A Study of Phosphopeptones from Casein

I. Methods of fractionation

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Recently, phosphoproteins have been the object of several chemical investigations and a considerable amount of speculation. Several authors have attempted to isolate and study phosphoproteins from liver and other organs [1-4], and the physiological significance of these is much discussed at present [5]. However, most of the studies have been made on hydrolytic products of the phosphorus-containing proteins of milk and egg [6,7]. These are, of course, convenient to study because of their ready availability.

After enzymatic hydrolysis of casein, Shinn and Nicolet [7,8] isolated a phosphorus-containing fraction with a total of 8 amino acids. Mellander [9], who has made an extensive review of the literature, prepared barium and calcium salts of casein phosphopeptones, using a modification of the method described by Damodaran and Ramachandran. His product contained 13 different amino acids [10,11]. Later, Mellander and Isaksson [12,13] demonstrated an interesting physiological property of their phosphopeptones: calcium phosphopeptones are resorbed and can be used to promote bone calcification in rachitic children.

In this laboratory we have also been interested in phosphoproteins, but it has become evident that before their physiological significance and metabolism can be unravelled much further, more work will have to be done toward the isolation of these substances in a purified form. We have therefore concentrated our efforts on the development of methods of isolation and analysis, using phosphopeptones from casein as suitable models. These have been obtained after hydrolysis with either hydrochloric acid or proteolytic enzymes. Phosphoserine and phosphothreonine have been crystallized from acid hydrolysates [14-16]. Moreover, some low molecular peptides from such hydrolysates have also been isolated [17]. The present paper deals mainly with methods used in attempts to fractionate phosphopeptones of higher molecular weight, obtained by enzymatic hydrolysis.

METHODS AND RESULTS

Material. The calcium phosphopeptones used in the following experiments were obtained through the courtesy of Professor Mellander, Institute of Physiological Chemistry, Gothenburg, and were prepared according to the method used by him [9]. In this method large amounts of casein are digested for 12 days at pH 2 and 37° C with three successive additions of pepsin. A constant amino nitrogen value is then reached. Subsequently, the pH of the digest is adjusted to 7.5 and it is exposed to the action of repeatedly added amounts of trypsin at 37° C for 8 days, at which time a constant amino nitrogen value is obtained. The phosphopeptones are then precipitated as lead salts, decomposed by means of hydrogen sulfide, and finally obtained as calcium salts. Our preparation had the following composition: N 8.8; P 4.0; Ca 10.7 %; and also contained small amounts of Pb and Fe. Standard micro-analytical methods have been employed for the analysis of the elements shown in our Tables. For nitrogen the common micro-modification of Kjeldahl's method (*cf.* Pregl-Roth [18]) was used. Phosphorus was determined according to Teorell [19]. For amino nitrogen the manometric method of van Slyke [20] was used. Calcium was determined by the method of Widmark and Vahlquist [21] and iron as ferro-*o*-phenanthrolin according to the modification described by Blix [22]. For analysis the phosphopeptones were dried to constant weight *in vacuo* over phosphorus pentoxide at 70° C.

Preliminary experiments showed that the phosphopeptone preparation not only contained several metals and some basic nitrogen impurities, but also that it was a mixture of many peptides. It therefore seemed advisable to desalt the material and to carry out a rough fractionation of the peptides before attempting a more sensitive chromatographic resolution.

Ionophoresis. The first step of this procedure utilized an ionophoresis apparatus composed of four large plexiglass compartments, and built on the same principle as that described by Sanger and Tuppy [23]. Compartment 1, the anode cell, was separated from compartment 2 by a membrane consisting of two cellophan sheets; compartments 2 and 3 were separated by a goat skin membrane; and compartment 3 was separated from compartment 4, the cathode cell, by a double paper parchment membrane. The electrodes were 4.5 × 4.5 cm platina sheets. Compartment 1 was filled with 0.3 N H$_2$SO$_4$, compartment 3 with a solution containing 2 g of calcium phosphopeptone, and compartments 2 and 4 were filled with distilled water. When thus filled and ready for use, the apparatus contained about 800 ml solution.

At the beginning of an experiment the voltage was adjusted so that the current was about 300 mA, and then, as the current dropped, was gradually increased to 500 V. The content of the cathode compartment was replaced with distilled water several times during the experiment. After the current strength had dropped to about 50 mA, the content of the anode compartment was replaced with 0.3 N H$_2$SO$_4$. Finally, after a few hours, the content of compartment 2 was replaced with distilled water. The experiment was discontinued after about 30 hours, when the current had reached a limiting value of about 20 mA.

With this method most of the metal and basic nitrogen impurities (peptides) migrated to the cathode, and a crude separation of the phosphopeptides into 4 fractions was obtained. About 150 mg of the original 2 g remained in compartment 3. Approximately half of this had precipitated out of solution during the experiment. This was taken as fraction 1. The phosphopeptone remaining in solution was taken as fraction 2. About 1 g phosphopeptone had migrated to cell 2, and about 150 mg could be recovered from the combined and neutralized solutions from compartment 1. To this end the solubility of several heavy metal salts of phosphopeptone was studied. Water solutions of phosphopeptones were not precipitated by the acetates of cadmium, cobalt, iron, or mercury, while insoluble salts were formed by those of bismuth, copper, lanthanum, silver, and uranium. The copper phosphopeptone was the salt most easily and quantitatively decomposed by electrodialysis, a procedure which we considered preferable to decomposition by hydrogen sulfide. Moreover, it was just as insoluble as the other salts, and had been used in the preparation of phosphopeptones by Rimington as early as 1927 [24]. Thus, to obtain fraction 4 in the form of a free acid, it was first precipitated from Na_2SO_4 solution at pH 3 as its copper salt, and then the latter was decomposed by electrodialysis in an apparatus similar to that described by Theorell and Åkesson [25].

The composition of the four fractions obtained by ionophoresis is listed in Tables 1 and 2. As can be seen, a considerable amount of iron has remained in compartment 3 and traces even appear om cell 2. This is of special interest, since the natural occurrence of iron phosphoproteins has already been established by several authors [26-28].

Table 1. Composition of fractions from ionophoresis of phosphopeptone.

Cell	N	P	Ca	Fe	Amino N in % of total N
1	10.8	6.9	0	0	7
2	10.0	4.9	0	trace	6
3 (soluble)	11.9	4.4	1.8	3.2	9
3 (insoluble)	11.3	2.1	1.6	1.9	7

Methods of Indication. Before attempting a further separation of the ionophoresis fractions, it was necessary to find suitable methods for the identification of phosphopeptones. Three different methods were chosen, the ninhydrin and biuret methods for the detection of peptides, and the ammonium molybdate-benzidine reaction for the specific detection of the phosphate ion. Each of these can be used as a spot test for the indication of peptides on paper chromatograms.

The usual ninhydrin reagent (0.25 % ninhydrin in butanol containing 1 % acetic acid) gave no color reaction with the phosphopeptones, even when spots containing 1 000 μg were dried onto a filter paper and sprayed with the reagent. However, a color reaction was obtained with the modification of the ninhydrin reagent recently described by Lewis [29]. With this reagent 5 μg phosphopeptone could be detected.

The biuret reaction used was a modification of that used by Killander [30] on paper chromatograms. 0.1 mg of the phosphopeptone, dried onto an area of 1 cm² of filter paper gave a violet spot with the combined reagents. It has not been possible to increase the sensitivity of the reaction. A reagent, stable for longer periods, is the following. A mixture of 10 ml of ethanol and 1 ml of glycerine is saturated with copper acetate. To this solution is added 40 ml of ethanol and 50 ml of a 25 per cent solution of tetra-methyl-ammonium hydroxide. With this reagent 100 μg of phosphopeptone could be detected.

The phosphate reaction used was the ammonium molybdate-benzidine spot test [31], modified for use with organic phosphate. The procedure is as follows: The papers are sprayed with 96 % ethanol saturated with potassium hydroxide, and are subsequently heated at 100° C for 10 minutes. This procedure is repeated once. This causes the papers to assume an intense yellow color, and causes hydrolysis of the phosphopeptone without destroying the paper (the paper used throughout in these experiments has been Munktell No. OB, washed with acetic acid and water according to Hanes and Isherwood [32]). Before applying the reagents the papers are suspended in a glass jar containing an atmosphere concentrated with hydrochloric acid for 30 to 40 minutes. This causes the papers to turn white, and the molybdate reagent can then be applied. The papers are lightly and evenly sprayed with the ammonium molybdate solution and allowed to dry for 15 minutes. Subsequently, they are sprayed with the benzidine solution and immediately afterwards suspended in a second glass jar, containing an atmosphere of concentrated ammonia. Within a very short time blue spots appear corresponding to the positions of phosphopeptides. The limit of identification on a chromatogram is about 25 μg of phosphopeptone with a phosphorus content of 5 per cent.

Of the three methods mentioned above the ninhydrin and ammonium molybdate-benzidine reactions have been of the greatest value. Usually these two reactions have been carried out in succession on the same paper, allowing the detection of phosphorus-free peptides as well as phosphorus-containing peptides.

Paper Chromatography

Paper chromatography was found to be of value not only as a resolution technique in its own right, but also as a means of indicating fractionation attained in experiments with ion exchange resins and carbon.

Before suitable solvent systems were found, a large number were tried which were miscible with water in both small and large proportions such as: ethanol, propanol, acetone, phenol, pyridine/amyl alcohol, pyridine, n-butanol, n-butanol/acetic acid, and benzyl alcohol. In many cases there was a tendency to form multiple-banded spots, but when the bands were separately eluted with water, electro-dialysed and again analysed by paper chromatography, it was shown that no real separation had occurred. The apparent separation seemed to have been caused by the formation of addition products of solvent and solute. Some of the solvent systems which did give a useful separation were the following: *iso*butyric acid/water, propionic acid/*iso*valeric acid

(1 : 1)/water, and propionic acid/water mixtures. At present the most generaliy used mixture is the following: 25 ml benzene / 25 ml water / 62 ml propionic acid.

For the development of one-dimensional chromatograms, solutions of phosphopeptone containing about 100 μg were applied to a filter paper strip as spots of 1 cm diameter at 3 cm intervals along the starting line. Ascending chromatography was generally used with the benzene/water/propionic acid mixture; the front reached the upper part of the paper in 9—10 hours. The papers were dried at room temperature.

Amino Acid Determinations

Fractionation experiments on ion exchange and carbon columns were controlled by analyses for total nitrogen, amino nitrogen, and phosphorus in order to determine differences in elementary composition. Moreover, it was felt that valuable complementary information would be disclosed by amino acid analyses. For that purpose a rapid method, capable of yielding a scanning estimation was necessary, and this seemed to be afforded by a modification of the colorimetric method, recently described by Lewis [29] for estimation of amino acids on twodimensional paper chromatograms.

An extensive comparison of washed [33] and unwashed Munktell No. OB, Whatman No. 1, and Whatman No. 4 papers was carried out. In all cases the washed papers gave a better and more rapid separation of the amino acids. The Munktell papers were more easily washed free from impurities and yielded as good a separation as the Whatman papers; they were therefore used in the following. The dimensions of the papers were 38 × 28 cm[33]. Ascending chromatography with phenol-ammonia was used in the long direction, and it required about 15 hours for the front to reach the upper edge of the paper. Freshly destilled phenol was always used. The sheets were then dried at room temperature for 24 hours in a cabinet with good ventilation, and the yellowish brown material deposited by the phenol front was cut away. Subsequently, the papers were developed in the second direction using descending chromatography with pyridine/amyl alcohol for 6 hours. This solution could be used 2—3 times without change. The papers were then dried, sprayed with the ninhydrin reagent described by Lewis, and allowed to dry again for 24 hours. For separation of leucine and isoleucine, one-dimensional chromatograms were developed with lutidine/tertiary amyl alcohol on washed papers for 35—40 hours. Glass containers of suitable sizes were employed for all runs, and control papers with amino acid mixtures resembling those of the samples both in quality and quantity were always run parallel in the same jar.

The spots were cut out and eluted from the dried papers as described by Lewis, and the extracts were transferred quantitatively to a colorimeter cell, and measured in the Beckman B spectrophotometer at 570 mμ (440 mμ for the proline extracts). The value read was corrected for the blank obtained by extracting a circle of the same size as the colored spot from the developed paper. The corresponding amino acid values were read from standard curves constructed for each amino acid by running large series of different amounts

of all the amino acids on two dimensional chromatograms. When necessary, a correction factor was used, calculated from the control, run in parallel with the sample. In most of the experiments the standard error of a single estimation was ± 10—15 per cent. Some values obtained from ionophoresis and Dowex 2 (see below) fractions are listed in Table 2. The recoveries are comparatively good, but in general about 20 % too low. The same result is obtained if the amino nitrogen is calculated in per cent of the total nitrogen.

Table 2. Amino acid composition of fractions from ionophoresis and Dowex 2. Values expressed as percentage for moisture-free material.

Preparation	Asp	Glu	Ser	Gly	Thr	Ala	Lys	Arg	His	Pro	Val	Ileu	Leu	α-amino butyr. ac.	Total	Total + PO₄
Cell 1	8.4	20.5	14.0	2.5	4.2	4.7	0.6	—	—	3.4	5.8	5.0	1.0	—	70.1	90.7
Cell 2	10.4	24.3	13.6	3.2	4.6	2.4	1.9	—	0.3	3.8	5.3	5.5	1.8	—	77.1	91.8
Cell 3 (soluble)	10.0	23.6	12.0	3.1	4.7	2.9	4.4	0.9	3.2	5.7	4.3	5.8	2.7	—	83.3	96.5
Cell 3 (insoluble)	9.9	27.5	12.3	1.7	1.6	1.8	3.1	0.9	3.2	6.3	4.2	1.9	1.7	—	76.1	82.4
Fraction 2 Dowex	10.7	26.5	13.0	1.8	7.8	2.6	5.1	—	1.9	7.5	3.5	2.0	0.5	—	82.9	96.7
Fraction 3 Dowex	20.2	33.6	11.0	2.6	4.4	1.9	8.9	—	0.8	0.9	2.3	3.6	1.4	7.2	98.8	107.2
Fraction 4 Dowex	13.9	28.4	15.7	3.1	5.2	2.4	3.6	. —	0.7	3.3	3.3	6.5	2.4	4.7	93.2	108.5
Fraction 5 Dowex	13.1	24.2	12.8	2.6	2.8	5.5	4.4	—	—	4.4	5.0	5.6	1.5	4.9	86.2	102.1
Fraction 6 Dowex	8.9	24.3	15.2	3.5	3.2	3.8	2.7	—	—	6.2	5.3	7.4	3.0	—	82.9	99.7
Fraction 7 Dowex	10.0	29.9	19.3	2.8	7.8	4.1	2.7	—	—	5.2	5.7	8.4	3.1	3.9	102.9	118.2

A comparison between the four ionophoretic fractions shows very clearly that the two fractions from cell 3 contain considerable amounts of all of the three basic amino acids, while the fraction from cell 2 only contains lysine and histidine and the material from cell 1 only inconsiderable amounts of lysine.

Ion Exchange Columns

Some preliminary experiments were carried out with the ion exchange resin, Dowex 50 (approx. 10 % cross linking, and 250—500 mesh) using 0.01—2 N HCl as displacer. However, this resin did not yield a satisfactory separation and about half of the phosphopeptone put onto the column could not be recovered from the effluent solution. This also diminished the value of Dowex 50 as a desalting material.

Experiments with Dowex 2 columns (approx. 10 % cross linking, and 200 —400 mesh) carried out according to a procedure similar to that described by Cohn [34,35] gave better results. In these experiments a large 6.5 × 50 cm glass column fitted with a glass filter was used for preparative work. 2 g of material from compartment 2 of the ionophoresis apparatus were dissolved by adding dilute NaOH to a volume of 20—30 ml and a pH of 5. The solution was transferred to the column, which was in the chloride form, and had been wahsed until the effluent was of pH 5. Fraction 1 was washed from the column with distilled water, fractions 2 and 3 were removed with 0.01 N HCl,

Table 3. Composition of fractions from separation on Dowex 2 columns.

Fraction	N	P	Amino N in % of total N	Yield in g from 4 g
1 (water)	11.7	3.7	—	0.20
2 (0.01 N HCl)	13.2	4.6	4	1.02
3 (0.01 N HCl)	13.2	2.8	7	0.17
4 (0.02 N HCl)	12.9	5.1	5	0.15
5 (0.05 N HCl)	12.4	5.1	7	0.28
6 (0.2 N HCl)	11.3	5.4	4	0.07
7 (2 N HCl)	11.3	5.4	—	0.08

and fractions 4, 5, 6, and 7 with 0.02 N HCl, 0.05 N HCl, 0.2 N HCl, and 2 N HCl, respectively. In each case the volume of displacer used was such that control papers sprayed with the phosphate reagent showed a nearly complete removal of the fraction. The fractions displaced with 0.02—2 N HCl were immediately brought to pH 3, precipitated with copper acetate, and electro-dialysed. Total nitrogen, phosphorus, and yield are summarized in Table 3. For amino acid composition of the fractions, see Table 2.

As can be seen from the Tables, Dowex 2 suffers from one of the same defects as Dowex 50, for about half of the material put onto the column cannot be displaced with the concentrations of HCl used. Experiments are now in progress with modified forms of Dowex 2 in order to improve the yield. Nevertheless, use of unmodified Dowex 2 does permit a fractionation of the mixture of peptides from compartment 2. There is a clear tendency toward the displacement of more highly acidic peptides with increasing concentrations of HCl. And there is also a tendency for the later fractions to contain higher concentrations of leucine and isoleucine. Moreover, the occurance of α-amino-butyric acid in some of the later fractions is to be noted. So far, however, no single pure peptide has been obtained directly by fractionation on Dowex 2.

Carbon Columns

At present the routine procedure used for the further separation of the ionophoresis fractions has employed chromatography on carbon columns as the second step. The carbon used has been Norit FNX Special. Before use, the carbon has been washed with several liters of 20 per cent acetic acid, 96 per cent alcohol, and finally with distilled water. In addition, to improve the yields of the fractions displaced from the column, the carbon has been pretreated with stearic acid in a manner closely similar to that described by Hagdahl, Williams, and Tiselius [36] and Hagdahl (personal communication). 100 g of the charcoal is suspended in an ether solution of stearic acid and stirred mechanically for 24 hours, adding more ether at intervals. The ether is then filtered off. Under these conditions the Norit takes up no more than 20—25 per cent of stearic acid even though an excess amount of the latter is present.

The carbon obtained by this procedure is suspended in distilled water and poured into a shallow glass column. Several liters of water are again run through the column before the start of the experiment. A water solution of 200 mg phosphopeptone is then transferred to the column, and the column is washed with 2 liters of distilled water, causing the removal of about 20 per cent by weight of the phosphopeptone. This fraction, as tested by paper chromatography, has been found to be quite different from the next fraction, displaced from the column by a saturated solution of *iso*valeric acid in water (neither 20 per cent acetic acid nor 20 per cent propionic acid cause a displacement of any of the phosphopeptone). A third fraction, rather similar to the second, can be displaced by a 10 per cent solution of *iso*valeric acid in 20 per cent acetic acid. Together, the two *iso*valeric acid fractions amount to about 40—45 per cent of the phosphopeptone put onto the column. A fourth fraction can be displaced with lauric acid and a fifth with stearic acid (both acids in the form of sodium salts in a saturated solution of 50 per cent ethanol). The phosphopeptones displaced with these solutions bring the total up to about 85—95 per cent of that originally put on the column. Chromatograms of the fractions have shown that a definite separation of the ionophoresis fractions is attained. Nevertheless, all of the fractions have been shown to contain several peptides.

Paper Powder Columns

In order to further separate the fractions obtained from the carbon columns, paper chromatography on columns has been used. Paper columns have been used in this laboratory for several years for the separation of amino acids. These columns have been largely of two sizes, 1.8 × 70 cm and 6.5 × 40 cm. The powder has been of the same quality as that used to manufacture Munktell No. OB paper, and has been ground several times in a Wiley mill and thoroughly washed with water, ethanol, and ethyl ether on large Buchner funnels.

The smaller sized columns have usually been filled with 0.5—1 g portions of dry powder and packed with flat stainless steel stamps, of a diameter a few mm less than that of the glass chromatographic tube. The large columns have been packed with 2—3 g portions of paper using alternately a flat and a rounded stamp. This procedure was shown to be necessary in order to obtain a uniform flow rate of solvent through the column, as controlled by observation of the movement of a band of methyl orange through the tube with *n*-butanol/acetic acid/water [37] as solvent.

The solvent to be used in a given experiment is run through the dry column until the effluent is colorless, and a mixture of the dry amino acids is packed evenly onto the upper surface of the column with a glass stamp. The amino acid mixtures are usually mixed with a small amount of paper powder to insure a more even distribution over the top of the column. After cautiously driving the amino acids into the column with small additions of solvent, the reservoir is connected, and the solution allowed to drop into the tubes of a fraction collector. Usually atmospheric pressure is all that is needed to obtain a satisfactory flow of solvent. Using this method and the *n*-butanol/acetic acid/water mixture, a satisfactory separation of up to 200 mg of leucine, valine,

α-amino-butyric acid, and alanine can easily be obtained on the larger column. It is our impression that the resolution is nearly as good as that obtained on paper strip chromatograms.

The paper column methods worked out for amino acids have worked equally well for phosphopeptones, and several of the Norit fractions have been further separated into fractions giving a ninhydrin reaction but no phosphate reaction, fractions giving a phosphate reaction but no ninhydrin reaction, and into fractions giving both a ninhydrin and a phosphate reaction. Up to now, only the smaller columns have been used, 150 mg samples of each fraction being put onto the column and eluted with the same benzene/water/propionic acid mixture used in the paper chromatograms mentioned earlier.

Using the methods outlined above it has been possible to obtain larger quantities of phosphopeptides, at least partially separated and purified from non-phosphorus containg peptides. Some chemical properties of these peptides will be described in a following paper.

SUMMARY

Some methods have been outlined for the separation and purification of phosphopeptones obtained from bovine casein. Those methods routinely used have been ionophoresis, and chromatography on carbon, ion exchange, and paper powder columns.

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