THE ISOLATION AND CHARACTERIZATION OF PHOSVITIN FROM AVIAN SERUM

by

Chi-Ching Mok

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

Department of Agricultural Chemistry,
McGill University,
Montreal.

April 1960

ACKNOWLEDGEMENTS

The author wishes to express her sincere thanks to Professor R.H. Common, Chairman of the Department of Agricultural Chemistry, for his untiring interest, suggestions and encouragement during the whole course of this work.

Acknowledgement is gratefully made to Professor N. Nikolaiczuk and Professor W.A. Maw, Macdonald College, for their unfailing co-operation in supplying experimental birds.

The author also wishes to express her gratitude to Dr. W.H. Cook and Dr. W.G. Martin, Division of Applied Biology, National Research Council, Ottawa, for most kindly undertaking the determination of the average particle size of serum phosvitin.

She wishes to thank her fellow students, Mr. H.F. MacRae and Mr. K.A.McCully, for their unfailing help and valuable advice with regard to various laboratory procedures.

The financial support of the Quebec Agricultural Research Council, which made this work possible, is gratefully acknowledged.

TABLE OF CONTENTS

SECTION I

SECTION II

SECTION I

GENERAL INTRODUCTION

The earliest studies of the avian egg-yolk protein appear to be those of Gobley and of Valenciennes & Fremy. These workers recognized the presence of a protein, which Dumas & Cahours named 'vitellin', in egg-yolk. The nature of vitellin was the subject of controversy for many years. Its content of phosphorus led Miescher to regard it as a nuclein or pseudonuclein. It was not until 1906 when Plimmer & Bayliss studied the action of trypsin and of 1% NaOH on the phosphorus of vitellin, that its nature and distinction from nuclein became clear. Plimmer also demonstrated the presence of a second protein of low phosphorus content in egg-yolk. He gave the name of 'livetin' to this second protein.

With the awakening of the interest of the composition of the avian serum and the changes that it undergoes with the onset of laying, attention was drawn to the partition of serum phosphorus. From these studies, it emerged that a phosphoprotein appeared in the serum when the hen entered reproductive activity. Laskowski isolated this protein fraction and gave it the name 'serum vitellin'. It was suggested that this material represented 'yolk vitellin' synthesized in the liver and then transported to the ripening follicles. Some preliminary studies provided evidence

that the new protein fraction behaved similarly to the phosphoprotein fraction of yolk, and that at least two proteins, or rather lipoprotein fractions, are involved.

Meanwhile Mecham & Olcott had isolated from egg-yolk the remarkable phosphoprotein which they called 'phosvitin'. An outstanding property of phosvitin is its high phosphorus content of about 10% phosphorus. It became of considerable interest, therefore, to ascertain whether or not the serum of the laying hen (or heavily estrogenized hen) contains phosvitin.

The present thesis describes the successful isolation of phosvitin from the serum of the estrogenized pullet.

SECTION II

HISTORICAL REVIEW

2.1- Recognition of 'vitellin' and its distinction from 'nucleins'.

It is difficult to say when the study of egg-yolk proteins began. Fourcroy, in 1782, examined egg-yolk by chemical methods, and stated that it was chiefly 'albumin', fat being the substance second in importance. At that time, however, the concept of proteins as it is known today, had not been formulated. Indeed, it was not until 1839 that the Dutch chemist, Mulder, coined the word 'protein', although he used it originally to denote a supposed nitrogenous radical common to all those substances that we now designate as proteins. Indeed, ideas on the nature of proteins only began to assume their modern form toward the end of the nineteenth century, as a consequence of the work of von Kossel, of F. Hofmeister and of Emil Fischer.

In 1841 Bence Jones coagulated egg-yolk by heat and then extracted the fatty constituents by means of ethyl-ether. The nitrogenous material that remains from this treatment was later named 'vitellin' by Dumas & Cahours (1842).

The nature of 'vitellin' was the subject of controversy for many years. Hoppe-Seyler (1865) believed that vitellin was a 'lecithoprotein' and showed that lecithin could be

removed from it by boiling alcohol. He also stated that 'vitellin' itself did not contain any phosphorus, but that the phosphorus which appeared in the analysis was due to the presence of lecithin. But at the same time Miescher obtained from the yolk a substance containing a great deal of phosphorus. This substance also possessed the properties of protein. These facts lead Miescher to believe that it was a nuclein. However, Kossel (1886) found subsequently that no trace of purine bases could be detected in the yolk. On the basis of this observation, he pointed out that vitellin was essentially different from the nucleins found in cells. However, it was not until the work of Plimmer & Bayliss (1906) appeared that the nature of vitellin and its distinction from the nucleins became clear.

In the meantime, the definition of vitellin had undergone modification. A reasonably pure form of vitellin was prepared by Osborne & Campbell (1900). They mixed egg-yolk with an equal volume of saturated NaCl solution, extracted the mixture repeatedly with ether, and dialyzed the fat-free residue with water. A heavy precipitate formed which could be dissolved in 10% NaCl to give a clear solution. Upon dialysis to remove the salt, a pure vitellin was precipitated. Vitellin prepared in this manner was found to contain 0.94% phosphorus and 15 - 30% lipid. It was stable in ether, but was readily broken down by contact

with alcohol with liberation of phospholipid. All the 'combined lipid' could be removed by extraction with 80% alcohol.

Plimmer & Bayliss (1906) subjected casein and vitellin to the action of trypsin, and studied the time taken under various conditions for the phosphorus to be split off in soluble forms. They found that vitellin was much more slowly digested than casein. A 1% NaOH solution, however, would bring all the phosphorus of casein into solution within 24 hours at 37°C. Later, Plimmer & Scott (1908) found that vitellin behaved in the same way to 1% NaOH as casein. All the phosphorus in vitellin was separated completely as inorganic phosphoric acid by the action of 1% NaOH within 24 - 28 hours.

Now treatment of nucleins with dilute alkali at 37°C for 24 hours does not split off any phosphate as inorganic phosphate. Hence this work gave a clear distinction of casein and vitellin from the nucleins. The two proteins were recognized, therefore, as members of a new class of 'phosphoproteins' in which the phosphoric acid radicals are combined quite differently to the state in which they are combined in nucleins or nucleoproteins. It was recognized that the latter carried the phosphorus in the so called 'prosthetic' group, whereas the phosphoric acid groups in the phosphoproteins were considered as being linked directly to the

protein itself.

Levene & Alsberg (1900) applied E. Fischer's methods of analysis to vitellin. Osborn & Jones (1909) also made similar analyses. Calvery & White (1931) prepared vitellin by dilution of the yolk with an equal volume of 10% sodium chloride solution and extraction of the resulting solution with ether containing about 2% ethanol. Yolk membranes were removed by filtering through cheesecloth. The yolk solution was then diluted with 20 volumes of water. The precipitate obtained on dilution was dissolved in 10% sodium chloride and diluted again with 20 volumes of water. The crude vitellin thus obtained was suspended in 80% alcohol, heated to boiling and kept nearly at the boiling point for several hours. It was then filtered, washed with ether and dried in a vacuum desiccator for several days. The yield of purified vitellin was 35 to 40 gm. from 24 eggs, and it contained 15.03% N and 0.92% P. Calvery & White (1932) also studied the nitrogen distribution in vitellin by the Van Slyke method. The contents of tyrosine, tryptophane and cystine were determined by colorimetric methods and arginine, histidine and lysine were determined by isolation as well-characterized crystalline derivatives.

Chargaff (1942) prepared the lipovitellin complex by dilution with an equal volume of saturated sodium chloride followed by extraction of the diluted egg-yolk solution with

ether. Repeated dialyses and ether extractions of the resulting precipitate in 10% NaCl yielded a final product containing 13.0% N, 1.5% P, 0.9% S, 23% total lipids and 17.9% phosphatides. He also showed that only a portion (18.8%) of the phosphatides present in egg-yolk was firmly bound to the protein. There is no essential difference in the composition of the phosphatides occurring in the free state and of those combined with vitellin. Yolk phosphatides were almost exclusively derivatives of choline and ethanolamine.

2.2- The 'second protein' of egg-yolk - livetin.

The presence of another protein in addition to that precipitated on dilution of egg-yolk was recognized by Valenciennes & Fremy (1854). They termed this protein 'albumin'. This fraction was not investigated further until Plimmer (1908) observed that after complete precipitation of vitellin by water, the aqueous solution gave a very strong biuret reaction and that on boiling and slightly acidifying with acetic acid, a heavy precipitate of protein was formed. This was at first regarded as unprecipitated vitellin. However, phosphorus determinations showed that it was not identical with vitellin, for it contained only 0.1% of phosphorus compared with 1.0% in vitellin. It seemed scarcely possible that this coagulable protein was identical with ovalbumin in the yolk, since ovalbumin was coagulable

by ether, and since the new coagulable protein contained less nitrogen than did ovalbumin. Thus it was concluded that this protein represented another constituent of egg-yolk. The name 'livetin' was given to this second protein of yolk. Plimmer (1908) suggested that livetin might be closely related to vitellin and that it might be, in fact, vitellin minus the phosphorus containing fraction of the molecule. Although Plimmer brought evidence to show that this new protein was not ovalbumin, he was not able to demonstrate clearly that the proteins of the white did not contribute to his coagulum.

In 1928, Kay & Marshall resumed the study of 'livetin' afresh. They obtained it in a fairly pure form. To be sure that the yolk was free of adherent white, they carefully removed the chalazae with scissors and washed the yolk with three or four changes of 0.9% sodium chloride. The yolk was further cleaned by allowing it to roll slowly down a strip of dry calico sheeting. It was finally dried, as far as possible, on the sheeting. In this way, they claimed that the white could be completely removed. Lecithovitellin was removed from the clean yolks by the method of Plimmer (1908). The supernatant from which vitellin had been precipitated was saturated with ammonium sulphate. Almost all the livetin could be precipitated by this treatment. The filtrate from this precipitate gave only a very slight opalescence with

trichloroacetic acid, thus demonstrating the absence of appreciable amount of albumin. The precipitate was taken up with water, the concentration of ammonium sulfate was brought up to 25%, and the slight precipitate that formed was filtered off. To the clear filtrate, sufficient saturated ammonium sulfate solution was added to bring the concentration of salt to half saturation. The precipitated livetin filtered off, redissolved and precipitated again. Solution and precipitation were repeated once more and the precipitated protein was dissolved in the minimal quantity of water. Lipid was removed by alcohol-ether extraction at -15°C. The extracted protein was then dried and dissolved in minimal quantity of water. The pH of the solution was adjusted to 5.0. It was then dialyzed at 3°C against repeated changes of distilled water till the dialysate had ceased to give the faintest opalescence with barium chloride. Although there were now only traces of salts in the dialysing sac, the livetin was still in solution. Its behaviour was, therefore, that of a pseudoglobulin. On continuing the dialysis for some weeks, a portion of the protein was precipitated. Livetin prepared in this way was found to contain 15.1% to 15.4% nitrogen, and 0.06% to 0.05% phosphorus. Amino acid analysis, using the method of Folin & Ciocalteu for tryptophane and that of Folin & Rooney for cystine, showed that there was no direct relationship between vitellin and livetin. In the fresh yolk, the ratio of vitellin to

livetin was fairly constant. Kay & Marshall considered that one-quarter to one-fifth of the yolk proteins of the hen's egg was livetin. (This proportion of livetin, of course, is much too low in the light of more recent work.)

Schjeide, Levi & Flickinger (1955) have reported that conversion of vitellin to livetin may occur in frog eggs by the action of the enzyme phosphoprotein phosphatase. No investigation appears to have been made as to whether or not this conversion would occur in hen eggs.

2.3- The recognition of phosvitin.

As early as 1900, Levene & Alsberg had prepared a phosphorus-rich 'avivitellenic acid' from vitellin. Vitellin was allowed to stand in dilute ammonia for two hours, and then neutralized with acetic acid after cooling in an ice bath. Picric acid solution was added, and the liquid then strongly acidified with acetic acid. The precipitate was filtered off. Several volumes of alcohol were added to the filtrate to obtain the precipitate of avivitellenic acid. This precipitate was dissolved in water, acetic acid was added and then 5% HCl in alcohol to acidify to congo red. The precipitate was subjected to this purification several times. This final material contained 13.0% N, 10.0% P and 0.57% Fe. This preparation represents a rather drastic treatment, but later work has supported the view that there is a

fraction of the yolk proteins that has a remarkably high content of protein phosphorus.

Mecham & Olcott (1949) obtained such a fraction by the following procedure: Egg-yolk was mixed with half its volume of 1.2 M $MgSO_4$ and the mixture was then diluted four-fold with water. The precipitate was dissolved in 0.4 M $(NH_4)_2SO_4$ and adjusted to pH 4.0 and the solution was extracted with cold ether to remove lipids and to throw unwanted proteins out of solution. The supernatant was again extracted with ether, filtered and finally saturated with $(NH_4)_2SO_4$ to precipitate a phosphorus-rich fraction which Mecham & Olcott called 'phosvitin'. This material contains about 9.7% P and 11.9% N, moreover 60 - 70% of all the protein phosphorus is present in this fraction. Six and one-half to seven per cent of yolk protein is phosvitin. According to Mecham & Olcott the presence of phosphorus in vitellin preparation is due to contamination with phosvitin. Reports of Blackwood & Wishart (1934) indicated that peptic or tryptic digestion yielded peptides of varying but high phosphorus content. Since phosvitin is resistant to peptic and tryptic digestions (Mecham & Olcott, 1949), Blackwood & Wishart's work does not support the view that phosvitin is responsible for the high phosphorus containing peptides obtained by enzymatic digestion of vitellin.

The works of Lipmann & Levene (1932) has thrown some light as to how the phosphorus is bound to the protein. Vitellinic acid, prepared by the method of Levene & Alsberg (1900), was subjected to hydrolysis for 10 hours with 2 N hydrochloric acid. It was then neutralized with barium carbonate and then made alkaline to phenolphthalein. About one-third of the bound phosphoric acid remained in solution and two-thirds, together with the inorganic phosphate, separated with the precipitate. The precipitate was filtered off. Addition of alcohol to the supernatant to a final concentration of about 50% precipitated some material which was soluble in water. The aqueous solution contained nearly all the organic phosphate. On adding alcohol up to a concentration of 25%, a substance was precipitated which had the composition of the dibasic salt of serinephosphoric acid. It is clear, therefore, that the phosphate group is linked to the protein through the hydroxyl group of serine.

Amino acid analyses on phosvitin showed that the serine accounted for 32.3% of the dephosphorylated protein. Phosphorus determination showed that phosphorus content and serine content are equivalent, thus indicating that all of the phosphorus in the protein is present as an O - ester of serine.

2.4- The influence of the ultracentrifuge and free electrophoresis on the study of yolk proteins.

2.4.1- The lipoproteins -lipovitellin and lipovitellenin.

Alderton & Fevold (1945) prepared lipovitellin by throwing out the lipovitellin by centrifuging egg-yolk diluted with two volumes of water in Sharples centrifuge, drying by lyophilization, extracting the dried precipitate with ether, dissolving the lipovitellin in 1% sodium chloride solution, and reprecipitating by removal of the salt by dialysis. The precipitated material contained approximately 90% of the total lipovitellin present in the yolk.

Chargaff (1942, 1944) had showed that ether does not break down the protein - lipid complex of yolk proteins; the lipoprotein can, therefore, be free of uncombined fat by ether extraction. Alderton & Fevold found that approximately 15 - 16% of the material they prepared was extractable with ether, leaving the lipovitellin free from uncombined lipid. Further extraction of the ether-insoluble product with alcohol removed the combined lipid. Vitellin obtained in this manner was insoluble in water and salt but could be dissolved in alkali. The nitrogen content of lipovitellin ranged from 12.70 - 13.05%, of vitellin 15.01 - 15.62%; phosphorus in lipovitellin was 2.34 - 1.30%, in vitellin 2.13 - 1.10%.

A second yolk lipoprotein was isolated by Fevold & Lausten (1946). They named this new protein 'lipovitellenin'. The separation of lipovitellin from lipovitellenin was based on the fact that, in the presence of yolk lipid, the lipovitellenin can not be thrown down in the Sharples centrifuge after dilution of egg-yolk with two volumes of water, whereas lipovitellin is readily deposited as a precipitate. After the removal of lipovitellin, the supernatant, containing livetin, fats, and lipovitellenin, was extracted with two volumes of ether. Three layers separated: (a) an aqueous layer containing the livetins; (b) an ether layer containing the lipids; and (c) a third layer of insoluble material containing the lipovitellenin and which separated between the other two. This lipovitellenin was separated, extracted with ether and purified by dissolving in 1% sodium chloride saturated with ether followed by reprecipitation by dialysis. The precipitated lipovitellenin differs from lipovitellin both in lipid and phosphorus content. Lipovitellenin contains twice as much lipid as lipovitellin. After alcohol extraction, vitellenin contains only about 1/3 as much phosphorus as does vitellin.

2.4.2- The livetins.

The application of free electrophoresis to the study of the water-soluble proteins in yolk, the livetins, was made by Shepard & Hottle (1949). These authors used

Longsworth's modification of the Tiselius apparatus for electrophoretic analysis. As has been pointed out by Tiselius, one of the great advantages of electrophoresis over that of the classical precipitation method for the study of protein is that precipitation involves rather drastic changes in the state of solutions which might bring about partial denaturation. Shepard & Hottle showed that livetin is not one substance but a group of substances. The material present in livetin preparation shows electrophoretic mobilities resembling those of the protein components of hen's plasma. Of the three peaks shown, the fastest has the mobility of serum albumin. The second peak is comparable to the slowest of the β-globulins and the slowest peak has a mobility not much different from that of the second of the two large peaks of hen's serum. Their findings were confirmed by Martin, Vendegaer & Cook (1957). The latter authors prepared livetin by shaking egg-yolk with two volumes of water and two volumes of carbon tetrachloride. The resulting emulsion was broken by centrifuging at 30,000 r.p.m. and the aqueous layer at the top decanted for re-extraction, dialyzed against distilled water and lyophilized. Electrophoretic analysis of this material at 1% concentration in glycine or phosphate buffer revealed three major components. According to their mobility, in decreasing order, they were designated as α-, β- and γ- livetin. This mixture has the solubility properties of globulin. Refractive index patterns

of the whole egg-yolk suggested that the mixed livetins may represent about half, and γ-livetin alone about a quarter, of the proteins in the yolk. γ-Livetin was separated from the mixture by precipitation with isopropanol at 20% $^{v}/_{v}$ at 0°C or with 37% saturated ammonium sulfate at 4°C. The precipitated γ-livetin was contaminated with α- and β-livetins. The α- and β-livetins could not be separated by differential precipitation. However, it has been separated on a small scale with Kekwick electrophoretic cell. α-Livetin has several of its properties comparable to those of serum albumins. Its electrophoretic mobility, sedimentation coefficient, molecular weight and tyrosine-tryptophane ratio are similar to those of serum albumins, but its solubility behaviour is not identical with those of albumin. The molecular weight of β-livetin is comparable to that of ovalbumin, but the former's mobility, tyrosine-tryptophane ratio and sedimentation coefficient are all lower. β-Livetin behaves as a pseudoglobulin and is largely precipitated at half-saturation with ammonium sulfate. γ-Livetin is the least stable of the livetins. A marked loss in solubility occurs at each purification step, on freeze-drying, or even on standing. It has a much higher molecular weight than the livetins.

2.4.3- Ultracentrifugal and electrophoretic studies of whole egg-yolk.

Vandegaer, Reichmann & Cook (1956) reported that egg-

yolk diluted with 5% sodium chloride showed one rising and two sedimenting fractions when examined in an analytical ultracentrifuge. Free electrophoresis showed that the major component in the rising fraction was the lipid-rich lipovitellenin, with a very small amount of a second component or impurity. Martin, Turner & Cook (1959) subsequently isolated vitelleinin from this fraction by extraction with chloroform-methanol at -23°C followed by washing with cold ether. The ether-saturated protein was then suspended in water at 0°C and dialyzed exhaustively against water to remove lipid solvent. The dialysate was freeze-dried. Vitellenin obtained in this manner was found to have two or more polypeptide chains that may be combined directly or through lipid by forces weaker than the peptide linkage. Most of the lipid extracted by ether is believed to be part of, or associated with, the protein in the native state. As to the sedimenting fraction, six definite components appeared on electrophoresis. This gives an extra fraction, since the three livetins, phosvitin and lipovitellin add up to five. The fastest moving peak is phosvitin. The next two peaks appear to be α- and β-livetins. α-Livetin and β-livetin alone account for about 42% of the sedimentary material. The other three less mobile components do separate into discrete peaks if the electrodes are large enough. The nature of the most mobile component among the three is unknown. It could be γ-livetin, γ-livetin adherent

to lipovitellin, or a second lipovitellin. One of the remaining peaks is lipovitellin.

McKinley, Oliver, Maw & Common (1953) made some preliminary studies of the behaviour of egg-yolk on zone electrophoresis. Yolk was suspended in 1% sodium chloride. This suspension was subjected to zone electrophoresis on paper in veronal buffer, pH 8.6, made up for ionic strength of 0.5 in 20% methanol. The paper was stained with Naphthalene Black for protein and Oil Red O for lipid. Five distinct zones appeared on the electropherogram. They were designated as X, Y, the lipid-rich zone, the phosphorus-rich zone and a non-mobile zone at the point of application. The X and Y zones could be possibly α- and β-livetins respectively, since these two zones also appeared in the supernatant from centrifugation of the suspension of egg-yolk in 1% sodium chloride.

Evans & Bandemer (1957) have also presented the results of electrophoresis of yolk protein in aqueous veronal buffer. They described seven zones and tentatively

identified them as follows:

Band	Identification	Strength	Lipid
A	Ovalbumin	faint, not always present	absent
B	Livetin	weak	absent
C	Livetin		
D	Lipovitellin + livetin	strong	strong
E	Lipovitellin (non-mobile)	strong	strong
F	Lipovitellenin (moved toward cathode and possibly phosvitin)		strong
G	Not a lipoprotein (moved toward cathode)	slight	absent

Their results agree in general with McKinley *et al.* (1953).

McCully, Maw & Common (1959) have described six fractions on zone electrophoresis of a suspension of yolk in 1% sodium chloride. These authors also used aqueous veronal buffer. The paper was stained with Amidoschwarz for protein. By comparing the mobility of pure phosvitin, α-, β- and γ-livetins, it could be concluded that the fastest moving

zone in whole egg-yolk is phosvitin. The next three zones are α-, β- and γ-livetins respectively. The two slow moving zones, which were designated as P_1 and P_2, are lipophosphoproteins. The protein nitrogen to protein phosphorus ratio in these two fractions were the same in spite of their electrophoretical separation. This may suggest that both of these fractions contain phosvitin.

2.5- Recognition of the role of divalent cations in separation of yolk proteins.

Joubert & Cook (1958) have recently studied the status of phosvitin by ultracentrifugation and electrophoresis. The major sedimenting fraction of egg-yolk proteins and lipoproteins was apparently homogeneous in 10% sodium chloride solution when centrifuged, but they succeeded in fractionating it into lipovitellin, phosvitin and γ-livetin. They believed that phosvitin, γ-livetin and lipovitellin interact strongly and the methods previously employed for preparing lipovitellin probably yield a complex of these three proteins. The phosphorus content of this complex has been reported to have varied from 1 to 2%. When phosvitin is removed, the protein phosphorus content quite consistently dropped to 0.5% or slightly lower. Joubert & Cook (1958) have proposed that 'lipovitellin' separated by the older methods, originating from the work of Osborne & Campbell and of Plimmer, should be called 'lipovitellin complex' and that the term

'lipovitellin' should be reserved for the 'lipovitellin' free from γ-livetin and phosvitin. They pointed out that all samples of lipovitellin prepared by dilution precipitation from sodium chloride is a complex of γ-livetin-lipovitellin-phosvitin complex and a divalent cation is effective in breaking down this complex. Subsequently they devised a method of preparing phosvitin by using magnesium sulfate. Phosvitin was precipitated from egg-yolk solution in 0.4 M $MgSO_4$ by adding an equal volume of water. The crude phosvitin was purified by repeat precipitation. It was then dissolved in 10% sodium chloride and centrifuged to get rid of lipid material. The subnatant was dialyzed against acetate buffer at pH 4.0, $\Gamma/2 = 0.1$, to remove the remaining lipovitellin. After dialyzing against several changes of water to get rid of the buffer, it was lyophilized. Material so prepared contains 9.6% phosphorus and has the other properties of phosvitin described by Mecham & Olcott (1949).

Williams & Sanger (1959) prepared phosvitin by the method of Mecham & Olcott (1949). The phosvitin was then subjected to partial hydrolysis by treatment with HCl conc. at 37°C for 12 hours. The HCl was then removed and the hydrolysate was examined by high voltage paper electrophoresis. The buffer was 20% formic acid, pH 1.5. At this pH the carboxyl group of peptides are uncharged so that the peptides will move towards the cathode unless they carry a strongly charged phosphate or sulfate group. The phosphate radical

of serine phosphate is still charged at pH 1.5, and peptides which contain one residue of serine phosphate are electrically neutral under these conditions. However, peptides which contain more than a single serine phosphate radical are negatively charged and move towards the anode. The hydrolysate yielded six major bands and a number of intermediate bands, all of which moved towards the anode. Hydrolysis of these fractions revealed that serine was the only amino acid present. The peptides of the main bands could be broken down by partial hydrolysis (conc. HCl at 37°C for 12 hours) to yield the slower moving bands. In this way, it was possible to demonstrate the presence of $(\text{Serine-P})_6$, but Williams & Sanger consider that even longer polyphosphoserine sequences are present. The analysis of Mecham & Olcott (1949) give approximately 49.3 amino acid residues per 10^4 gm. phosvitin and of these 29 are accounted for by serine. Their analyses indicate, moreover, that practically all of the serine must be present as phosphoserine. A total phosphorus content of 9.7%, therefore, corresponds to a phosphoserine anhydride content of approximately 52.5%. This together with the observation of Williams & Sanger, leads to the interesting notion that phosvitin may take the form of one or more relatively long polyphosphoserine sequences attached to a more normal type of polypeptide structure. It is quite evident that phosvitin has a most unusual structure.

2.6- The present state of knowledge of the yolk proteins in terms of reasonably well characterized entities.

Histochemical studies by Marza & Marza (1935) of yolk indicated the presence of several different types of yolk granules, to which he gave the names primodial yolk globules, yellow yolk globules, white yolk globules. The primodial yolk globules are observed to consist of a large quantity of material staining as 'vitellin' encapsuled by a thin layer of nucleo-protein. Yellow yolk granules are comprised nearly entirely of 'vitellin' staining materials, whereas white yolk granules are characterized by the presence of some materials having the staining properties of 'livetin'. Schjeide & Urist (1959) obtained such egg-yolk granules by centrifugation at 30,000 g for 8 hours. After three washings with distilled water, the egg-yolk granules were solubilized by addition of equal volumes of 20% NaCl. Filtration and centrifugation (12 hours at 90,000 g) of this material resulted in the separation of two components (X_1 and X_2). As resolved in the ultracentrifuge, X_1 had high phosphorus and serine contents, while X_2 had a high polysaccaride and lipid content.

Sugano (1957) attempted to clarify some of the numerous terms employed in the literature with respect to the insoluble fraction of yolk protein. Sugano repeated the manipulations of Osborne & Campbell (1900) as modified by

Chargaff (1942), of Alderton & Fevold (1945), of Fevold & Lausten (1946) and of Mecham & Olcott (1949). He compared these preparations electrophoretically, using the Tiselius apparatus and sodium carbonate-bicarbonate buffer (pH=9.8, $\Gamma/2$=0.15). It was shown that lipovitellin, prepared according to the method of Alderton & Fevold as modified by Chargaff, is a complex mixture. Four peaks appeared in the Schlieren diagram. The fastest moving peak was identified as phosvitin and the next two peaks were provisionally named α- and β-lipovitellin by Sugano. The nature of the slowest moving peak is not yet known. The concentration of these components in Alderton & Fevold 'lipovitellin' is 17.05% phosvitin, 38.0% α-lipovitellin and 27.5% β-lipovitellin. Other components account for the remaining 17.5%. From the phosphorus contents before and after alcohol extraction, it could be calculated that about 72% of the total phosphorus and about 83% of the protein phosphorus of the lipovitellin could be attributed to the 17% phosvitin which was present in the complex. About 50% of the total yolk phosvitin was precipitated in this fraction. Schjeide & Urist (1959) suggested that the lipovitellin of Fevold & Alderton corresponds to their centrifugally resolved components X_1 and X_2, and that the α-lipovitellin of Sugano is the same as their X_2. Sugano (1957) also devised an elaborate method to separate the α- and β-lipovitellins. He found that about 80% of the lipovitellenin prepared by the Fevold & Lausten method

is β-lipovitellin and that no phosvitin is present in this preparation. About 83% of the phosphorus of the lipovitellenin was due to the phospholipid, which was alcohol-extractable.

As mentioned before, Vandegaer, Reichmann & Cook (1956) have carried out an ultracentrifugal study of yolk in 10% NaCl. The ultracentrifugal examination of this diluted yolk indicated three components, viz., one rising fraction (fraction R) and two sedimenting fractions (S_1 and S_2). Fractions R and S_1 were lipoproteins. These two lipoproteins may correspond to the α- and β-lipovitellins of Sugano.

The phosvitin of Mecham & Olcott is a relatively pure protein preparation. The X_1 fraction obtained by Schjeide & Urist (1959) is stated by them to contain phosvitin.

2.7- 'Serum vitellin' - discovery and relation to reproductive activity.

2.7.1 - Changes in blood calcium, phosphorus, in relation to reproductive activity.

The dramatic changes of serum phosphorus, calcium and lipid content during the laying period have been the subject of research for many workers. Hughes, Titus & Smites (1927) reported that hens, whose ovaries are developing eggs, usually show double or triple the amount of serium calcium

that is characteristic for non-reproducing hens, pullets and cocks. Benjamin & Hess (1933) showed that the increase was due entirely to the increase in the level of the two non-filtrable fractions of calcium. The non-filtrable absorbable form is raised to a value of 6.5 to 12.5 mg. per 100 ml. - a 6-fold increase. The protein bound calcium is raised to a concentration of 7.2 to 13.3 mg. per 100 ml. which represents a 5-fold increase. The levels of the filtrable fraction remained practically unchanged. The first published fact concerning changes in phosphorus content of the blood in relation to egg laying was contained in Miescher's (1897) observation that at the time eggs ripen in the salmon, the blood serum showed 'increased amounts' of lecithin and fatty acids. The next contribution was by Lawrence & Riddle (1916), who showed that the total P contents of the plasmas of cocks, non-laying hens and laying hens were related to each other as are the values 100, 115 and 205. In 1932, Heller, Hunter & Thompson published data for the partition of phosphorus in the cells and the plasma of laying hens. Their data showed a discrepancy between the total plasma P and the sum of lipid P, acid soluble P and inorganic P, but no explanation was offered for this difference. In a subsequent more detailed study of the partition of Ca and P in blood during the life cycle, Heller, Paul & Thompson (1934) drew attention to the fact that the major part of the increase in total P with onset of laying is accounted for by increase in lipid P.

In 1935 Laskowski (1935a) made careful determinations of the P fraction in the serum of the laying hen. He showed that the sum of acid soluble P + ester P + lipid P accounted practically quantitatively for the total P in the serum of the non-laying bird, but that there was an appreciable discrepancy in the laying bird. His data may be summarized as follows:

Phosphorus, mg./100 ml. plasma, average values

	Acid Soluble	Ester	Lipid	Ester +lipid	Total	Total - (acid sol. +lipid)	Difference= Protein P
Non-laying	3.8	0.2	9.0	12.5	12.2	-0.6	traces
Layers	4.9	0.1	22.2	27.1	36.0	8.9	4.1-15.1

Laskowski correctly interpreted this discrepancy as due to the appearance of a new protein P fraction in association with laying.

2.7.2- Isolation of 'serum vitellin'.

The presence of phosphoprotein in the serum of laying hens were demonstrated independently by Roepke & Hughes (1935) and by Laskowski (1935b). Roepke & Hughes found that in the serum of males and non-laying hens, the total phosphorus is equal to the sum of the lipoid and acid soluble fractions. In the serum of laying hens the total phosphorus is 35.2 mg./100 ml. which is greater than the sum of the lipoid and acid soluble

fraction, indicating the presence of an additional fraction. This additional fraction is most likely phosphoprotein. Subsequently they obtained this phosphoprotein fraction by precipitation with trichloroacetic acid followed by repeated extractions with a 10% trichloroacetic acid solution and boiling alcohol-ether mixture. The protein phosphorus averaged about 10.52 mg./ 100 ml. of serum. The protein concerned was quite resistant to acid hydrolysis, being only slowly hydrolyzed by concentrated hydrochloric acid at room temperature.

Laskowski (1935b) attempted to isolate the serum phosphoprotein by precipitation with ammonium sulfate and by precipitation at the isoelectric point, but these methods were not satisfactory. However, he found that a ten-fold dilution of the blood of laying hens' plasma produced a precipitate. The occurrence of this precipitating fraction was so closely related to laying that it can be used as a diagnostic procedure for determination of the laying activity of the hen. Accordingly, he devised the following method for isolation of the phosphoprotein: the pH of the plasma was adjusted to 5.00—5.50 with hydrochloric acid and was then diluted ten-fold with water. The precipitate was purified by dissolving in 10% NaCl solution and adding solid NaCl to saturation. A precipitate of fibrinogen that formed was removed by centrifugation. The supernatant was then

diluted 100 folds with water. The precipitate as obtained at this stage contained 0.9% P. Laskowski named this new protein 'serum vitellin', since it had almost the same phosphorus content as 'vitellin' prepared from egg-yolk by similar methods. The serological properties of serum vitellin, as showed by Roepke & Bushell (1936), were found to be closely related to those of egg-yolk vitellin.

Riddle (1927) noted that the ovarian hormone, which is responsible for the growth of oviduct, is also responsible for the increase of calcium, phosphorus and lipid in the serum during egg production. Riddle & Dotti (1936) showed that estrone, estriol, estradiol, or estradiol benzoate, will produce hypercalcemia in normal, castrate, hypophysectomized and thyroidectomized pigeons and in normal doves and fowls. Laskowski (1938) found that serum vitellin was not only present in laying hens' serum but could also be detected in the serum of non-laying hens which had been treated with anterior pituitary hormone. It would appear that estrogen produced in the ovary under the stimulation of the anterior pituitary in turn stimulates the production of serum phosphoprotein.

It was found that serum phosphoprotein has a much higher calcium binding capacity than the usual serum proteins. McDonald & Riddle (1945) calculated that 1 gm. of serum vitellin (containing 0.95 to 0.99% P) can combine more than

7 mg. of calcium — a value 8 to 9 times higher than other serum proteins.

Since this 'serum vitellin' appears only during egg production, it becomes of interest to see if the actual level of serum protein is increased during this period. Greenberg, Larson, Pearson & Burmester (1936) found that the protein level does increase during the egg production period while Sturkie & Newman (1951) reported that there is no significant difference in serum protein level between laying and non-laying hens. This problem was clarified when Vanstone, Maw & Common (1955) determined the total serum protein level from the 14th day of incubation to the 13th week of egg production. These workers found that about three weeks before laying of the first egg, serum protein begins to increase from about 4 gm./100 ml., until it reached a value of 5.4 gm./100 ml. in the week before laying of the first egg. At this stage, two new zone electrophoretic fractions appeared in the serum and these were responsible for the elevation of serum protein level. The serum protein level fell off for a time to about 3.6 gm/100 ml. after laying had begun and then gradually recovered as laying progressed. These results agree in general with the more limited observations of Greenberg et al. (1936).

2.7.3- Site of formation of serum vitellin.

It was known that serum albumin, globulin and fibrinogen and other proteins are produced in the liver (Dury & McMaster, 1929; Taurog, Lorenz, Entenman & Chaikoff, 1944). Hosoda et al. (1955) showed that neither ovary nor spleen is the site of formation of serum vitellin. Flickinger & Rounds (1956), who injected $Na_2H^{32}PO_4$ into the laying hen, found that at the 6th hour after the injection the radioactivity of the liver phosphoprotein phosphorus was greater than that of the blood, which in turn had a greater activity than the yolk phosphoprotein phosphorus. The egg is increasing rapidly in mass while the liver maintains a constant size. At the 12th hour after the injection, a large amount of phosphoprotein was found to be present in the yolk itself whereas the amounts of phosphoprotein in blood and serum declined. The declining activity in liver and blood and increasing activity in yolk led Flickinger & Rounds(1956) to conclude that serum vitellin is synthesized in the liver and is transported through the blood stream to the egg. Vanstone et al, (1957) have also submitted direct evidence that plasma phosphoprotein in the estrogenized cockerel is exclusively hepatic in origin. That proteins can be transported from blood stream to egg-yolk was shown as early as 1934 by Jukes, Fraser & Orr. They reported that subcutaneous injections of laying hens with diphtheria toxoid resulted in the

appearance of a low but definite antitoxin potency in the serum. Diphtheria antitoxin appeared simultaneously in the egg. It is to be expected, therefore, that if a protein appears in the serum, it may also be found in the yolk, or vice versa. Numerous attempts have been made to identify serum proteins with yolk proteins.

2.7.4- Recent studies of the lipophosphoprotein complex of hen's serum.

As mentioned earlier, Shepard & Hottle (1948) showed that livetin preparation shows electrophoretic mobilities resembling those of the protein components of hen's serum. Serological studies have also indicated that serum vitellin is similar to egg-yolk vitellin (Roepke & Bushell, 1936).

More recent studies of serum protein were made by using electrophoretic technique. Deutsch & Goodloe (1945), showed that a fast moving protein fraction, moving ahead of albumin, appears in the electrophoretic pattern of the serum of the laying hen. Moore (1948) reported that hen and cock serum patterns could be reversed by contra-sex hormones. The fast moving fraction is ether-extractable. Brandt, Clegg & Andrew (1951) concluded that this fast moving fraction is related to egg production, since it does not appear in non-laying hens or non-treated cockerel serum. The appearance of this fast-moving component was accompanied by variation of

serum calcium and phosphorus. Clegg & Heins (1953), using ^{32}P, showed that this fast-moving fraction and another fraction, which migrates in the same region as β-globulin, were responsible for the high phosphorus content of sera from laying hens or diethylstilbestrol treated normal chicken. Clegg et al. (1956) showed that the extra non-diffusible calcium in the serum of diethylstilbestrol treated cockerel was present in combination with this fast moving, high phosphorus content fraction. If calcium was added to the serum of the diethylstilbestrol treated cockerel, this fast-moving fraction disappeared. Comparison of the mobilities of the components of the normal and diethylstilbestrol-treated sera shows that a component with slower mobility appears and migrating in the area of β-globulin. The effect of a divalent cation in complexing with serum proteins was thus fully demonstrated.

Ultracentrifugal studies also have thrown some light on the nature of this protein fraction. Schjeide (1954) has observed the occurrence of a large amount of lipoprotein, in laying hen serum corresponding to β-lipoprotein ($D_{1.003}^{1.063}$) and lighter components. Hillyard, Entenman & Chaikoff (1956) have examined the serum of cocks treated with estrogen and found large amounts of a fraction having a high triglyceride content floating at a lower specific gravity than does β-lipoprotein. It is not present in normal cock serum. They also observed an increase in the β-lipoprotein fraction and a decrease in

α-lipoprotein ($D_{1.063}^{1.21}$) fraction.

Schjeide & Urist (1956) also reported that two uncharacterized serum protein components X_1 and X_2 appeared in the estrogenized cockerel in the ultracentrifuge. Component X_1 appeared to be a phosphoprotein containing little, if any lipid and with a sedimentation constant (S_{20}) of 7.5. Component X_2 was a very dense lipoprotein with a S_{20} value of 15.0. These fractions are responsible for the binding of calcium and the elevation of phosphorus content in laying hen serum. In fact, all the calcium in the estrogenized bird was found to be complexed with X_1 component. X_1 plus a variable amount of X_2 could be co-precipitated in uncontaminated form simply by dilution of the serum with water. Thus Laskowski's 'vitellin' would seem to correspond to a mixture of the X_1 fraction and a variable proportion of the X_2 fraction. Most of the protein bound calcium was released during dilution. The precipitate can be redissolved in 1% NaCl or a lesser amount of $CaCl_2$. When X_1 is separated from X_2 in the ultracentrifuge, it is soluble in distilled water. However, when X_1 was combined with X_2 in water, a precipitate results. This precipitate is an aggregation of the two types of molecules. They claimed that egg-yolk vitellin can be resolved ultracentrifugally into two components, one is a phosphoprotein and the other is a relatively dense lipoprotein. Therefore, Schjeide & Urist concluded that X_1 and X_2 in serum are similar to egg-yolk vitellin, probably they

have a common origin. Since phosphoprotein and lipoprotein usually occur in the same relative proportion in serum and egg-yolk, it is reasonable to suppose that most of the X_1 and X_2 molecules in the serum are destined to be deposited in the egg-yolk. The difference between the X_1 component of serum and the phosphoprotein of egg-yolk appear to be that the X_1 component of serum is a soluble and discrete substance heavily complexed with calcium; the phosphoprotein of egg-yolk is largely insoluble because it is complexed with X_2 lipoprotein. X_1 and X_2 are soluble in plasma only as long as sufficient dialyzable material (probable calcium or similar ions) is present to keep them separate. The high capacity of these fractions for calcium was assumed to be due to the presence of phosphate groups of the phosphorylated amino acid, as suggested by Urist, Schjeide & McLean (1959). There was no evidence for the presence of a colloidal calcium-phosphate complex. Recalling that phosphoserine makes up about 1/3 of the phosvitin molecule, one would suspect that these two fractions were contaminated with phosvitin.

McIndoe (1959) isolated a lipophosphoprotein complex from laying hen serum which may be related to X_1 and X_2 of Schjeide & Urist (1956). This fraction was obtained by dilution of citrate plasma with 9 volumes of water. After standing in the refrigerator for one and a half to two hours, the precipitate of crude lipophosphoprotein was collected by centrifugation at 3000 g. for 20 - 30 minutes at 2° to 5°C.

The precipitate was dissolved in water and reprecipitated by bringing the NaCl concentration to 0.015 M. After standing at 2°C for fifteen minutes, the precipitate (PLP) was centrifuged as before for 40 minutes. The yield was about 2 gm. per 100 ml. of plasma. This fraction appears in hen plasma about 7 days before the first ovulation and disappears within a few days when hens go off laying. PLP has an N/P ratio (by weight) close to 3.5. It is insoluble in NaCl solutions between 0.01 M and 0.04 M. It contains 20% protein and 80% lipid. The protein moiety is similar to Laskowski's serum vitellin in that it has a rather high (0.74%) phosphorus content. Of the 80% lipid, 25% is phospholipid, 4% is cholesterol while the remainder is triglyceride. All plasma triglyceride appears to be in the PLP fraction. Ultracentrifuge studies showed that PLP is not homogenous. It consists of two components; one of which is a light lipoprotein and the other is a protein or a denser lipoprotein. Investigation of the supernatant from which PLP had been separated showed that only 10% of the plasma protein nitrogen and approximately one-third of the protein phosphorus is contained in the PLP fraction. McIndoe (1959) suggested that the high protein phosphorus content of the supernatant during the pre-ovulating period without any corresponding increase in protein nitrogen may be explained if the protein phosphorus is present as phosvitin, which contains approximately 10% P.

McKinley et al. (1953) were the first to apply zone electrophoresis to separate avian serum proteins. Their separations were based on the use of veronal buffer of pH 8.6 and ionic strength of 0.05. Five serum protein fractions were distinguished in sera from the male and sexually immature pullets. These five fractions were designated as: albumin, α_1-, α_2-, β- and γ-globulins. On examining sera from estrogenized immature pullets, a broad new zone ahead of the β-globulin region was observed. This zone was heavily associated with lipid. It can be resolved into two fractions by using methanolic buffer. They thus confirmed the results of Schjeide & Urist (1956) in so far as the latter had shown that there are two major lipoprotein fractions in the serum of estrogenized birds. McCully et al. (1959) have also reported the presence of two lipoprotein zones, which they called P_1 and P_2, in the serum of laying hen. These workers suggested that P_1 is possibly a lipovitellenin complex while P_2 is possibly a lipovitellin complex (a complex of lipovitellin-phosvitin-γ-livetin). Phosvitin, the phosphorus rich egg-yolk protein isolated by Mecham & Olcott (1949) was shown to be present in the serum in the following manner: Serum from heavily estrogenized birds was diluted ten-fold with distilled water and the crude lipovitellin complex was separated on the centrifuge and redissolved in 0.15 M NaCl. This solution was then treated with oxalate to precipitate calcium. The precipitate was removed by centrifugation and the super-

natant was subjected to zone electrophoresis. The electropherograms showed no indication of albumin or of α_1-, α_2- or α_3-globulins. A lipoprotein zone corresponds to the P_1 fraction and a broad protein zone appeared. In addition, there was a very faintly stained band which had a mobility, under the same conditions, greater than that of albumin and the same as that of a sample of egg-yolk phosvitin run concurrently. This observation appears to be the first demonstration by zone electrophoresis of the presence of phosvitin in the serum of the estrogenized hen and it was evident that this phosvitin forms a complex with calcium and lipovitellin.

The present thesis describes the isolation and characterization of phosvitin in pure form from the serum of the estrogenized hen. It may be mentioned here that this isolation was achieved prior to the electrophoretic demonstration of the presence of phosvitin in such sera by McCully *et al.* (1959).

SECTION III

EXPERIMENTAL

3.1- Analytical methods.

3.1.1- Determination of dry matter.

The protein solution in each preparation was lyophilized in the Virtis freeze-drying unit. In drying from the frozen state, there was no continual mixing of solution or any movement of solvent. The surface of the evaporating frozen ice layer gradually receded and left more and more of the highly porous residue of dry protein particles exposed. Case-hardening was carefully avoided. If the freeze-drying unit was operated under 50 micron of pressure, 50 ml. of solution could be dried within 24 hours. The dried product was voluminous — in fact, the volume corresponded closely to the volume of the original frozen state. However, it still contained a considerable amount of moisture. Therefore, the freeze dried product was transferred from the freeze-drying unit to a vacuum desiccator and dried over conc. H_2SO_4 till its weight was constant. No attempt was made to do a moisture determination on the sample using the vacuum oven, because the amount of product obtained was small and drying in the oven would have denatured the protein and hence made it unsuitable for further studies.

3.1.2- Determination of phosphorus and nitrogen.

Phosphorus was determined on all samples by the method of Berenblum & Chain (1938a, b), using the following reagents:

1. 10 N sulfuric acid (approx.): 28% conc. H_2SO_4.
2. N sulfuric acid (approx.): sol. (1) diluted 10 times.
3. 5% ammonium sulfate.
4. Stock solution of stannous chloride: 10 gm. of stannous chloride was dissolved in 25 ml. conc. HCl.
5. Dilute stannous chloride solution: sol. (4) diluted 200 times with 1 N H_2SO_4.
6. iso-Butanol.
7. Ethanol.

A standard phosphate solution was prepared by dissolving 2.193 gm. KH_2PO_4 in 500 ml. water (= 1 mg. P per ml.). Suitable dilutions of this solution would give solutions with various phosphorus concentrations. These solutions were used for comparison with the unknown.

In each case, the organic protein phosphorus was digested with 60% perchloric acid, followed by a few drops of 30% H_2O_2. Fifteen to twenty mg. of protein sample was used. Digestion was completed within 4 hours. The digest was made up to suitable volume according to the phosphorus content of the preparation. One ml. of this dilute solution was measured

into a separatory funnel and the following added: 2ml. of distilled water, 2.5 ml. of 5% ammonium molybdate and 10 ml. of iso-butanol. The mixture was shaken for one to two minutes and the aqueous layer was discarded. The alcoholic solution was washed by shaking with two lots of 5 ml. N H_2SO_4 and then shaken with about 15 ml. of dilute stannous chloride solution for about 30 seconds. The aqueous layer was discarded. The blue solution was poured into a 10 ml. volumetric flask. The separatory funnel was washed with ethanol and the solution made up to mark with the washings. The percentage transmittance of the blue color solution was measured in an Evelyn photoelectric colorimeter (660 mμ filter) and converted to optical density. The phosphorus content of the sample was then determined from the standard curve prepared by using standard phosphorus solutions.

Nitrogen was determined according to micro-Kjeldahl method (A.O.A.C). To 10-20 mg. of the sample in a 30 ml. Kjeldahl flask was added 1.30 gm. K_2SO_4 + 40 gm. HgO and 2.0 ml. conc. H_2SO_4. This mixture was heated on an electric burner until colorless. Digestion was continued for another four hours. The digest was transferred to the Parnas-Wagner micro-Kjeldahl distillation apparatus. Ten ml. of NaOH-$Na_2S_2O_3$ (50 gm. NaOH and 5 gm. $Na_2S_2O_3$ in 100 ml. H_2O) was added and steam distilled. The distillate was collected in an Erlenmeyer flask containing 10 ml. of 4% boric acid. The

ammonia was titrated with 0.02 N HCl, using methyl red-bromocresol green (5 parts 0.2% alc. bromcresol green solution with 1 part 0.2% alc. methyl red solution) as indicator. A blank determination was conducted, using the same quantities of reagents and the same length of time for digestion. The % N was calculated according to the formula:

$$\frac{(\text{ml. HCl in detn.} - \text{ml. blank}) \times \text{normality} \times \text{eq.wt. of N}}{\text{wt. of sample (mg.)}} \times 100$$

3.1.3- Determination of phosphoprotein.

Protein phosphorus in serum was determined according to the method of Laskowski as modified by Common, Rutledge & Bolton (1947). The method is as follows: to 9 ml. of 10% trichloroacetic acid in a 15 ml. centrifuge tube was added 1 ml. of serum with agitation. The precipitate was separated from the solution by centrifugation. The supernatant was discarded. The precipitate was washed twice in the centrifuge tube with 4% w/v trichloroacetic acid. The precipitate was then thoroughly broken down and extracted with 12 ml. of Bloor's mixture (3 vol. ethanol + 1 vol. ethyl ether), stoppered and left overnight. Next morning, the precipitate was spun down and the lipid extract decanted. This lipid extraction was repeated four times. The precipitate was then combusted with conc. H_2SO_4, using $HClO_4$ to complete the combustion. Phosphorus was determined by the method of Berenblum & Chain (1938a, b).

3.1.4- Technique of zone electrophoresis.

The apparatus used for electrophoresis was essentially the same as that described by Flynn & De Mayo (1951). In this apparatus the paper is held in a horizontal position rather than draped over a rod so as to elevate the central portion of the paper strip as in the apparatus of Durrum (1950).

All electropherograms were run for 24 - 30 hours at 5°-7° C at a constant voltage of 200 volts with filter paper (Whatman 3 MM) 17 inches in width and 16 inches in length.

Buffer: Veronal buffer of pH 8.6 and ionic strength 0.05 was prepared by dissolving 1.84 gm. diethylbarbituric acid and 10.30 gm. sodium diethylbarbiturate in 1.0 liter of distilled water.

Staining: 1. For protein. Strips cut from the electropherogram for protein staining were air dried. Lipid was extracted from the paper by soaking overnight with a ethanol-ethyl ether mixture (3:1 v/v) containing 5% trichloroacetic acid. The strips were next refluxed for three successive half hour periods with ethanol-chloroform (1:1 v/v) containing 5% trichloroacetic acid. The paper was then stained with a saturated solution of Amidoschwarz (Chromagesellschaft, Stuttgurt, Germany) prepared by dissolving the dye in acetone containing 10% acetic acid, as described

by Kawerau (1954). After staining for 20 minutes the strips were washed several times with a solution of methanol + water + 1.0 N HCl (90:10:1 v/v) till the background was freed from dyestuff.

2. For lipid. Strips cut from the electropherogram were stained for lipid with Oil Red O (saturated solution of Oil Red O in 60% ethanol) for 16 hours, as described by Durrum et al. (1952).

3.2- The isolation of phosvitin from egg-yolk and avian serum.

3.2.1- The preparation of phosvitin from egg-yolk.

The following experiments were merely repetitions and confirmations of the procedure of Joubert & Cook (1958) for preparation of phosvitin, but were necessary in order to obtain satisfactory samples of phosvitin. (Samples of purchased phosvitin were found to be unsatisfactory both as regards to phosphorus content, solubility and electrophoretic behaviour.) Since phosvitin itself is a highly charged molecule, it reacts strongly with other proteins with opposite charge. Obviously it reacts strongly with lipovitellin and γ-livetin. Joubert & Cook found that divalent cations are effective in breaking down this complex. Magnesium ion was used for this purpose.

3.1.1a- Experiment 1.

To 400 gm. egg-yolk was added 1200 ml. 0.4 M $MgSO_4.7H_2O$ and 37.44 gm. solid $MgSO_4.7H_2O$. This mixture was stirred for an hour at 6°C. It was left at that temperature for about half an hour. Some lipid material floated on the surface and this was removed by centrifugation for 30 minutes at 2500 r.p.m. and filtering through cheesecloth. The filtrate was slowly diluted with an equal volume of cold water. The magnesium ion concentration at this stage was 0.2 M. It was left at 6°C overnight. Next morning, the precipitate was collected by centrifugation. On the basis of its P content, this precipitate was about 70% phosvitin. It was redissolved in 800 ml. 0.4 M $MgSO_4.7H_2O$ and reprecipitated as before. The precipitate was dissolved in 100 ml. of 10% sodium chloride and centrifuged for 2 hours at 4400 r.p.m. Lipid materials which floated on the surface were removed. Sodium chloride was used to obtain the necessary high density. The subnatant was diluted with 200 ml. of water and dialyzed against several changes of acetate buffer, pH 4.0, $\Gamma/2=0.1$ (prepared by dissolving 8.20 gm. sodium acetate in water, adding 33.50 ml. glacial acetic acid and diluting to 1 liter with water). This precipitated the remaining lipovitellin, which was removed by centrifugation. The supernatant was then dialyzed against several changes of distilled water to remove the buffer. The removal of buffer was considered to be completed when the diffusate no longer gave a positive sodium flame test. The dialysate was freeze-

dried and then dried over conc. H_2SO_4 *in vacuo*. The yield was 1.52 gm., with a phosphorus content of 11.5% and a nitrogen content of 12.9%. This preparation was considered to be satisfactory except that in the final freeze-drying, the material was obtained in a hard, rather horny mass; besides, the yield was rather low.

3.2.1.b- Experiments 2 and 3.

Two further lots of egg-yolk were worked up for phosvitin as described above. This time the lyophilization was successful and the preparation was friable and easily powdered.

	Expt. 2	Expt. 3
Yield	1.62 gm.	0.53 gm.
Phosphorus content	9.3%	8.7%
Nitrogen content		13.0%

The yields in these experiments were rather low compared with that reported by Mecham & Olcott (1949), who claimed that 10 gm. of phosvitin was recovered from 1 kg. of egg-yolk (Joubert & Cook had not reported their yield). Mecham & Olcott pointed out that 6.5% - 7.0% of yolk protein is phosvitin. If 400 gm. of egg-yolk was used, one should have got 400 gm x 16% x 7% = 4.48 gm. of phosvitin (16% being

the percentage of protein in yolk). The actual yields were low compared with this. This low yield may be due to the co-precipitation of phosvitin with lipovitellin as suggested by Francis (1952). By studying the exchange of radioactivity between lipovitellin and ^{32}P phosvitin in solution, Francis was able to show that phosvitin may be dissociated from lipovitellin in 10% NaCl, but when lipovitellin was precipitated from 10% NaCl solution, it was found to be radioactive, indicating ^{32}P phosvitin was co-precipitated with it.

In the present experiments, it was observed that a large amount of lipovitellin was precipitated when the protein solution was dialyzed against the pH 4 acetate buffer (the isoelectric point of phosvitin is not known; the isoelectric point of lipovitellin is 3.4 - 3.5 according to Young & Phinney (1951). Joubert & Cook (1958) reported that the value for lipovitellin is 5.6. These values would seem to be the value applicable for lipovitellin-phosvitin complex). It is not surprising that some of the phosvitin must have been co-precipitated down with lipovitellin at this stage.

3.2.1c- Experiment 4.

In view of the fact that the total phosphoprotein in serum is low, it was considered advisable to try to separate phosvitin from egg-yolk on a small scale in order to test the

practicability of the method. In this experiment, the entire process of isolation of phosvitin was carried out on a reduced scale on two egg-yolks.

Yield of phosvitin	45 mg.
Phosphorus content	6.8%
Nitrogen content	10.2%

This small phosvitin preparation was obviously impure, as shown by the low N and P contents. However, the N/P ratio is approximately the same as that for phosvitin, and the experiment at least demonstrated the feasibility of work on this scale.

3.2.1d- Experiment 5.

The object of this experiment was to test the electrophoretic homogeneity of the phosvitin preparation.

Preparation No. 2 was examined by zone electrophoresis in veronal buffer. The phosvitin ran as a single band. This was in agreement with Mecham & Olcott (1949) who used free electrophoresis and showed that phosvitin ran as a single band in veronal buffer. Staining with Oil Red O indicated that the material was free of lipid.

In the course of this work, it was observed that phosvitin took up the Amidoschwarz dyestuff readily but that

most of the dye tended to remove again when the papers were being washed with methanol and HCl to remove the dyestuff from the background. This property of phosvitin means that small amounts may easily be missed on paper electropherograms, but at the same time it provides a useful qualitative test for phosvitin.

3.2.2- The attempted isolation of phosvitin from sera of laying hens.

3.2.2a- Experiment 1.

This experiment was done under the hypothesis that phosvitin in laying hens' serum exists essentially in the same form as it is in egg-yolk, and that Joubert & Cook's (1958) method could be applied directly for the separation of phosvitin in plasma. In view of the fact that protein concentration is lower in plasma than in egg-yolk, the plasma was concentrated by freeze-drying before any attempt at isolation of phosvitin was made.

Blood was drawn from the wing veins of hens through a size 18 needle into a centrifuge tube containing an appropriate amount of potassium oxalate (0.5 ml. of 10% w/v oxalate for each 10 ml. of blood). In all, 230 ml. of laying hens' blood was collected in this manner. After removal of the corpuscles, the plasma was frozen quickly and put in the

freeze-drying unit. The freeze-drying of plasma was not as successful as expected. The frozen plasma tended to thaw within 3 hours. Foaming occurred when this happened. The freeze-drying process was then perforce discontinued and the plasma had to be removed from the freeze-drying unit and frozen again. By repeating this process, the volume of plasma was reduced to 130 ml. This concentrated plasma was worked up for phosvitin as though it were egg-yolk. An equal volume of 0.4 M $MgSO_4.7H_2O$ and 11 gm. of solid $MgSO_4.7H_2O$ was added with stirring. After standing at 6°C for 5 hours, it was centrifuged to remove lipid material. The subnatant was diluted with an equal volume of water. No precipitate of crude phosphoprotein could be observed after the solution was left standing at 6°C for 24 hours.

It was concluded that the plasma system and egg-yolk system are entirely different in behaviour and that the method of Joubert & Cook could not be applied directly for the isolation of phosvitin from plasma.

3.2.2.b- Experiment 2.

It was thought that Laskowski's (1935b) serum vitellin might be a complex of phosvitin-lipovitellin and other proteins and that it might be possible to separate phosvitin from this complex by the method of Joubert & Cook (1958).

One hundred ml. of blood was drawn from the wing veins of 8 laying hens. After centrifugation for the removal of corpuscles, 70 ml. of oxalated plasma was recovered. The pH of the plasma was 7.35. An equal volume of cold distilled water was added. Approximately 20 ml. of 0.1 N HCl was added carefully to bring the pH of the dilute plasma solution to 5.30. This acidified dilute plasma was left standing at 6°C overnight. A large amount of precipitate was collected by centrifugation next day. This precipitate was dissolved in 20 ml. of 5% NaCl followed by adding solid NaCl to saturation. The solution was again left standing in the cold for 24 hours. The precipitate (fibrinogen) was removed by centrifugation. The supernatant was diluted 100-fold with distilled water. After standing at 6°C for 24 hours, the precipitate, which corresponded to Laskowski's serum vitellin, was collected by centrifugation. This serum vitellin was redissolved in 5% NaCl and reprecipitated as before. The precipitate thus obtained was worked up for phosvitin as though it were egg-yolk as follows. Eighty ml. of 0.4 M $MgSO_4.7H_2O$ was added. The material dissolved fairly rapidly. An equal volume of distilled water was then added with stirring. No precipitate of crude lipovitellin-phosvitin complex could be obtained after the solution had been left standing at 6°C for 24 hours. Another 24 hours was allowed for the precipitate, if there was any, to settle down; no precipitate could be obtained by the end of this period. The failure of lipovitellin-phosvitin

complex to precipitate under these conditions raised the following possibilities:-

1. That there is no phosvitin present in laying hen's serum. This was considered highly improbable even though McCully et al. (1959) had not at the time demonstrated by paper electrophoretic technique that phosvitin is present in the estrogenized hen serum. It was thought most likely that the amount present is so small that it was lost during the manipulation of isolation.

2. That there are certain factors in the plasma which prevent lipovitellin-phosvitin from precipitation under these experimental conditions.

3. That the phosvitin-lipovitellin complex was broken down after the removal of calcium in the blood by oxalate. The role of calcium vis-à-vis serum protein complexes has been fully demonstrated by Clegg et al. (1956) and McCully et al. (1959). The latter workers have shown that when calcium is removed from the serum, free phosvitin is liberated from the phosvitin-lipovitellin complex, but this information was in the future at that time. It was known, however, that phosvitin when not combined with other proteins, is soluble in water or dilute salt solution. Thus it might tend to be removed together with the albumin when the plasma was diluted. This may explain why no phosvitin-lipovitellin complex was precipitated down in 0.2 M $MgSO_4 . 7H_2O$ solution.

4. The solubility of crude phosvitin-lipovitellin complex in NaCl. According to McIndoe (1959), the lipophosphoprotein in hen plasma is practically insoluble between 0.01 and 0.04 M NaCl. It is soluble in more dilute or more concentrated salt solution. In the foregoing experiment, NaCl was added to saturation to remove fibrinogen followed by 100-fold dilution to precipitate crude serum vitellin. The salt concentration after 100-fold dilution was not known. It may be possible that the concentration fell in the range where phosvitin-lipovitellin complex was insoluble.

To avoid (3) and (4), serum, instead of plasma, should be used.

3.2.2.c- Experiment 3.

Before further attempts to isolate phosvitin from laying hen serum were undertaken, it was thought advisable to use cock's serum as a control to see if any protein would precipitate down under the same experimental conditions. Accordingly, 50 ml. of blood was drawn from the cock. The blood was allowed to clot. The serum was worked up for phosvitin, following the same procedure as in Experiment 2. No precipitate of serum vitellin was obtained when the solution was diluted 100-fold after saturation with NaCl.

3.2.2.d- Experiment 4.

It was felt that some factors in the serum of laying

hens might have prevented the phosvitin, if there was any, from separating from the other serum proteins. Accordingly, 100 ml. of laying hen serum was mixed with two egg-yolks. This mixture was set aside at 6°C for about 5 hours. At the end of this period, this mixture was worked up for phosvitin by the method of Joubert & Cook (1958). The final product contained 11.3% nitrogen and 6.2% phosphorus. The phosphorus content was rather high compared with other egg-yolk proteins. It was considered probable that the major component of this material was phosvitin. Therefore, it was concluded that the presence of serum did not prevent this phosvitin from egg-yolk to precipitate in Joubert & Cook's method.

3.2.2.d- Experiments 5 and 6.

Laying hen's serum was used in these experiments. Laskowski's method for preparation of serum vitellin was slightly modified. Since there was no fibrinogen in the serum, the saturation of solution with NaCl to remove fibrinogen was unnecessary; the volume of solution to be handled was greatly reduced.

Two hundred and ninety ml. of laying hen serum was obtained from 23 laying hens by cutting their jugular veins. The pH of the serum was brought down to 5.50 with 88 ml. 0.1 N HCl and distilled water was added to this acidified serum. This dilution did not effect the pH of the serum.

It was then left standing at 6°C for 48 hours to allow the precipitate to settle down. The precipitate was dissolved in 50 ml. of 1% NaCl solution. Distilled water was added to bring the salt concentration to 0.1%. The precipitate thus obtained was believed to be Laskowski's serum vitellin. It was worked up for phosvitin as though it were egg-yolk. The yield was minute (3 - 4 mg.).

Another lot of laying hen serum (250 ml.) was worked up for phosvitin as described above in the hope of getting a better yield. The yield this time was 20mg. It was not feasible to do any analysis on it, but it was possible to show by electrophoresis that the final product displayed the same mobility and staining properties as egg-yolk phosvitin on zone electrophoresis.

From these two experiments, it could be concluded that phosvitin is present in the serum of normal laying hens in concentrations that are rather small for ease of separation from the relatively large amount of other serum proteins.

3.2.3- The isolation and characterization of phosvitin from serum of estrogenized hens.

It was suspected that the relatively low phosphoprotein in serum was due to the transfer of this constituent from liver to the ovary where formation of yolk takes place.

Accordingly, it was desired to work with birds in which the level of serum phosphoprotein had been raised to a high level by treatment with estrogen.

3.2.3.a- Isolation of serum phosvitin.

Four laying hens were each given 2 mg. of estradiol benzoate (as 'Progynon B' Schering) intramuscularly for 5 successive days. The birds were then killed and 200 ml. of serum was recovered. The serum was adjusted to pH 5.3 by adding 80 ml. 0.1 N HCl slowly. Distilled water was then added to this acidified serum slowly with stirring until the volume reached 400 ml. The solution was left standing at 6°C overnight. Next morning, the precipitation of crude lipovitellin complex was separated on the centrifuge (3000 r.p.m. for 30 minutes), redissolved in 1% NaCl and re-precipitated by 10-fold dilution with distilled water. The precipitate was worked up for phosvitin by the method of Joubert & Cook (1958) as though it were egg-yolk. At the same time phosvitin was being separated from one egg-yolk as a control.

The precipitate was taken up in 25 ml. 0.4 M $MgSO_4.7H_2O$ and sufficient solid $MgSO_4.7H_2O$ was added to give 0.4 M $MgSO_4.7H_2O$ with the water held in the precipitate. The clear solution was then diluted with an equal volume of distilled water to give 0.2 M $MgSO_4.7H_2O$. The precipitate of lipovitellin complex was separated on the centrifuge, again taken up

in 0.4 M $MgSO_4.7H_2O$ and reprecipitated by dilution. All supernatants to this stage were discarded. The pH of the supernatants from which crude lipovitellin complex was separated did not differ much from that of the egg-yolk preparation, and in no case was the difference in pH greater than 0.6.

The partially purified lipovitellin complex was then dissolved in 10% NaCl and centrifuged at the highest speed possible (app. 4400 r.p.m.) in an International Refrigerated Centrifuge PRI. The lipid that separated on the surface was removed by filtration through a packed wad of cheesecloth. The filtrate was diluted with twice its volume of water and then dialyzed against several changes of acetate buffer (pH=4, $\Gamma/2$=0.1). At this stage the crude lipovitellin was precipitated down while most of the phosvitin remained in solution.

The supernatant in the dialysis sac was separated by centrifugation and then dialyzed against distilled water to remove the buffer. It was then lyophilized, and the residue was dried to constant weight <u>in vacuo</u> over conc. H_2SO_4.

Yield	—	150 mg.
N	—	12.3%
P	—	10.1%

The nitrogen and phosphorus content of this material agreed with published value for yolk phosvitin. The yield corresponded to 75 mg./100 ml. serum, or approximately to 7.5 mg.phosvitin P per 100 ml. serum. On zone electrophoresis, the preparation displayed the same behaviour as egg-yolk protein.

It was concluded that phosvitin may be isolated from the serum of the hen by the method of Joubert & Cook (1958) provided that the level of serum phosphoprotein has been increased to a sufficiently high level by treatment with exogenous estrogen.

This result, together with the results of Experiment 6, Section 3.2.2.e, may be considered to have demonstrated the presence of phosvitin in the serum of laying hens.

At this stage it remained only to prove that the result obtained described in the proceeding paragraph was reproducible. Accordingly, the entire procedure was repeated four times with results that may be summarized in tabular form:-

TABLE I

Expt.	No. of birds	Estrogen treatment	Vol. of serum ml.	Total serum phosphoprotein P expressed as mg.P/100 ml.	Mg. phosvitin obtained	Phosvitin P obtained expressed as mg.P/100 ml. serum
1	5	2 mg. estradiol benzoate per day for 5 days	185	20	10.0	0.55
2	6	"	250	18	27.6	1.11
3	*48	0.5 mg. estradiol benzoate per day for 14 days	720	13	80.0	1.11
4	12	2 mg. estradiol benzoate per day followed by 4 mg. stilbestrol per day for 3 days	360	25	150.0	4.15

*Not all the serum from each bird was available in this experiment

The foregoing results left no doubt that the isolation was reproducible, but did not provide much indication as to the extent of the recovery of the total serum phosvitin.

SECTION IV

SUMMARY

The work described above shows fairly conclusively that phosvitin can be recovered from the serum of laying hens in which the serum lipophosphoprotein complex has been raised to high levels by administration of estrogen. It represents, so far as the author is aware, the first occasion on which phosvitin has been obtained from serum.

It is highly probable that phosvitin could be isolated from the sera of estrogenized immature pullets or of roosters, for the pattern of blood composition in such birds is indistinguishable from that of the laying hen, apart from quantitative aspects that depend on levels of estrogen used. Thus it is fairly certain that the phosvitin is formed in the liver.

The demonstration by Vanstone *et al.* (1957) that serum phosphoproteins were formed exclusively in the liver of the estrogenized cockerel points in the same direction, *viz.*, that the ovary, quiescent or active, is in no way necessary for phosphoprotein formation in the bird. True, some phosphoprotein may be formed in the active ovary as well as in the liver, but all the evidence suggest that the liver is the sole site of formation in both the estrogenized bird and the normal laying hen, and that it is transferred to the ovary in the blood, probably in association with lipoproteins.

SECTION V

REFERENCES

Alderton, G., & Fevold, H.L. 1945.
Preparation of the egg-yolk lipoprotein, lipovitellin.
Arch. Biochem. 8: 415-419.

Association of Official Agricultrual Analysts: Official and Tentative Methods of Analysis. Eighth Edition. 1955. p. 805-806.
Association of Official Agricultural Chemists.
Washington 4, D.C.

Bence-Jones, H. 1841.
Cited by Jukes, T.H., & Hay, H.D. 1932.
Egg-yolk proteins.
J. Nutrition 5: 81-101.

Benjamin, H.R., & Hess, A.F. 1933.
The forms of the calcium and inorganic phosphorus in human and animal sera.
J. Biol. Chem. 103: 629-641.

Berenblum, I., & Chain, E. 1938a.
Studies on the colorimetric determination of phosphate.
Biochem. J. 32: 286-294.

Berenblum, I., & Chain, E. 1938b.
An improved method for the colorimetric determination of phosphate.
Biochem. J. 32: 295-298.

Blackwood, J.H., & Wishart, G.M. 1934.
The hydrolysis of lecithovitellin by pepsin and by trypsinkinase.
Biochem. J. 28: 550-558.

Brandt, L.W., Clegg, R.E., & Andrew, A.C. 1951.
The effect of age and degree of maturity on the serum proteins of the chicken.
J. Biol. Chem. 191: 105-111.

Calvery, H.O., & White, A. 1931.
Vitellin of hen's egg.
J. Biol. Chem. 94: 635-639.

Chargaff, E. 1942.
A study of lipoproteins.
J. Biol. Chem. 142: 491-504.

Chargaff, E. 1944.
Lipoproteins.
Advances in Protein Chemistry. Vol. 1, p. 1-24.
Academic Press Inc., Publishers
New York, N.Y.

Clegg, R.E., & Hein, R.E. 1953.
P^{32} distribution in serum proteins of the chicken.
Science 117: 714-715.

Clegg, R.E., Ericson, A.T., Hein, R.E., McFarland, R.H., & Leonard, G.W. 1956.
An electrophoretic component responsible for calcium binding in the blood sera of chickens.
J. Biol. Chem. 219: 447-454.

Common, R.H., Rutledge, W.A., & Bolton, W. 1947.
The influence of gonadal hormone on serum riboflavin and certain other properties of blood and tissues in the domestic fowl.
J. Endocrinology 5: 121-130

Deutsch, H.F., & Goodloe, M.B. 1945.
An electrophoretic survey of various animal plasma.
J. Biol. Chem. 161: 1-20.

Dumas, J., & Cahours, A. 1842.
Cited by Jukes, T.H., & Hay, H.D. 1932
Egg-yolk proteins.
J. Nutrition 5: 81-101.

Durrum, E.L. 1950.
A microelectrophoretic and micronophoretic technique.
J. Am. Chem. Soc 72: 2943-2948.

Durrum, E.L., Paul, M.H., & Smite, E.R.B. 1952.
Lipid detection in paper electrophoresis.
Science 116: 428 - 430.

Dury, D.R., & McMaster, P.D. 1929.
The liver as the source of fibrinogen.
J. Exp. Med. 50: 569-578.

Evans, R.J., & Bandemer, S.L. 1957.
Separation of egg-yolk proteins by paper electrophoresis.
Agri. and Food Chem. 5: 868-872.

Fevold, H.L., & Lausten, A. 1946.
Isolation of a new lipoprotein, lipovitellenin, from egg-yolk.
Arch. Biochem. II: 1-7.

Flickinger, R.A., & Rounds,D.E. 1956.
The material synthesis of egg-yolk proteins as demonstrated by isotopic and serological means.
Biochim. Biophys. Acta 22: 38-42.

Flynn, F.V., & De Mayo, P. 1951.
Micro-electrophoresis of proteins on filter paper.
Lancet 261: 235-239.

Fourcroy, A.F., 1782.
Cited by Jukes, J.H., & Kay, H.D. 1932.
Egg-yolk proteins.
J. Nutrition 5: 81-101

Francis, G.F. 1952.
Immunological and serological properties of phosvitin.
Biochem. J. 51: 715-720.

Greenberg, D.M., Larson, C.E., Pearson, P.B., & Burmester, B.R. 1936.
The state and partition of calcium and inorganic phosphorus in the serum of the fowl: effect of growth and ovulation.
Poultry Sci. 15: 483-489.

Heller, V.G., Hunter, K.R., & Thompson, R.B. 1932.
Phosphorus distribution in chicken blood as affected by the diet.
J. Biol. Chem. 97: 127-132.

Heller, V.G., Paul, H., & Thompson, R.B. 1934.
Changes in the blood calcium and phosphorus partition during the life cycle of the chicken.
J. Biol. Chem. 106: 357-364.

Hillyard, L.A., Entenman, C., & Chaikoff, I.L. 1956.
Concentration and composition of serum lipoprotein of cholesterol fed and stilbestrol injected birds.
J. Biol. Chem. 223: 359-368.

Hoppe-Seyler, G.K.F. 1865.
Cited by Jukes, T.H., & Kay, H.D. 1932.
Egg-yolk proteins.
J. Nutrition 5: 81-101.

Hosoda, T., Kaneko, T., Mogi, K., & Abe. T. 1955.
Serological studies on egg production in the fowl.
I. On the locus of serum vitellin production.
Poultry Sci. 34: 9-15.

Hughes, T.S., Titus, R.W., & Smites, B.L. 1927.
The increase in the calcium of hen's blood accompanying egg production.
Science 65: 264.

Joubert, F.J., & Cook, W.H. 1958.
Preparation and characterization of phosvitin from hen egg-yolk.
Can J. Biochem. Physiol. 36: 389-408.

Jukes, T.H., & Kay, H.D. 1932.
Egg-yolk proteins.
J. Nutrition 5: 81-101.

Jukes, T.H., Fraser, D.T., & Orr, M.D. 1934
The transmission of diphtheria antitoxin from hen to egg.
J. Immunol. 26: 353-360.

Kawerau, E., 1954.
Electrophoresis of serum and urine proteins on filter paper strips and agar jelly wi th bridge unit.
Analyst 79: 681-686.

Kay, H.D., & Marshall, P.G. 1928.
The second protein (livetin) of egg-yolk.
Biochem. J. 22: 1264-1269.

Kossel, A., 1886.
Cited by Jukes, T.H., & Kay, H.D. 1932.
Egg-yolk proteins.
J. Nutrition 5: 81-101.

Laskowski, M. 1935a.
Uber die Phosphorverbindugen im Blutplasma der Legehenne.
Biochem. Z. 278: 293-300.

Laskowski, M. 1935b.
Darstellungsmethode des Serumvitellins.
Biochem. Z. 278: 345-348.

Laskowski, M. 1938.
The gonadotropic hormone and level of blood phosphorus in the hen.
Biochem. J. 32: 1176-1180.

Lawrence, J.V., & Riddle, O. 1916.
Studies on the physiology of reproduction in birds. VI. Sexual differences in the fat and phosphorus content of the blood of the fowls.
Am. J. Physiol. I: 430-437.

Levene, P.A., & Alsberg, C.L. 1900.
The cleavage products of vitellin.
J. Biol. Chem. 2: 127-133.

Lipmann, F.A., & Levene, P.A. 1932.
Serinephosphoric acid obtained on hydrolysis of vitellinic acid.
J. Biol. Chem. 98: 109-114.

Martin, W.G., Vandegaer, J.E., & Cook, W.H. 1957.
Fractionation of livetin and the molecular weights of the α- and β-components.
Can. J. Biochem. Physiol. 35: 241-250.

Martin, W.G., Turner, K.J., & Cook, W.H. 1959.
Macromolecular properties of vitellenin from egg-yolk and its parent complex with lipid.
Can. J. Biochem. Physiol. 37: 1197-1207.

Marza, V.D., & Marza, E.V. 1935.
The formation of the hen's egg.
V. Histochemistry of yolk-formation: Proteins.
Quart. J. Microscop. Sci. 78: 191-250

McCully, K.A., Maw, W.A., & Common, R.H. 1959.
Zone electrophoresis of the proteins of the fowl's serum and egg-yolk.
Can. J. Biochem. Physiol. 37: 1457-1468.

McDonald, M.R., & Riddle, O. 1945.
The effect of reproduction and estrogen administration on the partition of calcium, phosphorus and nitrogen in pigeon plasma.
J. Biol. Chem. 159: 445-464.

McIndoe, W.M. 1959.
A lipophosphoprotein complex in hen plasma associated with yolk production.
Biochem. J. 72: 153-159.

McKinley, W.P., Oliver, W.F., Maw, W.A., & Common, R.H. 1953.
Filter paper electrophoresis of serum proteins of the domestic fowl.
Pro. Soc. Exptl. Bio. Med. 84: 346-351.

Mecham, D.K., & Olcott, H.S. 1949.
Phosvitin, the principle phosphoprotein of egg-yolk.
J. Am. Chem. Soc. 71: 3670-3679.

Miescher, F. 1897.
Cited by Riddle, O. 1942.
Cyclic changes in blood calcium, phosphorus, and fat in relation to egg laying and estrogen production.
Endrocrinology 31: 498-506.

Moore, D.H. 1948.
Effect of reciprocal steroid treatment on the electrophoretic patterns of fowl sera.
Endrocrinology 42: 38-45.

Osborne, T.B., & Campbell, G.F. 1900.
The proteids of the egg-yolk.
J. Am. Chem. Soc. 22: 413-422.

Osborne, T.B., & Jones, D.B. 1909.
Hydrolysis of vitellin from the hen's egg.
Am. J. Physiol. 24: 153-160.

Plimmer, R.H.A., & Bayliss, W.H. 1906.
The separation of phosphorus from caseinogen by the action of enzymes and alkali.
J. Physiol. 33: 439-461.

Plimmer, R.H.A. 1908.
The proteins of egg-yolk.
J. Chem. Soc. 93: 1500-1505.

Plimmer, R.H.A. & Scott, F.H. 1908.
A reaction distinguishing phosphoprotein from nucleoprotein and the distribution of phosphoprotein in tissue.
J. Chem. Soc. 93: 1699-1721.

Riddle, O. 1927.
Cited by Fleischmann, W., & Fried, I.A. 1945.
Studies on the mechanism of the hypercholesterolemia and hypercalcemia induced by estrogen in immature chicks.
Endrocrinology 36: 406-415.

Riddle, O., & Dotti, L.B. 1936.
Blood calcium in relation to anterior pituitary and sex hormones.
Science 84: 557-559.

Roepke, R.R., & Hughes, J.S. 1935.
Phosphorus partition in the blood serum of laying hens.
J. Biol. Chem. 108: 79-83.

Roepke, R.R., & Bushell, L.D. 1936.
Serological comparison of phosphoprotein of the serum of the laying hen and vitellin of the egg-yolk.
J. Immunol. 30: 109-113.

Schjeide, O.A. 1954.
Studies of the New Hampshire chickens embryo.
III. Nitrogen and lipid: analyses of ultracentrifugal fractions of plasma.
J. Biol. Chem. 211: 355-362.

Schjeide, O.A., Levi, E., & Flickinger, R.A. 1955.
A study of the yolk proteins of the frog eggs by physical and chemical means.
Growth 19: 297-306.

Schjeide, O.A., & Urist, M.R. 1956.
Proteins and calcium in serum of estrogen-treated roosters.
Science 124: 1242-1244.

Schjeide, O.A., & Urist, M.R. 1959.
Proteins and calcium in egg-yolk.
Exptl. Cell Research 17: 84-94.

Shepard, C.C., & Hottle, G.A. 1949.
Studies of the composition of livetin fraction of the yolk of hen's eggs with the use of electrophoretic analysis.
J. Biol. Chem. 179: 349-357.

Sturkie, P.D., & Newman, H.J. 1951.
Plasma proteins of chickens as influenced by time of laying, ovulation, number of blood sample taken and plasma volume.
Poultry Sci. 30: 240-248.

Sugano, H. 1957.
Studies on yolk proteins.
II. Electrophoretic studies on phosvitin, lipovitellin and lipovitellenin.
J. Biochem. 44: 205-215.

Taurog, A., Lorenz, F.W., Entenman, C., & Chaikoff, I.L. 1944.
The effect of diethylstilbestrol on the in vitro formation of phospholipids in the liver as measured with radioactive phosphorus.
Endrocrinology 35: 483-487.

Urist, M.R., Schjeide, O.A., & McLean, F.C. 1959.
The partition and binding of calcium in the serum of the laying hen and of the estrogenized rooster.
Endrocrinology 63: 570-585.

Valenciennes, A., & Fremy, E. 1854.
Cited by Jukes, T.H., & Kay, H.D. 1932.
Egg-yolk proteins.
J. Nutrition 5: 81-101.

Vandegaer, J.E., Reichmann, M.E., & Cook, W.H. 1956.
Preparation and molecular weight of lipovitellin from egg-yolk.
Arch. Biochem. Biophy. 62: 328-337.

Vanstone, W.E., Maw, W.A., & Common, R.H. 1955.
Levels and partition of the fowl's serum proteins in relation to age and egg production.
Can. J. Biochem. Physiol. 33: 891-903.

Vanstone, W.E., Dale, D.G., Oliver, W.F., & Common, R.H. 1957.
Sites of formation of plasma phosphoprotein and phospholipid in the estrogenized cockerel.
Can. J. Biochem. Physiol. 35: 659-665.

Williams, J., & Sanger, F. 1959.
The grouping of serine phosphate residue in phosvitin and casein.
Biochem. Biophy. Acta 33: 294-296.

Young, E.G., & Phinney, J.I. 1951.
On the fractionation of the proteins of egg-yolk.
J. Biol. Chem. 193: 73-80.

A P P E N D I X

Through the courtesy of Dr. W.G. Martin and Dr. W.H. Cook, Division of Applied Biology, National Research Council, Ottawa, a sample of the serum phosvitin was examined at the laboratories of the Council for behaviour in the ultracentrifuge. The data obtained, together with the published values for egg-yolk phosvitin (Joubert & Cook, 1958), are as follows:

	Sedimentation coefficient(S°_{20})	Weight average molecular weight
Serum phosvitin	3.17 ± 0.05	3.5×10^4
Egg-yolk phosvitin	3.14 ± 0.05	3.1×10^4

From the above date, it could be concluded that the sedimentation behaviour of the serum phosvitin preparation did not differ significantly from that of egg-yolk phosvitin.