring the radioactivity of the protein fractions eluted from the columns and (2) measuring the total ^{32}P-activity of the phosphoserine and phosphothreonine fractions isolated from partial hydrolysates of the fractionated proteins using Dowex 50. The determinations have thus necessarily been only approximate and have not included phosphoproteins in which there is a negligible uptake of radioactive phosphate under the conditions of these experiments. Yet they have been of value here since the phosphoproteins which have been of greatest interest have been those in which the phosphorus itself is metabolically active, with extent and rapidity of uptake of radioactive phosphate being taken as a provisional measure of metabolic activity.

Fractionation methods. It seems likely that the difficulty in obtaining reproducible precipitation of the cell sap phosphoproteins using the method of Cohn may be the result of the very factor, *viz.* co-precipitation, which has made the method so selective when used for the fractionation of serum proteins in the hands of Cohn and collaborators. Thus, small differences in the protein and inorganic cation concentrations of the cell sap caused by irregular perfusion or an altered adsorption of soluble proteins onto the intracellular particles as result of changes in the pH of the cell sap during the preparation could conceivably result in significant changes in the fractionation pattern. This would be especially true for the more highly charged proteins which would normally show a strong tendency to form co-precipitates. To reduce these effects in the subsequent ammonium sulfate fractionation experiments the following steps were taken. 0.01 M versene was included in the homogenization medium (and perfusion media). The precipitation was carried out at

pH 7.0 and only a few fractions were taken to facilitate the subsequent zone electrophoresis.

In the experiments with the strongly basic ion exchangers it was found that a considerable amount of phosphoprotein + nucleoprotein material ostensibly became strongly bound to the column (the fractions eluted with NaOH). Experiments designed to prevent this effect were not successful. It seems possible that denaturation of the proteins has occurred here. To further investigate this possibility experiments with more weakly basic ion exchange columns would be of value. Another possibility is that all or a major part of the strongly "adsorbed" material is in fact submicroscopic particulate matter [19] which has not been sedimented in the centrifugal fields employed here. It is perhaps significant that roughly proportional amounts of phosphoprotein + nucleoprotein material is "denatured" or rendered insoluble by (1) treatment with low concentrations of ethanol, (2) passage through ion exchange columns, (3) precipitation with low concentrations of $MgCl_2$ (unpublished experiments).

The ion exchange experiments described here have been of a preparative nature, and no attempt has been made to obtain a finer separation of the less strongly bound, non phosphothreonine or phosphoserine-containing proteins. Some experiments have been carried out where the purified phosphoamino acid-containing fractions were rechromatographed on TEAE columns with gradient elution but so far neither these nor re-electrophoresis experiments have yielded a subfractionation of the main phosphorus-containing peaks shown in Figs. 4 and 5. However, additional attempts to purify the phosphoprotein fractions are in progress as well as incubation experiments with the purified fractions. These experiments will be reported in a later paper.

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