Isolation of Phosphorylserine from the Plasma Phosphoproteins of the Laying Hen

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Although it has been well established that the onset of laying in the domestic fowl is accompanied by marked increases in protein-bound phosphorus in the blood, the chemical nature of this phosphorus has received relatively little attention. The initial observations of Laskowski (1935) and Roepke & Hughes (1935) demonstrating such an increase have since been confirmed by others both in the domestic fowl (Clegg & Hein, 1953; McKinley, Oliver, Maw & Common, 1953; Clegg, Ericson, Hein, McFarland & Leonard, 1956) and in the pigeon (McDonald & Riddle, 1945). This increase has been associated both with the ability of plasma, or sera, to yield a precipitate when diluted with water (Laskowski, 1935) and with the presence of a new component when sera are examined by electrophoresis (Brandt, Smith, Andrews & Clegg, 1952; McKinley *et al.* 1953; Clegg *et al.* 1956). A recent examination of the precipitate formed on dilution (McIndoe, 1959) showed, over the laying period, that the gross composition was fairly constant, that it contained 70–80% of the total plasma lipids, and that the protein-bound phosphorus was released as inorganic phosphorus when treated with dilute alkali. Even so the precipitate was a mixture of at least two components, separable by ultracentrifuging in saline solution. McIndoe also showed that a large percentage of the total protein-bound phosphorus of the plasma still remained in solution after removal of the precipitate.

The presence of protein-bound phosphorus in the plasma of the laying hen is generally assumed to be associated with the deposition of phosphoprotein in the yolk. Early work of Roepke & Bushnell (1936) suggested that sera from laying hens contained substances capable of inducing the production of antibodies, in the rabbit, to crude preparations of egg-yolk proteins containing the protein phosphorus of the yolk. More recently Mok, Martin & Common (1961) isolated and characterized a small quantity of phosvitin from blood taken from laying hens after administration of oestradiol benzoate. No phosvitin was obtained when oestradiol was not administered.

Apart from this work there appears to be no unequivocal demonstration that the protein-

bound phosphorus of the plasma is indeed organically bound to an amino acid as it is in phosvitin. In this paper, the isolation and identification of *O*-phosphorylserine from the plasma phosphoproteins are described.

MATERIALS AND METHODS

Collection of plasma, and preparation and treatment of plasma fractions

The birds used were a Rhodes-Sussex cross, bred at the National Institute for Research in Dairying, Shinfield, Reading, and maintained on a standard commercial laying mash. The state of the birds, i.e. whether in full lay or not, was determined from the daily records of egg production. Blood was obtained by severing the neck vessels and collecting the blood in beakers containing either heparin (10 units/ml. of blood) or trisodium citrate dihydrate (5 mg./ml. of blood). Where heparin was used birds were also pretreated by an intravenous injection of 1000 units of heparin. The blood was centrifuged at 5° for 30 min. at 2000g and the plasma removed and stored at 2°. During transport to the laboratory the flasks were surrounded by ice. Where the incorporation of radioactive phosphate into plasma phosphoproteins was studied, radioactive phosphate (300 μ c in 0.9% sodium chloride) was administered to each bird via the wing vein. This route was also used for the withdrawal of samples of blood of up to 10 ml.

Preparation of phosphoprotein fractions. Lipophosphoprotein was precipitated from the plasma of laying hens by diluting with 9 vol. of ice-cold water (cf. McIndoe, 1959). After standing for 1–2 hr. at 2° the precipitate was centrifuged. With plasma from the citrate-treated blood the precipitate collected at the bottom of the tubes. With plasma from heparinized blood the precipitate floated to the surface and required centrifuging at 59000g in the no. 21 head of the Spinco Model L to ensure a firm packing of the material. In these experiments the supernatant was carefully removed. The lipophosphoprotein obtained from plasma subjected to either treatment was fatty and yellow in appearance. It was soluble in water and was stored as an aqueous solution. It was reprecipitated either by adjusting the salt concentration to 20 mM-sodium chloride (McIndoe, 1959) or by adding trichloroacetic acid to a final concentration of 10% (w/v). The supernatants remaining after removal of the lipophosphoproteins from the plasma were treated with trichloroacetic acid (to 10%, w/v), and after chilling for 1 hr. the precipitates were removed and washed with trichloroacetic acid (5%, w/v) before further treatment.

Extraction of lipids. Lipids were extracted by treating the precipitates with 20 vol. of chloroform-methanol (2:1, v/v) (Folch, Ascoli, Lees, Meath & LeBaron, 1951) and repeating the extraction with a further 10 vol. of extractant. The residues were washed with ethanol and ether and dried first by suction on a filter funnel and finally under vacuum over phosphorus pentoxide.

Isolation of phosphorylserine. The dried lipid-free residues from 20–50 ml. samples of plasma were hydrolysed for 6.5 hr. at 100° in 5–9 ml. of 2*N*-hydrochloric acid, and, after centrifuging, the supernatants were dried under vacuum over phosphorus pentoxide and potassium hydroxide. The dark-brown solids were dissolved in approximately 2.0 ml. of water, and fractionated on a column (3 cm.² × 20 cm.) of Dowex 50 (H⁺ form; X 12), with 0.04*N*-hydrochloric acid as eluent (Kennedy & Smith, 1954). The total phosphorus content of each 5 ml. fraction was determined. Under these conditions inorganic phosphorus was eluted between the 20th and the 35th ml., authentic phosphorylserine was eluted from the column between the 70th and the 100th ml., and authentic phosphorylthreonine between the 100th and the 150th ml. of effluent. The fractions corresponding to the position occupied by phosphorylserine were combined and freeze-dried over phosphorus pentoxide and potassium hydroxide. The residues were taken up in water (0.1–0.2 ml.) and subjected to paper electrophoresis on Whatman no. 3 paper in formic acid–water (1:4, v/v), adjusted to pH 1.5 with sodium hydroxide (Williams & Sanger, 1959), with a potential gradient of 40 v/cm. for 2 hr. at 11°. After electrophoresis the papers were dried at 60° and the areas corresponding to phosphorylserine cut out and eluted by capillary elution with water. The eluates were freeze-dried and stored. When required, samples were hydrolysed at 100° in 6*N*-hydrochloric acid for 18 hr. and the hydrolysates dried over phosphorus pentoxide and potassium hydroxide. These hydrolysates were used either for the determination of serine and phosphorus or examined by paper chromatography for the presence of amino acids.

Analytical methods

Phosphorus. Total phosphorus and inorganic phosphorus were determined by methods previously described (Heald, 1959).

Phosphoprotein phosphorus. This was determined as the inorganic phosphorus released on incubation of a phosphoprotein with alkali. Samples of phosphoprotein were digested in *N*-sodium hydroxide (4 ml.) at 37° for 18 hr. The solutions were cooled and made acid by the addition of 0.2 vol. of trichloroacetic acid (50%, w/v) and 0.1 vol. of sulphuric acid (10*N*). After chilling for 20–30 min. the precipitate was centrifuged and the supernatant, or a sample, removed for the determination of inorganic phosphorus.

Total lipids. These were determined by first extracting the appropriate material with 20 vol. of chloroform-methanol (2:1, v/v). The extract was then washed by first shaking with 0.2 vol. of water, and, after removal of the upper phase, by shaking twice more with a further 0.2 vol. of the upper phase of a mixture of 0.2 vol. of water and 1 vol. of chloroform-methanol (cf. Folch, Lees & Sloane-Stanley, 1954). The washed solution containing the lipids was brought to volume and samples taken for the determination of dry wt. at 100°.

Phospholipids. These were estimated as total phosphorus

in samples of the washed lipid extracts. A factor of 25 was used to convert mg. of phosphorus to mg. of phospholipid.

Serine. This was determined in hydrolysates of phosphorylserine, obtained as above, by the method of Troll & Cannan (1953). Authentic phosphorylserine subjected to the same hydrolysis procedure was included to provide reference standards.

Determination of radioactivity. This was determined by counting the solutions used to estimate phosphorus in a M.6.H. liquid counter (20th-Century Electronics Ltd.). Corrections were made for decay, background and dead-time where necessary.

Paper chromatography of amino acids. This was performed by the ascending technique, with phenol–water (6:4, w/v) and the upper layer of butan-1-ol–acetic acid–water (4:1:5, by vol.) as the solvents.

Chemicals

Phosvitin, O-phosphorylserine and O-phosphorylthreonine. These were obtained from Nutritional Biochemicals Corp., California, U.S.A. The phosvitin contained 10.1% of phosphorus.

Radioactive phosphate. This was obtained in 0.9% sodium chloride solution from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Table 1 shows the general composition, in terms of phosphorus, of the various fractions obtained from the plasma of laying and non-laying birds. The increased quantities of phosphoprotein phosphorus, phospholipid and total lipids in plasma from laying birds have been described previously, and variation such as that between the two experimental groups shown in Table 1 has been noted [for review see Sturkie (1954)]. The distribution of lipids and of phosphoprotein phosphorus in the lipophosphoprotein fractions differs in the two types of experimental groups. Thus in Expt. 1, in which heparin was used as an anticoagulant, the lipophosphoprotein contained 41% of the total plasma lipid and 16.7% of the total phosphoprotein phosphorus. In Expt. 2, where citrate was the anticoagulant, the lipophosphoprotein contained 75% of the total plasma lipid and 72% of the phosphoprotein phosphorus. This latter distribution is similar to that described by McIndoe (1959) who used citrate as an anticoagulant. The injection of a polyanion is known to induce the appearance of a lipoprotein lipase in the blood (Korn, 1959; Robinson & French, 1960), and the differences in the distribution of lipids and phosphoprotein in the lipophosphoprotein fractions derived from heparinized blood are probably due to such an action on the lipoprotein complexes in the plasma of the laying hen. In agreement with McIndoe (1959) no precipitate of lipophosphoprotein could be obtained from plasma of the cockerel or the non-laying hen.

Identification of phosphorylserine in hydrolysates of the plasma phosphoproteins. When the lipid-free-phosphoprotein fractions were partially hydrolysed with hydrochloric acid and the aqueous solutions, containing 85–96% of the total phosphorus of the fractions, were separated on Dowex 50, only two phosphorus-containing fractions were obtained from any hydrolysate. The first was eluted at a point corresponding to inorganic phosphate and the second fraction at a point corresponding to authentic phosphorylserine. Similar hydrolysates prepared from trichloroacetic acid precipitates of plasma from non-laying hens or cockerels contained little phosphorus, none of which appeared as a distinct fraction at any point other than that occupied by inorganic phosphate.

The recoveries of phosphorus added to the columns in the various hydrolysates are shown in Table 2. The greatest recoveries were obtained with hydrolysates derived from the lipophosphoproteins, which also yielded the greater proportion of their organic phosphorus as presumptive phosphoryl-

serine. When an authentic specimen of phosvitin was subjected to the same procedures, the recovery of phosphorylserine was intermediate between those obtained from the two plasma phosphoprotein fractions. Total hydrolysis of the presumptive phosphorylserine fraction followed by paper chromatography of the hydrolysate revealed the presence of many amino acids in addition to serine. The fraction was therefore subjected to electrophoresis at pH 1.5 as described by Williams & Sanger (1959). Under these conditions all amino acids other than those containing one or more phosphate groups migrate towards the cathode and those containing phosphate groups remain negatively charged and migrate towards the anode. Peptides containing one phosphate group are either electrically neutral, or weakly basic, depending on the amino acids present. When the presumptive phosphorylserine fractions were treated in this way two areas, reacting positively with ninhydrin and with the reagent of Wade & Morgan (1954) for the identification of phosphates, were detected

Table 1. *Composition of plasma and plasma fractions from laying hens, non-laying hens and cockerels*

For details of preparation and analysis see the Materials and Methods section. In Expt. 1, the blood was heparinized, and, in Expt. 2, was treated with citrate before separation of the plasma. All values are expressed as mg./100 ml. of plasma. In each experiment 6 laying hens, 1 non-laying hen and 1 cockerel were used.

Fraction	Expt. 1			Expt. 2		
	Laying hens	Non-laying hen	Cockerel	Laying hens	Non-laying hen	Cockerel
Plasma						
Total P	39.7	13.4	10.5	120.0	19.2	16.2
Inorganic P	4.6	4.9	—	9.8	9.6	4.0
Phosphoprotein P	9.6	<0.5	<0.5	16.5	4.0	1.6
Lipid P	18.8	5.5	6.4	51.5	7.9	7.9
Phospholipids (Lipid P × 25)	468.0	138.0	160.0	1285.0	196.0	196.0
Total lipids	1590.0	560.0	520.0	4200.0	420.0	420.0
Lipophosphoprotein						
Total P	8.0	0	0	—	0	0
Phosphoprotein P	1.6	0	0	11.9	0	0
Lipid P	7.3	0	0	33.0	0	0
Phospholipids (Lipid P × 25)	183.0	0	0	825.0	0	0
Total lipids	650.0	0	0	3140.0	0	0
Supernatant						
Phosphoprotein P	7.9	<0.5	<0.5	4.5	4.0	1.6

Table 2. *Recovery of phosphorus after fractionation of hydrolysates of phosphoprotein fractions on Dowex 50*

For experimental details see the Materials and Methods section.

Fraction hydrolysed	P added to column (μg.)	P recovered (%)	Organic P added (μg.)	Organic P in fraction corresponding to phosphorylserine (%)
Lipophosphoprotein (1)*	210.0	93.0	130.0	54.0
Lipophosphoprotein (2)*	550.0	99.0	246.0	54.0
Supernatant phosphoprotein (1)*	2600.0	72.5	2116.0	24.8
Supernatant phosphoprotein (2)*	3640.0	64.0	3080.0	21.0
Phosvitin	620.0	76.0	422.0	33.6
Phosvitin	353.0	99.0	258.0	46.5

* The numbers 1 and 2 refer to the experiments listed in Table 1.

Table 3. *Specific radioactivity of phosphoprotein phosphorus and phosphorylserine derived from plasma fractions of laying hens after injection of ^{32}P*

Birds were injected with 300 μC of ^{32}P in 0.9% sodium chloride and bled at the intervals stated below. Blood was treated with citrate and the various fractions were prepared and analysed as described in the Materials and Methods section. Specific activity is expressed as counts/100 sec./ μg . of phosphorus. —, Not estimated.

Time after injections (hr.)	Specific activity of phosphorus					
	Alkali-labile phosphorus			Phosphorylserine		
	From total phosphoprotein	From phospholipoprotein	From supernatant phosphoprotein	From total phosphoprotein	From phospholipoprotein	From supernatant phosphoprotein
2	26.3	—	—	27.4	—	—
4	61.9	63.7	61.9	—	63.1	63.3
6	81.4	81.2	76.4	—	82.3	85.4

moving towards the anode. The most rapidly moving of these migrated at a rate equivalent to that of authentic phosphorylserine and contained 84% (range 72–100%, 10 determinations) of the phosphorus added to the paper. The slower-moving fraction migrated at half this rate and contained 22% (range 16–28%) of the phosphorus added to the paper.

The areas of paper corresponding to the position of these fractions were cut out, eluted with water, subjected to total hydrolysis and the hydrolysates were chromatographed on paper. The major fraction yielded only serine, the minor fraction principally serine and glutamic acid together with traces of alanine and possibly leucine. Determinations of the serine:phosphorus ratio in samples of the major fractions after total hydrolysis gave the values 0.90 and 0.95 for fractions derived from the phospholipoproteins of Expts. 1 and 2 (Table 1) and 1.18 and 1.08 for fractions derived from the corresponding supernatant phosphoproteins. It was concluded that the major phosphorus-containing amino acid present in hydrolysates of the phosphoproteins was phosphorylserine. The phosphorus:serine ratio was not determined in the minor fraction but, since a glutamylphosphate stable to acid hydrolysis has not been described, it was considered that the phosphorus in the minor fraction was present in a phosphoserylpeptide.

Equivalence of phosphorylserine to the phosphoprotein phosphorus. Since the recovery of phosphorus as phosphorylserine amounted to only 25% of the total protein phosphorus at the most, it was not certain that phosphorylserine was representative of the total phosphoprotein phosphorus, determined as inorganic phosphorus liberated by incubation of the phosphoprotein with alkali. Further, although phosphorylserine was present in hydrolysates of both the plasma-phosphoprotein fractions there was no indication whether these fractions were similar or separate entities.

In an attempt to answer these points laying hens were injected with radioactive phosphate and bled

at suitable intervals after injection. The plasma-phosphoprotein fractions were separated and the specific radioactivity of the alkali-labile phosphorus and of the phosphorylserine obtained after hydrolysis were determined. Table 3 shows that the specific radioactivities of the phosphorylserine of the phosphoprotein fractions were almost identical and were also very close to the specific radioactivities of the alkali-labile phosphorus both of the total phosphoproteins and of the individual fractions.

DISCUSSION

The results described above establish *O*-phosphorylserine as the sole major phosphorylated amino acid detected in acid hydrolysates of the plasma phosphoproteins of the laying hen. In this respect the plasma phosphoproteins are similar to other phosphoproteins so far described and in particular to phosvitin from egg yolk, in which the total phosphorus appears to be present in the *O*-seryl linkage (Mecham & Olcott, 1949; Williams & Sanger, 1959).

The extent to which the phosphorus of the plasma phosphoproteins is also present in the *O*-seryl form can be partly assessed from the data in Tables 2 and 3. Thus the percentage of the total plasma-phosphoprotein phosphorus recovered as phosphorylserine was similar to that when a purified specimen of phosvitin was subjected to the same procedure. Further, the experiments with radioactive phosphorus showed that the specific radioactivity of the phosphorylserine present in hydrolysates of the phosphoprotein fractions obtained at different times after injection was identical with that of the alkali-labile phosphorus commonly employed as a means of estimating phosphoproteins. It seems reasonable to conclude that phosphorylserine is, at the least, representative of the total phosphoprotein phosphorus. Conversely, the determination of phosphorus in the phosphoproteins of laying-hen plasma by means of alkali lability would appear to yield phosphorus only

from phosphoprotein and not from some other source. Since it has been shown (McIndoe, 1959) that no further quantities of alkali-labile phosphorus are released from the plasma phosphoproteins after incubation for 18 hr. it is considered that this method of determination of phosphoprotein phosphorus is likely to yield an accurate value for the total quantities present.

Experiments such as the above, though establishing the nature of part at least of the protein-bound phosphorus, do not yield information about the overall nature of the phosphoprotein. Tentative evidence has been presented suggesting that phosvitin may be the only phosphoprotein present, part combining with lipid to form a precipitate on dilution of the plasma and part remaining in solution (McIndoe, 1959; Mok *et al.* 1961). Such a suggestion offers a simple explanation for the different composition of the lipophosphoprotein complexes obtained with different anticoagulants during the collection of blood (Table 1), and for the similar specific radioactivities of the phosphoprotein phosphorus derived from the two fractions. However, the accuracy of the suggestion can be decided only by isolation and further study of the phosphoproteins.

SUMMARY

1. Phosphorylserine has been isolated and identified as the sole phosphorylated amino acid present in acid hydrolysates of the plasma phosphoproteins of the laying hen. No phosphorylserine was detected in hydrolysates of plasma from non-laying hens or from cockerels.

2. After the injection of radioactive phosphate the specific radioactivity of phosphorylserine, isolated after different times from two plasma-phosphoprotein fractions, was the same in both fractions and similar to that of inorganic phosphate released on incubating the phosphoproteins with dilute alkali.

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The Influence of Adjacent Phosphate and Hydroxyl Groups on Amino Acid Esters

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Perhaps the most remarkable property of teichoic acids is the considerable reactivity of their D-alanine ester residues. Teichoic acids from bacterial walls or from intracellular material all possess these reactive ester groups and, although their

number varies somewhat in samples prepared from different organisms and by different techniques, values as high as 0.75 have been reported for the ratio alanine:P in the polymers.

The glycerol teichoic acids are polymers of