ZONE ELECTROPHORETIC STUDIES OF THE PROTEINS OF AVIAN SERUM AND EGG YOLK

A Thesis

by

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GENERAL INTRODUCTION

It has been realized for many years that egg yolk is remarkable for its high content of phosphoprotein. However, considerable uncertainty has existed as to the number and nature of the proteins present in egg yolk. Most of the more recent studies have distinguished two lipoprotein fractions, which have been designated as 'lipovitellin' and 'lipovitellenin'; at one time lipovitellin was regarded as a phospholipophosphoprotein, but more recently it has been realized that the older lipovitellin preparations included a phosphoprotein of relatively very high (about 10%) phosphorus content. This phosphoprotein, or degradation products thereof, has been variously designated as 'haematogen', 'vitellinic acid', or 'phosvitin'. In addition, egg yolk is known to contain at least three different water-soluble proteins which are ordinarily designated as 'livetins'. The distinction of the foregoing six major protein fractions does not in any way imply that all of these fractions are molecular species; indeed, certain of the foregoing protein fractions have already been resolved further, and more such resolutions may be expected in the future.

Avian blood serum contains albumins, globulins, and

in the case of the laying hen or estrogenized fowl, is characterized by the presence of a phosphoprotein constituent ('serum vitellin'). These various protein fractions can be differentiated by salting-out techniques and by electrophoretic analysis.

Zone electrophoresis was applied to the study of the proteins of fowl's serum and egg yolk in this laboratory in the year 1951. Six protein fractions could be distinguished in the serum of the cock or sexually immature pullet by paper electrophoresis in aqueous veronal buffer pH 8.6. The six zones were designated, in order of decreasing mobility under the given conditions, as follows: albumin, α_1-globulin, α_2-globulin, α_3-globulin, β-globulin, and γ-globulin. These six zones were distinguished also in buffer made up in the same proportions but with 20% v/v methanol instead of water. Studies with sera from laying hens or from estrogenized fowl have shown the presence of an additional lipid-rich zone that moved slowly in aqueous veronal buffer pH 8.6. This zone was designated provisionally as 'presumptive lipovitellin' (PLV). It proved difficult to distinguish this PLV zone from other zones or to resolve it further when the buffer was aqueous veronal, but use of veronal buffer made up with 20% methanol permitted the resolution of two distinct

lipoprotein zones on the resultant electropherograms. The slower of these zones was apparently rich in protein phosphorus and was designated as zone PP ('presumptive phosphoprotein'). The faster lipoprotein zone, which was also the wider of the two, was designated 'fraction 8'.

The methanolic buffer technique was also applied to the examination of egg yolk proteins. The electropherograms showed two lipoprotein zones, of which one evidently was analogous to the PP zone of the serum proteins of laying hens or estrogenized fowl, while the other was analogous to the second lipoprotein zone (fraction 8) of such sera. The electropherograms also showed two lipid-free zones 'X' and 'Y' that represented non-vitellin components of yolk.

The foregoing studies were based on the use of apparatus constructed and operated according to the directions of Flynn and de Mayo (1951) and most of the observations were made with the use of methanolic veronal buffer. However, although methanolic veronal buffer had proved useful in the original separation of two lipoprotein zones on electropherograms of avian sera and of egg yolk, it seemed likely that its use must involve undue complexity of the conditions of electrophoresis, e.g., through

differential evaporation. Hence it was obviously desirable, if possible, to resolve the lipoproteins in aqueous veronal buffer.

The existence of the phosphorus-rich phosphoprotein phosvitin in egg yolk, and the view that the yolk constituents are synthesized in the liver and transferred thence to the ovary in the blood plasma, suggested that the sera of laying hens or estrogenized fowl would contain phosvitin. When the present studies were begun, phosvitin had not been separated, by paper electrophoresis either from whole egg yolk solutions or from the sera of either laying or estrogenized birds. Phosvitin was obtained subsequently from serum by Miss Chi-Ching Mok, working in this laboratory. Therefore, it was desirable to devise electrophoretic techniques whereby phosvitin could be separated electrophoretically from the serum or yolk proteins. Consequently, the present thesis is concerned with:

(a) the resolution of avian serum and egg yolk proteins by paper electrophoresis in various aqueous buffers;

(b) the distribution of total phosphorus and protein-bound phosphorus on paper electropherograms of the fowl's serum,

of whole egg yolk proteins and of certain ultracentrifugal fractions of egg yolk;

(c) some quantitative observations on the major phosphoprotein zones of electropherograms of egg yolk.

PART I

Zone Electrophoretic Studies of Avian Serum Proteins

1. HISTORICAL REVIEW

1.1. Puberal and Estrogen-induced Changes of Certain Serum Constituents of the Fowl

1.1.1. Calcium.

Blair Bell (1908) was the first to note an elevation of the blood calcium level in the laying hen. The increase in serum calcium level associated with ovarian activity was first demonstrated with pigeons by Riddle and Honeywell (1925), with doves and pigeons by Riddle and Reinhart (1926) and with chickens by Parhon (1926).

Riddle (1927) suggested that the ovarian hormones, which are responsible for the cyclic growth of the oviduct, are responsible also for the increased levels of plasma calcium, phosphorus and fat found during the egg-production cycle of pigeons. Hughes, Titus and Smits (1927) have reported plasma calcium values for normal chickens ranging in age from day-old chicks to mature laying pullets. These workers also suggested that the increase in the blood calcium of the laying hen appeared to be due to the same complex of hormonal activities that brings about the development of secondary sexual characteristics.

Riddle and Dotti (1936) were the first to

demonstrate that injections of estrogenic substances produce an increase in the serum calcium of pigeons and fowls; injections of androgen were without effect. Several other investigators (Common, Rutledge and Bolton, 1947; Common, Rutledge and Hale, 1948) have found that treatment with exogenous estrogen increased the level of serum calcium of immature pullets and that this increase was related directly to the dosage of estrogen administered. Concurrent administration of estrogen and androgen to immature pullets has been found to increase serum calcium to levels equal to those attained by estrogen treatment alone (Common et al., 1947; Common, Rutledge and Hale, 1948). Other observations (Common, Maw and Jowsey, 1953; Common, McCully, Steppler and Maw, 1956) have suggested that the hypercalcemia evoked by estrogen might be enhanced by concurrent administration of androgen, but subsequent ad hoc experiments in this laboratory have failed to substantiate this suggestion (Common, 1962).

The increase in blood calcium due to the onset of egg production or to estrogenization, which has been confirmed many times (vide Gardner and Pfeiffer, 1943; Lorenz, 1954; Riddle, 1942), occurs mainly in the non-ultrafiltrable or bound fraction, there being little change in the ultrafiltrable fraction (Benjamin and

Hess, 1933; Correll and Hughes, 1933; Laskowski, 1933; Heller, Paul and Thompson, 1934; Taylor and Russell, 1935; McDonald and Riddle, 1945; Riddle and McDonald, 1945; Schjeide and Urist, 1956; Urist, Schjeide and McLean, 1958). The nature of the material responsible for the binding of the increased calcium will be discussed in sections 1.2. and 1.3.

1.1.2. Lipids.

The relationship of high blood lipid to egg laying was first established by Lawrence and Riddle (1916) and by Warner (1916) and also in a more extensive study by Warner and Edmond (1917). These workers showed that blood lipid levels are increased in the hen during ovarian activity. This early work has been repeatedly confirmed and extended to show that there is a definite correlation between ovarian activity and the blood lipid level (Heller *et al.*, 1934; Roepke and Hughes, 1935; Greenberg, Larson, Pearson and Burmester, 1936; Lorenz, Entenman and Chaikoff, 1938; Chaikoff, Lorenz and Entenman, 1941; Riddle, 1942; Walker, Taylor and Russell, 1951).

Laskowski (1938) showed that gonadotropic hormones

(pituitary or mare serum but not those of urinary origin) were effective in increasing the plasma phospholipid level in non-laying hens. Striking increases in blood lipid levels were observed by Entenman, Lorenz and Chaikoff (1938) following the administration of crude gonadotrophin to immature pullets, and by Lorenz, Chaikoff and Entenman (1938) who administered 'crude estrin' to immature birds of both sexes. Estrin increased cholesterol, phospholipids and neutral fat, the most pronounced effect being the increase of neutral fat. Subsequent observations by several investigators have shown that lipemia could also be obtained in the male and immature female bird and in castrates by the injection of a variety of estrogenic compounds (Zondek and Marx, 1939 a, b; Landauer, Pfeiffer, Gardner and Man, 1939; Entenman, Lorenz and Chaikoff, 1940; Flock and Bollman, 1942; Fleischmann and Fried, 1945; Common et al., 1947; Common, Rutledge and Hale, 1948; Common, Bolton and Rutledge, 1948; Baum and Meyer, 1956; Hillyard, Entenman and Chaikoff, 1956; Schjeide and Urist, 1956; Urist et al., 1958).

All lipid fractions (cholesterol, phospholipids and neutral fat) share in the response of blood lipid

to exogenous estrogen. According to Flock and Bollman (1942) the response of the phospholipid fraction includes increases of cephalins as well as choline-containing phospholipids. Increases of lecithins make up most of the latter; increases of sphingomyelins were small and inconsistent (Ranney, Entenman and Chaikoff, 1949). McKinley, Oliver, Maw and Common (1953) and McKinley (1954) observed that the zones on paper electropherograms of avian sera which stained for lipid with Oil Red O also gave a positive plasmalogen reaction (see section 1.4.).

1.1.3. Total Protein.

In his review of the earlier work on proteins of chicken blood, Howe (1925) stated that many of the values reported were unreliable because of faulty techniques. Moreover, many of the studies had been based upon small numbers of birds and no details had been given concerning the age, sex, state of reproductive activity or other conditions. Some of the later methods used have included the kjeldahl method, the calculation of total protein from refractive index or specific gravity, and the biuret method.

It has been shown repeatedly that the plasma protein

level is higher in the estrogenized or laying bird than in the male or immature female (Benjamin and Hess, 1933; Rochlina, 1934a; Mandel, Clavert and Mandel, 1947 a, b; Brandt, Clegg and Andrews, 1951; Sturkie and Newman, 1951 a, b; McKinley, Maw, Oliver and Common, 1954; Common, Maw, Layne and McCully, 1958). However, there has been some disagreement as regards the influence of reproductive activity on the level of plasma proteins in normal female birds. Sturkie and Newman (1951a) found no significant difference in the total plasma protein level as between laying and non-laying hens. They also reported that the plasma proteins of non-laying birds appeared to be higher than that of some of the laying birds in the study, although the value was no higher than that reported for some groups which were laying. Hence, the observations of Sturkie and Newman (1951a) suggested the possibility that the level of plasma protein in the female may be dependent on the stage of the reproductive cycle. This was also suggested by the results of Greenberg et al. (1936) who reported the following total serum protein values for the pullet:

0.5 to 2.0 months before laying	3.8%
1 to 2 days before laying	5.0%
after 2 weeks laying	4.2%

McDonald and Riddle (1945) reported similar results for total plasma protein in the pigeon. The results reported by the two latter groups afforded some support for the view that endogenous estrogen secretion in the fowl is sufficient to increase the level of serum protein at some stages of reproductive activity.

Vanstone, Maw and Common (1955) followed the serum protein levels of a single group of fowl from the embryonic stage to full laying activity. Their results, in general, confirmed those of Greenberg et al. (1936) and those of McDonald and Riddle (1945). Vanstone et al. (1955) found that about six weeks before laying the first egg, the total serum protein level began to increase from about 4.0 gm. per 100 ml. and that it attained a value of about 5.4 gm. per 100 ml. in the week before the first oviposition. It is during this puberal period that serum calcium, phosphorus and lipid begin to increase, phosphoprotein to appear in the serum and the oviduct to hypertrophy. All these latter effects, as well as the increase of total serum protein, may be attributed to endogenous estrogen activity. The PP fraction and 'fraction 8' (see section 1.4.) appeared in the serum at this stage in amounts sufficient to account for most of the increase in total serum protein. With the

beginning of laying, the total serum protein level fell rather sharply to about 3.4 gm. per 100 ml. The level gradually recovered as laying progressed and after eleven weeks it had regained the pre-puberal level of 4.0 gm. per 100 ml. These authors presented their results as evidence for the view that endogenous estrogen activity is sufficiently great to increase the total serum protein level in the puberal pullet in the week before the first oviposition. The absence of such a positive effect in the laying period may be related to the speed with which serum protein is being depleted for egg formation, rather than to the absence of any influence of endogenous estrogen on serum protein.

1.2. Recognition and Isolation of Serum Phosphoproteins

Lawrence and Riddle (1916) appear to have made the first observations on non-lipid blood phosphorus. Their results indicated that an alcohol-insoluble phosphorus fraction increased with the increase of ovarian activity in the fowl. The results of Heller, Hunter and Thompson (1932) for the partition of phosphorus in the cells and the plasma of laying hens revealed a discrepancy between the total plasma phosphorus and the sum of lipid phosphorus, acid-soluble phosphorus and inorganic

phosphorus. However, no explanation was offered for this difference. The results of Heller et al. (1934) showed a similar discrepancy but this discrepancy was shown to appear first at the beginning of egg laying. The presence of a phosphoprotein fraction in the blood of laying hens was noted independently by Laskowski (1935a) and by Roepke and Hughes (1935). This discovery was the result of studies of the partition of the serum phosphorus of the fowl. These investigators demonstrated that the total phosphorus of the serum of either males or non-laying hens was practically equal to the sum of the lipid and acid-soluble phosphorus, whereas in sera from laying hens the total phosphorus was much greater than the sum of the lipid and acid-soluble phosphorus.

Laskowski (1935a) and Roepke and Hughes (1935) regarded the discrepancy between total phosphorus and acid-soluble plus lipid phosphorus in laying hen sera as being entirely due to phosphoprotein phosphorus. However, Mandel, Clavert and Bieth (1947) and Mandel and Mandel (1948) demonstrated the presence of a relatively small amount of nucleic acid in avian sera. This has subsequently been confirmed for estrogenized pullets by Common, Chapman and Maw (1951). Hence, nucleic acid phosphorus will be included in values

reported for phosphoprotein phosphorus. Roepke and Hughes (1935) found that the protein phosphorus content of laying hens sera averaged 10.52 mgm. phosphorus per 100 ml. This phosphoprotein was found to have properties similar to those of vitellin. These workers also stated that serological tests gave some indication that the vitellin of egg yolk was similar to the phosphoprotein of hen serum.

Laskowski (1935b) subsequently isolated this phosphoprotein material in fairly pure form and suggested for it the name 'serum vitellin'. He found that it contained 15.1% nitrogen and 0.91% phosphorus, values which were in good agreement with those found for 'vitellin' prepared from egg yolk by similar methods. The serological properties of serum vitellin were found to be closely related to those of egg yolk vitellin (Roepke and Bushnell, 1936). Laskowski (1936) examined the blood of several other species of vertebrates (excluding mammals) for the presence of a vitellin fraction. He found in nearly every case the appearance or an enormous increase of a serum vitellin fraction in association with the maturing of the eggs. Plasma protein phosphorus was absent from the blood of birds and reptiles during reproductive rest or else was present in only trace amounts;

during egg formation the level increased to values ranging from 9 mgm. per 100 ml. to 23 mgm. per 100 ml. Recently, Urist and Schjeide (1961) have shown that massive doses of estrogen cause the elevation of phosphoprotein phosphorus in certain oviparous vertebrates including species of Amphibia, Aves, Reptilia, and Teleostei, but not in Elasmobranchii or Mammalia.

Laskowski (1938) found that a single injection of gonadotrophin led to the appearance of serum vitellin in the plasma of non-laying hens. The other phosphorus fractions of the plasma, viz., total lipid and acid-soluble phosphorus increased at the same time. Laskowski (1938) suggested that the appearance of serum vitellin in the physiologically normal laying bird is also due to gonadotrophic hormone, since the changes in the individual phosphorus fractions were of the same relative order, although different in actual amounts. He also suggested that the chief difference was that the serum vitellin normally goes to form the developing ova, which was not possible under his experimental conditions.

The increase of serum phosphoprotein by treatment with estrogen was demonstrated in chicks by Fleischmann and Fried (1945) and in pigeons by McDonald and Riddle (1945). Fleischmann and Fried (1945) found that the

injection of estradiol dipropionate into normal immature chicks of both sexes resulted in large concomitant increases of serum calcium, inorganic phosphorus, lipid phosphorus and protein phosphorus. McDonald and Riddle (1945) demonstrated that estradiol benzoate, but not androstenedione, was effective in increasing serum calcium and phosphoprotein in mature and immature normal, parathyroidectomized, or hypophysectomized pigeons of both sexes. These workers also reported that serum phosphoprotein had a much higher calcium-binding capacity (8 to 9 times higher) than the usual serum proteins. Riddle (1942) correlated the appearance of serum vitellin with the elevated calcium level that is observed in the serum of laying birds.

Observations of the increase of serum phosphoproteins by estrogen treatment have been made by several other workers (Mandel et al., 1947; Common et al., 1947; Common, Rutledge and Hale, 1948; Common, Bolton and Rutledge, 1948; Hosoda, Kaneko, Mogi and Abe, 1955 a, b). These observations are consistent with the theory that estrogen produced in the ovary under the stimulus of the anterior pituitary in turn stimulates the production of serum phosphoprotein and perhaps other serum proteins.

McKinley et al. (1953) and McKinley, Maw, Oliver

and Common (1954) diluted the serum of estrogenized pullets with an equal volume of water. The precipitate which formed was found to contain the phosphoprotein fraction of the serum.

Schjeide and Urist (1956, 1960) and Urist *et al.* (1958) have reported the ultracentrifugal isolation of fractions designated as X_1-phosphoprotein and X_2-lipoglycoprotein from the serum of the laying hen or estrogenized rooster. They reported that all the calcium in the estrogenized rooster serum was found bound to the X_1-phosphoprotein fraction. A more complete treatment of these results will be given in section 1.3. These workers also found that component X_1 plus variable amounts of component X_2 could be coprecipitated in uncontaminated form simply by dilution of the serum with distilled water.

Abe, Tanabe, Kaneko, Mogi and Hosoda (1958) diluted the sera of laying hens 1:20 with an antisera. A precipitate formed on dilution and was removed by centrifugation. This precipitate was found to contain approximately 95% of the radioactivity of the P^{32} in the protein phosphorus fraction.

McIndoe (1957, 1959a) found that a lipophosphoprotein

complex (PLP) could be precipitated from the plasma of laying hens by dilution with 9 volumes of water. This material was not present in the plasma of non-laying hens or of cocks. This complex is soluble in water and is reprecipitated by bringing the sodium chloride concentration to 0.15 M. PLP is insoluble in sodium chloride solutions between 0.01 M and 0.04 M. The yield of PLP was about 2 gm. per 100 ml. of plasma. This fraction appears in the plasma about a week before the first ovulation and disappears within a few days when hens go off lay. This is in agreement with the results of Hosoda et al. (1955b), who showed immunologically that 'serum vitellin' disappears within 5 days of the last egg being laid. PLP has a N:P ratio (by weight) of about 3.45. It contains about 20% protein, containing about 14.3% nitrogen and 0.75% phosphorus, and 80% lipid of which 25% is phospholipid and 4% is cholesterol, the remainder is triglyceride. About 80% of the plasma lipid is associated with the PLP and this indicates that almost all the plasma triglyceride is in the PLP fraction.

Ultracentrifugal studies (McIndoe, 1959a) indicated that the PLP fraction was not homogeneous. Two ultracentrifugal components separated, one being

a light lipoprotein and the other a protein or a dense lipoprotein.

Only 10% of the plasma-protein nitrogen and approximately one-third of the protein phosphorus was contained in the PLP fraction. The latter observation was in agreement with that of Roepke and Bushnell (1936), who found that the material precipitated by dilution contained less than half the total plasma-protein phosphorus. McIndoe (1959a) suggested that the high protein phosphorus content of the supernatant from PLP preparation during the pre-ovulatory period without an increase in protein nitrogen may be explained if the protein phosphorus is present as phosvitin, which contains about 10% phosphorus. Support for this suggestion was given by Mok, Martin and Common (1961), who showed that agar electrophoresis of the supernatant from dilution precipitation of lipophosphoprotein gave a phosvitin zone just in advance of the albumin zone.

Common and Mok (1959) and Mok *et al.* (1961) have prepared a phosvitin preparation from the serum of estrogenized laying hens. The method was as follows: serum was adjusted to pH 5.3 with 0.1 N hydrochloric acid solution and water was added until the serum was diluted two-fold. After standing the precipitate of

crude lipovitellin complex was removed by centrifugation, redissolved in 1% sodium chloride solution, and reprecipitated by ten-fold dilution with distilled water. The precipitate was taken up in 0.4 M magnesium sulphate solution and sufficient of the solid salt was added to provide a final concentration of 0.4 M magnesium sulphate. The resultant clear solution was diluted with an equal volume of distilled water. The precipitate of crude lipophosphoprotein was separated, dissolved in 0.4 M magnesium sulphate solution, and precipitated by dilution as before. The partially purified lipophosphoprotein material was dissolved in 10% sodium chloride solution and centrifuged. The lipid material which separated at the surface was removed by filtration. The filtrate was diluted with twice its volume of water and then dialyzed against several changes of acetate buffer pH 4.0, μ = 0.1. The resultant precipitate was considered to be crude lipovitellin and was removed by centrifugation. The supernatant was dialyzed against distilled water to remove the acetate buffer. The clear solution was then lyophilized and the fluffy pure white product (phosvitin) was dried to constant weight *in vacuo* over concentrated sulphuric acid.

This phosvitin preparation contained 12.3% nitrogen,

10.1% phosphorus, 31.0% serine, and 0.56% tryptophan. Serum phosvitin and a yolk phosvitin preparation contained the same N-terminal groups. Alanine was the major N-terminal amino acid with minor amounts of N-terminal lysine. The sedimentation constant $S^{o}_{20,w} = 3.7$, weight-average molecular weight 4.2×10^4 (Archibald method), and number-average molecular weight 4.0×10^4 (calculated from the tryptophan content) of the serum phosvitin were similar to those of phosvitin prepared from egg yolk by the same preparative procedure. The two preparations behaved similarly when subjected to paper electrophoresis.

Mok (1960) and Mok *et al*. (1961) also isolated a presumptive phosvitin from the serum of laying hens. The yield of the material from the serum of laying hens was so small that it was impractical to do any analyses on it, but it was possible to show by paper electrophoresis that this material displayed the same mobility and staining properties as egg yolk phosvitin on similar electropherograms. It was suggested that the low yield of phosvitin might be due to a relatively low level of phosphoprotein in the sera of hens in full lay, for the level of lipophosphoprotein in the sera of hens in full lay may be much lower than it is just before laying begins (Vanstone *et al*., 1955).

1.3. Ultracentrifugal and Free Electrophoretic Studies of Avian Serum Proteins

Sanders, Huddleson and Schaible (1944) used veronal buffer pH 8.6, μ = 0.1, to study the plasma and serum of normal and leucotic chickens. The normal sera showed the following peaks: albumin, α_1-, α_2-, β-, and γ-globulin. A protein component closely associated with γ-globulin appeared in the serum of the leucotic chicken and was designated as the L-component.

Deutsch and Goodloe (1945) found only slight differences in the electrophoretic patterns of adult male and female chicken plasmas when the buffer was veronal-citrate pH 8.6. Both male and female plasma contained a pre-albumin component but Deutsch and Goodloe (1945) did not state whether the female was ovulating or not. In both plasmas turbidity moved with the α_2- and β-components. These workers also stated that the α_1-globulin appeared as a more distinct entity in the male than in the female plasma.

Chick embryo plasma was studied electrophoretically and ultracentrifugally by Moore, Shen and Alexander (1945) who used a phosphate-sodium chloride buffer pH 7.4 and showed that embryonic plasma differed widely

from adult chicken plasma. The electrophoretic analysis showed rapid changes in the plasma during embryonic development. Their component 3, which had the mobility of β-globulin in adult serum, was predominant in the 8-day to 11-day embryos but gradually receded. Component 1 or albumin was predominant in all embryos after 13 or more days of incubation and appeared to reach a maximum at about the time of hatching. Ultracentrifugation of unfractionated chick plasma revealed only a single component. These workers analyzed several samples of serum from embryos of various ages for non-diffusible carbohydrate, and found several times as much per unit nitrogen as was found in adult chicken serum. Electrophoretically separated components contained carbohydrate, but considerably more carbohydrate was associated with the faster fraction.

Moore (1945) employed the same phosphate buffer and showed that there was a marked difference between the serum patterns of the male and female chicken. Sera from laying hens contained a pre-albumin component; an α-globulin (component 3) was absent and the two slowest moving components were greatly increased. Moore (1948) showed that the pronounced sex difference in the sera does not appear in the chicken until maturity. Exogenous

estrogen administered to cocks and capons resulted in electrophoretic patterns that were similar to those of laying hens. The pre-albumin component in the sera of the laying hen or the estrogenized bird and component 2 in the serum of the normal cock were removed by preliminary ether extraction. The β-γ-globulin region was reduced in both sexes by the ether extraction. It was also shown that administration of androgen to the laying hen quickly altered its serum so that the electrophoretic pattern of the serum was almost identical to the typical pattern of cock serum.

Marshall and Deutsch (1950) studied the blood serum proteins of the developing chick embryo by electrophoresis in veronal buffer pH 8.6, $\mu = 0.1$. They found that all the major electrophoretic components which were present in the electrophoretic pattern of the adult chicken serum were present in the serum from 10-day embryos, but there was a gradual change in the relative amounts of the components as the embryo approached maturity. However, the patterns of the embryo serum showed components with mobilities greater than that of serum albumin. These three components were found to be rich in phospholipid. It was also stated that there was little, if any, phosphoprotein in embryonic serum.

The pre-albumin components disappeared from the serum shortly after the chick had hatched. Marshall and Deutsch (1950) showed that embryonic serum was ultracentrifugally heterogeneous. These results do not agree with the results of Moore et al. (1945), who did not find any pre-albumin components and found embryonic serum to be ultracentrifugally homogeneous.

In their investigation of the effects of age and degree of maturity on the serum proteins of the chicken, Brandt, et al. (1951) subjected the sera to free electrophoresis in a borate buffer pH 8.6. In general, the electrophoretic analyses confirmed the results obtained by salt fractionation, i.e., as the birds matured, the total protein and the γ -globulin fraction of the serum increased. A pre-albumin fraction was present in the sera of laying hens but was not present in the sera of immature birds, of adult roosters or of non-laying hens, and the electrophoretic pattern of laying hen sera was greatly enlarged in the β-γ-globulin region. These authors suggested that there is a relationship between the pre-albumin component and egg formation because of its sudden appearance in hens' sera just starting egg production and its presence in laying hens' sera but not in the sera of the other groups studied. The

pre-albumin component consisted of about 5.1% of the total serum proteins of the laying hen. Component 3 (α_1-globulin) was absent from the serum of all but one of the laying hens examined.

Clegg, Sanford, Hein, Andrews, Hughes and Mueller (1951) demonstrated that the electrophoretic patterns of the serum proteins of diethylstilbestrol-treated cockerels, 8 weeks and 2 years of age, were similar to those for the serum proteins of laying hens. They concluded that treatment with diethylstilbestrol would cause changes, normally associated with egg formation, to occur in the serum proteins of male birds.

Hein and Clegg (1952) and Clegg and Hein (1953a) measured the distribution of phosphorus in the electrophoretic components of sera from chickens previously treated with P^{32} by an electrophoretic-radiochemical technique. These workers modified the Tiselius electrophoretic apparatus so that the radioactivity associated with each protein fraction could be measured as the protein fronts migrated past the observation point, and at the same time the electrophoretic pattern, the protein mobilities and the concentration of the separated protein fractions could be determined. Hein and Clegg (1952) stated that more than 95% of the P^{32}

in the serum of 8 week-old chickens was present as phosphoprotein and less than 5% existed as ionic phosphate. The above proportions were determined by means of dialysis of whole serum; hence, the serum phospholipids would be present and the phospholipid phosphorus would be labelled, presumably, by the P^{32}. In the control 8 week-old chickens, P^{32} was observed in the albumin, α_1-, α_2-, β-, and γ-globulin regions and the highest radioactivity was associated with the albumin. Clegg and Hein (1953a) found that the P^{32} of the albumin and α-globulins remained unchanged after treatment of similar birds with diethylstilbestrol. However, the estrogen-induced pre-albumin fraction displayed much radioactivity and the radioactivity of the enhanced β-γ-globulin region was greatly increased. These results of P^{32} distribution have been confirmed recently by Clegg, Ericson and Misra (1960). The P^{32} distribution in the laying hen serum was similar to that found in the estrogenized bird except that the level of P^{32} was much lower.

Heim and Schechtman (1954) used borate buffer pH 8.6, μ = 0.112 for electrophoretic examination of chicken serum during development. Components with mobilities exceeding that of albumin were found in embryonic and laying hen sera, but not in the sera of males or immature females. The albumin component maintained a relatively

constant percentage composition during embryonic development and in young chicks. The α-globulins declined and β-globulin increased during embryonic development. The γ-globulin component increased toward the end of the development period and over several days after hatching.

The lack of a direct relationship between the calcium-binding capacity of chicken serum and the albumin concentration was demonstrated in two ways by Clegg and Hein (1953b). In the first experiment chicks were injected with varying levels of diethylstilbestrol. The non-diffusible calcium was determined and electrophoretic analyses in borate buffer were carried out on the sera. The level of non-diffusible calcium increased from approximately 10 mgm. per 100 ml. in the non-estrogenized bird to approximately 60 mgm. per 100 ml. in the heavily estrogenized bird, with no appreciable change in the serum albumin level. Estrogenization evoked the appearance of the pre-albumin component and the usual increase in the β-γ-globulin fraction, the increases depending on the dosage of diethylstilbestrol. In their second experiment, these investigators utilized the fact that the albumin content of the chick's serum increases with age. Sera were obtained from chicks at 0, 1, 2, 3 and 4 weeks of age and analyzed as in the first experiment.

The level of serum albumin increased from 1.7 gm. per 100 ml. in the newly-hatched chick to 2.5 gm. per 100 ml. at 3 weeks and then decreased to 2.3 gm. per 100 ml. at 4 weeks. At the same time the levels of total and non-diffusible calcium remained relatively constant.

When calcium was added to the borate buffer, the electrophoretic mobilities of the components of normal cockerel serum were reduced slightly but neither the number nor the order of the components was altered (Ericson, Clegg and Hein, 1955; Clegg, Ericson, Hein, McFarland and Leonard, 1956). The mobilities of the albumin, α_1-, α_2-, β-, and γ-globulin fractions of sera from estrogenized cockerels were reduced slightly, but the mobility of the pre-albumin fraction was drastically reduced by the addition of calcium, so that at a concentration of approximately 14 mgm. calcium percent the pre-albumin component migrated in the already enlarged β-γ-globulin region. Clegg et al. (1956) used P^{32}-labelled sera from estrogenized birds to show that added calcium combined with the pre-albumin fraction and caused it to move to the β-γ-globulin region. These same workers also added Ca^{45} to estrogenized serum and demonstrated that the Ca^{45} radio-activity was concentrated in the β-γ-globulin region.

Clegg et al. (1956) concluded that it was the pre-albumin component with its mobility reduced which combined with the added calcium, and that this fraction may be responsible for the extra non-diffusible calcium present in the blood sera of laying hens and diethylstilbestrol-treated cockerels.

Schjeide and Deutsch (1953) quantitatively separated chick embryo serum proteins of low specific gravity (i.e., those lipoproteins which float in a sodium chloride solution of density 1.063) from proteins of higher specific gravities. The sedimentation rates and relative concentrations of the dense protein components were then determined. In sera from 8-day to 15-day embryos, two components sedimenting at approximately 2.0 S and 5.3 S at infinite dilution were observed. At 18 days, an additional fast component of 18.4 S was detected, and in the newly-hatched chick two additional relatively fast-sedimenting components were seen (8.1 S and 15.5 S). Four of these components prevailed in the 7-week chick, the 18.4 S component having disappeared. In the laying hen, all five components were observed, including a small amount of 17.9 S component, and, in addition, a component was seen which sedimented at a rate intermediate between those of the 8.1 S and 14.7 S components.

The 5.0 to 5.3 S component was present in greatest concentration at all stages with the exception of sera from 8-day and 21-day embryos. In general, the 5.0 to 5.3 S component increased relative to the 2.0 S species during development, although there was also an absolute increase in the 2.0 S material. Lipoprotein was demonstrated in the 2.0 S material at all stages, but lipoprotein was not present in more rapidly sedimenting classes. The dense serum fractions of the 21-day embryo were analyzed electrophoretically. Components migrating at rates comparable to albumin, α_1-, α_2-, β_2-, and γ-globulin were observed. Only a trace of γ-globulin-like protein was present, but the other globulin-like and albumin-like proteins were present as 54% and 46% respectively, of the total. The 5.0 S component was tentatively identified as consisting mainly of albumin and the 2.0 S component "to be a composite of proteins migrating in the regions of the α_1-, α_2-, and β_2-globulins". (The authors do not make it clear whether this refers to globulins of avian sera or of human serum).

Schjeide (1954) separated two ultracentrifugal lipoprotein fractions (Lipoproteins I and II) from the plasma of chickens. In embryos, 18 days and younger,

most of the plasma lipid was present in the lighter fraction, Lipoprotein I. However, by the time of hatching, the lipid was almost equally divided between the lighter and denser lipoproteins. Up to three days before hatching, Lipoprotein II was found to be present in relatively small but increasing amounts. However, between 18 days of incubation and the time of hatching, Lipoprotein II almost tripled in amount and remained in relatively high concentration in the plasma of all older birds. An appreciable amount of carbohydrate appeared to be present in this fraction.

Schjeide (1955) divided the plasma lipoproteins of chicken embryos in various stages of development into three groups on the basis of their flotation in aqueous media of different densities. The lightest lipoproteins were characterized by a predominance of triglyceride up until nearly the time of hatching, when sterol ester became the major lipid component of this group. The median (sic) density group of lipoproteins contained phospholipid as the major component early in development, but a shift to sterol ester occurred after the fifteenth day of incubation. The lipids of the more dense lipoproteins were composed of over 50% phospholipid, but increasing amounts of sterol ester appeared

as development proceeded.

Hillyard et al. (1956) have shown that subcutaneous implants of diethylstilbestrol increased the total serum lipoprotein level of cockerels. By means of differential centrifugation of whole serum, these investigators obtained five lipoprotein fractions from the sera of estrogenized cockerels, viz., A1 or $D^{1.006}$, A2 or $D_{1.006}^{1.063}$, B or $D_{1.063}^{1.107}$, C or $D_{1.107}^{1.220}$ and D or $D_{1.220}$ in order of increasing densities at 20°C. Fraction A1 consisted of low density lipoproteins and included the chylomicrons; fraction A2 consisted of the low density lipoproteins which have been classified as β-lipoproteins in electrophoretic studies; fractions B and C were the α_2- and α_1-lipoproteins, respectively (Hillyard, Entenman, Feinberg and Chaikoff, 1955); fraction D was a residue in the procedure and included albumin and globulins in addition to lipoprotein. No further separation of lipoprotein from fraction D could be achieved by flotation at densities higher than 1.2 because these higher densities approach those of the non-lipid-containing proteins. Estrogenization increased fractions A1 and A2, and these increases accounted for practically all of the increase of the total lipoprotein level. Estrogen treatment had no effect on the level of fraction B, but

it decreased the level of fraction C and increased that of fraction D.

Schjeide and Urist (1956) utilized ultracentrifugal techniques in a study of the serum lipoproteins of the heavily estrogenized rooster. Beginning within 24 hours of estrogen administration and rising nearly to a maximum within 5 days, there was a large increase in serum chylomicrons ($D^{1.003}$) and β-lipoproteins ($D_{1.003}^{1.063}$), a nearly complete disappearance of α-lipoproteins ($D_{1.063}^{1.21}$), and the appearance of at least two new components, X_1 and X_2. The concentration of albumin was practically the same in injected and control birds. Component X_1 appeared to be a phosphoprotein containing little, if any, lipid and with a sedimentation rate of S 7.5 whereas component X_2 was a very dense lipoprotein with a sedimentation rate of S 15.0. Analyses for calcium in chylomicrons, in β-lipoproteins, and in α-lipoproteins revealed that these bound less than 5% of the total calcium in the serum. Similar analyses indicated that albumin and the denser globulins also bound comparatively small amounts of serum calcium in estrogenized roosters. The elevated serum calcium appeared to be mainly associated with the X_1 component and these investigators could account for nearly all the calcium in the serum of estrogenized birds as complexed with X_1, albumin and lipoprotein.

Component X_1 plus variable amounts of component X_2 could be coprecipitated in uncontaminated form by dilution of the serum with distilled water. Most of the protein-bound calcium was released during the process. The precipitate could be redissolved in 1% sodium chloride solution or in a lesser amount of calcium chloride. When X_1 was separated from X_2 by ultracentrifugation, it was soluble in distilled water. However, when X_1 was combined with X_2 in distilled water, a precipitate resulted which was composed of the two types of 'molecules'.

Urist *et al.* (1958) subsequently found that the X_1 component was the protein chiefly responsible for binding the large increase of serum calcium in estrogenized roosters. X_1 differed from other proteins in the serum in that it contained a high percentage of protein phosphorus. These workers stated that the X_1 and X_2 proteins are kept in solution by the electrolytes of the serum. They are easily precipitated from the serum by dilution, and are presumably separated from the normal protein constituents of the serum by this process. They may then be redissolved by the addition of salts of calcium and/or sodium. However, the proteins in this reconstituted solution are stated to have lost their electrophoretic mobility as well as a large part of their

ability to combine with calcium. These authors stated, therefore, that the X_1 and X_2 proteins were denatured in these respects.

The serine content of serum was markedly increased following estrogen treatment. Urist et al. (1958) have suggested that serine is the characteristic amino acid and that phosphoserine is the predominant building stone of egg yolk protein and perhaps, therefore, of the X_1 component of serum as well.

Schjeide and Urist (1959) reported molecular weights of 154,000 for serum X_1 and 400,000 for serum X_2. These values were twice those reported for the X_1 and X_2 components of egg yolk. These authors suggested that, if the egg yolk proteins are derived from their serum counterparts, then it would appear that they may be split exactly in half shortly before, during or after transfer from the circulation to the egg cell.

In a later communication, Schjeide and Urist (1960) state that they have used the term X_1-phosphoprotein in place of phosvitin and the term X_2-lipoglycoprotein in place of lipovitellin. They also state that the amount of calcium associated with serum X_1-phosphoprotein in the native state is sufficient to neutralize the

negative charges of the phosphoserine in the molecule so that no complex is formed, and that phosphorus and calcium analyses of the X_2 component (lipovitellin) of serum show it to be uncontaminated with X_1. Again, they state that no significant amount of phosphoprotein was actually complexed with lipovitellin. However, Cook (1961) has pointed out that a sample containing 5% phosvitin is not likely to be detected by sedimentation although this amount is highly significant analytically.

Schjeide, Binz and Ragan (1960) have shown that injection of estrogen into embryonated eggs resulted in the production of only one of the three serum fractions that characteristically result from estrogen treatment of the adult fowl and this in relatively low quantity in the younger embryos. The protein was light lipoprotein (S_f 10-100). X_1-phosphoprotein and X_2-lipoglycoprotein appeared in the serum for the first time when estrogen was injected into the yolk sacs of newly-hatched chicks.

Tanabe, Abe, Kaneko and Hosoda (1961) centrifuged the serum of laying hens at 40,000 r.p.m. for 8 hours. They found that almost all the protein phosphorus was sedimented in their fraction LSIII (the bottom sediment).

1.4. Zone Electrophoretic Studies of Avian Serum Proteins

Filter paper electrophoresis was first applied to the separation of proteins by Cremer and Tiselius (1950), by Durrum (1950) and by Turba and Enenkel (1950). So far as the author is aware, Common, McKinley and Maw (1953) were the first to apply the paper electrophoretic technique to the study of avian serum proteins. The technique and apparatus used were essentially the same as that described by Durrum (1950) as modified by Flynn and de Mayo (1951), except that the papers remained in a horizontal position rather than draped over a rod so as to elevate the central portion of the paper strip. These studies were based on the use of veronal buffer pH 8.6, $\mu = 0.05$. Five serum protein fractions were distinguished in sera from the male and the sexually immature pullet. These fractions were designated in order of decreasing mobility as follows: albumin, α_1-, α_2-, β-, and γ-globulin.

On examining sera from estrogenized immature pullets, a broad new zone was apparent. This zone was well ahead of the β-globulin region although its trailing edge showed "tailing" that tended to stretch back into the leading edge of the β-globulin zone, the γ-globulin zone was still clearly visible. The new band was

associated with heavy lipid staining and it was designated 'presumptive lipovitellin' (PLV). The α_1-globulin zone could not be detected in some of the electropherograms of the serum of the estrogenized pullets.

It should be noted here that Common, McKinley and Maw (1953) and McKinley (1954) have shown that the serum proteins of androgen treated pullets, of sexually immature pullets, and of immature cockerels, all gave similar electrophoretic patterns. McKinley (1954) also showed that birds treated with estrogen plus androgen gave the same electrophoretic patterns as birds that were treated with estrogen only.

Attention was given to the possibility of resolving the PLV zone, or of altering its mobility so that its presence would not obscure the β-globulin zone. It was found that a buffer made up in the same proportions as the aqueous veronal, but with 20% v/v methanol instead of water, resulted in the separation of a new slow-moving fraction near the line of application.

McKinley *et al*. (1953) described more fully the use of the methanolic veronal buffer that resolved the PLV fraction into a 'presumptive phosphoprotein' (PP) fraction that moved in the region of the trailing edge

of the γ-globulin zone. At the same time it was noted that while the β-globulin zone was now distinct, the γ-globulin zone was greatly enhanced in amount. They also noted the peculiar double nature of the PP zone. The faster lipoprotein zone, which was also the wider of the two, was later designated 'fraction 8' by Vanstone et al. (1955).

Using the methanolic veronal buffer, McKinley et al. (1953) found it possible to describe the occurrence, in the sera of both immature pullets and estrogenized pullets, of a third α-globulin fraction, α_3-globulin. The mobilities, relative to that of albumin, of the α_1-, α_2-, α_3-, β-, and γ-globulins were found not to be appreciably altered by the use of the methanolic veronal buffer as compared with the aqueous veronal buffer, in spite of the marked effects on the behavior of the PLV constituents.

Studies based on the use of P^{32}-labelled sera showed that the PP zone and fraction 8 were both associated with phospholipid (McKinley et al., 1953). However, fraction 8 had a relatively low protein phosphorus content while the PP zone contained a relatively large proportion of the protein-bound P^{32}.

Staining for lipid with osmic acid (Common, McKinley and Maw, 1953) or with Oil Red O (McKinley et al., 1953) showed the presence of two major lipid staining zones on electropherograms of sera from males or immature pullets. The position of these lipid-staining regions, one at the line of application and the other associated with the α_1-globulin zone, were not altered when methanolic veronal buffer was substituted for the aqueous veronal buffer. Electropherograms, obtained with aqueous veronal buffer, of the sera of estrogenized or laying pullets displayed lipid staining at the point of application and in the PLV region. However, electropherograms of these sera obtained with methanolic veronal buffer, displayed lipid-staining at the line of application, in the PP zone and in the fraction 8 region. A remarkable feature of these electropherograms was the absence of a lipid-staining zone from the α_1-globulin region despite the intense lipemia of the sera.

McKinley et al. (1953) and McKinley (1954) showed the presence of plasmalogen on electropherograms of avian sera in the same regions that stained with the lipid stains. They also found a large increase of the plasmalogen reaction in sera from estrogenized or laying birds. McKinley, Grice and Connell (1955) subsequently

applied the plasmalogen reaction to the problem of detecting whether or not poultry carcasses were from birds that had been treated with estrogen within a short time of slaughter.

McKinley et al. (1953) and McKinley, Maw, Oliver and Common (1954) diluted the serum of estrogenized pullets with an equal volume of water. The precipitate was removed by centrifugation, suspended in 1% sodium chloride solution and then subjected to electrophoresis in methanolic veronal buffer. The electropherogram displayed a double band in a position closely approximating that of the double PP band of the electropherograms of sera of estrogenized pullets or of laying hens. Mok et al. (1961) subjected the supernatant from dilution precipitation of lipophosphoprotein to zone electrophoresis in agar and were able to show a phosvitin zone which moved just in advance of the albumin zone. This result showed that only part of the phosvitin was thrown down with the crude lipophosphoprotein.

Vanstone et al. (1955) used the methanolic veronal buffer technique in their study of the changes in the serum proteins from the stage of the 14-day embryo to that of full reproductive activity. The electropherograms of the sera from males or immature pullets showed

the usual six zones (an albumin plus five globulin zones). The sera from the blood samples drawn during the week before the first oviposition and from laying birds gave, in addition, the PP and fraction 8 zones described by McKinley *et al.* (1953) for estrogenized or laying birds. An α_1-globulin zone was distinguishable in about half of the samples but the associated lipid zone was no longer perceptible.

The protein fractions from the sera of embryos differed markedly from that of adult males and females. The serum from 14-day embryos contained a pre-albumin fraction, albumin, α_2-, and β-globulin zones. Similar zones occurred on the electropherograms of serum from the 18-day embryo but the proportions of material in the various zones had changed. The serum of the day-old chick showed the appearance of the α_1- and γ-globulin zones, but the pre-albumin zone was by then very faint. By 7 days post-hatching, all the fractions characteristic of the prepuberal female or male had appeared.

Staining the electropherograms of embryo sera for lipid with Sudan Black B showed positive staining in the pre-albumin region and at the line of application. The chick sera showed lipid-staining in the usual regions on

electropherograms of immature birds, i.e., in the α_1-globulin region and at the line of application.

Recently, Yesair, Goldstein and Daniel (1959), using a veronal buffer pH 8.6, $\mu = 0.075$ and the apparatus of Durrum (1950), investigated the serum of the chick embryo. For 12-, 15-, 18-, and 21-day chick embryos they described five paper-electrophoretic serum protein fractions. These included a pre-albumin fraction for all samples. They also showed that fraction 2 (albumin) and fraction 5 (β-globulins) increased during development with a concomitant decrease in fractions 3 and 4 (α-globulins). Yesair et al. (1959) separated the so-called α- and β-lipoproteins by a two-hour electrophoretic run and then stained the papers with Oil Red O. This procedure did not permit the association of positive lipid-staining with the corresponding protein zones.

Perk, Perek, Loebl and Allalouf (1960) studied the effects of androgen, of estrogen and of estrogen plus androgen on the serum proteins of the young chicken by paper electrophoresis. They used a Michaelis buffer (sodium barbiturate, sodium acetate and hydrochloric acid) pH 8.6, $\mu = 0.1$ in a horizontal type chamber. The electrophoretic patterns displayed six bands as follows, in order of decreasing mobility: albumin, α_1-, α_2-, β_1-, β_3-, and

γ-globulin. Androgen-treated chickens showed the same electrophoretic patterns as did the controls, a result previously noted by Common, McKinley and Maw (1953) and by McKinley (1954). The estrogen treated birds showed increases in the α_2- and β_3-globulin fractions. However, Perk et al. (1960) did not report the staining of any of their electropherograms for lipid, nor did they report the appearance of any lipophosphoprotein components in their estrogenized sera that would correspond to the PP and fraction 8 zones reported by McKinley et al. (1953) and by Vanstone et al. (1955).

Sherman and Hull (1961) examined the ammonium sulphate and water dialysis fractions of chicken serum by paper electrophoresis. These workers used the horizontal open-strip method with veronal buffer pH 8.6, μ = 0.05. Five zones were distinguished on electropherograms of unfractionated serum, viz., albumin, two α-globulins, β-, and γ-globulin, in order of decreasing mobility. The euglobulin, or precipitate at 33.3% saturation of serum with ammonium sulphate, showed a diffuse band, which corresponded to the regions occupied by the β- and γ-globulins, rather than being composed of a single electrophoretic entity. The pseudoglobulin, or precipitate formed between 33.3% and 50% saturation,

demonstrated approximately equal amounts of the α- and β-globulins and albumin. The albumin, or supernatant at 50% ammonium sulphate saturation, revealed α- and β-globulins and albumin with the latter predominating. These electrophoretic results demonstrated that serum protein fractions prepared by ammonium sulphate precipitation are relatively heterogeneous.

It is of interest to note that paper electrophoretic techniques are being used to study the serum proteins of chicks affected with various diseases (vide Goldstein and Scott, 1956; Bieri and Pollard, 1959; Darcel and Klassen, 1960; Shelton and Olson, 1960; Sherman and Hull, 1960).

2. EXPERIMENTAL

2.1. Electrophoretic Techniques

Paper electrophoresis was done in a horizontal Matthew cell, model PC-103 (Matthew Laboratories, Yonkers, N.Y.) and in a ridgepole type cell, Spinco model R (Beckman Instruments Inc., Belmont, California).

(a) The Matthew cell was simplified in operation by supporting the paper on a sheet of pebbled "plexiglas" (size P4) instead of on the supporting racks supplied by the manufacturer (Sehon, Richter, Harter and Rose, 1956).

Whatman 3MM paper was cut into strips 7.0 in. wide by 19.5 in. long. One such strip was used for each electropherogram. A light pencil line was drawn across the strip 3.5 in. from the end which served as the cathodic end of the electropherogram. The strip was dipped in the required buffer and then put in the electrophoresis cell and allowed to equilibrate. After equilibration the serum sample was applied in a narrow transverse band across the strip on the pencil line at the rate of 400 µl. per 7.0 in. width of paper. The current density was maintained at 0.57 ma. per in. width of paper. The period of electrophoresis was usually 24 hours. The apparatus was kept throughout in a cold room maintained

at approximately 5°C.

(b) The Spinco-Durrum cell was used according to the instructions of the manufacturer without modification. Fifty-five microliters of serum was applied to each paper strip. (The standard strips of S & S #2043A paper were 1.2 in. in width). The current density was maintained at 0.60 ma. per in. width of paper. The period of electrophoresis was 18 hours. The apparatus was kept throughout in a cold room maintained at approximately 5°C.

2.2. Buffers

(a) Veronal buffer pH 8.6, μ = 0.05. Prepared by dissolving 1.84 gm. diethylbarbituric acid and 10.30 gm. sodium diethylbarbiturate in distilled water and diluting to 1.0 liter volume.

(b) Borate buffer pH 8.6 (Brandt et al., 1951). Prepared by dissolving 3.09 gm. boric acid, 3.73 gm. potassium chloride and 0.48 gm. sodium hydroxide in distilled water and diluting to 1.0 liter volume.

(c) Borate buffer pH 9.0 (Block, Durrum and Zweig, 1958). Prepared by dissolving 7.63 gm. sodium borate and 0.62 gm. boric acid in distilled water and diluting

to 1.0 liter volume.

(d) Veronal-citrate buffer pH 8.6 (Deutsch and Goodloe, 1945). Prepared by dissolving 9.21 gm. diethyl-barbituric acid and 2.25 gm. sodium citrate dihydrate in 1.0 liter distilled water and adjusting to pH 8.6 with sodium hydroxide.

2.3. Staining Methods

On completion of an electrophoretic run, each main strip was air-dried and then cut longitudinally into substrips which were stained for protein, or for lipid, or for ester-linked protein phosphorus (ELPP) as follows:

(a) Strips cut from the electropherograms for protein staining were left overnight in ethanol-ether (3:1 v/v) acidified with 5% trichloroacetic acid. Next morning the solvent was heated to boiling and then poured off. The strips were next refluxed for three successive one-half hour periods with ethanol-chloroform (1:1 v/v) containing 5% trichloroacetic acid. After the papers were air-dried, they were stained for 20 minutes in a saturated solution of Amidoschwarz-10B (Chromagesellschaft, Stuttgart, Germany) in acetone containing 10% by volume glacial acetic acid. The excess dye was removed by washing the strips with a solution of methanol (90 vols.), water

(10 vols.) and 1.0 N hydrochloric acid (1 vol.). The final rinse was in methanol. This staining procedure is essentially that of Kawerau (1954) except that he used samples of Naphthalene Black 12B that required purification, a procedure which was unnecessary with the Amidoschwarz-10B used in the present study.

(b) Untreated strips were stained for 16 hours in a saturated solution of Oil Red O in 60% ethanol as described by Durrum, Paul and Smith (1952).

(c) ELPP was stained on strips that had been freed from lipid by the method described above. The method for ELPP staining was either that of Hanes and Isherwood (1949), or that of Wade and Morgan (1953), or that of Harrap (1960). Of these three methods, the first was sensitive, but the papers were very fragile and the buff background of correctly-stained papers soon turned blue on exposure to air. The second method called for careful control of the pH of the papers. The colors were stable once the papers had dried, but in the course of drying, colorless zones indicative of the presence of phosphate tended to turn pink as was the background. The method of Harrap (1960) was found to be most generally satisfactory, but the blue zones indicative of ELPP became paler after the final treatment with α-benzoinoxime, and the stained strips were not usually suitable for photography.

2.4. Blood Sampling

Blood samples were drawn from the wing vein through a No. 18 needle and allowed to clot spontaneously. The serum was separated by centrifugation. Sera were stored at 5°C. for not longer than two days before electrophoresis. It was noted, however, that it was possible to obtain satisfactory electropherograms of samples of cock serum that had been kept for as long as two weeks at 5°C. Serum from estrogenized fowl may be kept for some days at 5°C. without alteration of the paper electrophoretic pattern.

2.5. Measurement of Radioactivity

Electrophoretic technique and buffers for the experiments with P^{32}-labelled sera were as described above. A substrip 0.75 in. wide was cut from the air-dried main electropherogram. The distribution of P^{32} on this strip was then measured by means of a Forro radiochromatogram scanner (Forro Scientific Co., Evanston, Illinois) coupled to an automatic recorder (Esterline-Angus Company, Inc., Indianapolis, Illinois). An adjoining strip was freed from lipid as described in section 2.3. and scanned to give the distribution of protein-bound P^{32}. The tracings were transferred to rectangular co-ordinates for

presentation as figures.

2.6. Analytical Methods

2.6.1. Serum Protein Nitrogen.

Protein nitrogen was determined by precipitation of the serum proteins with trichloroacetic acid and a subsequent microkjeldahl determination (A.O.A.C., 1955) on the precipitated material. The figure for protein, therefore, includes the lipid nitrogen of the lipoproteins.

2.6.2. Serum Calcium.

Serum calcium was determined on trichloroacetic acid filtrates of the serum by the method of Patton and Reeder (1956). Sucrose was added before titration, as suggested by Bond and Tucker (1954). The method was as follows: Five milliliters of serum was diluted with 10 ml. distilled water in a 25 ml. volumetric flask and 5 ml. 25% trichloroacetic acid was added slowly with shaking. The contents of the flask were made to volume, mixed, allowed to stand for at least one-half hour and finally filtered through Whatman No. 30 filter paper. Suitable aliquots of the filtrate were diluted to a volume of approximately 15 ml. To this solution was added 2 ml. 20% sucrose solution and

4 ml. 8 N potassium hydroxide solution. After mixing, the solution was allowed to stand for 3 to 5 minutes with occasional swirling, followed by the addition of approximately 30 mgm. potassium cyanide and approximately 30 mgm. hydroxylamine hydrochloride. The flask was swirled until the reagents dissolved; 'Cal Red' (Scientific Services Laboratories, Inc., Dallas, Texas) indicator was added and the solution was titrated with 0.005 N Complexone III* to the pure blue end point.

2.6.3. Serum Phosphorus.

Serum inorganic phosphorus, acid-soluble phosphorus, and lipid phosphorus were determined by the standard procedures described by Hawk, Oser and Summerson (1954), except that the determinations of phosphorus were done by the method of Martin and Doty (1949). Serum total protein phosphorus was estimated by the method described by Common et al. (1947).

2.7. Results and Discussion

2.7.1. Comparison of Sera from the Cock, from the Laying Hen, and from the Estrogenized Non-laying Hen by Use of the Matthew Electrophoresis Cell.

The sera were run in aqueous veronal buffer pH 8.6,

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* Complexone III is the proprietary name of the disodium salt of ethylenediaminetetraacetate made by B.Siegfried, Zofingen, Switzerland.

μ = 0.05. The results are exemplified in Figure 1. Good resolution of the usual six zones (albumin, α_1-, α_2-, α_3-, β-, and γ-globulin zones in order of decreasing mobility) was secured for the cock's serum (Figure 1A). The α_1-globulin was reasonably well distinguished. Lipid staining was present in a region slightly ahead of the α_1-globulin zone and at the line of application, as has been observed previously (Common, McKinley and Maw, 1953; McKinley et al., 1953; Vanstone et al., 1955).

The results for the laying hens' sera (Figure 1B) were satisfactory. The albumin, α_2-, α_3-, β-, and γ-globulin zones were well defined, but an α_1-globulin zone was not clearly resolved nor was there any lipid-staining in this region. The latter observation confirms previous similar observations (McKinley et al., 1953; Vanstone et al., 1955) on sera of laying hens and estrogenized pullets. The most striking result was the satisfactory resolution of two distinct lipoprotein zones in aqueous veronal buffer. These zones were designated P-1 and P-2 (Figure 1, B and C) in order of decreasing mobility. These zones both showed positive staining for lipid. "Channelling" and confusion of zones in the β-γ-globulin region had hampered earlier studies (Common, McKinley and Maw, 1953) based on the use of aqueous veronal buffer, but these troubles were overcome by the technique used in

FIGURE 1

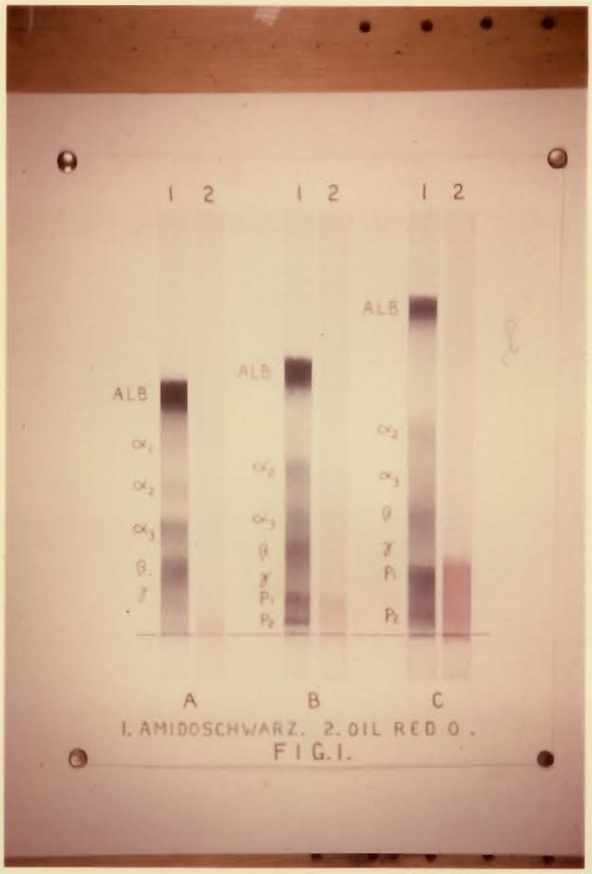
Electrophoretic Separation in Aqueous Veronal Buffer of Protein Zones of Sera from the Cock, from the Laying Hen, and from the Estrogenized Non-laying Hen.

Stains: (1) Amidoschwarz.
(2) Oil Red O.

(A) Cock's serum: Matthew cell with veronal buffer pH 8.6, μ = 0.05. Sample applied at cathodic end of paper strip.

(B) Laying hen's serum: conditions as for strip A.

(C) Estrogenized non-laying hen's serum: conditions as for strip A.



1. AMIDOSCHWARZ. 2. OIL RED O.

FIG. 1.

the present studies.

The results with the sera of the estrogenized birds (Figure 1C), in which the serum phosphoprotein level was high, were equally satisfactory. These electropherograms showed zones similar to those displayed by the electropherograms of laying hens' sera. The staining for both protein and lipid in the P-1 and P-2 zones was increased. It is possible that the P-2 zone corresponds with the PP zone of Vanstone et al. (1955) and that the P-1 zone corresponds with the fraction 8 described by the same authors. The PP zone and fraction 8 were resolved in methanolic veronal buffer. Zones P-1 and P-2 also appear to correspond with the two major peaks described and designated lipovitellenin and lipovitellin by Evans and Bandemer (1957) for electropherograms of egg yolk run in aqueous veronal buffer. The relation of the two lipoprotein zones P-1 and P-2 to the X_1 and X_2 components, of laying hen and estrogenized rooster serum, distinguished in the ultracentrifuge by Schjeide and Urist (1956, 1960) and Urist et al. (1958) was obscure at this stage of the study. However, Urist and Schjeide (1961) had presented a figure of a paper electropherogram of their estrogenized rooster serum in one paper. They did not state the type of apparatus or the buffer used for the electrophoresis.

The X_1-X_2 components were shown to move in a single broad zone just ahead of their β-globulin region. It may be remarked here that the X_1-X_2 region on this electropherogram bears a considerable resemblance to the PLV zone of electropherograms of estrogenized pullet serum run in aqueous veronal buffer and described by Common, McKinley and Maw (1953).

The foregoing electropherograms did not provide any evidence of the presence of phosvitin, although phosvitin has recently been isolated from the serum of the laying hen and of the estrogenized laying hen (Common and Mok, 1959; Mok, 1960; Mok et al., 1961).

The major conclusion from the foregoing experiments was that two lipoprotein components (zones P-1 and P-2) can be distinguished on electropherograms of sera from laying or estrogenized birds by direct paper electrophoresis in aqueous veronal buffer in a cell of the Matthew type.

2.7.2. Effect of Position of Application of Sample on Resolution and a Comparison of Results Obtained with the Matthew Cell and with the Spinco-Durrum Cell.

Cock's serum was applied to paper strips in the

Matthew cell (a) in the normal position near the cathodic end (Figure 2D) and (b) midway between the cathodic and anodic ends (Figure 2E). A sample of the same serum was run simultaneously in the Spinco-Durrum cell by the technique ordinarily used with this cell (Figure 2F). Aqueous veronal buffer pH 8.6, μ = 0.05 was used in all electrophoretic runs.

Strip D showed resolution of the usual six protein zones. The resolution of the β- and γ-globulin fractions was less distinct on strip E than on strip D. The results with strip F (Spinco-Durrum cell) showed well-defined zones for albumin, α_1-, α_2-, and α_3-globulins, but the resolution of the β- and γ-globulins was relatively poor.

Similar comparisons were made for samples of serum from laying hens (Figure 2, G,H,I). Strips H and I showed that the resolution of β- and γ-globulins and zones P-1 and P-2 was extremely poor when electrophoresis was done in the Spinco-Durrum cell or in the Matthew cell with application of the sample midway between the electrodes.

The above results show that the resolutions by the Spinco-Durrum technique or by the Matthew cell technique with application of the sample midway between the electrodes were inferior to that secured by use of the

FIGURE 2

Comparison of Electrophoretic Results Obtained with the Matthew Cell and with the Spinco-Durrum Cell.

Stains: (1) Amidoschwarz.
(2) Oil Red O.

(D) Cock's serum: Matthew cell with veronal buffer pH 8.6, $\mu = 0.05$. Sample applied at cathodic end of paper strip.

(E) Cock's serum: conditions as for strip D but sample applied midway between cathodic and anodic ends of paper strip.

(F) Cock's serum: Spinco-Durrum cell with veronal buffer pH 8.6, $\mu = 0.05$. Sample applied in normal position for Spinco-Durrum operation.

(G,H,I) Laying hen's serum: conditions as for strips D, E and F respectively.

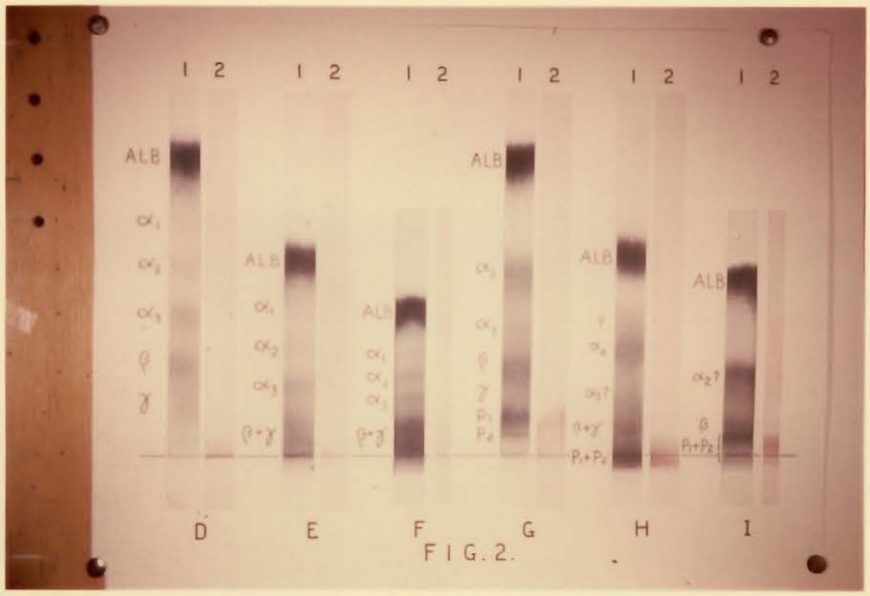

1 2
1 2
1 2
1 2
1 2
1 2
ALB
α1
α2
α3
β
γ
ALB
α1
α2
α3
β+γ
ALB
α1
α2
α3
β+γ
ALB
α2
α3
β
γ
Pr
Pa
α+γ
P+Pa
ALB
γ
α2
α1?
α+γ
P+Pa
ALB
α2?
β
P+Px
D
E
F
G
H
I

FIG. 2.

Matthew cell with application of the sample near the cathodic end of the paper strip.

Accordingly, the Matthew cell technique with application of sample near the cathodic end of the paper strip was adopted for all the electropherograms of this study that are discussed below.

It may be mentioned that Narain, Lyman, Deyoe and Couch (1961) have also commented briefly on poor resolution of chick serum proteins when subjected to paper electrophoresis by the Spinco-Durrum procedure.

2.7.3. Paper Electrophoretic Demonstration of Phosvitin in Crude Serum 'Lipophosphoprotein' Complex.

Serum from heavily estrogenized birds was diluted ten-fold with distilled water. The precipitate of the crude lipovitellin complex was separated by centrifugation and was redissolved in 0.15 M sodium chloride solution. This solution was then treated with sufficient ammonium oxalate to precipitate all the calcium. The precipitated calcium oxalate was removed next morning by centrifugation and the supernatant was subjected to paper electrophoresis in veronal buffer pH 8.6. The electropherograms showed (a) no indication of albumin or of α_1-, α_2-, or α_3-globulins

and (b) the presence of a lipoprotein zone corresponding in position with zone P-1 and a broad protein zone in advance thereof. These latter two fractions may be related to the two ultracentrifugal fractions of the serum lipophosphoprotein complex mentioned by McIndoe (1959a). In addition, there was a very faintly-staining protein zone which had a mobility, under the given conditions, greater than that of serum albumin and the same as that of a sample of egg yolk phosvitin (kindly furnished by Miss Chi-Ching Mok) that was run concurrently. A similar result, i.e., a phosvitin zone, a broad protein zone and a zone corresponding in position with zone P-1, was obtained if the calcium was complexed by means of added Complexone. However, if the solution of the serum lipophosphoprotein complex was run in veronal buffer without pretreatment to remove the calcium, it was impossible to detect a phosvitin zone with any degree of certainty. At this stage, however, it had not yet been found possible to demonstrate the presence of phosvitin in the serum of the fowl by paper electrophoresis of untreated serum.

The major conclusion from this experiment was that paper electrophoresis of a solution of crude lipophosphoprotein complex (obtained by dilution precipitation of estrogenized serum, solution in 0.15 M sodium chloride

solution and subsequent removal of calcium) yields a lipoprotein zone, a second wider lipid-free zone, and a very faintly-staining phosvitin zone.

2.7.4. Staining Behavior of Phosvitin with Amidoschwarz-10B.

Paper electropherograms of egg yolk phosvitin preparations were run in veronal buffer pH 8.6. The paper strips were processed in the usual manner. It was observed that phosvitin zones stained with Amidoschwarz in a fashion that is characteristic of phosvitin. The dye tended to wash out from stained phosvitin zones rather readily whenever background staining was being removed. Partially washed papers showed relatively strong protein-staining of the phosvitin zones. As the washing was continued, however, the dye tended to be washed out from the phosvitin zones. By the time the washing was complete, only a relatively weak protein stain remained in the phosvitin zone. It was observed also that phosvitin zones on paper strips that were not put through the usual lipid extraction procedure stained very faintly when compared to phosvitin zones on paper strips that had been lipid extracted.

It was observed also that as the Amidoschwarz dye solution aged, the dye solution became more acid due to evaporation of the acetone solvent and that the final

phosvitin stain became more distinct, while the hue tended toward a mauve color rather than the characteristic blue of the dye. Further investigation of this point revealed that the intensity of staining of the phosvitin zone increased as the proportion of acetic acid in the solvent was increased. It is also of interest to note that phosvitin did not give a positive stain with either Azocarmine B or with Bromophenol Blue.

2.7.5. Paper Electrophoresis of Protein-bound Phosphorus of Fowl's Serum as Shown by Means of ELPP Staining Reactions.

2.7.5.1. Introduction.

A phosvitin zone was distinguishable on paper electropherograms of crude 'lipophosphoprotein' obtained from the serum of estrogenized birds (see section 2.7.3.). Comparable presumptive phosvitin zones could not be distinguished on similar electropherograms of whole serum from laying or estrogenized fowl, although by this time phosvitin had been prepared from such sera (Common and Mok, 1959; Mok, 1960; Mok et al., 1961). The mobility of phosvitin by itself is greater than that of serum albumin in veronal buffer pH 8.6. These observations suggested that the phosvitin fraction of serum might be

associated with the lipoprotein fraction in ways not easily broken in the course of paper electrophoresis in veronal buffer. This prompted a study of the possibility of resolving a phosvitin zone on paper electropherograms of whole serum by use of veronal-citrate buffer, by pretreatment of the serum with ethylenediaminetetraacetate to chelate calcium, or by removal of the calcium by oxalate. The effects of borate buffers were also studied because Brandt et al. (1951), Clegg et al. (1951) and Clegg and Hein (1953a) have reported the presence of a component (Clegg's component 1) in sera of laying hens or estrogenized fowl which moved ahead of the albumin zone (Clegg's component 2) in free electrophoresis in borate buffer pH 8.6.

2.7.5.2. Reactivity of Phosvitin with Reagents for ELPP.

Electropherograms of phosvitin prepared from laying hen's and estrogenized hen's serum and from egg yolk were run in veronal buffer pH 8.6. The phosvitin zones stained for protein with Amidoschwarz in the way that is characteristic of phosvitin, i.e., the dye tended to wash out from the stained zones rather readily whenever background staining was being removed. The phosvitin zones gave positive reactions for ELPP with each of the three methods used, viz., that of Hanes and Isherwood

(1949), that of Wade and Morgan (1953) and that of Harrap (1960).

Similar experiments in which egg lecithin (Nutritional Biochemicals) or phosphatidyl serine (Nutritional Biochemicals) were subjected to electrophoresis showed that both these lipid materials also gave a positive reaction for ELPP. For this reason, all electropherograms were thoroughly extracted to remove phospholipid (see section 2.3.) before the phosphate reaction was applied. Such extraction was found to remove reactive phospholipid.

2.7.5.3. Paper Electrophoresis of Sera from Estrogenized Pullets in Different Buffer Systems.

Two pullets aged 12 weeks were each given 1.0 mgm. estradiol benzoate ('Progynon-B', Schering, Ltd., Montreal) per day for 5 successive days. Blood samples were withdrawn and the serum separated. The serum calcium level was 34.2 mgm. per 100 ml. for one pullet and 31.9 mgm. per 100 ml. for the other.

Electropherograms of these sera were run in the following buffers:

(A) Veronal pH 8.6.

(B) Borate pH 8.6.

(C) Borate pH 9.0.

(D) Veronal-citrate pH 8.6.

Representative results are presented in Figure 3.

The protein resolution in veronal buffer (Figure 3A) conformed in detail with resolutions described previously (sections 2.7.1. and 2.7.2.). Two lipoprotein zones (P-1 and P-2) were resolved and showed the usual dense staining for lipid. Faint staining for lipid was present also in regions corresponding to the α_2- and α_3-globulins. P-1 and P-2 zones gave strong staining reactions for ELPP but no reaction for ELPP was perceptible elsewhere.

Resolution of protein zones in borate buffer pH 8.6 (Figure 3B) was relatively poor. Only an albumin zone, a general globulin zone (α) and a single lipoprotein zone (L) were distinguishable. The lipoprotein zone stained heavily for lipid and faint diffuse staining for lipid extended from this zone as far as the trailing edge of the albumin zone. A reaction for ELPP occurred in a zone (PT) just ahead of the maximum of the albumin zone, but no reaction for ELPP was observed in the lipoprotein zone. The position of this ELPP zone suggested that it might be due to a component similar to Clegg's component 1 (Brandt et al., 1951; Clegg et al., 1951; Clegg and Hein, 1953a). The separation of this PT zone from albumin

FIGURE 3

Electropherograms of Sera of Estrogenized Pullets in Different Buffers.

Stains: (1) Amidoschwarz.
(2) Oil Red O.
(3) Reagent of Harrap (1960) for ester-linked protein phosphorus (ELPP).

(A) Veronal buffer pH 8.6.

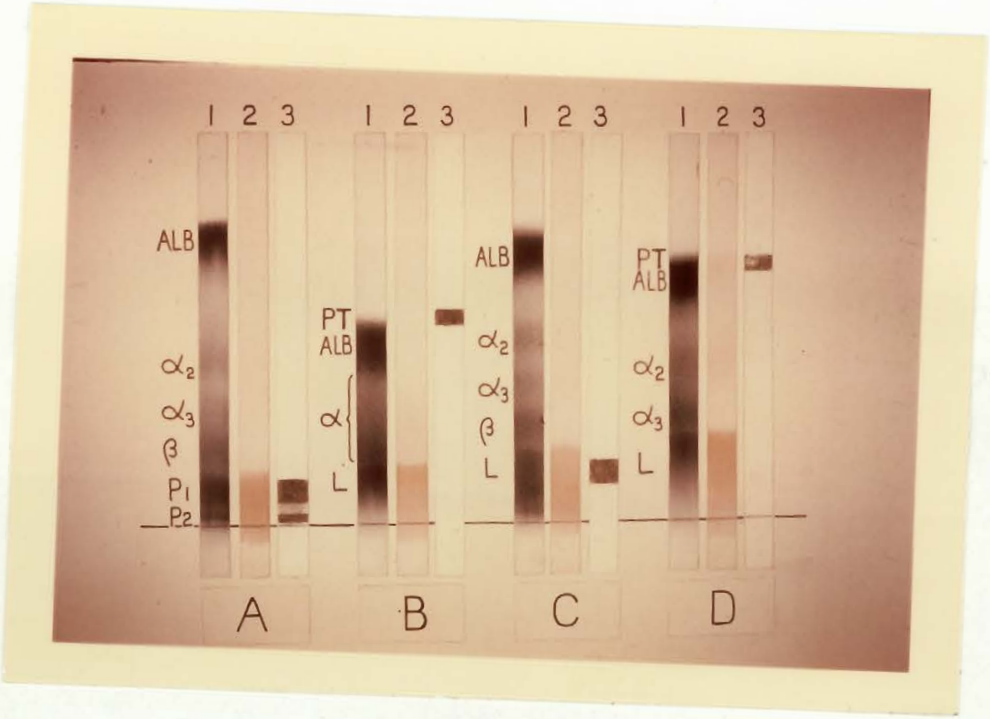
(B) Borate buffer pH 8.6.

(C) Borate buffer pH 9.0.

(D) Veronal-citrate buffer pH 8.6.

Key to zones: PT = phosvitin; other protein zones designated as suggested in section 2.7.1.

Note: Colors of strips stained for ELPP were unsuitable for photography, and position of reaction has been indicated by heavy pencil shading.


1 2 3
1 2 3
1 2 3
1 2 3
ALB
α2
α3
β
P1
P2
PT
ALB
α
L
ALB
α2
α3
β
L
PT
ALB
α2
α3
L
A
B
C
D

was relatively slight, so that the two zones overlapped. The use of borate buffer pH 8.6 was not studied further because of the relatively poor resolution of the protein zones on the paper electropherograms.

Borate buffer pH 9.0 gave resolution of protein zones (Figure 3C) similar to that obtained with veronal buffer, with the exception that the lipoprotein zone (L) was not resolved into P-1 and P-2 zones. In addition to the heavy lipid staining of the single broad lipoprotein zone, there were faint zones of lipid staining corresponding to the α_2- and α_3-globulin zones. There was a reaction for ELPP in the lipoprotein zone, but an ELPP reaction could not be detected with certainty elsewhere. The latter result was unexpected in view of the results with borate buffer pH 8.6, but it was confirmed in later work (see section 2.7.6.).

Veronal-citrate buffer pH 8.6 (Figure 3D) gave clear resolution of an albumin zone and of two globulin zones. Only a single broad lipoprotein zone (L) was observed. Staining for lipid gave the usual dense staining in the lipoprotein zone and faint staining in zones corresponding with the α_2-globulin and the leading third of the albumin zone. A reaction for ELPP was not perceptible in the lipoprotein zone, but there was a well-defined

reaction for ELPP in a zone (PT) just ahead of the albumin peak. It is suggested that it was due to phosvitin liberated from its association with other proteins of the 'lipophosphoprotein' complex as a consequence of chelation of calcium by the citrate of the buffer. This presumptive phosvitin carried with it a small amount of lipid. It may be noted again that its position corresponded approximately with that taken by Clegg's component 1 in free electrophoresis of sera of hens or estrogenized fowl in borate buffer (Brandt et al., 1951; Clegg et al., 1951; Clegg and Hein, 1953a).

It is of interest to note that Brandt et al. (1951), in their free electrophoretic studies of laying hens' sera, demonstrated the presence of a pre-albumin component when the buffer was borate pH 8.6 or veronal-citrate. However, when veronal buffer was used a pre-albumin component was not observed. The foregoing paper electrophoretic studies showed similar results, i.e., borate buffer pH 8.6 and veronal-citrate buffer permitted resolution of an ELPP reactive pre-albumin component whereas a similar component could not be demonstrated when veronal buffer was used.

2.7.5.4. Effects of (a) Pretreatment of Serum with Ethylenediaminetetraacetate (as Complexone III) and of (b) Removal of Calcium by Precipitation with Oxalate.

If the appearance of a presumptive phosvitin (PT) zone in Figure 3, B and D were due to chelation of calcium, then somewhat similar results should follow pretreatment of the serum with Complexone or removal of the calcium by precipitation with oxalate. Samples of serum from the estrogenized pullets (section 2.7.5.3.) and from an untreated cock were treated with Complexone (5 mgm. Complexone III per ml. serum) and then subjected to paper electrophoresis as follows:

Strip	Serum	Buffer
E	Estrogenized pullet	Veronal pH 8.6.
F	Cock	Veronal pH 8.6.
G	Estrogenized pullet	Borate pH 9.0.
H	Cock	Borate pH 9.0.
I	Estrogenized pullet	Veronal-citrate pH 8.6.
J	Cock	Veronal-citrate pH 8.6.

The cock's serum was included in order to check possible effects of the Complexone and different buffers on the resolutions ordinarily obtained with sera of cocks or unestrogenized immature pullets. The results for these

Complexone-treated sera are exemplified in Figure 4.

Figure 4E shows that pretreatment of the estrogenized pullet's serum with Complexone followed by electrophoresis in veronal buffer gave the same results for staining of protein zones and of lipid zones as those obtained in the absence of Complexone, except that in some of the electrophoretic runs the resolution of distinct P-1 and P-2 zones was abolished. Staining for ELPP showed the presence of reactive material (PT) just ahead of the albumin zone in strip E. This zone was not observed in strip A (Figure 3) or in any runs in plain veronal buffer, i.e., without pretreatment of the serum with Complexone. The staining for ELPP in the lipoprotein region of strip E (Figure 4) was fainter and was not resolved between two zones as compared with strip A (Figure 3). These observations suggested that chelation of divalent ions by Complexone had an effect similar to that of inclusion of citrate in the buffer in so far as both measures resulted in the appearance of an ELPP zone just ahead of the albumin zone, reduction or disappearance of ELPP staining in the lipoprotein zone and a diminution or abolition of the resolution of two distinct lipoprotein zones, viz., P-1 and P-2.

Figure 4F shows the pattern obtained when cock's

FIGURE 4

Electropherograms of Sera of Estrogenized Pullets and of a Cockerel in Different Buffers with Pretreatment of the Sera with Complexone III.

Stains: (1) Amidoschwarz.
(2) Oil Red O.
(3) Reagent of Harrap (1960) for ester-linked protein phosphorus (ELPP).

(E) Estrogenized pullet serum, veronal buffer pH 8.6.

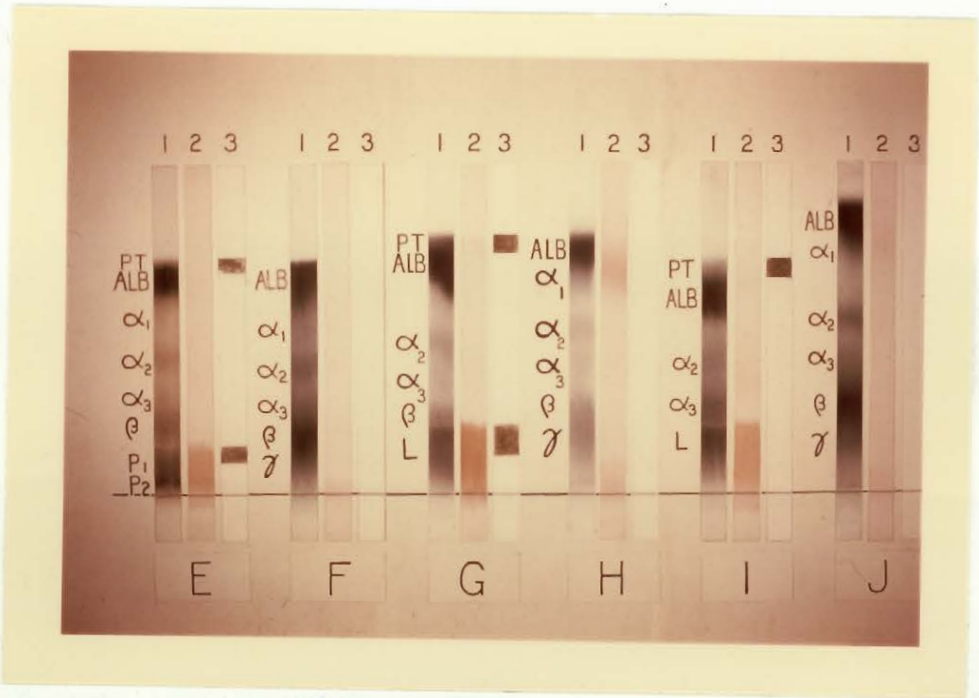
(F) Cockerel serum, veronal buffer pH 8.6.

(G) Estrogenized pullet serum, borate buffer pH 9.0.

(H) Cockerel serum, borate buffer pH 9.0.

(I) Estrogenized pullet serum, veronal-citrate buffer pH 8.6.

(J) Cockerel serum, veronal-citrate buffer pH 8.6.


1 2 3
PT
ALB
α1
α2
α3
β
P1
P2
E
ALB
α1
α2
α3
β
γ
F
PT
ALB
α2
α3
β
L
G
ALB
α1
α2
α3
β
γ
H
PT
ALB
α2
α3
L
I
ALB
α1
α2
α3
β
γ
J

serum was treated as was the estrogenized pullet's serum for Figure 4E. The resolution of protein zones was the same as that obtained without addition of Complexone (see Figures 1A and 2D), and no reaction for ELPP was perceptible. This showed that the presence of Complexone did not modify the electrophoretic behavior of the zones ordinarily resolved on electropherograms of sera from cocks or from immature pullets. Hence, the observed modifications of electrophoretic patterns by Complexone were a function of the presence of the estrogen-induced proteins.

Figure 4G shows that pretreatment of the estrogenized pullet's serum with Complexone followed by electrophoresis in borate buffer pH 9.0 gave essentially the same pattern as that shown in Figure 4E, except the resolution of distinct P-1 and P-2 zones was completely abolished and only one lipoprotein zone (L) was resolved.

Figure 4H shows the pattern of the cock's serum treated as was that of the estrogenized pullet's serum exemplified in Figure 4G. The results were substantially the same as for Figure 4F and a similar description applies.

Figure 4I shows the results for Complexone-treated estrogenized pullet's serum run in veronal-citrate buffer. The results for protein resolution, for lipid staining,

and for reaction for ELPP were substantially the same as those obtained by use of veronal-citrate buffer alone, as shown in Figure 3D. There was, however, fairly strong staining with Amidoschwarz just ahead of the albumin region and corresponding in position with the zone of reaction for ELPP. This region also stained faintly but distinctly for lipid. The protein staining reaction and presence of lipid indicated that the PT zone was not pure phosvitin but contained other material as impurities, because serum phosvitin preparations run alone stained only faintly for protein and did not show any lipid staining. Furthermore, it will be shown in Part II, section 2.8.1. of this thesis that phosvitin zones resolved from whole egg yolk suspensions stained only faintly for protein and did not show any lipid staining.

Figure 4J shows the results for cock's serum treated as was the estrogenized pullet's serum as shown in Figure 4I. The patterns were similar to those for Figure 4, F and H.

Sera from which calcium had been removed by precipitation with oxalate gave results so closely similar to those obtained for sera pretreated with Complexone that presentation of relevant figures has been deemed unnecessary.

The foregoing experiments demonstrated that the movement of ELPP on paper electropherograms of avian sera was greatly affected by the nature of the buffer, and especially by the presence or absence of chelating agents. Sera from which calcium had been removed gave results closely similar to those obtained when the divalent ions had been chelated with a chelating agent.

2.7.6. Movement of Total P^{32} and Protein-bound P^{32} on Paper Electropherograms of Avian Sera.

The observations described above in sections 2.7.5.3. and 2.7.5.4. showed that the movement of ELPP on electropherograms of avian sera may be greatly affected by the nature of the buffer, and especially by the presence or absence of chelating agents. There remained the possibility that minor amounts of protein-bound phosphorus might not have been detected by reason of insufficient sensitivity of the reaction for ELPP. Hein and Clegg (1952), Clegg and Hein (1953a) and Clegg *et al*. (1960) have shown that appreciable amounts of P^{32} were present throughout the region of protein migration in free electrophoresis of avian sera labelled with P^{32}. Their results showed that a large amount of the P^{32} was associated with the pre-albumin component

of estrogenized or laying hen sera. However, their observations did not distinguish protein-bound P^{32} from lipid-bound P^{32}. The above considerations prompted experiments based on the use of P^{32}-labelled sera, because it is relatively easy to distinguish protein-bound P^{32} from total P^{32} on paper electropherograms.

Two pullets were each given 1.0 mgm. estradiol benzoate ('Progynon-B', Schering, Ltd., Montreal) per day for 5 successive days. In addition, 0.5 millicuries P^{32}-labelled orthophosphate in sterile isotonic saline (Merck & Co., Montreal) was injected intramuscularly each morning. On the fifth day, and 6 hours after the last injection, the pullets were bled and serum samples were separated. A third pullet was similarly injected with P^{32}-labelled orthophosphate but was not estrogenized. This bird served as a control. Some relevant analytical results for the sera are set out in Table I. The estrogenized pullets showed increases of total serum protein, of serum calcium, of lipid phosphorus, and of protein phosphorus that are characteristic of birds treated with exogenous estrogen. Pullet No. 1 showed a somewhat greater response to the estrogen treatment than did pullet No. 2 but the difference was such as is frequently encountered in such experiments.

TABLE I

ANALYSES OF SERA FROM PULLETS TREATED WITH P^{32}-LABELLED ORTHOPHOSPHATE SO AS TO LABEL SERUM LIPID PHOSPHORUS AND SERUM PROTEIN PHOSPHORUS

	Control pullet (unestrogenized)	Pullet No. 1 (estrogenized)	Pullet No. 2 (estrogenized)
Total crude protein (N x 6.25) gm. per 100 ml.	3.40	5.04	4.76
Serum Ca, mgm. per 100 ml.	12.4	36.7	24.4
Serum P, mgm. per 100 ml.			
Inorganic	6.1	14.9	12.5
Acid-soluble (total)	6.8	16.1	13.6
Lipid	9.8	56.1	29.7
Protein	0.31	14.0	6.8

The following six electrophoretic runs were made on each serum.

(1) Veronal buffer pH 8.6.

(2) Serum pretreated with Complexone; veronal buffer pH 8.6.

(3) Borate buffer pH 9.0.

(4) Serum pretreated with Complexone; borate buffer pH 9.0.

(5) Veronal-citrate buffer pH 8.6.

(6) Serum pretreated with Complexone; veronal-citrate buffer pH 8.6.

The experimental results for pullet No. 1 are presented in Figures 5, 6 and 7.

The results for veronal buffer (Figure 5) agreed with those obtained by staining for ELPP (Figure 3A) in so far as there were two main protein-bound P^{32} zones near the line of application. However, there were indications of another P^{32} peak between the two major protein-bound P^{32} peaks. The scanning further revealed that minor amounts of protein-bound P^{32} were present on the electropherograms as far as a point just short of the albumin zone. All of the protein-bound P^{32} was accompanied by at least some phospholipid-P^{32}, but there was no clear association of minor P^{32} peaks with individual globulin zones. Pretreatment of the serum with Complexone did not appreciably modify the resolution of the protein zones as shown by staining, but it did result in a striking movement of most of the protein-bound P^{32} to a position that straddled the albumin zone. This main protein-bound P^{32} zone was accompanied by very little phospholipid-P^{32} or lipid-staining material. There was only a small single broad protein-bound P^{32} zone in the lipoprotein region. These observations as a whole confirmed and extended those made by staining for ELPP (Figure 4E).

The results with untreated serum and borate buffer

FIGURE 5

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Serum of Estrogenized Pullet No. 1 in Veronal Buffer pH 8.6 in Absence and Presence of Complexone III.

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

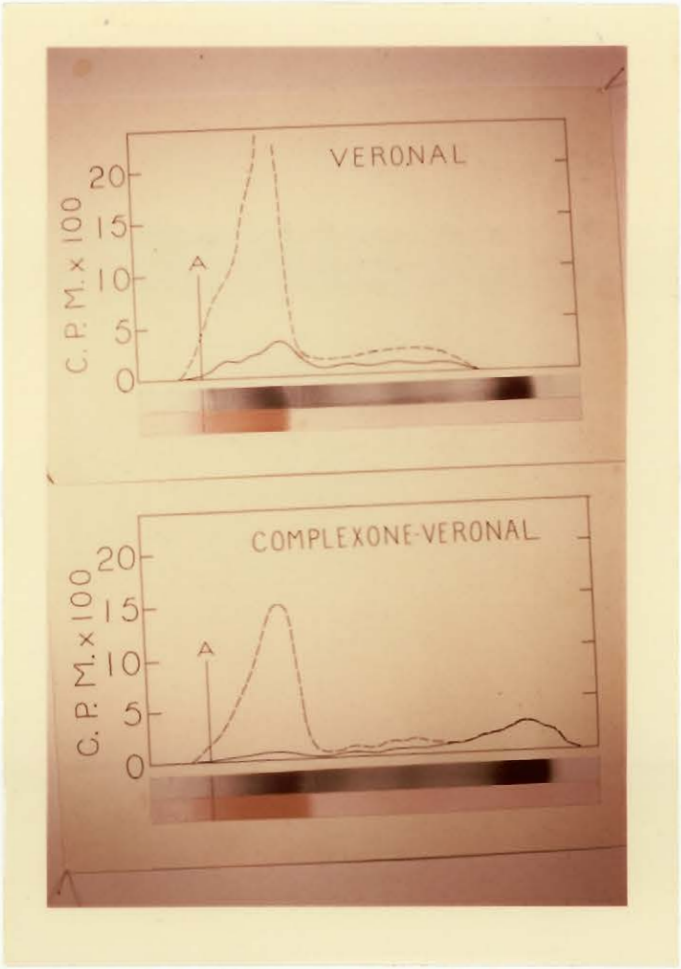

VERONAL
C.P.M. x 100
20
15
10
5
0
A
COMPLEXONE-VERONAL
C.P.M. x 100
20
15
10
5
0
A

FIGURE 6

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Serum of Estrogenized Pullet No. 1 in Borate Buffer pH 9.0 in Absence and Presence of Complexone III.

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

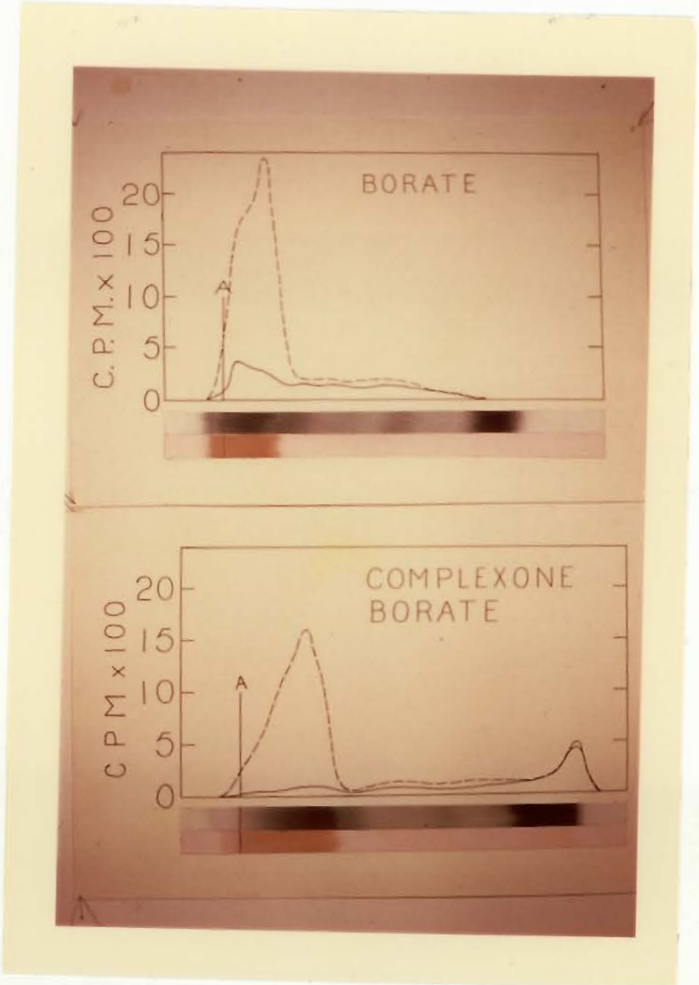

BORATE
C.P.M. x 100
20
15
10
5
0
A
COMPLEXONE
BORATE
CPM x 100
20
15
10
5
0
A

FIGURE 7

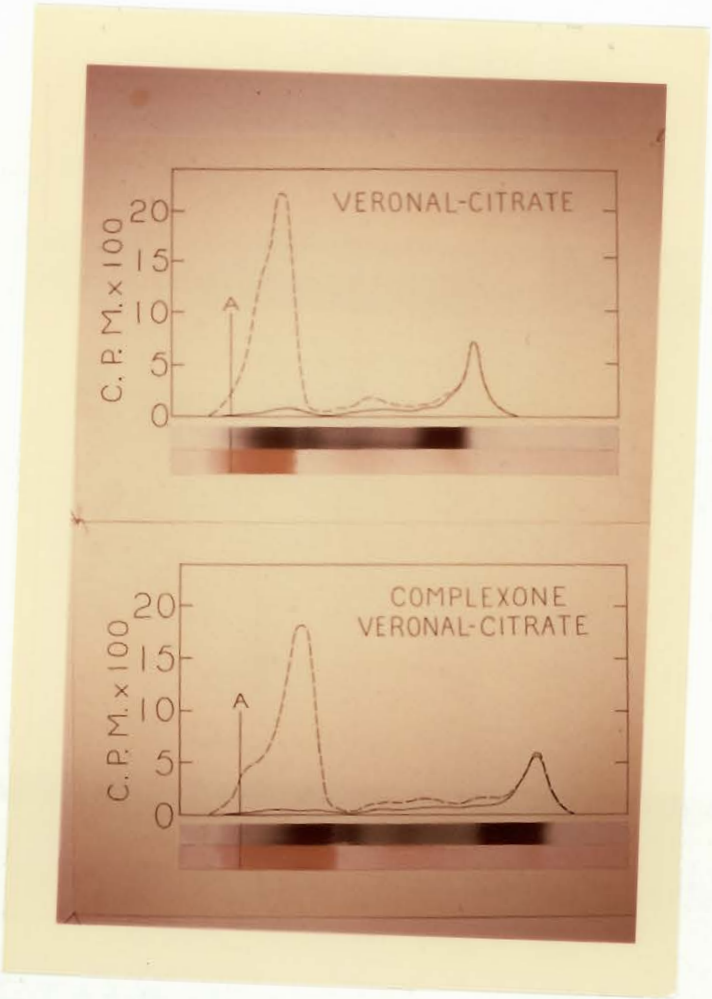
Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Serum of Estrogenized Pullet No. 1 in Veronal-citrate Buffer pH 8.6 in Absence and Presence of Complexone III.

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.


VERONAL-CITRATE
C.P.M. x 100
20
15
10
5
0
A
COMPLEXONE
VERONAL-CITRATE
C.P.M. x 100
20
15
10
5
0
A

pH 9.0 (Figure 6) resembled those obtained with untreated serum and veronal buffer, except that there were indications of protein-bound P^{32} and lipid-P^{32} associated with the α_2-globulin and α_3-globulin regions, and the distribution of protein-bound P^{32} in the lipoprotein regions was reversed, i.e., the larger peak was associated with the P-2 zone. Pretreatment of the serum with Complexone resulted in most of the protein-bound P^{32} moving forward to give a sharply defined peak (presumptive phosvitin) just ahead of the albumin zone. A slight amount of phospholipid-P^{32}, as well as faint lipid staining, was associated with this peak. There were two minor protein-bound P^{32} zones in the lipoprotein regions.

Electrophoresis of untreated serum in veronal-citrate buffer (Figure 7) afforded resolutions and movement of protein-bound P^{32} similar to those given by pretreatment with Complexone and use of borate buffer (Figure 6). The protein-bound P^{32} peak was ahead of the albumin peak and gave only very faint staining with Amidoschwarz. As mentioned above, the dyestuffs ordinarily used in staining proteins are rather easily washed out from phosvitin zones. The results with Amidoschwarz staining, therefore, agree with the suggestion that the major protein-bound P^{32} peak was due to phosvitin. When Complexone-treated serum was

used with veronal-citrate buffer (Figure 7), the results were broadly similar to those obtained without pretreatment with Complexone. There were indications of a small amount of phospholipid-P^{32} in association with the main protein-P^{32} peak, and there was fairly strong staining for protein in this zone, in contrast with the results in the absence of Complexone.

The behavior of the presumptive phosvitin, as shown in Figures 5, 6 and 7, and its relative freedom from phospholipid-P^{32} when it appeared along with, or just ahead of, the albumin region, suggest that Clegg's component 1 (Hein and Clegg, 1952; Clegg and Hein, 1953a; Clegg et al., 1960) may have represented phosvitin and that most of its P^{32} may have been due to protein-bound phosphorus.

Certain quantitative aspects of the results of this series of experiments are presented in Table II. Agreement between strips was satisfactory, having regard to the difficulty of ensuring even distribution of serum across the entire strip. Agreement was very good, however, so far as the ratios of protein-bound P^{32} to total P^{32} were concerned.

The results with pullet No. 2 confirmed those obtained with pullet No. 1, except in so far as the levels of lipid phosphorus and protein-bound phosphorus were lower (see

TABLE II

PROPORTIONS OF TOTAL P^{32} AND PROTEIN-BOUND P^{32} ON ELECTROPHEROGRAMS OF FOWL'S SERA IN DIFFERENT BUFFERS. RADIOACTIVITY EXPRESSED AS C.P.M. PER 0.75 IN. WIDTH STRIP EQUIVALENT TO APPROXIMATELY 43 µL. SERUM. SLIT WIDTH ON COUNTER 0.25 IN.

		Veronal buffer		Borate buffer pH 9.0		Veronal-citrate buffer		
		None*	Complexone*	None	Complexone	None	Complexone	Average
Control Pullet	Total radioactivity	1004	1408	1112	1153	1250	1033	1160
	Protein radioactivity	0	0	0	0	0	0	0
	Protein rad. x 100 / Total rad.	0	0	0	0	0	0	0
Pullet No. 1	Total radioactivity	15120	10494	11349	11075	10546	10795	11563
	Protein radioactivity	3168	2728	3230	2923	2435	2618	2850
	Protein rad. x 100 / Total rad.	21.0	26.0	28.5	26.4	23.1	24.3	24.6
Pullet No. 2	Total radioactivity	4931	4653	4966	4688	4822	4515	4763
	Protein radioactivity	1202	1102	1078	1117	1029	947	1079
	Protein rad. x 100 / Total rad.	24.4	23.7	21.7	23.8	21.3	21.0	22.7

* None = no pretreatment of serum. Complexone = pretreatment of serum with Complexone.

Tables I and II). The experimental results for pullet No. 2 are shown in Figures 8, 9 and 10.

The results for veronal buffer (Figure 8) agreed with those obtained for pullet No. 1 and with those obtained by staining for ELPP, in so far as there were two major protein-bound P^{32} zones near the line of application. However, the protein staining revealed only one lipoprotein region in the position of zone P-1. After pretreatment of the serum with Complexone, most of the protein-bound P^{32} moved to a region just behind the albumin zone. The mobility of the presumptive phosvitin of the serum of pullet No. 2 was not as great as it had been for pullet No. 1 after pretreatment of the serum with Complexone. The results for the serum of pullet No. 2 run in borate buffer pH 9.0 (Figure 9) were in agreement with those obtained for pullet No. 1. Similarly, agreement between the results for the sera of both pullets run in veronal-citrate buffer was observed, except that more phospholipid-P^{32} radioactivity was associated with the presumptive phosvitin zone of the serum of pullet No. 2 (Figure 10).

A point of the greatest interest which may be described at this stage was the occurrence of a very fast P^{32} zone ('zone A') that was observed on electropherograms

FIGURE 8

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Serum of Estrogenized Pullet No. 2 in Veronal Buffer pH 8.6 in Absence and Presence of Complexone III.

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

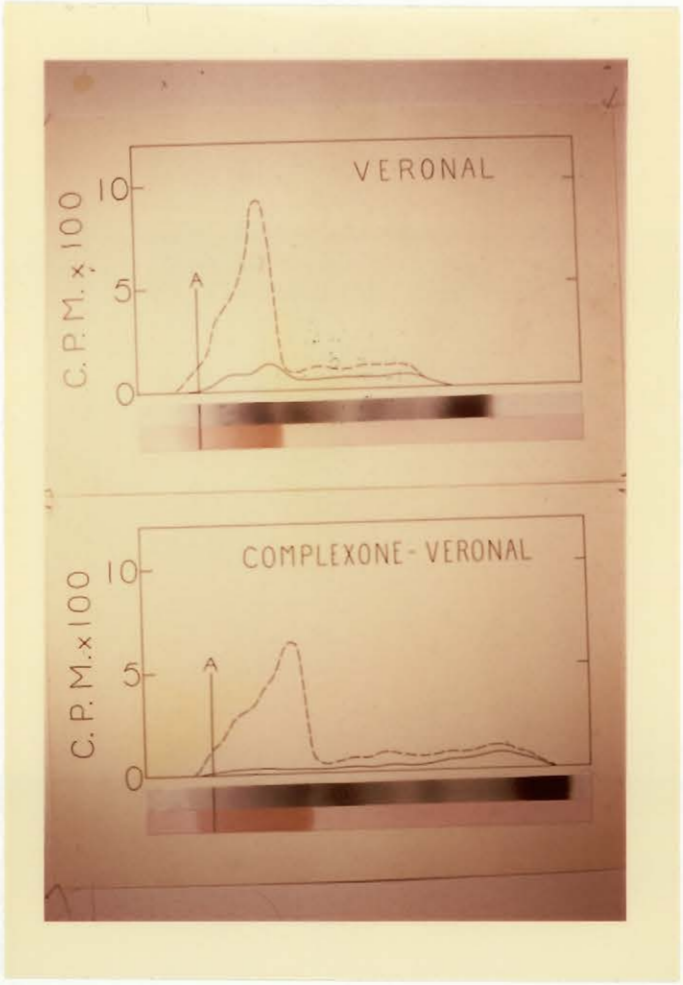

VERONAL
C.P.M. x 100
10
5
0
A
COMPLEXONE - VERONAL
C.P.M. x 100
10
5
0
A

FIGURE 9

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Serum of Estrogenized Pullet No. 2 in Borate Buffer pH 9.0 in Absence and Presence of Complexone III.

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

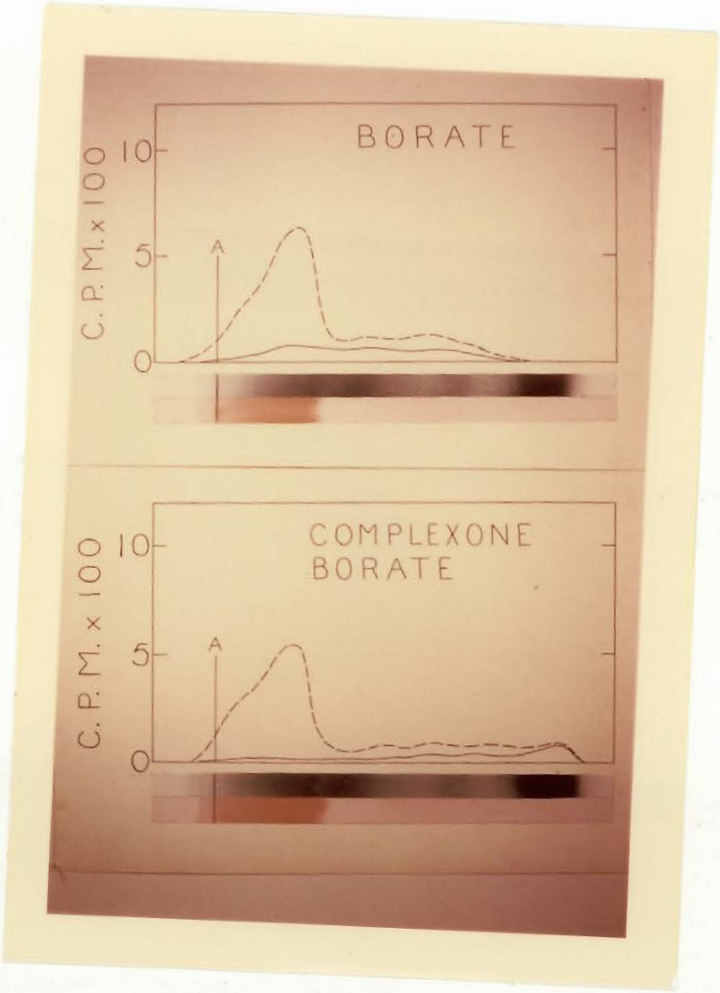

BORATE
C.P.M.x100
10
5
0
A
COMPLEXONE
BORATE
C.P.M.x100
10
5
0
A

FIGURE 10

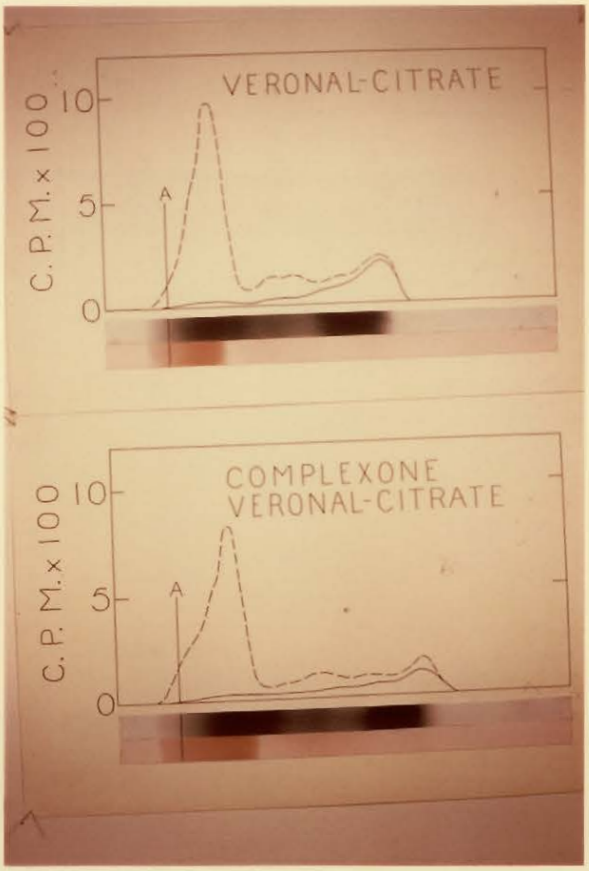
Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Serum of Estrogenized Pullet No. 2 in Veronal-citrate Buffer pH 8.6 in Absence and Presence of Complexone III.

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.


VERONAL-CITRATE
C.P.M.x 100
10
5
0
A
COMPLEXONE
VERONAL-CITRATE
C.P.M.x 100
10
5
0
A

of the serum of pullet No. 2 run in veronal-citrate buffer, both in the absence and presence of Complexone. This fast zone A was not observed on electropherograms of the serum of pullet No. 2 run in either veronal or borate buffers, and it was not observed on any of the electropherograms of the serum of pullet No. 1 or of the control pullet. This zone A was detected only on unextracted strips, since it was removed by the reagents used for lipid extraction. The relative mobility of zone A was approximately 3 (3.1 for veronal-citrate and 2.9 for Complexone-veronal-citrate) if the mobility of the serum albumin zone be taken as 1.

Zone A did not stain with Amidoschwarz or with Oil Red O but it gave a positive ELPP reaction. The ninhydrin reaction was doubtful and was at best only very faintly positive. The periodate-Schiff test of Köiw and Grönwall (1952) for carbohydrates gave a negative result. The material of zone A showed no fluorescence and gave no indication of ultraviolet absorption.

The quantity of P^{32} involved was of the same order of magnitude as that of the protein-bound P^{32}. It can be seen from the results in Table II that zone A was probably absent from the other electropherograms because the amounts of total P^{32} and protein-bound P^{32} were of similar orders of magnitude for all the electropherograms

of pullet No. 2. If the material of zone A had been present in the electropherograms run in veronal and borate buffers, then the extra radioactivity of the material of zone A would be sufficient to have been observed. The radioactivity of the material of zone A was present in amounts equivalent to approximately 30% of the total P^{32} radioactivity, i.e., 1490 and 1339 c.p.m. per 43 µl. serum for the serum samples run in veronal-citrate and Complexone-veronal-citrate respectively. The values for the A zones were actually somewhat greater than those for total protein-bound P^{32} (Table II).

The material of zone A was not inorganic phosphate, for in an ad hoc experiment it was found that, by the end of a six-hour run, inorganic phosphate had moved completely off the paper. Zone A was not colored and did not fluoresce, hence there is no evidence that it was a nucleotide (flavin mononucleotide (Nutritional Biochemicals), flavin-adenine dinucleotide (Schwarz Bioresearch) and diphosphopyridine nucleotide (Schwarz Bioresearch) were run and their mobilities were less than that of the material of zone A). In addition, electrophoretic runs in all three buffers, viz., veronal, borate and veronal-citrate, were made on the following compounds: adenosine 3-monophosphate (Schwarz Bioresearch), adenosine 5-monophosphate (Schwarz

Bioresearch), adenosine diphosphate (Schwarz Bioresearch), and inosine monophosphate (Nutritional Biochemicals). These compounds had mobilities less than that of the material of zone A, although the mobility of the adenosine diphosphate approached that of zone A.

No further attempts were made to identify the material of zone A. Its high mobility suggested a relatively small molecule containing several phosphate residues. Since it was removed by the reagents used for lipid extraction, it was not a protein. It would appear not to be a nucleotide and it gave a negative test for carbohydrate. The mobilities of the serum proteins were not as great in the veronal-citrate buffer as in the veronal and the borate pH 9.0 buffers. Hence, the zone A component of pullet No. 2 serum had apparently run completely off the paper in the veronal and borate buffers used since the zone could not be detected in the electropherograms run in these buffers. The question also arises as to whether the material of zone A is a component of all avian sera, or whether it is present only in estrogenized or laying hens sera, or whether it occurs only in the serum of a particular bird, e.g., pullet No. 2.

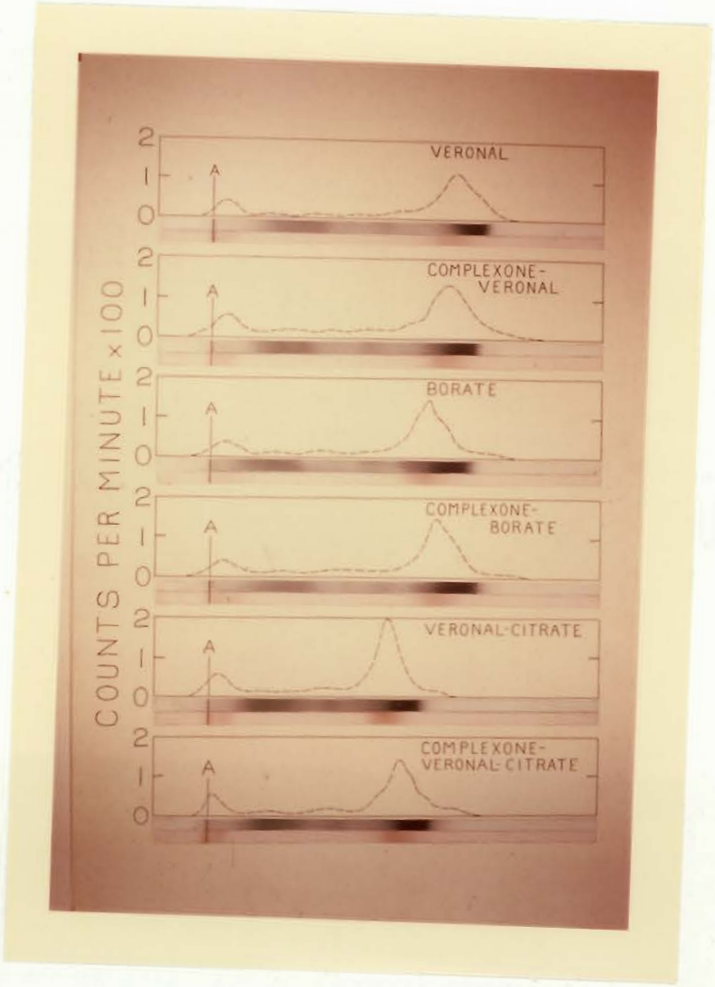
The results for the serum of the control pullet are presented in Figure 11. The data provided no indication

FIGURE 11

Distribution of Total P^{32} on Electropherograms of Serum of an Unestrogenized Pullet in Various Buffers as Indicated. (Slight but doubtful indications of protein-bound P^{32} noted on two only of the electropherograms were not inserted on the figures.)

Upper strips stained with Amidoschwarz.

Lower strips stained with Oil Red O.


COUNTS PER MINUTE x 100
2
1
0
A
VERONAL
COMPLEXONE-
VERONAL
BORATE
COMPLEXONE-
BORATE
VERONAL-CITRATE
COMPLEXONE-
VERONAL-CITRATE

of protein-bound P^{32} in four of the tests (Complexone-veronal, borate, Complexone-borate and Complexone-veronal-citrate) and only very small and doubtful traces in the remaining two tests (veronal and veronal-citrate). It was concluded that the protein phosphorus content of the control serum was negligibly small, if any (see also Table I). This is in agreement with the results of Tanabe et al. (1961), who stated that protein phosphorus was not present in the serum of cocks. It was not astonishing, therefore, that the radioactivity corresponded almost entirely with phospholipid-P^{32}. In each of the six electropherograms, there was a minor phospholipid-P^{32} peak near the line of application and a major phospholipid-P^{32} peak just short of the albumin zone. The position of the major peak corresponded rather closely with the position of a maximum of staining with Oil Red O. The scanning patterns did not display marked differences as between the different buffer systems, but the sharpest resolution of the major phospholipid-P^{32} peak was obtained with veronal-citrate buffer, i.e., with the buffer which gave the clearest resolution of protein-bound P^{32} in the cases of the sera of the estrogenized pullets.

2.7.7. The Presence of a Non-protein, Ninhydrin-positive Zone on Paper Electropherograms of Fowl Serum.

Representative strips were taken from all the electropherograms of the sera of the three pullets (control, pullet No. 1 and pullet No. 2) described in section 2.7.6. These strips were dipped in acetate buffer pH 5 in order to obtain optimal pH for the ninhydrin reaction. Ninhydrin staining confirmed all the protein zones observed on Amidoschwarz staining. In addition, a relatively fast, ninhydrin-positive zone was observed well in advance of the phosvitin zone on strips that had not been extracted to remove lipid. The ninhydrin-positive zone was observed on electropherograms of the sera of all three birds except those sera that were run in borate buffer pH 9.0. The ninhydrin reaction for the protein zones of all sera run in borate buffer, was relatively unsatisfactory. It was not determined whether the absence of the ninhydrin-positive zone on electropherograms run in borate buffer was due to unsatisfactory ninhydrin staining conditions or whether this zone was not resolved on electropherograms run in borate buffer.

The ninhydrin-positive zone stained neither with Amidoschwarz nor with Oil Red O nor with ELPP reagents. It was not, therefore, a protein or a lipid or a compound

containing phosphorus. It is suggested that the ninhydrin-positive zone may be due to a peptide but it was not identified further.

2.7.8. The Absence of ELPP from the Pre-albumin Components of Embryonic Fowl Serum.

The serum of the chick embryo contains one or more components that move ahead of the albumin in free electrophoresis (Marshall and Deutsch, 1950; Heim and Schechtman, 1954) or paper electrophoresis (Vanstone et al., 1955), whereas no such pre-albumin component is seen when sera of immature pullets or of cockerels are subjected to electrophoresis under the same conditions. Heim and Schechtman (1954) concluded from their free electrophoretic studies that the pre-albumin components observed by them in embryonic sera were probably not identical with the pre-albumin components which they observed in sera of laying hens. Marshall and Deutsch (1950) have reported that embryonic sera contain little or no protein-bound phosphorus and they further suggested that this fact would appear to exclude the possibility that the faster migrating components of embryo serum are derived intact from the yolk. Absence of ELPP from the pre-albumin component of embryonic serum resolved on paper

electrophoresis would provide some further evidence for the non-identity of pre-albumin components of embryonic sera with those of sera from laying hens.

Blood samples were drawn by heart puncture from a number of chick embryos at 15 days of incubation and bulked. Similar bulked samples were obtained from embryos at 18 days of incubation, from day-old chicks, from 2-day chicks, and from 7-day chicks. Total serum protein and serum calcium determinations were made on the resulting sera. The values obtained are presented in Table III. In general the total serum protein values agreed closely with the values reported by Vanstone et al. (1955) for comparable samples except for the 2-day chick. The abnormally high value for the latter was almost certainly a reflection of hemoconcentration due to the circumstance that these birds were held in the incubator until the third day after hatching. The serum calcium values are somewhat questionable because only very small samples of serum were available and only a single analysis was done on each serum sample.

Each of the serum samples was divided into two subsamples. One subsample was subjected to electrophoresis in veronal buffer pH 8.6. The other subsample was treated with Complexone (3 mgm. Complexone III per ml. serum) and then subjected to electrophoresis in veronal buffer. It

TABLE III

ANALYSES OF SERA FROM EMBRYOS AND YOUNG CHICKS

Age	Total serum protein gm. per 100 ml.	Serum calcium mgm. per 100 ml.
Embryos		
15-day	1.17	-
18-day	1.73	10.6
Chicks		
1-day	2.24	9.4
2-day	3.00	8.0
7-day	2.16	12.6

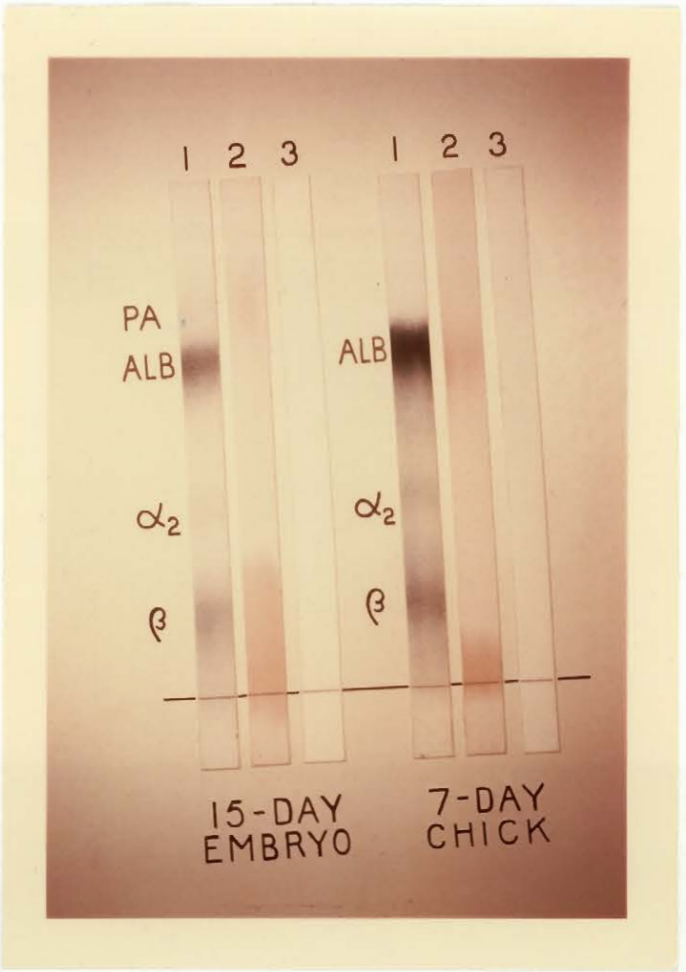
was found that pretreatment with Complexone did not alter the electrophoretic patterns of these sera. Typical results for 15-day embryos and for 7-day old chicks are shown in Figure 12. The resolutions correspond closely with those reported by Vanstone et al. (1955) for comparable sera so far as staining for protein and lipid were concerned. Staining for ELPP was uniformly negative. This negative result could be a consequence of lower protein concentrations, but against this it should be emphasized that a positive ELPP reaction has been obtained in every instance where the ELPP reagents have been applied to a phosvitin or presumptive phosvitin zone that stained as deeply with Amidoschwarz as did the pre-albumin zones of

FIGURE 12

Electropherograms of Sera from 15-day Embryos and from 7-day Chicks.

Stains: (1) Amidoschwarz.
(2) Oil Red O.
(3) Reagent of Harrap (1960) for ester-linked protein phosphorus (ELPP).

Electrophoresis in veronal buffer pH 8.6. (Similar patterns were obtained whether or not the serum was pretreated with Complexone III.)


1 2 3
1 2 3
PA
ALB
ALB
α2
α2
β
β
15-DAY
EMBRYO
7-DAY
CHICK

the embryo sera. It may be concluded that the pre-albumin zone of embryonic sera is not phosvitin and that it is different from the pre-albumin zones observed when sera from estrogenized fowl are resolved by use of suitable chelating agents or buffers. This conclusion is in agreement with the suggestions of Heim and Schechtman (1954) and with the observations of Marshall and Deutsch (1950).

2.8. General Discussion

Ultracentrifugation is the method of choice for resolution of serum proteins in so far as it is the technique least liable to alter the proteins from their native states. Free electrophoresis involves the use of buffer solutions. Zone electrophoresis introduces additional complications associated with the actions of the supporting medium (Martin, 1959), although it is the most readily available technique of the three. It is highly desirable that some correlation between results from the three techniques should be available, but this problem is greatly complicated by the appearance of the lipophosphoprotein complex in the serum of the bird in response to estrogenic activity.

As already mentioned, Schjeide and Urist (1960) have distinguished two major ultracentrifugal protein

components in the serum of the laying hen or estrogenized bird and have named these serum X_1-phosphoprotein and serum X_2-lipoglycoprotein. These authors have stated that they used the term X_1-phosphoprotein rather than phosvitin and the term X_2-lipoglycoprotein rather than lipovitellin. Schjeide and Urist (1960) have distinguished two analogous protein components in egg yolk and have named these yolk X_1-phosphoprotein and yolk X_2-lipoglycoprotein. If the X_1 and X_2 components are phosvitin and lipovitellin respectively, then the X_1 component would correspond to the PT or presumptive phosvitin zone described in the present study. It will be demonstrated in Part II section 2.8.5.2. of this thesis that the P-2 zone of egg yolk proteins corresponds to lipovitellin plus some phosvitin. Hence, it would appear that the serum X_2 component of Schjeide and Urist (1960) corresponds in general with the P-2 component of paper electropherograms if the P-2 zones of serum and egg yolk comprise the same components, although serum lipovitellin has not hitherto been characterized satisfactorily.

Yolk phosvitin may be separated from yolk lipovitellins by manipulation of the concentration of magnesium ions (Joubert and Cook, 1958b) and a serum phosvitin may be obtained by similar manipulation of the serum proteins of

the estrogenized or laying fowl (Common and Mok, 1959; Mok, 1960; Mok *et al.*, 1961). The present work has shown that serum phosvitin can probably be dissociated from the lipoprotein components of the serum of estrogenized fowl by paper electrophoresis when the conditions are such as to chelate calcium. When this resolution was achieved, the resolution of P-1 and P-2 lipoprotein zones was abolished as phosvitin was removed from the lipoprotein complex by electrophoresis. If zone P-2 is a serum lipovitellin-phosvitin complex, the removal of phosvitin from the complex with lipovitellin may render the lipovitellin soluble in the buffer and hence increase its mobility so that it moves with the P-1 zone. In veronal buffer the P-2 zone may be due to a calcium complex of phosvitin and lipovitellin that is relatively insoluble in the buffer and hence relatively immobile on electrophoresis. The precise reasons for the resolution of these two zones on paper electrophoresis of serum in veronal buffer pH 8.6 calls for further study. The two zones (P-1 and P-2) appear not to be resolved on paper electrophoresis when the ionic strength of the veronal buffer is increased to $\mu = 0.3$ by suitable addition of sodium chloride. In fact, resolution of the protein fractions fails at this ionic strength and all the protein moves in a single zone with a mobility approximately equal

to that of the P-1 zone in ordinary veronal buffer.

Ericson *et al*. (1955) and Clegg *et al*. (1956) have demonstrated that the presence of calcium ions did not alter the number or order of the free electrophoretic components of normal cockerel serum, but that it reduced drastically the mobility of the leading component (Clegg's component 1) of sera from estrogenized pullets. The results reported in this study are the converse of these observations, in so far as chelation of calcium led to the appearance of a protein zone, viz., presumptive phosvitin, immediately ahead of the albumin zone on paper electropherograms of whole sera from estrogenized pullets. The results of the present study tend to support the view that the presumptive phosvitin zone, as resolved in veronal-citrate buffer, may correspond with Clegg's free electrophoretic 'component 1'.

Urist *et al*. (1958) have related the non-diffusible calcium of the serum of the laying hen to the function of transport of yolk material synthesized in the liver to the ovary for deposition in the maturing yolks rather than to the function of shell secretion. Attempts to relate total serum calcium to the stage of egg shell formation (e.g., Charles and Hogben (1933), Rochlina (1934b)) have not always taken account of this possibility.

More recently Winget and Smith (1958) have detected a diurnal fluctuation of total plasma calcium depending on shell gland activity, but they have pointed out that the association is not a simple one and that the changes, which are mainly in the non-diffusible calcium of the plasma, may not be a direct result of shell mineralization. Winget, Smith and Hoover (1958) have demonstrated that a shell gland which is actively secreting a shell may reduce the total plasma calcium level by approximately 18% as the blood passes through the shell gland. The level of non-diffusible calcium in the plasma was reduced from 24.7 mgm. percent to 19.8 mgm. percent and the diffusible calcium from 10.4 mgm. percent to 9.4 mgm. percent. It is interesting to speculate as to the possible effect of this reduction of plasma calcium on the state of the plasma lipophosphoprotein complex. Fluctuations in serum calcium level are presumably affected not only by rate of removal for shell secretion but also by rates of production of yolk material in the liver and its deposition in the maturing follicles, rates of absorption of calcium from the gut and excretion of calcium in the urine and by rates of resorption and deposition of skeletal calcium, especially in the endosteal bone formed in response to estrogenic action. The large increase of non-diffusible calcium in the sera of laying or estrogenized birds may be closely related to the

powerful polyelectrolyte properties of phosvitin.

Apart from the foregoing general considerations, certain of the details of the results reported in this thesis may have some significance in connection with the interpretation of paper electropherograms of sera from estrogenized or laying fowl in terms of alterations from the levels of components resolved on electropherograms of sera from unestrogenized or immature or male fowl. Although protein-bound phosphorus remained mainly in the lipoprotein zone on paper electropherograms of sera from estrogenized birds in veronal buffer, minor amounts were detected in other zones. Again, even though chelation of calcium led to most of the protein-bound phosphorus moving to the pre-albumin zone, small amounts of protein-bound phosphorus remained in other zones, presumably as contaminants. The peculiar behavior of phosvitin towards staining with Amidoschwarz suggests the desirability of appropriate reservations in the interpretation of densitometric measurements on filter paper electropherograms of sera from laying or estrogenized birds. Phosphoprotein may be present in various zones of paper electropherograms of sera from laying or estrogenized birds without necessarily revealing itself through dyestuff staining procedures, and without obviously changing the

resolution of the protein zones as gauged by staining.

With respect to the interpretation of densitometric measurements, it is of interest to note some of the reports appearing in the literature with reference to quantitative dye-binding by proteins on zone electrophoresis. Franglen and Martin (1954) used Bromocresol Green and studied the dye-binding characteristics of human albumin and human γ-globulin, both individually and in mixtures of known composition. They stated that the plot of the dye bound to a protein against concentration differs from one protein to another. Hence, the results suggested that quantitative methods of analysis by paper-strip electrophoresis required reassessment. Jencks, Jetton and Durrum (1955) reported that, in order to obtain a stable and reproducible dye (Bromophenol Blue) uptake by proteins on paper, it was necessary to control the heat denaturation of the protein, the time of staining, and the rinsing of background dye from the paper; variations in these factors made the comparison of various staining techniques difficult. For example, varying the time of denaturation may alter the apparent albumin:globulin ratio through an almost two-fold range due to the different effects of heating on albumin and globulins, and excessive rinsing of dyed

papers may lower the globulin value considerably because dye is rinsed more easily from the globulins than from albumin. Abdel-Wahab and Laurence (1955) reported that the dye uptake of plasma proteins and albumin:globulin ratios as found with Amidoschwarz and Lissamine Green were independent of the time of dyeing and dye concentration over at least a ten-fold range. Results with Azocarmine B were not reproducible because the dye uptake was dependent on the dye concentration. Von Frijtag Drabbe and Reinhold (1955) reported that the average uptake of Amido Black 10B by human serum albumin was 1.28 times greater than for γ-globulin. The length of time that the proteins were exposed to the dye solution influenced the uptake of the dye and the dye uptake differed for different proteins. Formusa, Benerito, Singleton and White (1957) used human sera and horizontal paper strip electrophoresis. They stated that, under standardized conditions, the dye (Bromophenol Blue) uptake was proportional to the concentration of protein nitrogen, regardless of the type of serum protein. Strickland, Podleski, Gurule, Freeman and Childs (1959) used agar electrophoresis for human serum. The dyes Ponceau 2R, Amido Black 10B, and Bromophenol Blue were used for staining. It was found that the various serum protein fractions behaved differently toward the different dyes,

and corresponding protein fractions from different sera varied widely in their abilities to bind the same dye. Osborn (1960) found that the uptake of dye, measured by elution, was found to vary according to the method of denaturation (heat, freezing-ethanol, salicyl-sulphonic acid), the nature of the dye solvent (especially the salt and ethanol concentration) and with the duration and temperature of the subsequent washing procedure. Different denaturing agents gave different albumin:globulin dye uptake ratios.

It should be emphasized that the paper electropherograms of the present study were further complicated by the appearance of phosvitin and lipophosphoproteins in the sera of estrogenized and laying birds. As shown above, phosvitin has a peculiar staining behavior with Amidoschwarz and, in addition, phosvitin does not stain with Azocarmine B or with Bromophenol Blue. However, so far as the author is aware, no reports have appeared in the literature on the dye-binding characteristics of the lipoproteins of avian sera.

3. SUMMARY

In addition to the six protein fractions (albumin, α_1-, α_2-, α_3-, β-, and γ-globulin) distinguishable in sera from cocks or sexually immature pullets by paper electrophoresis in aqueous veronal buffer, two lipoprotein zones (P-1 and P-2) have been distinguished in sera from laying hens or estrogenized pullets by this same technique. Phosvitin was first detected in serum of estrogenized hens by paper electrophoresis of a saline solution of crude lipovitellin obtained from the serum. Phosvitin zones stained with Amidoschwarz in a way that is characteristic of phosvitin.

The movement of protein-bound phosphorus on paper electropherograms of fowl sera was studied (a) by use of reactions for ester-linked protein phosphorus (ELPP) and (b) by use of sera in which the phosphorus had been labelled with P^{32}. Serum from estrogenized pullets yielded two major lipoprotein zones, P-1 and P-2, on electrophoresis in veronal buffer pH 8.6. Most of the ELPP or protein-bound P^{32} was associated with these two zones, although minor amounts of protein-bound P^{32} were detected in other zones with the exception of the albumin zone. Somewhat similar results were obtained with borate buffer pH 9.0.

Use of veronal-citrate buffer pH 8.6, or pretreatment of the serum with ethylenediaminetetraacetate (Complexone III), or removal of the calcium by oxalate precipitation abolished the resolution of two lipoprotein zones, which were replaced by a single lipoprotein zone. At the same time, most of the ELPP or protein-bound P^{32} moved to a zone just ahead of the albumin zone, although minor amounts of protein-bound P^{32} were still associated with the other zones.

It is suggested that chelation or removal of the serum calcium led to dissociation of phosvitin from the lipoproteins of the serum and the independent migration of this phosvitin to a zone slightly ahead of the albumin zone.

Paper electropherograms of the P^{32}-labelled serum of an estrogenized pullet showed a P^{32} zone (zone A) of very high mobility when electrophoresis was done in veronal-citrate buffer. This phosphorus component was present in amounts equivalent to one-third of the total serum P^{32}. It was not inorganic phosphate and it was removed from the paper by the reagents used for lipid extraction. A strong ELPP reaction was observed but zone A stained neither with Amidoschwarz nor with Oil Red O nor with ninhydrin. It was, therefore, neither a protein nor a lipid but it was not identified further.

The pre-albumin zones of sera from chick embryos did not give any staining reaction for ELPP. This observation supports the conclusion that the pre-albumin components of sera from embryos and of sera of estrogenized pullets or laying hens are due to different proteins, and that phosvitin is either absent from embryo serum, or present in amounts below the limit of sensitivity of the reaction used for its detection.

PART II

Zone Electrophoretic Studies of Egg Yolk Proteins

1. HISTORICAL REVIEW

1.1. The 'Two Proteins' of Egg Yolk

The earlier literature on egg yolk proteins has been reviewed by Needham (1925, 1931) and by Jukes and Kay (1932a).

The earliest reference to egg yolk proteins appears to be that of Fourcroy (1782). He stated that yolk was chiefly 'albumin', fat being the substance second in importance. Bence-Jones (1841) coagulated egg yolk by heat and extracted fat with ether. The protein material that remained was later named 'vitellin'* by Dumas and Cahours (1842). Lehmann and Messerschmidt (1842) pointed out the unusual physical properties of vitellin. They demonstrated that the precipitate, which formed when egg yolk was mixed with water, was easily soluble in either sodium chloride or ammonium chloride solutions, was re-precipitated from such solutions by the addition of water, and could be taken up again in salt solutions.

Valenciennes and Frémy (1854) were the first to

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* The terms vitellin and lipovitellin were used by the earlier workers to denote various preparations which are now known to have been complex or denatured. No attempt has been made in this review to bring the significance of the terms as used by the earlier workers into line with the significances usually assigned to them at present, and which will be elucidated in a later section of this thesis.

recognize the existence of two proteins in egg yolk. They stated that vitellin in egg yolk was always found associated with a certain quantity of 'albumin'. Hence, to prepare vitellin, they treated egg yolk with cold water. The albumin remained in solution while the vitellin was precipitated. The precipitated vitellin was washed with water, with alcohol and with ether, and the residue was regarded as a very pure protein.

Hoppe-Seyler (1865) stated that vitellin was a lecithoprotein and showed that the lecithin could be split off by boiling alcohol. He also stated that vitellin contained no phosphorus, but that the phosphorus that appeared in the analysis was due to contamination with lecithin. However, at the same time Miescher (1866) obtained from egg yolk a substance containing a great deal of phosphorus. This substance possessed certain properties of a protein and these facts led Miescher (1866) to believe that it was a nuclein. Subsequently, Kossel (1886) found that no purine bases could be detected in the so-called nuclein of egg yolk (vitellin). He pointed out that vitellin was essentially different from the nuclein of the cell. However, it remained for Plimmer and Bayliss (1906) to show the nature of vitellin and its distinction from the nucleins.

In the meantime, the definition of vitellin had undergone modification. Osborne and Campbell (1900) prepared a reasonably pure form of vitellin. Egg yolk was mixed with an equal volume of saturated sodium chloride solution. The mixture was extracted repeatedly with ether containing a little alcohol and the fat-free residue was dialyzed against water, whereby the vitellin was precipitated. By re-solution in 10% sodium chloride solution and dialysis against water to remove the salt, followed by extraction with alcohol and ether to remove lecithin, a relatively pure vitellin was obtained. Vitellin prepared in this manner was found to contain 0.94% phosphorus and 15% to 30% lipid. Osborne and Campbell (1900) reported that the lipoprotein was stable to ether but that the phospholipid was readily liberated by contact with alcohol. All the 'combined lipid' could be removed by extraction with alcohol. These workers suggested the name 'lecithin-vitellin' for the lipoprotein.

Plimmer and 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. However, treatment with a 1% sodium hydroxide solution for

24 hours at 37°C. brought all the phosphorus of casein into solution as inorganic phosphoric acid. Plimmer and Scott (1908) found that vitellin behaved in a similar manner when submitted to the action of a 1% sodium hydroxide solution, i.e., all the phosphorus in the vitellin was completely separated as inorganic phosphoric acid by the action of 1% sodium hydroxide at 37°C. for 24 to 48 hours.

Treatment of nucleins with dilute alkali at 37°C. for 24 to 48 hours does not split off any phosphorus as inorganic phosphate, and hence the work of Plimmer and Scott (1908) gave a clear distinction of casein and vitellin from the nucleins. Accordingly, these two proteins were recognized 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.

Levene and Alsberg (1906-07) determined the amino acid distribution of vitellin by Fischer's methods. Osborne and Jones (1909) made a similar analysis. Both of the above vitellin preparations were judged to be free from livetin (Jukes and Kay, 1932a); livetin being the name which Plimmer (1908) had proposed for 'the second protein' of egg yolk (see below). Calvery and White (1931-32) 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. After removal of the yolk membranes by filtration, the solution was diluted with 20 volumes of water. The resulting precipitate was dissolved in 10% sodium chloride solution and the vitellin was reprecipitated by dilution with water. This crude vitellin was suspended in 80% alcohol, heated to boiling and kept near the boiling point for several hours. It was then filtered, washed with ether and dried in a vacuum desiccator. The yield of vitellin was 35 gm. to 40 gm. from 24 eggs. This preparation contained 0.92% phosphorus and 15.03% nitrogen. These workers also determined the nitrogen distribution in their vitellin by the procedure of Van Slyke. The tyrosine, tryptophan and cystine contents were determined by colorimetric methods. Arginine, histidine and lysine were determined by isolation as well-characterized, crystalline derivatives.

Plimmer (1908) observed that the aqueous solution remaining after complete precipitation of vitellin by water still gave a very intense biuret reaction. He found that acidification with acetic acid, followed by boiling, yielded a heavy precipitate of coagulated protein. This was at first regarded as unprecipitated vitellin, but a phosphorus determination showed that it contained only

0.1% phosphorus as compared with 1.0% phosphorus in vitellin. Both fractions contained about 15% nitrogen. Plimmer (1908) concluded that this protein material represented another constituent of egg yolk. He suggested that this second protein of yolk may have been vitellin without the phosphorus-containing portion and he provisionally named it 'livetin'.

Kay and Marshall (1928) prepared livetin in a fairly pure, undenatured form. In order to remove all the egg white from the yolk, it was found necessary to wash the yolk several times with 0.9% sodium chloride solution, and also to remove the chalazae with scissors. The yolk was further cleaned by allowing it to roll slowly down a strip of calico sheeting. Lecitho-vitellin was prepared by the method of Plimmer (1908). Livetin was precipitated from the filtrate by half-saturation with ammonium sulphate. Almost all the livetin could be precipitated by these means. The precipitate was taken up with water, the concentration of ammonium sulphate was brought up to 25%, and the slight precipitate that formed was filtered off. To the clear filtrate, sufficient saturated ammonium sulphate solution was added to bring the concentration of the salt to half-saturation. The precipitated livetin was filtered off, and the solution and precipitation were repeated twice.

Lipid was removed by alcohol-ether extraction at -15°C. The behavior of the livetin prepared by the above method was that of a pseudoglobulin with an isoelectric point at pH 4.8 to pH 5.0. It contained about 15% nitrogen, 0.05% phosphorus and 1.8% sulphur. These analyses, together with amino acid (tyrosine, tryptophan, cystine) analyses, showed that this livetin was unrelated to vitellin. Kay and Marshall (1928) considered that livetin represented one-quarter to one-fifth of the yolk proteins of the hen's egg.

Jukes and Kay (1932b) determined the contents of the basic amino acids (arginine, histidine, lysine) in both livetin and vitellin. The differences were not very striking. Jukes (1933) reported the fractionation of the amino acids of livetin.

Chargaff (1942) prepared the lipid-protein complex lipovitellin by dilution of egg yolk with an equal volume of saturated sodium chloride solution. This solution was extracted in the cold with ether. The aqueous phase was dialyzed to precipitate the lipovitellin. The precipitate was dissolved in 10% sodium chloride solution, and the ether extraction and dialysis were repeated twice. This preparation of lipovitellin contained 13.0% nitrogen, 1.5% phosphorus, 0.86% sulphur, 23% total lipids and

about 18% phosphatides. Chargaff (1942) also reported that only 18.8% of the phosphatides present in egg yolk were firmly bound to the protein. Phosphatides isolated from the alcohol extract (combined) and from the ether extract (free state) had essentially the same composition.

1.2. The Phosphorus-rich Phosphoprotein 'Phosvitin'

Miescher (1870-1) found that peptic digestion of the yolk protein split off an insoluble product which Bunge (1882) named 'haematogen'. Hugounenq and Morel (1905, 1906) investigated this material and found that it contained 0.455% iron and 8.7% phosphorus. Levene and Alsberg (1900) isolated a compound, with a similar iron to phosphorus ratio, from vitellin by a method that did not entail an enzymic hydrolysis. Vitellin was suspended in water and treated with strong ammonium hydroxide solution which was allowed to remain in contact with the protein for 2 hours. The solution was slowly neutralized with acetic acid, an excess of picric acid was added and the mixture was filtered. The precipitate which formed on the addition of alcohol to the filtrate was purified by repeated solution and reprecipitation, and finally extracted with a boiling mixture of alcohol and ether. The product, which was named 'vitellinic acid', contained

13% nitrogen, 10.0% phosphorus and 0.57% iron. Posternak and Posternak (1927a) prepared a derivative of vitellin that was similar to vitellinic acid. They called it ovotyrine-β. Hydrolysis of this fraction revealed the presence of large amounts of L-serine (Posternak and Posternak, 1927b). These workers calculated that there was sufficient serine present to combine with all the phosphorus. They suggested, therefore, that the main phosphorus-containing unit of vitellin was serine-phosphoric acid.

Mecham and Olcott (1948, 1949) extracted from lipoprotein precipitates of egg yolk a phosphoprotein which they named 'phosvitin'. Egg yolk was mixed with half its volume of 1.2 M magnesium sulphate solution and the mixture was diluted four-fold with water. The precipitate was dissolved in 0.4 M ammonium sulphate solution and adjusted to pH 4.0. The solution was extracted at room temperature with ether to remove lipids and to coagulate the non-phosvitin proteins. The supernatant was extracted with ether, filtered and finally saturated with ammonium sulphate to precipitate the phosvitin. The phosvitin was purified by dialysis after solution in 0.25 M sodium chloride solution. The purified phosvitin contained 9.7% phosphorus and 11.9% nitrogen. This fraction accounted

for 6.5% to 7.0% of the yolk protein and 60% to 70% of the phosphoprotein phosphorus of egg yolk. Hence, it was evident that phosvitin contained much of the protein phosphorus ordinarily associated with vitellin and that it must be a contaminant of most vitellin preparations. Francis (1952) has confirmed this inference by means of an exchange reaction of P^{32} between lipovitellin in 10% sodium chloride solution and phosvitin labelled with P^{32}.

Amino acid analysis of Mecham and Olcott's (1949) phosvitin showed the presence of just sufficient serine to permit all the phosphorus to be present as the serine phosphate ester. This type of combination was assumed because serine phosphate esters have been isolated from yolk proteins (Lipmann and Levene, 1932; Levene and Schormüller, 1933).

1.3. The More Recent Studies of Egg Yolk Proteins

1.3.1. The Lipoproteins - Lipovitellin and Lipovitellenin.

Alderton and Fevold (1945) prepared lipovitellin by the following method: egg yolk was diluted with 2 volumes of water and then centrifuged in a Sharples centrifuge. The lipoprotein was deposited as a waxy, translucent solid while most of the fat and the water-soluble proteins were

thrown off as an aqueous emulsion. The precipitate was extracted with ether and the lipovitellin was purified by solution in 10% sodium chloride solution and reprecipitation by dialysis. The precipitated material contained approximately 90% of the total lipovitellin present in the yolk.

Chargaff (1942, 1944) has shown that ether does not break up the protein-lipid complex of yolk protein; the lipovitellin can, therefore, be freed from uncombined lipid by ether extraction. Approximately 15% to 16% of the material precipitated by the method of Alderton and Fevold (1945) could be removed by extraction with ether. Subsequent alcohol extraction of the ether-insoluble product removed an amount of material (combined lipid) equal to approximately 16.8% of the total. The vitellin thus obtained was insoluble in water and salt solutions, but could be dissolved in alkali. The nitrogen content of lipovitellin ranged from 12.70% to 13.18%, and that of vitellin from 15.01% to 15.65%; the phosphorus content of lipovitellin ranged from 1.30% to 2.37%, and that of vitellin from 1.01% to 2.13%.

Fevold and Lausten (1946) have isolated a second yolk lipoprotein which they named 'lipovitellenin'. The method of separation of lipovitellin from lipovitellenin

depended on the fact that, in the presence of yolk lipids, the lipovitellenin was not thrown down in the Sharples centrifuge after dilution of the egg yolk with 2 volumes of water, whereas lipovitellin was readily deposited as a precipitate. After removal of the lipovitellin, the supernatant was extracted with 2 volumes of ether. Three layers separated: (a) an aqueous layer containing the livetins; (b) an ether layer containing the free lipid; and (c) a layer of insoluble material containing the lipovitellenin, which separated between the other two layers. The lipovitellenin was separated, extracted with ether and purified by solution in 10% sodium chloride solution saturated with ether, followed by reprecipitation by dialysis. This lipovitellenin differed from lipovitellin both in lipid and phosphorus contents. Lipovitellenin contained about twice as much combined lipid as lipovitellin. The protein component of lipovitellenin, vitellenin, was regarded as a phosphoprotein, similar to vitellin in general solubility behavior. However, it contained less than one-third as much phosphorus as vitellin.

1.3.2. The Livetins.

Shepard and Hottle (1949) have applied the technique

of free electrophoresis to the study of the water-soluble proteins of yolk. They prepared the livetin fraction by two methods. One method involved ether extraction and the second method employed dialysis and adjustments of salt content and pH. Both methods of preparation yielded material that gave similar electrophoretic results. Electrophoretic examination of these materials in phosphate buffer pH 7.95 showed that livetin was not one substance but a group of substances. The material present in these livetin preparations showed mobilities resembling those of the protein components of hen's plasma. The fastest of the three major peaks had a mobility similar to that of serum albumin. The second peak compared with the slowest of the β-globulins, and the slowest peak had a mobility not much different from that of the second of the two large peaks of hen serum. Shepard and Hottle (1949) attempted to separate the components of the livetin fraction by alcohol precipitation. They were able to obtain the slowest major electrophoretic component in a form essentially free from the other observed components.

Knight and Schechtman (1954) prepared a livetin fraction. Egg yolk was diluted with 10% sodium chloride solution, homogenized, ether extracted 6 to 7 times, and then dialyzed against an excess of 0.9% sodium chloride

solution. The solution was centrifuged to remove the insoluble vitellin fraction. The clear, colorless livetin fraction was dialyzed to remove any ether. Paper electrophoresis in veronal-acetate buffer pH 8.6 resolved this livetin fraction into four components.

Martin, Vandegaer and Cook (1957) examined the water-soluble proteins of egg yolk. These were prepared by diluting the yolk with 3 volumes of water and then removing the lipovitellin fraction by centrifugation. The supernatant was shaken with an equal volume of ether and left to separate. The lower aqueous layer, containing the livetin fraction, was separated from the intermediate emulsified layer of lipovitellenin and the ethereal layer. A livetin fraction was also prepared by shaking egg yolk with 2 volumes of water and 2 volumes of carbon tetrachloride. The resulting emulsion was broken by centrifuging at 30,000 r.p.m. The aqueous layer was decanted for re-extraction, dialyzed against distilled water and lyophilized. Both isolation procedures yielded similar products.

Electrophoretic examination of the livetin fraction, at 1% concentration in glycine buffer pH 9.0 or in phosphate buffer pH 7.9, revealed three major components. These fractions were designated α-, β-, and γ-livetin in order of decreasing mobility. Three components were also separated

in the ultracentrifuge. The α- and β-livetins were separated and purified electrophoretically after removal of the γ-livetin by precipitation from 37% saturated ammonium sulphate solution or 20% v/v isopropanol solution*. The α- and β-livetins could not be separated by differential precipitation. Several of the properties of α-livetin were found to be comparable to those of serum albumins. α-Livetin was similar to serum albumin in sedimentation coefficient, molecular weight, and tyrosine:tryptophan ratio, but its solubility behavior was not identical with that of albumin. The molecular weight of β-livetin was comparable with that of ovalbumin, but mobility, tyrosine:tryptophan ratio and sedimentation coefficient of the former were all lower.

The α-, β-, and mixed livetins resembled pseudo-globulins in solubility, but γ-livetin was unstable. Molecular weights determined were 8.0×10^4 for α-livetin and 4.2×10^4 for β-livetin. Martin et al. (1957) found that, under suitable conditions of sedimentation and electrophoresis, whole egg yolk yielded three components having the same ultracentrifugal and electrophoretic properties as the three livetins prepared from the water-soluble fraction. This provided some evidence that the

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* In point of fact, it is difficult to separate all of the γ-livetin from the α- plus β-livetin by these means, although the γ-livetin can thus be freed from α- and β-livetins fairly easily (Mok 1962).

method of preparation had not involved serious denaturation.

Later, γ-livetin was characterized by Martin and Cook (1958). It had the solubility of a euglobulin but lost its solubility irreversibly on prolonged dialysis, on freeze-drying or even on standing. It could be preserved for reasonable periods as a precipitate in saturated ammonium sulphate solution. γ -Livetin had a molecular weight of 1.5×10^5.

Mandeles (1960) separated egg yolk into three fractions by the method of Martin et al. (1957). The water-soluble or livetin fraction was chromatographed on DEAE-cellulose. Ten components were separated by the DEAE-cellulose chromatography but these components were not characterized.

1.3.3. The 'Lipovitellin Complex'.

Joubert and Cook (1958a) diluted egg yolk with 4 volumes of 10% sodium chloride solution. They subjected this solution to ultracentrifugation thereby separating a rising fraction, R, from a clear subnatant that contained two sedimenting fractions, S1 and S2. The major sedimenting fraction, S1, appeared to be ultracentrifugally homogeneous in 10% sodium chloride solutions. When examined in other salt solutions, however, fraction S1

could be resolved into lipovitellin, phosvitin and γ-livetin fractions. On dissolving egg yolk in 0.4 M magnesium sulphate solution and diluting to 0.2 M, much of the phosvitin was precipitated. Further dilution of the supernatant yielded lipovitellin, from which most of the γ-livetin and contaminating phosvitin could be removed by further treatment. Separation and recovery of both lipovitellin and phosvitin by this procedure indicated that phosvitin is a separate protein and not an integral part of the lipovitellin molecule. When the sedimenting fraction of egg yolk was dissolved in buffer solutions at pH 9.0, γ-livetin could be resolved ultracentrifugally from the remainder of this fraction, but the components could not be separated by dilution precipitation or by means of ammonium sulphate or ethanol. Lipovitellin prepared by the above method, and containing about 10% γ-livetin and 20% lipid, had a molecular weight of 3.7×10^5 and a phosphorus content of 0.49%.

Joubert and Cook (1958a) proposed that the lipovitellin separated by the older methods, originating from the work of Osborne and Campbell (1900) and of Plimmer (1908), should be called 'lipovitellin complex' and that the term lipovitellin should be reserved for the 'lipovitellin' freed from phosvitin and γ-livetin.

They pointed out that all samples of lipovitellin prepared by dilution precipitation from sodium chloride solutions must have contained phosvitin and γ-livetin as impurities. Further support for the notion of a 'lipovitellin complex' was given by Sugano (1957) who showed that lipovitellin prepared by the method of Alderton and Fevold (1945) was not homogeneous electrophoretically. This preparation contained three fractions, viz., phosvitin and two electrophoretic components which Sugano (1957) termed 'α-lipovitellin' and 'β-lipovitellin'.

Joubert and Cook (1958b) subsequently published a simplified method of obtaining phosvitin from egg yolk. Phosvitin was precipitated from egg yolk solution in 0.4 M magnesium sulphate solution by the addition of an equal volume of water. The crude phosvitin was purified by repeated solution and reprecipitation. It was then dissolved in 10% sodium chloride solution and centrifuged to remove any lipid material. The subnatant was dialyzed against acetate buffer pH 4.0 to remove the remaining lipovitellin. After the precipitate had been removed, the supernatant solution was dialyzed against water and then lyophilized. This material contained 9.6% phosphorus, 12.6% nitrogen, and had the chemical and physical properties of the phosvitin described by Mecham and Olcott (1949). In addition, it had a molecular weight of 3.6×10^4 at pH 4.0.

Phosvitin is a strong polyelectrolyte with properties that are dependent on both the concentration of the solute and the composition of the solvent. Phosvitin forms complexes with magnesium and calcium salts. Joubert and Cook (1958b) considered that their work had demonstrated the existence of lipovitellin and phosvitin as separate entities.

Williams and Sanger (1959) have subjected phosvitin, prepared by the method of Mecham and Olcott (1949), to a partial hydrolysis by treatment with concentrated hydrochloric acid at 37°C. for 12 hours. The hydrolysate was examined by high-voltage paper electrophoresis using 20% formic acid buffer pH 1.5. At this pH the carboxyl groups of peptides are uncharged, and hence the peptides will migrate toward the cathode unless they carry strongly acidic phosphate or sulphonic acid groups. At pH 1.5 the phosphate radical of serine phosphate is still charged and peptides which contain one residue of serine phosphate are electrically neutral under these conditions. Peptides which contain more than one serine phosphate residue are negatively charged and move toward the anode. The phosvitin hydrolysate revealed six major bands and a number of intermediate bands, all of which moved towards the anode. Total hydrolysis of the material of these main bands showed that serine was the only amino acid present. Partial hydrolysis (concentrated

hydrochloric acid at 37°C. for 12 hours) of the peptides of the main bands showed that these peptides broke down to yield materials of lesser electrophoretic mobilities on paper. In this way it was possible to demonstrate the presence of (serine phosphate)$_6$ (band 6). The authors suggested that even longer polyphosphoserine sequences are probably present.

Recently Connelly and Taborsky (1961) have fractionated phosvitin, prepared by the method of Mecham and Olcott (1949), into two fractions by ion exchange chromatography on DEAE-cellulose. The fractions differed from each other in metal content, amino acid composition, and chemical stability at alkaline pH, although they were quite similar in terms of the gross aspects of their composition.

Sundararajan, Sampath Kumar and Sarma (1960) have described a method for the preparation of phosvitin that involved the use of butanol to disrupt the lipid-protein complex of lipovitellin. This procedure rendered the vitellin insoluble and released all the phosvitin into solution from which it could be recovered by isoelectric precipitation. This phosvitin preparation and one prepared by the method of Mecham and Olcott (1949) were examined by paper electrophoresis in buffers of different pH. Both samples gave one major band together with two

minor slower-moving components.

McIndoe (1961) prepared phosvitin by a method that involved the use of butanol. He discussed the conditions for the precipitation of phosvitin by the use of Mg^{2+}, Ca^{2+} and cetyl pyridinium chloride. Phosvitin was also found to react in various ways with basic dyes.

1.3.4. Electrophoretic and Ultracentrifugal Studies of Whole Egg Yolk.

Young and Phinney (1951) reported the results of free electrophoretic studies of ether-extracted egg yolks. Using veronal buffer pH 8.5, $\mu = 0.32$, they were able to distinguish three peaks. The peaks were designated in order of decreasing mobility as, vitellomucoid or phosvitin, livetin, and lipovitellin. These electrophoretic results did not confirm the finding of Fevold and Lausten (1946) of the existence of a second lipoprotein, lipovitellenin.

Clegg, Hein, Suelter and McFarland (1955) reported free electrophoretic examinations of ether-extracted egg yolk labelled with P^{32}. They were able to distinguish four major peaks when the buffer was borate-citrate pH 7.5 or glycine-phosphate pH 9.4. The results showed that with both buffers the fast moving components (1 and A)

contained a high percentage of the P^{32}. These components represented approximately 39% of the total P^{32}, although they represented only approximately 10% of the total material. In the case of the glycine-phosphate buffer, components 2 and 3 represented approximately 5% and 9% respectively of the total material, and in the same manner contained small amounts of P^{32}, 6% and 4%, respectively. Component 4 represented 70% of the total material and contained 50% of the P^{32}. Borate-citrate buffer afforded a different distribution of the components and of the P^{32} in the three slower moving fractions. Component B contained 28% of the total material and 12% of the P^{32}, component C represented 54% of the total material and 32% of the P^{32}, and component D represented 8% of the total components and 11% of the P^{32}. No identifications were suggested for any of these fractions.

However, the agreement between the component percentage and the P^{32} percentage in the case of components 1 and A indicated that identical substances were involved. In the light of later work, it would be reasonable to suggest that this component was phosvitin.

Sugano (1958b) reported that he had recognized nine components in egg yolk from the results of free electrophoretic studies using sodium carbonate-bicarbonate buffer

pH 9.8, $\mu = 0.15$. These components were phosvitin, α- and β-lipovitellin (as defined by Sugano, 1958a), three livetin components and three minor components. From the results of his electrophoretic analysis, he reported that whole egg yolk contained 8.5% phosvitin, 20.3% α-lipovitellin, 43.2% β-lipovitellin, 14.2% livetins, 3.0% 'γ-component' and 10.8% others. Sugano (1958b) calculated that about 75% of the total phosphorus of egg yolk proteins was accounted for by the phosphorus of phosvitin and β-lipovitellin. Phosvitin phosphorus was protein phosphorus and about 75% of the total protein phosphorus was accounted for by phosvitin. Approximately 80% of the lipid phosphorus was contained in the β-lipovitellin fraction.

Vandegaer, Reichmann and Cook (1956) reported that egg yolk diluted with 5% sodium chloride solution showed one rising (R) and two sedimenting fractions (S1 and S2) when examined in an analytical ultracentrifuge. Two of these, the rising and one sedimenting fraction (S1), were lipoproteins, and the solubility and stability of both were impaired by ether extraction of the free lipid. The observed properties and lipid content indicated that fraction R (the rising fraction) was the lipid-rich protein lipovitellenin and that S1, the main sedimenting fraction, contained lipovitellin. This lipovitellin, precipitated

by dilution and separated from 'free lipid' by high speed centrifugation, yielded material having a lipid content of 18.2%. The molecular weight of this lipovitellin was calculated to be 4.0×10^5.

Subsequent work by Turner and Cook (1958) on the lipoprotein from the low-density floating fraction (90% lipid) showed that the lipoprotein termed lipovitellenin by Fevold and Lausten (1946) was probably derived from a natural entity of even higher lipid content. About two-thirds of this lipoprotein was insoluble after ether extraction and this portion contained 43% lipid. The soluble material contained 53% lipid, and was polydisperse and apparently heterogeneous in lipid content. Since lipovitellenin was evidently a derived product of intermediate lipid content, the entire floating fraction and lipid-free protein (vitellenin) derived from it were investigated further. Martin, Turner and Cook (1959) found that this floating fraction (density 0.98) appeared to be heterogeneous but that no significant fractionation took place during centrifugation in several solvents of density 0.98 or higher. Most of the so-called 'free' lipid extracted by ether was apparently part of, or associated with, the lipoprotein in the native state.

The lipid-free vitellenin, prepared by chloroform-methanol extraction, was not soluble in mild aqueous solvents. Accordingly, most of the physical measurements were made in 88% formic acid solutions. The most reliable estimate of the molecular weight was 9.3×10^4 for the lipid-free protein or about half the minimum estimate for the size of the protein moiety of the lipid-containing materials. Martin et al. (1959) suggested that the vitellenin has two or more polypeptide chains that may be combined directly or through lipid by forces weaker than the peptide linkage.

Bernardi and Cook (1960a) have recently completed an extensive electrophoretic and ultracentrifugal study of the proteins of the high density fraction (HDF) of egg yolk. The HDF was prepared as follows: 1 volume of egg yolk was diluted to 3 volumes with 2 M sodium chloride solution and enough solid sodium chloride was added to bring the final concentration to 2 M sodium chloride. The suspension was stirred for 1 hour at 4°C. and then centrifuged at 30,000 r.p.m. in a preparative ultracentrifuge for 24 hours. Four zones were evident in the tube: A, the top layer was a firm layer of yellow gel; B, the middle layer was a clear colorless solution; C, the bottom layer was a viscous yellow solution grading

to a firm pellet at the bottom; D, a fluffy yellow suspension that separated under layer A. Layer A and the suspended material in layer D constituted the low density fraction (LDF) and the remainder constituted the HDF.

The HDF represented 31% of the non-dialysable egg yolk solids. Electrophoretic analyses showed that the HDF contained six proteins in the proportions indicated below in order of decreasing mobility: phosvitin 15%; α-livetin 7%; β-livetin 17%; α-lipovitellin 30%; β-lipovitellin 21%; and γ-livetin 10%.

The three leading boundaries of the electrophoretic patterns were phosvitin when the electrophoresis of the HDF was done in veronal buffer pH 9.0, μ = 0.3; or in glycine buffer pH 10.5, μ = 0.2; or in veronal buffer pH 8.5, μ = 0.3. This anomalous electrophoretic behavior of phosvitin was studied by determinations on purified material in veronal buffer pH 9.0; μ = 0.1 and in this buffer plus: 0.1 M magnesium sulphate; 0.01 M calcium chloride or 0.005 M sodium Versenate. At this lower ionic strength, only two components were detected in purified phosvitin; both magnesium and calcium complexes were electrophoretically homogeneous; and the Versene-treated material showed three components. Hence, the electrophoretic behavior of phosvitin depends on the

ionic strength and bivalent cation content of the solution.

The two lipoproteins, α- and β-lipovitellins in the terminology of Cook's group, were present in the HDF and were resolved electrophoretically. Hence, there are three lipoproteins in whole egg yolk since another lipoprotein (lipovitellenin) is present in the LDF. The three lipoproteins were demonstrated electrophoretically by the addition of a small proportion of the LDF to the HDF solution.

The α- and β-lipovitellins described by Bernardi and Cook (1960a) were not the same as those described by Sugano (1958a), which were prepared from ether extracted whole yolk and were considered by him to be purified forms of lipovitellin and lipovitellenin, respectively. The lipovitellins of Sugano (1959) were heterogeneous mixtures ultracentrifugally and electrophoretically, and they differed both qualitatively and quantitatively from the α- and β-lipovitellins of Bernardi and Cook (1960a).

Bernardi and Cook (1960a) found that lipovitellin and its two electrophoretic components all sedimented as a single boundary in 1 M sodium chloride solution. However, the sedimentation behavior of their α- and β-lipovitellins showed that they both dissociate into

smaller entities. β-Lipovitellin dissociates at pH 9.0 and α-lipovitellin at higher pH values, but both appear to behave as reversible association-dissociation systems. Lipovitellin may occur, therefore, in at least four forms (Cook, 1961).

Bernardi and Cook (1960b) separated the α- and β-lipovitellins from each other and from the livetins and phosvitin in the HDF by chromatography on hydroxyapatite columns. The HDF, dissolved in 0.2 M potassium phosphate buffer, was chromatographed on a column of hydroxyapatite. The first fraction left the column without being absorbed. Ultracentrifugal and electrophoretic analyses of this fraction showed that it was made up of three livetins in the proportion $\alpha:\beta:\gamma = 2:5:3$. Preliminary experiments indicated that the three livetins could be fractionated on hydroxyapatite columns at phosphate buffer concentrations between 0.05 M and 0.15 M.

The second chromatographic component, obtained with 0.6 M phosphate buffer, was β-lipovitellin, since it was dissociated when examined ultracentrifugally at pH 9.0. Electrophoretic analysis showed that it was contaminated with a small amount of α-lipovitellin, which was removed on rechromatographing.

The third chromatographic component obtained with 2 M phosphate buffer was α-lipovitellin contaminated with a small amount of β-lipovitellin which was removed on rechromatographing. Phosvitin was retained on the column and did not appear in any of the eluates.

Analyses of α- and β-lipovitellins showed that they had the same lipid content, nitrogen content, amino acid composition and molecular weight (4.0×10^5). A further similarity between α- and β-lipovitellin was suggested by a similarity of their N-terminal amino acids, which were arginine and lysine (Neelin and Cook, 1961). However, they differed in their protein phosphorus content; electrophoretic mobility, absorption on hydroxyapatite, ultracentrifugal behavior in alkaline media and in solubility.

McKinley, Oliver, Maw and Common (1953) made a preliminary study of the behavior of egg yolk proteins on paper electrophoresis. Egg yolk was diluted with 1% sodium chloride solution (1:2.5 v/v). This suspension was subjected to paper electrophoresis in veronal buffer pH 8.6, μ = 0.05 containing 20% v/v methanol. The papers were stained for protein with Naphthalene Black 12B 200 and stained for lipid with Oil Red O. The electropherograms showed five distinct zones. In order of decreasing

mobility these zones were designated as X, Y, a lipid-rich zone, a phosphorus-rich zone (PP) and a non-mobile zone at the line of application. McKinley (1954) suggested that zones X and Y were possibly livetin fractions. (In the light of the present work it would appear that they were, in fact, α- and β-livetin respectively). The lipid-rich and the phosphorus-rich zone (PP) apparently corresponded to two similar zones that were resolved electrophoretically. from the sera of laying hens or estrogenized birds.

McKinley *et al*. (1953) suggested that the heavy deposit of lipoprotein at the line of application might be associated with the physical structure of the yolk emulsion. Accordingly, yolk samples were diluted with 1% sodium chloride solution (1:2.5 v/v) and centrifuged. The precipitate of lipoprotein was dissolved in 10% sodium chloride solution and then diluted with 10% sodium chloride solution to the same total volume as the volume of the original suspension in 1% sodium chloride solution. Both the supernatant and precipitate fraction were submitted to electrophoresis in methanolic veronal buffer. The supernatant contained the X and Y components as well as the lipid-rich lipoprotein fraction. The precipitate showed the double PP band which contained some lipid. There was retention of lipid at the line of application but no marked retention

there of protein, as was the case when the suspension of whole egg yolk was examined.

McKinley, Oliver, Maw and Common (1954) dissolved egg yolk in a 10% sodium chloride solution and diluted ten-fold with water. A precipitate formed which was removed and dissolved in 10% sodium chloride solution. Electrophoretic analysis showed that the supernatant contained the X and Y components as well as the lipid-rich lipoprotein fraction. The precipitate contained the material responsible for the double PP band. These results were similar to those that were obtained by McKinley *et al*. (1953) when they separated the precipitate from a suspension of egg yolk in 1% sodium chloride solution and then subjected the dissolved precipitate and the supernatant to electrophoresis.

Evans and Bandemer (1957) have studied the egg yolk proteins by the ridgepole paper electrophoresis technique of Durrum (1950) using aqueous veronal buffer pH 8.6, $\mu = 0.05$. They reported the separation of seven electrophoretic protein fractions and compared the mobilities of these fractions with the mobilities of various preparations of lipovitellin, of lipovitellenin and of the livetins. Six of the zones were correlated with ovalbumin, two livetins, lipovitellenin, lipovitellin and a lipoprotein which they

suggested might be phosvitin, although this latter protein is not ordinarily regarded as a lipoprotein. Densitometric measurements of the proportions of livetin (8.6%), of lipovitellenin (41.7%), of lipovitellin (46.4%) and of other proteins (3.3%) in egg yolk were reported.

Evans and Bandemer (1957) also reported that ether extraction of egg yolk, either in the cold or at room temperature, changed the paper electrophoretic behavior of the yolk proteins. Two lipoprotein zones, lipovitellenin and lipovitellin, were obtained with unextracted yolks, but only one lipoprotein zone was obtained with ether-extracted yolks. They suggested that the difference in electrophoretic behavior by ether-extracted yolk proteins and non-extracted yolk proteins was caused by the breaking of an actual lipid-protein bond by the ether. Extraction of lipovitellenin with ether changed its properties so that it gave a zone in the same position as lipovitellin.

Evans and Bandemer (1957) suggested that the use of ether-extracted yolk samples by Young and Phinney (1951) accounted for their inability to confirm the presence of lipovitellenin in egg yolk. Evans and Bandemer (1957) also suggested that their results indicated that most of the egg yolk lipids are combined with the proteins and that at least two types of combinations exist. The first

is a relatively stable combination which is not broken by ether. A weaker lipoprotein-lipid complex also appears to occur, but this complex is broken by ether even in the cold. From the results of ultracentrifugation studies with unfractionated egg yolk, Weinman (1956) has also concluded that nearly all egg yolk lipids are bound to protein.

1.3.5. The Egg Yolk Granules.

Egg yolk contains a variety of microscopic particles belonging to two main groups: the 'yolk globules', which are relatively large particles, many of them resembling oil droplets; and the 'granules' which are much smaller and more uniform in size but less regular in shape (Romanoff and Romanoff, 1949).

In connection with their instructive studies of the soluble phase of egg yolk, Schmidt, Bessman, Hickey and Thannhauser (1956) separated the egg yolk granules from undiluted egg yolk by centrifugation at 20,000 G for 12 hours at 25°C. The sedimented granules were packed sufficiently well to permit the clear yellow supernatant solution to be decanted. Approximately 40% of the yolk proteins were present in the particulate fraction. These workers found that all of the phosphoproteins and at least

85% of the iron and calcium of yolk were present in the particulate fraction. They also found that more than 70% of the phospholipids occurred in the supernatant fraction and hence were not associated with the phosphoproteins. This was confirmed by Tanabe, Abe, Kaneko and Hosoda (1961), who stated that approximately 90% of the phospholipids of yolk were not associated with the phosphoprotein, which they found to be almost completely sedimented on ultracentrifugation of yolk at 40,000 r.p.m.

McIndoe (1959b) diluted egg yolk with 0.15 M sodium chloride solution and centrifuged to remove the particulate material. This particulate material contained about 5% of the lipid, 40% of the protein of yolk and virtually all the phosphoprotein, in agreement with the results of Schmidt *et al*. (1956) and with the results of Tanabe *et al*. (1961).

Urist, Schjeide and McLean (1958) separated the supernatant fluid and granules of egg yolk by centrifugation at 30,000 r.p.m. for 4 hours. After washing with distilled water, filtering and centrifuging at 70,000 G for 2 hours, the granules consisted of highly concentrated samples of phosphoprotein (X_1) and a sulphur-rich phospholipid lipoprotein (X_2).

In a more complicated study, Schjeide and Urist (1959) separated the granules from the yolk fluid by centrifugation. The yolk fluid contained ovalbumin, which they defined as 'one of the components of livetin', and a lipoprotein component. The yolk granules were washed in distilled water and dissolved in an equal volume of 20% sodium chloride solution. Filtration and centrifugation of this material resulted in a protein yield which consisted of two components (X_1 and X_2). The slower sedimenting component (X_1) was calculated to comprise 7% of the total proteins of egg yolk; the faster sedimenting species (X_2) amounted to approximately 40% of the total yolk proteins. The protein-bound calcium and protein-bound phosphorus in the yolk granules were associated almost exclusively with the X_1 moiety. No lipid was detected in the isolated X_1 fraction. The X_2 species was found to be an unusually dense lipoprotein which contained most of the polysaccharide of the granules. Hence, Schjeide and Urist (1959) termed these proteins X_1-phosphoprotein and X_2-lipoglycoprotein. They have stated that the terms 'X_1' and 'X_2' were used as a device to describe egg granule components resolved on the basis of density, and they also suggested that the phosvitin of Mecham and Olcott (1949) is a relatively pure preparation of the centrifugally resolved X_1-phosphoprotein. Schjeide and Urist (1959) further suggested that the α-lipovitellin

of Sugano (1958a) appeared to be synonymous with their term 'X_2' and that the β-lipovitellin of Sugano (1958a), which was said to correspond with the lipovitellenin of Fevold and Lausten (1946), is the predominant lipoprotein of egg yolk fluid.

In a later paper, Schjeide and Urist (1960) state that they used the term X_1-phosphoprotein in place of phosvitin and the term X_2-lipoglycoprotein in place of lipovitellin. They also state that yolk X_2-lipoglycoprotein splits into smaller molecules when placed in an alkaline medium. If it be assumed that the X_2-lipoglycoprotein is lipovitellin, then this finding is in agreement with those of Bernardi and Cook (1960 a, b), who found that lipovitellin dissociates in alkaline solvents.

Burley and Cook (1961) isolated egg yolk granules by diluting yolk with an equal volume of 0.16 M sodium chloride solution or with water and then centrifuging for 10 minutes at 29,000 r.p.m. The supernatant liquid was decanted and the pellet of granules was dispersed in 0.16 M sodium chloride solution or in water by grinding the mixture in a mortar. The granules were then recovered by centrifuging as before.

The granules represented on a dry basis 23% of the

yolk solids. Further analyses showed that the granules were 30% lipid and 70% protein, and that they contained about 90% of the protein phosphorus, 95% of the iron, and nearly 70% of the calcium in yolk. Separation of the macromolecular constituents of the granules showed that they were 70% α- and β-lipovitellins in the approximate ratio 1:1.8, 16% phosvitin, and 12% low-density lipoprotein. Therefore, Burley and Cook (1961) concluded that one or more low-density lipoproteins form an integral part of the granules. They also suggested that phosvitin exists in the granules as a high molecular weight complex.

The properties and composition of phosvitin and the lipovitellins isolated from the granules were the same as those isolated from solutions of whole yolk. New purification procedures reduced the protein phosphorus in α-lipovitellin to 0.50% and in β-lipovitellin to 0.27%. This confirmed that α-vitellin has the higher phosphorus content.

1.4. A Summary of the Present Knowledge of Egg Yolk Proteins

The extensive series of free electrophoretic and ultracentrifugal studies of egg yolk proteins carried out by Cook and his co-workers and summarized by Cook (1961) provides the most complete and reliable data on the preparation and properties of the yolk proteins. They have

isolated seven reasonably well-characterized protein fractions. A summary of their preparations, isolated from egg yolk by mild procedures based on ultracentrifugation, is given in Table IV.

As mentioned before, Schjeide and Urist (1960) state that they used the term X_1-phosphoprotein in place of phosvitin. They claim that their X_1-fraction is nearly pure phosphoprotein, containing 10% phosphorus and 50% serine of which almost 50% was phosphoserine. Hence, it would appear that the phosvitin of Mecham and Olcott (1949), of Joubert and Cook (1958b) and the X_1-phosphoprotein of Schjeide and Urist (1959) are more or less identical entities although the reported molecular weights differ greatly.

The term X_2-lipoglycoprotein was used by Schjeide and Urist (1960) in place of lipovitellin. They state that their X_2-fraction contains approximately 20% lipid, but give no protein phosphorus content. Hence, it is difficult to know how far the X_2-component is contaminated with X_1 or phosvitin or both. Schjeide and Urist (1959) also state that their X_2-lipoglycoprotein "seems to be synonymous with" the α-lipovitellin of Sugano (1958a), or lipovitellin.

TABLE IV

PREPARATIVE PROCEDURES AND PROPERTIES OF EGG YOLK PROTEINS

Preparative procedures Egg yolk dispersed in 0.16 M sodium chloride; centrifuged to remove granules and then centrifuged at high speed	Designation	Characteristic properties Molecular weight ($M \times 10^{-4}$)	Principal N-terminal amino acids	Lipid content (%)	Protein phosphorus content (%)	References
Granules Soluble in 0.7 M sodium acetate Separated from lipovitellins by column chromatography on Dowex 1	Phosvitin	3.6	Alanine and minor amounts of lysine	-	9.6 to 10.1	Mecham and Olcott (1949) Joubert and Cook (1958b) Neelin and Cook (1960) Burley and Cook (1961) Mok, Martin and Common (1961)
Soluble in 0.2 M phosphate buffer α- and β-forms separated by column chromatography on hydroxyapatite	α-lipovitellin β-lipovitellin	40 40	Arginine, lysine Arginine, lysine	20 20	0.50 0.27	Joubert and Cook (1958a) Bernardi and Cook (1960a,b) Burley and Cook (1961) Neelin and Cook (1961)
Low-density floating fraction: no known method of fractionation	This fraction yields lipovitellenin (about 40% lipid) on ether extraction	480	Arginine, lysine (C-terminal amino acid, glutamic acid)	90	0.13	Turner and Cook (1958) Martin et al. (1959) Cook (1961) Neelin and Cook (1961) Martin (1962)
Subnatant Soluble in 0.16 M sodium chloride Soluble in 37% saturated ammonium sulphate α- and β-forms separated electrophoretically	α-livetin β-livetin	8.0 4.2	Arginine, lysine Lysine plus two others	0.1 0.1	0.10 0.10	Martin et al. (1957) Neelin and Cook (1960)
Precipitated by 37% saturated ammonium sulphate	γ-livetin	15	Alanine	0.5	0.003	Martin and Cook (1958) Neelin and Cook (1960)

From the above it would appear that Schjeide and Urist (1959) have separated two fractions, X_1-phosphoprotein and X_2-lipoglycoprotein, which are impure forms of phosvitin and lipovitellin respectively.

Schjeide and Urist (1959) further state that the lipovitellenin of Fevold and Lausten (1946) is the predominant lipoprotein of egg yolk fluid which Schjeide and Urist (1959) have described as having a flotation rate of S_f 21, and that the β-lipovitellin of Sugano (1958a) is synonymous with their floating material. Cook (1961) stated that the floating fraction of Schjeide and Urist (1959) had a similar flotation pattern as the low-density fraction (about 90% lipid) of Vandegaer *et al*. (1956). Turner and Cook (1958) and Martin *et al*. (1959) have shown that this LDF yields lipovitellenin (about 40% lipid) on ether extraction.

2. EXPERIMENTAL

2.1. Electrophoretic Apparatus

Paper electrophoresis was done in a horizontal Matthew cell (Matthew Laboratories, Yonkers, N.Y.) as described in Part I, section 2.1. of this thesis.

2.2. Buffers

(a) Veronal buffer pH 8.6, $\mu = 0.05$.

(b) Borate buffer pH 8.6.

(c) Borate buffer pH 9.0.

(d) Veronal-citrate buffer pH 8.6.

The above buffers were prepared as described previously in this thesis (Part I, section 2.2.).

2.3. Staining Methods

On completion of an electrophoretic run, each main strip was air-dried and then cut longitudinally into substrips which were stained for protein, or for lipid, or for ester-linked protein phosphorus (ELPP) as follows:

(a) Staining for protein with Amidoschwarz-10B (Chromagesellschaft, Stuttgart, Germany) was done as described in Part I, section 2.3. of this thesis.

(b) Staining for lipid with Oil Red O on untreated strips was done according to the directions of Durrum, Paul and Smith (1952).

(c) Staining for ELPP on strips that had been freed from lipid was done by the method of Harrap (1960).

2.4. Egg Yolk

Egg yolks were separated from freshly-laid, sterile eggs. The chalazae were snipped off and any adherent albumen was removed from the yolk by rolling it on dry filter paper. Samples were then withdrawn from the yolks by hypodermic syringe.

Egg yolks in which the phosphorus was labelled with P^{32} were obtained as follows: a White Leghorn laying hen was given 0.5 millicuries P^{32}-labelled orthophosphate by intramuscular injection on each of five successive days. The hen laid on the day of the first injection (day 0) and on days 2,3,4,5,6,8,9,10,11. The yolks from the eggs laid on days 6 and 8 were bulked and mixed to furnish yolk sample A. The yolks from the eggs laid on days 9 and 10 were similarly bulked to furnish yolk sample B.

2.5. Ultracentrifugation

Ultracentrifugal separations were made on a Spinco model L preparative ultracentrifuge (Spinco Specialized Instrument Corp., Belmont, California) fitted with rotor No. 40.

2.6. Measurement of Radioactivity

The counting technique has been described previously in Part I, section 2.5. of this thesis. The date of the first injection of P^{32} was adopted as zero time for correction of radioactivities to a common basis.

2.7. Analytical Methods

2.7.1. Protein Nitrogen.

Protein nitrogen was determined by precipitation of the yolk protein with trichloroacetic acid and a subsequent microkjeldahl determination (A.O.A.C., 1955) on the precipitated material. The figure for nitrogen, therefore, included the lipid nitrogen of the lipoproteins.

2.7.2. Phosphorus.

Phosphorus was determined by the method of Martin and

Doty (1949). Protein phosphorus was determined by the procedure described by Common, Rutledge and Bolton (1947). Lipid phosphorus was determined on a suitable aliquot of an alcohol-ether extract obtained as described by Hawk, Oser and Summerson (1954) for blood serum.

2.7.3. Determination of Nitrogen and Phosphorus in Phosphoprotein Zones of Paper Electropherograms.

Electrophoresis was carried out in the Matthew cell as described in section 2.1. Lipid was extracted from the papers by the method described in Part I, section 2.3. The protein zones were located by Amidoschwarz staining of strips cut from the edges of the main electropherogram. The zones corresponding to the phosphoprotein zones (P-1 and P-2) were then cut out and analyzed for nitrogen by the microkjeldahl method. It should be noted that the accuracy of these nitrogen determinations was relatively low due to inability to get consistent blank determinations on the filter paper, e.g., the relationship between the quantity of paper digested and the nitrogen blank value determined was not linear and, in addition, the digestion of similar quantities of filter paper did not give similar nitrogen blank values. The reason for the inconsistency remains obscure. Levin and Oberholzer (1952) have estimated the protein contents of serum protein zones by kjeldahl analyses

of the filter paper. They stated that if an accurately measured amount of serum protein was used initially, the fractions were thus directly estimated in terms of protein nitrogen. The method gave duplicate results which appeared to be as accurate as those obtained by the much longer procedure of elution of the dye from stained electropherograms.

The phosphorus of the phosphoprotein zones was determined by the method of Martin and Doty (1949) after digestion of the paper with nitric and perchloric acids. The blank correction values for the filter paper were consistently low (below 1.0 γ phosphorus).

2.8. Results and Discussion

2.8.1. Paper Electrophoresis of Egg Yolk Proteins.

As already mentioned (Part I, section 2.7.1.), it was considered that the P-1 and P-2 components noted in the sera of laying and estrogenized fowl might correspond broadly with two major lipoprotein fractions noted in such sera by McKinley *et al*. (1953), by McKinley, Oliver, Maw and Common (1954) and by Vanstone, Maw and Common (1955) and in egg yolk by McKinley *et al*. (1953), even although these workers all used a methanolic veronal buffer. It was also thought likely

that the two zones might correspond with the two major lipoprotein zones noted by Evans and Bandemer (1957) on electropherograms of egg yolk run in aqueous veronal buffer. In order to study this point further, a sample of yolk (1 volume) from freshly laid eggs was dispersed in 1.0% sodium chloride solution (2.5 volumes). The mixture was centrifuged. The supernatant was reserved for electrophoretic examination (see below). The deposit was dissolved in 10% sodium chloride solution and made up to a volume of 3.5 ml. with the same solution of sodium chloride.

Electropherograms were run in veronal buffer pH 8.6 on (a) the dispersion of whole yolk in 1.0% sodium chloride solution (Figure 13J); (b) a sample of α- plus β-livetins prepared by free electrophoresis and containing traces of γ-livetin (Figure 13K); (c) the supernatant from centrifugation of the dispersion of egg yolk (Figure 13L); and (d) the deposit from centrifugation of the dispersion of egg yolk (Figure 13M) dissolved in 10% sodium chloride solution.

The electropherogram of the diluted whole egg yolk (Figure 13J) showed clearly two lipoprotein zones which have been designated P-1 and P-2 on account of their correspondence with the P-1 and P-2 zones of the sera of laying or estrogenized fowl. At this time zone P-1 was identified tentatively as lipovitellenin or, more probably, a complex thereof; and

FIGURE 13

Paper Electropherograms of Egg Yolk Proteins.

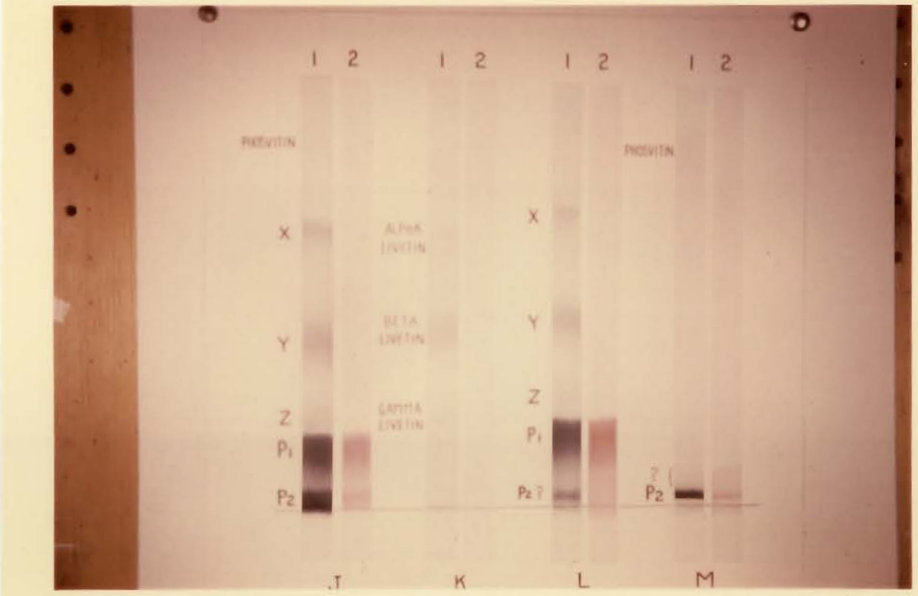
Stains: (1) Amidoschwarz.
(2) Oil Red O.

(J) Whole egg yolk dispersed in 1.0% sodium chloride solution.

(K) Mixture of livetins isolated by free electrophoresis and dissolved in 1.0% sodium chloride solution.

(L) Supernatant from centrifugation of dispersion of egg yolk in 1.0% sodium chloride solution.

(M) Deposit from centrifugation of dispersion of egg yolk in 1.0% sodium chloride solution, dissolved in 10% sodium chloride solution.


1 2
1 2
1 2
1 2
PROVITIN
PROVITIN
X
Y
Z
P1
P2
X
Y
Z
P1
P2 ?
P2
ALPHA LIVETIN
BETA LIVETIN
GAMMA LIVETIN
J
K
L
M

zone P-2, which was believed to correspond with the PP fraction noted by McKinley *et al*. (1953), was identified tentatively as lipovitellin or, rather, 'lipovitellin complex' (Joubert and Cook, 1958a). The two prominent lipid-free protein zones were obviously identical with the X and Y zones of yolk proteins noted by McKinley *et al*. (1953). A third less intensive zone was noted and was designated zone Z. With reference to Figure 13K, strip K was run in parallel with strip J and it will be noted that zone X corresponded in position with the α-livetin of Martin *et al*. (1957) and Martin and Cook (1958) and that zone Y corresponded in position with the β-livetin of the same workers. Zone Z likewise corresponded in position with a third faint zone of the livetin preparation. Zone Z may be identified, therefore, with γ-livetin (Martin and Cook, 1958), since it was known that the livetin preparation contained traces of the latter protein.

A point of major interest in connection with strip J was the presence of a lipid-free zone that stained faintly but distinctly with Amidoschwarz and that ran well in advance of the α-livetin zone. The position of this zone and the faintness of its staining with Amidoschwarz corresponded with the position and staining properties of phosvitin preparations isolated from egg yolk, as will be shown in section 2.8.2. This zone by

no means necessarily represents all of the phosvitin of the yolk sample. It may merely represent a part of the phosvitin that is readily liberated from the 'lipovitellin complex' (Joubert and Cook, 1958a) under the conditions of paper electrophoresis, as will be shown later in section 2.8.5.2. A further point of possible practical significance is that the phosvitin zone appeared to be more distinct when the dilution of egg yolk had been left to stand in the cold at 5°C. for 2 or 3 days, but further work is needed on this particular point.

Figure 13L, the electropherogram of the supernatant from the dispersion of egg yolk in 1.0% sodium chloride solution, showed clearly the presence of the P-1 (presumptive lipovitellenin) zone, of X (identified as α-livetin), of Y (identified as β-livetin), and of Z (identified as γ-livetin). A relatively slight P-2 zone was probably due to incomplete removal of this fraction, since an ultracentrifuge was not available at the time.

Figure 13M, the electropherogram of the solution (in 10% sodium chloride solution) of the material deposited on centrifugation of a dispersion of egg yolk in 1.0% sodium chloride solution, showed the presence of a strong P-2 lipoprotein zone and a faint, lipid-free phosvitin zone. Phosvitin was detected on strip M but not on strip L. This

observation suggested that the phosvitin zone on strip M represented phosvitin that was originally associated with the material that settled on centrifugation of the diluted egg yolk, but that dissociated readily from this material under the conditions of paper electrophoresis. (In point of fact, and as will be discussed later, the fact that the material deposited on centrifugation was redispersed in 10% sodium chloride solution must have been a major factor in determining this dissociation.) Joubert and Cook (1958a) considered that phosvitin and γ-livetin are associated with lipovitellin to form a 'lipovitellin complex', but that phosvitin is not associated with lipovitellenin. However, Cook (1961) has stated recently that γ-livetin does not interact strongly with the lipovitellins, as originally suggested. The circumstance that phosvitin appeared on the electropherogram in which zone P-2 was present but not on those from which P-2 was absent might be thought, therefore, to point to the identification of P-2 as lipovitellin or lipovitellin complex (lipovitellin and phosvitin) and of P-1 as lipovitellenin. The P-1 and P-2 zones resolved in the present study appear to be similar to the two major lipoprotein zones resolved by Evans and Bandemer (1957) and identified by them as lipovitellenin and lipovitellin. The resolution of their zones appears to have been relatively poor, possibly

because they used the Spinco-Durrum technique, but they did use an aqueous veronal buffer similar to that used in the present study. However, as will be shown in section 2.8.3., the protein nitrogen:protein phosphorus ratios of zones P-1 and P-2 were very similar, so that at this stage of the investigation it was not yet possible categorically to identify P-1 with lipovitellenin and P-2 with lipovitellin. It was considered that both zones were probably complexes and, as will be shown later in section 2.8.5.2., that both contained phosvitin even though zone P-2 obviously could be separated at least in part from phosvitin by paper electrophoresis.

Strip M showed the presence of yet another zone which lay just ahead of the P-2 zone and which is marked with an interrogation mark in Figure 13M. This zone stained faintly with Amidoschwarz and also with Oil Red O.

The foregoing experiments demonstrated that it was possible to distinguish six protein zones on paper electropherograms of egg yolk. One of the zones was due to phosvitin; three were due to livetins, viz., α-, β-, and γ-livetin; and the two major lipoprotein zones may have represented lipovitellenin and lipovitellin complexes. A third minor lipoprotein zone was of uncertain significance.

2.8.2. A Comparison of the Phosvitin Zones on Electropherograms of Whole Egg Yolk and of the Material Deposited on Centrifugation of Egg Yolk with the Phosvitin Zone of Egg Yolk Phosvitin.

Egg yolk was diluted with 1.0% sodium chloride solution (1:2.5 v/v). A sample of this dispersion was centrifuged; the deposit was dissolved in 10% sodium chloride solution and this solution was run in veronal buffer pH 8.6 in parallel with the whole diluted egg yolk. A sample of phosvitin that had been prepared from egg yolk by the method of Joubert and Cook (1958b) was dissolved in 10% sodium chloride solution and run in parallel with the foregoing two samples. The results are presented in Figure 14.

The electropherogram of the dispersion of whole egg yolk in 1.0% sodium chloride solution (Figure 14N) showed the usual six protein zones, including a faintly-staining phosvitin zone. Figure 14P, the electropherogram of the solution of the material deposited on centrifugation of a dispersion of egg yolk in 1.0% sodium chloride solution, showed the usual P-2 lipoprotein zone and a phosvitin zone. There was also a minor lipoprotein zone immediately in advance of the P-2 zone and similar to a corresponding zone shown in Figure 13M. Figure 14O, the yolk phosvitin preparation, showed clearly a faintly-staining phosvitin zone which had

FIGURE 14

Comparison of the Mobility of the Presumptive Phosvitin Zone of a Dispersion of Egg Yolk in 1.0% Sodium Chloride Solution with that of Phosvitin Prepared from Egg Yolk.

Stains: (1) Amidoschwarz.
(2) Oil Red O.

(N) Egg yolk dispersed in 1.0% sodium chloride solution.

(O) Phosvitin (egg yolk) dissolved in 10% sodium chloride solution.

(P) Deposit from centrifugation of a dispersion of egg yolk in 1.0% sodium chloride solution, dissolved in 10% sodium chloride solution.

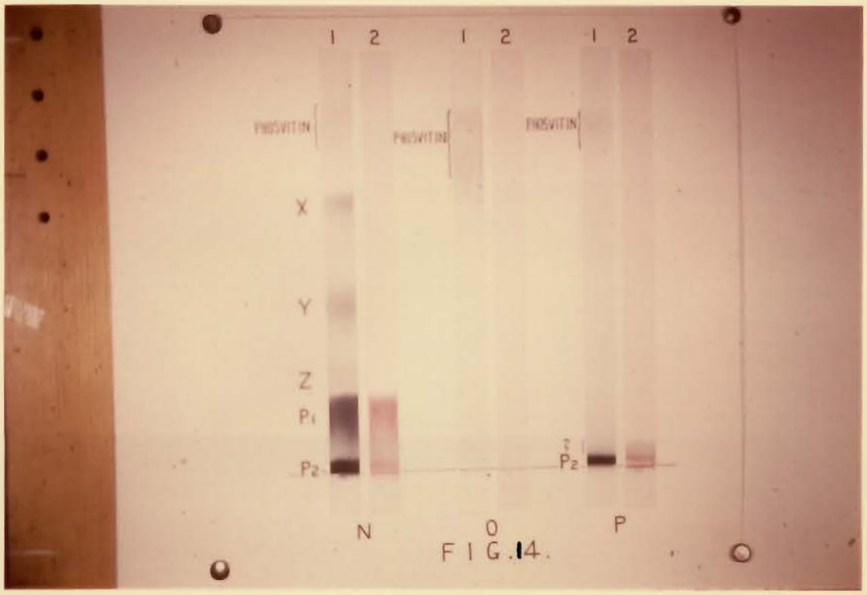


FIG. 14.

the same mobility as the phosvitin of strip P under the given conditions.

The egg yolk phosvitin preparation (Figure 140) gave a leading faint zone which moved at the same rate under the given conditions as the similar zones on strips N and P. This also confirmed the identification of the phosvitin zones on strips J and M, Figure 13 (section 2.8.1.). The phosvitin on strip O also gave one (and possibly two) slower zones. However, Bernardi and Cook (1960a) found that phosvitin forms complexes depending on the solvent and that it may then run as two or three zones or as a single broad zone, i.e., the electrophoretic behavior of phosvitin depends on the ionic strength and the bivalent cation content of the solution. The peculiar staining behavior of phosvitin with Amidoschwarz (see Part I, section 2.7.4.) complicates the study of the phosvitin zones but is useful, at the same time, in their identification. For the same reason, absorptiometric measurements on phosvitin zones are probably of relatively little value for purposes of estimating the amounts of phosvitin.

2.8.3. The Proportions of Protein Nitrogen and Protein Phosphorus in Zones P-1 and P-2 of Paper Electropherograms of Egg Yolk Protein.

The P-1 and P-2 zones of alcohol-ether extracted paper electropherograms of dispersions of whole yolk in 1.0% sodium chloride solution were analyzed for nitrogen and for phosphorus. The results are presented in Table V.

TABLE V

PROPORTIONS OF PROTEIN NITROGEN AND PROTEIN PHOSPHORUS IN ZONES P-1 AND P-2 OF PAPER ELECTROPHEROGRAMS OF EGG YOLK PROTEIN

Constituent	Zone P-1	Zone P-2
Protein N, mgm. per ml. yolk	6.70	10.74
Protein N, % of total yolk N	24.2	39.1
Protein P, mgm. per ml. yolk	0.45	0.68
Ratio, protein N:protein P	14.9	15.8

Two facts emerged from these preliminary analytical data. Firstly, the lipoproteins accounted for only about two-thirds of the total protein nitrogen of the yolk. This was a higher proportion of livetins than had been reported by various earlier workers, but it was in general accord with the results of the sedimentation studies of Martin et al. (1957). Secondly, the ratio protein nitrogen:protein phosphorus was

practically the same for both P-1 and P-2 fractions in spite of their electrophoretic separation. This fact, and the size of the ratios, suggested that both P-1 and P-2 may have contained a considerable proportion of phosvitin. Although the above results were of a very preliminary nature and were obtained from the analyses of a small number of electropherograms, the suggestion that both zones P-1 and P-2 contained phosvitin has been confirmed in later work (see sections 2.8.4., 2.8.5. and 2.8.6.).

2.8.4. The Distribution of Ester-linked Protein Phosphorus (ELPP) on Paper Electropherograms of Egg Yolk in Various Buffers.

One volume of whole egg yolk was diluted by admixture with 2.5 volumes of 1.0% sodium chloride solution. Portions each of 400 µl. of this diluted yolk were applied to strips of paper 7.0 in. wide and subjected to paper electrophoresis in each of the four buffers listed in section 2.2. Each main strip was air-dried and then cut longitudinally into sub-strips, of which at least one was stained for protein, another for lipid and still another for ELPP. The qualitative results are exemplified in Figure 15.

(a) Veronal buffer pH 8.6 (Figure 15 A, B, C). Some yolks did not yield a distinct 'fast' phosvitin zone (PT)

FIGURE 15

Paper Electropherograms of a Dispersion of Egg Yolk in 1.0% Sodium Chloride Solution.

Stains: (1) Amidoschwarz.
(2) Oil Red O.
(3) Reagent of Harrap (1960) for ester-linked protein phosphorus (ELPP).

(A) Veronal buffer pH 8.6. 'Fast' phosvitin zone not detected.

(B) Veronal buffer pH 8.6. 'Fast' phosvitin zone detected (different yolk from (A)).

(C) Egg yolk dispersion treated with Complexone and then run in veronal buffer pH 8.6.

(D) Borate buffer pH 8.6.

(E) Borate buffer pH 9.0.

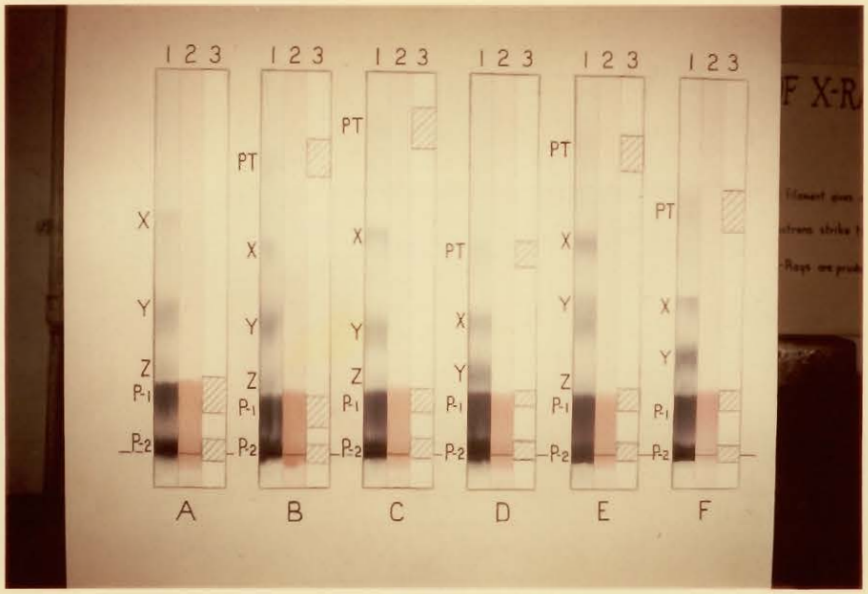
(F) Veronal-citrate buffer pH 8.6.

Key to zones: X = α-livetin; Y = β-livetin;

Z = γ-livetin; PT = 'fast' phosvitin;

P-1 and P-2 = lipoprotein zones.

Note: Strips stained for ELPP were unsuitable for photography, and positions of positive reactions have been indicated by cross-hatching.


1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
PT
X
Y
Z
P-1
P-2
A
B
C
D
E
F

and staining for ELPP was observed only in zones P-1 and P-2 (Figure 15A). Other yolks yielded a distinct PT zone that stained both for protein and for ELPP (Figure 15B) and, in addition, staining for ELPP was observed in zones P-1 and P-2. This difference in behavior was not due to differences in the age of the yolks, for all yolks were examined within a few hours of oviposition. It is suggested that the difference in behavior of the different egg yolks may be due to the bivalent cation (calcium) content and/or the physical structure of the egg yolk, since it has been demonstrated that the movement of the phosvitin of the sera of laying or estrogenized birds is greatly affected by the presence or absence of complexing agents in the buffer (Part I, section 2.7.5.); similar observations on the movement of egg yolk phosvitin are shown below. The α- and β-livetin zones were well-marked, but the visible γ-livetin zone (Z) was not so well defined. Results for separate runs on mixtures of α-, β-, and γ-livetin suggested that the visible Z zone corresponded in position with the leading region only of the γ-livetin, and that the main γ-livetin region was masked by the densely-staining material of zone P-1. Mok (1962) studied this point by immunoelectrophoresis as follows: an adult white male rabbit was immunized against a mixture of α-, β-, and some γ-livetin by the method of Kabat and Mayer

(1948). Paper electropherograms of (i) the mixture of livetins and of (ii) whole egg yolk dispersed in 1.0% sodium chloride solution were run in veronal buffer pH 8.6 and were then examined by immunoelectrophoresis against the rabbit immune serum. The electropherograms of the mixture of livetins gave well-defined precipitin reactions with each of the three livetin zones, thus demonstrating that the immune serum contained antibodies for each of the three livetin fractions. The α- and β-livetin zones (X and Y) of electropherograms of the whole egg yolk gave well-defined precipitin reactions against the immune serum. In addition there was a well-defined precipitin reaction associated not only with the γ-livetin zone (Z) visible on the stained electropherograms, but also with the entire P-1 zone.

Mok (1962) also demonstrated that when the lipoprotein constituent (low density fraction) responsible for the heavy protein- and lipid-staining of zone P-1 was separated by ultracentrifugation, as described in section 2.8.5.1., and then subjected to a similar examination by immunoelectrophoresis, it gave no precipitin reaction whatever with the rabbit antiserum. Hence the precipitin reaction in the Z and P-1 regions of electropherograms of whole egg yolk was not due to the lipoprotein of the P-1 zone but was due entirely to the γ-livetin fraction. The observations of

Mok (1962), therefore, confirmed that the visibly resolved Z zone represented part only of the γ-livetin and that most of the γ-livetin zone was masked on stained electropherograms by the heavy staining of the lipoprotein moiety of zone P-1. There was no indication of any livetin precipitin reaction in association with zone P-2. If any livetin was associated with zone P-2, therefore, it must have been non-diffusible.

When the dispersion of yolk was treated with ethylenediaminetetraacetate (Complexone III, B. Siegfried Limited, Zofingen, Switzerland) at the rate of 20 mgm. Complexone per ml. of original whole egg yolk, and electrophoresis was done in veronal buffer pH 8.6, a well defined PT zone was obtained consistently (Figure 15C). Positive staining for ELPP was observed in the PT, P-1 and P-2 zones although the staining for ELPP was relatively stronger in the PT zone and relatively weaker in the P-1 and P-2 zones when compared with the ELPP staining observed in Figure 15B. This observation indicated that the presence of a complexing agent resulted in the movement of some of the phosvitin from association with the P-1 and P-2 zones to the 'fast' phosvitin zone (PT).

(b) Borate buffer pH 8.6 (Figure 15D). The use of borate buffer pH 8.6 was prompted by the observations of

Brandt, Clegg and Andrews (1951), of Clegg, Sanford, Hein, Andrews, Hughes and Mueller, (1951), of Clegg and Hein (1953 a, b) and by the experience with this buffer for paper electrophoresis of sera from laying hens or estrogenized fowl (Part I, section 2.7.5.3.). A typical pattern for a dispersion of egg yolk in 1.0% sodium chloride solution is shown in Figure 15D. The P-1 and P-2 zones were well resolved as were also α- and β-livetin zones (X and Y). A Z zone (γ-livetin) was not apparent, a circumstance almost certainly due to this fraction being completely masked by the P-1 zone in consequence of the lesser mobilities of the proteins in this buffer. There was staining for ELPP in the 'fast' phosvitin zone (PT) and also in zones P-1 and P-2.

(c) Borate buffer pH 9.0 (Figure 15E). The results with this buffer were similar to those obtained with veronal buffer, except that a PT zone was observed consistently with all yolks studied and the Z zone appeared relatively faint.

(d) Veronal-citrate buffer pH 8.6 (Figure 15F). This buffer gave results for protein staining similar to those observed with veronal buffer, except that a Z zone was only faintly visible and that the staining for ELPP was relatively stronger in the PT zone and relatively weaker in the P-1 and P-2 zones. The latter observation suggested that veronal-citrate buffer was relatively effective in liberating

phosvitin from the lipoprotein zones, presumably in consequence of chelation of calcium.

The foregoing results showed that zones P-1 and P-2, as resolved in veronal buffer and certain other buffers, contained appreciable amounts of ELPP and that they were associated, therefore, with an appreciable fraction of the yolk phosvitin. Some ELPP remained with zones P-1 and P-2 with all the buffers used, but the veronal-citrate buffer appeared to permit the migration of more ELPP from these zones to the PT zone than did the other buffers.

2.8.5. Partition of Total Phosphorus and Protein-bound Phosphorus Between Ultracentrifugal Fractions of Egg Yolk.

2.8.5.1. Preparation and Analyses of Egg Yolk Fractions.

In spite of the foregoing qualitative observations on the positions of the livetins and of the ELPP positive zones on paper electropherograms of egg yolk, the exact nature of zones P-1 and P-2 called for further clarification.

Accordingly, certain preparations numbered I to VI were prepared from each of the two P^{32}-labelled egg yolk samples, A and B (section 2.4.), as follows:

I One volume yolk diluted by the addition of 2.5 volumes 1.0% sodium chloride solution.

II One volume yolk diluted by the addition of 1 volume distilled water and 1 volume of 3.0 M sodium chloride solution to give a final concentration of 1.0 M sodium chloride.

Another portion of the yolk sample was diluted with an equal volume of distilled water and centrifuged at approximately 40,000 r.p.m. (approximately 100,000 G) for one hour. This packed the granule material at the bottom of the tube but did not cause any visible separation of the low density fraction (LDF). The granule material was dispersed in 1.0 M sodium chloride solution for subsequent electrophoresis. Part of the supernatant was reserved for electrophoresis and the remainder was diluted with half its volume of 3.0 M sodium chloride solution and centrifuged at approximately 100,000 G for 24 hours. The gel of LDF was separated from the subnatant and dispersed in 1.0 M sodium chloride solution for subsequent electrophoresis. The subnatant was carefully mixed before sampling for paper electrophoresis, because the prolonged centrifugation for separation of the LDF was sufficient to throw most of the γ-livetin to the bottom of the tube. The foregoing simplified separation was essentially that described by Bernardi and Cook (1960a) and by Burley

and Cook (1961).

The foregoing fractions were numbered as follows:

III Granule material dispersed in 1.0 M sodium chloride solution.

IV Supernatant from segregation of granule material.

V LDF dispersed in 1.0 M sodium chloride solution.

VI Subnatant from separation of LDF.

All six preparations from each yolk sample were analyzed for nitrogen and for phosphorus. The average analytical data are presented in Table VI. The data show that the recoveries of both protein nitrogen and protein phosphorus in fractions III and IV were satisfactory (95.4% and 99.1% respectively). The further ultracentrifugal separation of fraction IV to give fractions V and VI involved an appreciable loss of nitrogen (equivalent to 5.3% of the total yolk nitrogen) but very little, if any, loss of protein phosphorus. The losses of lipid phosphorus were relatively greater, perhaps on account of the greater technical difficulty of handling the lipid material.

Over 95% of the protein phosphorus was recovered in the granule material, which corresponds to lipovitellins plus phosvitin (Burley and Cook, 1961). The high recovery of protein phosphorus in the granule material was in agreement with the results reported by Schmidt et al. (1956), by McIndoe

TABLE VI

NITROGEN AND PHOSPHORUS CONTENTS OF PREPARATIONS OF EGG YOLK.
AVERAGE VALUES FOR YOLK SAMPLES A AND B.

	Whole yolk I & II	Granule material III	Supernatant from granules IV	LDF V	Subnatant from LDF VI	LDF (purified)
Protein N* mgm. per ml. yolk	26.2	12.0	13.0	7.3	4.3	5.5
Protein N % of total	100	45.8	49.6	27.9	16.4	-
Protein P mgm. per ml. yolk	1.54	1.47	0.055	0.040	0.013	0.030
Protein P % of total	100	95.5	3.6	2.6	0.84	-
Lipid P mgm. per ml. yolk	4.67	0.61	3.54	2.97	0.032	2.59
Lipid P % of total	100	13.1	75.8	63.6	6.9	-
Ratio protein N:protein P	17.0	8.2	236	183	331	183

* Determined on the total trichloroacetic acid-precipitable material.

(1959b), by Tanabe et al. (1961) and by Burley and Cook (1961). Purification of the LDF by redispersion in 1.0 M sodium chloride solution and recentrifugation did not appreciably reduce its content of protein phosphorus or its ratio protein nitrogen:protein phosphorus. Evidently the single initial ultracentrifugal separation had effected a high degree of separation of phosvitin from the LDF.

2.8.5.2. Paper Electrophoretic Results in the Absence of Complexone.

The paper electrophoretic resolution of phosvitin of the sera of estrogenized pullets is profoundly affected by pretreatment of the serum with Complexone or by removal of calcium ions by oxalate. Accordingly, it was decided to examine the various yolk preparations after pretreatment with Complexone as well as in its absence. The subsamples of the preparations of yolk sample A which were not treated with Complexone were numbered Ia to VIa, and the subsamples which were pretreated with Complexone were numbered Ib to VIb. Paper electrophoresis was done in veronal buffer pH 8.6 on each subsample of each preparation. Strips cut from each main electropherogram were stained for protein and for lipid. Yet other strips were scanned for total P^{32} and strips from which lipid had been extracted were scanned

for protein-bound P^{32}. All radioactivities were corrected to zero time (the date of the first injection of P^{32}) and all radioactivities were calculated to a basis of 1 ml. of the original whole yolk sample so that the curves might be directly comparable. After counting for radioactivity, the strips were divided into zones and the phosphorus contents of the major phosphoprotein zones were determined.

The electrophoretic results, except those for lipid staining, for preparations Ia to Va are presented diagrammatically in Figure 16 and in Figure 17.

Staining of the electropherograms of the dispersion of whole egg yolk in 1.0% sodium chloride solution with Amidoschwarz (Figure 16, Ia) revealed the usual X,Y,Z, P-1 and P-2 zones. There was no perceptible stained PT zone, but the scanning for protein-bound P^{32} did reveal that a small proportion of the protein-bound P^{32} was present in the region where phosvitin might be expected to show up. It was clearly evident that the P-1 zone included a rather high proportion of the protein-bound P^{32}, thus confirming the results of staining for ELPP (section 2.8.4.) and the surmise that the P-1 zone, as ordinarily resolved, contained phosvitin (section 2.8.3.).

The P-2 zone represented material that did not move

FIGURE 16

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Whole Yolk Sample A.

(Ia) Whole yolk dispersed in 1.0% sodium chloride solution.

(IIa) Whole yolk dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

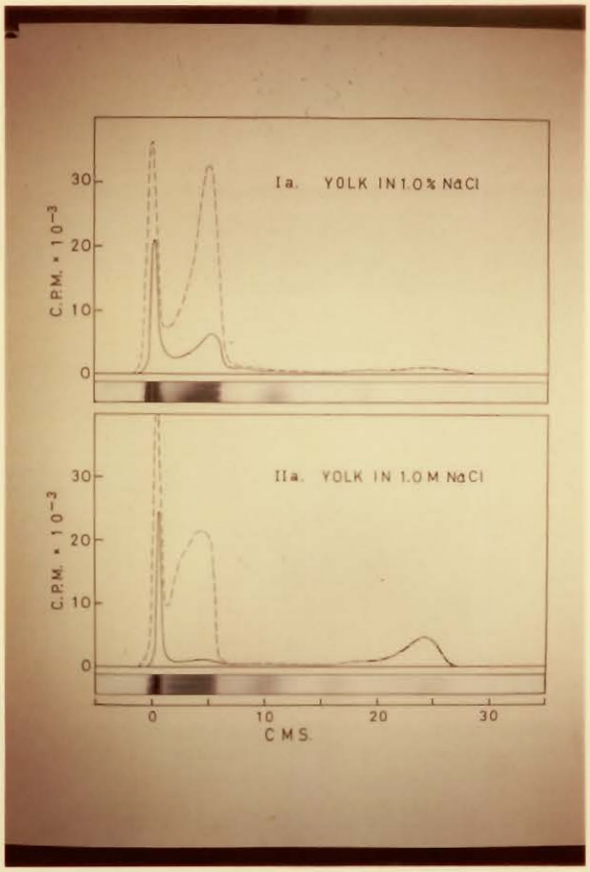

Ia. YOLK IN 1.0% NaCl
C.P.M. × 10⁻³
30
20
10
0
IIa. YOLK IN 1.0 M NaCl
C.P.M. × 10⁻³
30
20
10
0
0
10
20
30
CMS.

FIGURE 17

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Centrifugal Fractions of Whole Yolk Sample A.

(IIIa) Granule material dispersed in 1.0 M sodium chloride solution.

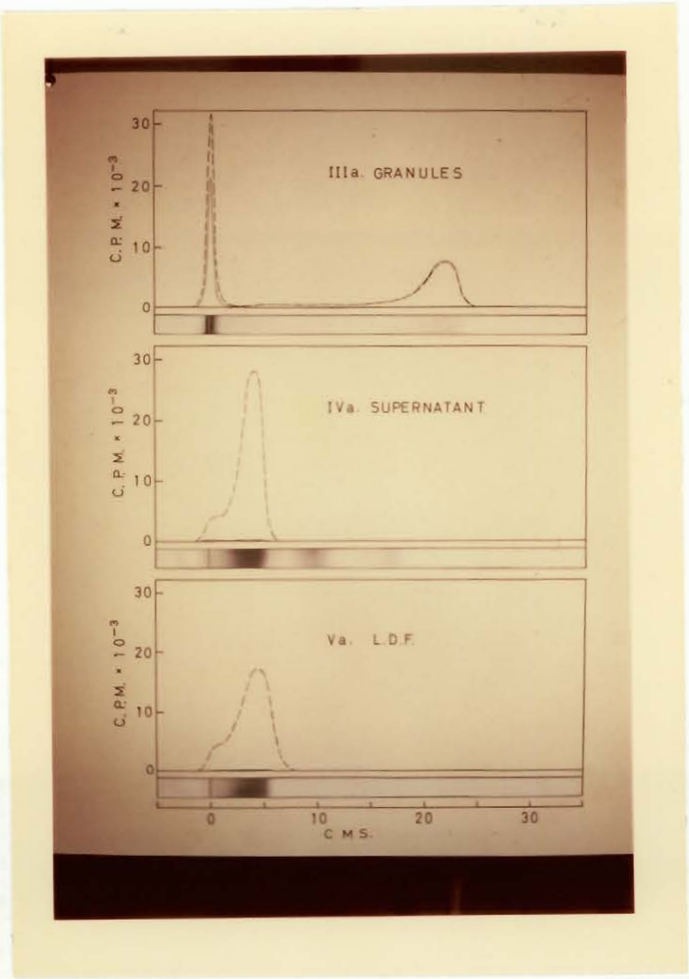
(IVa) Supernatant from segregation of granule material.

(Va) Low density fraction dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.


IIIa. GRANULES
IVa. SUPERNATANT
Va. L.D.F.
C.P.M. × 10⁻³
30
20
10
0
0
10
20
30
C.M.S.

appreciably from the line of application. It included a fair proportion of the protein-bound P^{32}. As will be shown below, this zone represented at least a large part of the lipovitellin fraction of the yolk.

The Amidoschwarz staining of the electropherograms of the dispersion of yolk in 1.0 M sodium chloride solution (Figure 16, IIa) revealed a pattern broadly similar to that obtained with the dispersion of yolk in 1.0% sodium chloride solution, except that the dispersion in 1.0 M sodium chloride yielded a distinctly stained PT zone which corresponded in position with a rather high proportion of the total protein-bound P^{32}. All the radioactivity in the PT zone was accountable as protein-bound P^{32}, at least within the limits of experimental error. At the same time, the proportion of the total protein-bound P^{32} in the P-1 zone was sharply reduced as compared with that observed for the P-1 zone of the yolk dispersion in 1.0% sodium chloride solution.

As regards zone P-2, there was some indication of a slight movement of the major materials of this zone from the line of application. This was perhaps a consequence of local and temporary solution of the lipovitellins in the sodium chloride solution used for dispersion, with subsequent reprecipitation as the salt concentration in

the region of application decreased during electrophoresis. It is seen from these results that dispersion of yolk in 1.0 M sodium chloride solution liberated much more phosvitin from the materials of zones P-1 and P-2 than did dispersion in 1.0% sodium chloride solution and that the phosvitin thus liberated was able to migrate relatively independently during electrophoresis.

Since the granule material was practically insoluble in 1.0% sodium chloride solution, electrophoretic examination of the granule material was restricted to the dispersion thereof in 1.0 M sodium chloride solution (Figure 17, IIIa). Amidoschwarz staining revealed a fast phosvitin or PT zone, as did also the distribution of protein-bound P^{32}. The densely-stained P-2 zone did not move appreciably from the line of application, but there were indications that a small fraction of it had proceeded for a short distance in the direction of the anode. This again suggested an effect of a temporary local ionic strength sufficient partly to dissolve the lipovitellins. The phosvitin zone was devoid of lipid staining and was also free from phospholipid P^{32}, within the limits of experimental error.

Amidoschwarz staining of the electropherogram of the supernatant (Figure 17, IVa) from the separation of granule

material showed the presence of α-, β-, and γ-livetin zones and of a deeply-staining lipoprotein zone corresponding in position with zone P-1. Zones corresponding to zones P-2 or PT were not perceptible. The results for distribution of P^{32} showed that the lipid phosphorus of the lipoprotein zone accounted for practically all of the P^{32} (see Figure 17, IVa and Table VII). Part of the small amount of protein-bound P^{32} that was present may have been due to the protein phosphorus of the LDF, although the phosphorus content of the lipid free part of the LDF may be as low as 0.06% (Martin, 1962).

A comparison of the distribution of P^{32} on electropherograms of fractions IIIa and IVa showed that the centrifugation had been remarkably efficient for the initial separation of the granule material (lipovitellins plus practically all the phosvitin) from the other proteins of egg yolk.

The paper electropherograms of the dispersion of LDF in 1.0 M sodium chloride solution (Figure 17, Va) revealed one lipoprotein zone corresponding in position with the P-1 zone of whole egg yolk and no perceptible trace of livetin zones was observed. As already mentioned (section 2.8.4.), the ultracentrifugally separated LDF gave no precipitin reaction whatever with rabbit antiserum

for α-, β-, and γ-livetins. The amount of protein-bound P^{32} in this LDF was relatively slight and corresponded to approximately 2% of the total protein-bound P^{32} of the whole yolk. It is evident that the centrifugal separation of this LDF has yielded the fraction in a state essentially free from phosvitin and γ-livetin. In this connection, the egg yolk lipoprotein prepared by McIndoe (1959b) would appear to be similar to the LDF described above. McIndoe (1959b) diluted yolk with 0.15 M sodium chloride solution, centrifuged to remove the particulate material and then diluted the supernatant with 9 volumes of water. A precipitate formed which was centrifuged to the surface. This lipoprotein material contained 88% lipid but only a trace of phosphoprotein. The centrifugal separation of the granule material described above would appear to correspond with the centrifugal separations described by Schmidt et al. (1956), by McIndoe (1959b), by Schjeide and Urist (1959), and by Burley and Cook (1961), and probably also with the separation of the 'lipovitellin' material as obtained by Evans and Bandemer (1961) after their initial centrifugation of a dilution of yolk with twice its weight of water. The lipovitellenin of Evans and Bandemer (1961) was separated by centrifugation (5000 r.p.m.) of the supernatant from the initial separation of 'lipovitellin'

after this supernatant had been dialyzed against distilled water and adjusted to pH 6.2. This lipovitellenin was deposited at the bottom of the centrifuge tube and the vitellenin obtained from it contained 0.5% phosphorus. These facts suggest that the lipovitellenin of Evans and Bandemer (1961) may have been slightly contaminated with denser material.

The results for electropherograms of subsamples IIa, IIIa, IVa, and Va provided strong evidence that the LDF of egg yolk accounts for most of the protein-staining material of zone P-1 and that the lipovitellin fraction accounted for most of the protein-staining material of zone P-2. The results also confirmed that zones P-1 and P-2 of whole egg yolk, as ordinarily resolved, both contained some of the phosvitin of egg yolk.

The paper electropherograms of the subnatant (subsample VIa) from the separation of LDF showed the presence of three livetin zones, corresponding in positions with α-, β-, and γ-livetins, and of no other zone. The electropherograms were devoid of more than minute traces of P^{32} which were mainly present at the line of application and probably corresponded with minute amounts of phospholipid. Clearly the separation of the livetin fractions from lipoprotein and phosvitin had been

remarkably effective despite the simplicity of the procedure. It was deemed unnecessary to present figures of the electropherograms of preparation VIa since the distribution of the zones was completely similar to that of the electropherogram shown in Figure 13K (section 2.8.1.).

Similar electrophoretic runs as the foregoing were performed on corresponding preparations of yolk sample B. The electrophoretic results, except those for lipid staining, for preparations I'a to V'a of yolk sample B are presented diagrammatically in Figure 18 and in Figure 19. The results were in close agreement with those for yolk sample A, apart from a slightly lower general level of radioactivity and similar descriptions apply.

2.8.5.3. Paper Electrophoretic Results in the Presence of Complexone.

The electrophoretic results for the preparations of Sample A that had been treated with Complexone (Ib to Vb), at the rate of 20 mgm. per ml. of original whole yolk, are presented diagrammatically in Figure 20 and in Figure 21. Addition of Complexone to serum is known to result in migration of the greater part of the phosvitin from the serum P-1 and P-2 zones to the 'fast' phosvitin

FIGURE 18

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Whole Yolk Sample B.

(I'a) Whole yolk dispersed in 1.0% sodium chloride solution.

(II'a) Whole yolk dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

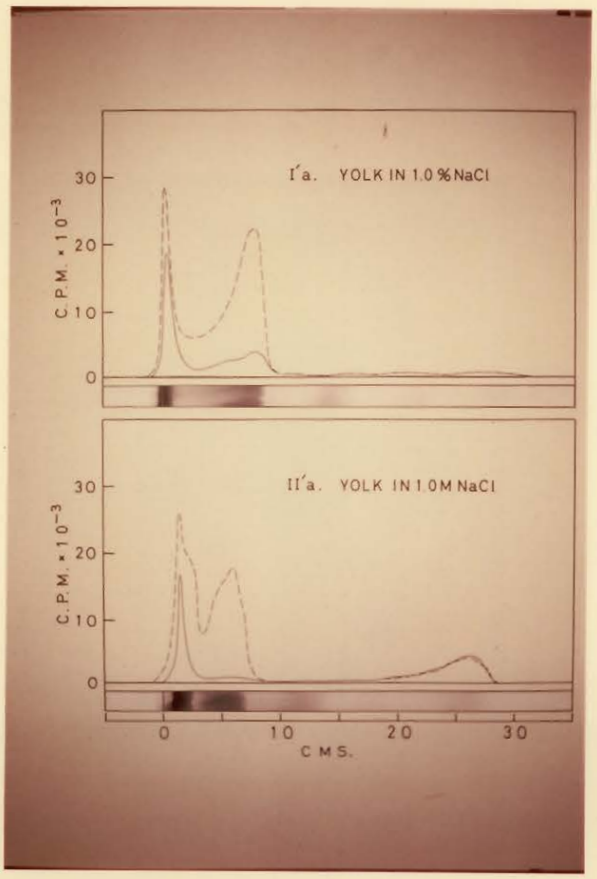

I'a. YOLK IN 1.0 % NaCl
II'a. YOLK IN 1.0 M NaCl
C.P.M. • 10⁻³
30
20
10
0
0
10
20
30
C M S.

FIGURE 19

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Centrifugal Fractions of Whole Yolk Sample B.

(III'a) Granule material dispersed in 1.0 M sodium chloride solution.

(IV'a) Supernatant from segregation of granule material.

(V'a) Low density fraction dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

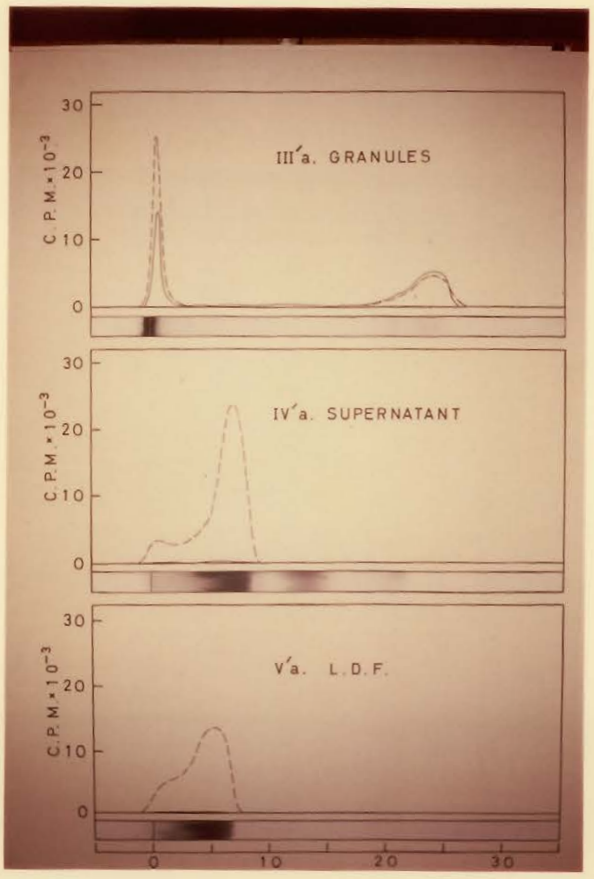

III'a. GRANULES
IV'a. SUPERNATANT
V'a. L.D.F.
C.P.M.×10⁻³
30
20
10
0
0
10
20
30

FIGURE 20

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Whole Yolk Sample A Pretreated with Complexone.

(Ib) Whole yolk dispersed in 1.0% sodium chloride solution.

(IIb) Whole yolk dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

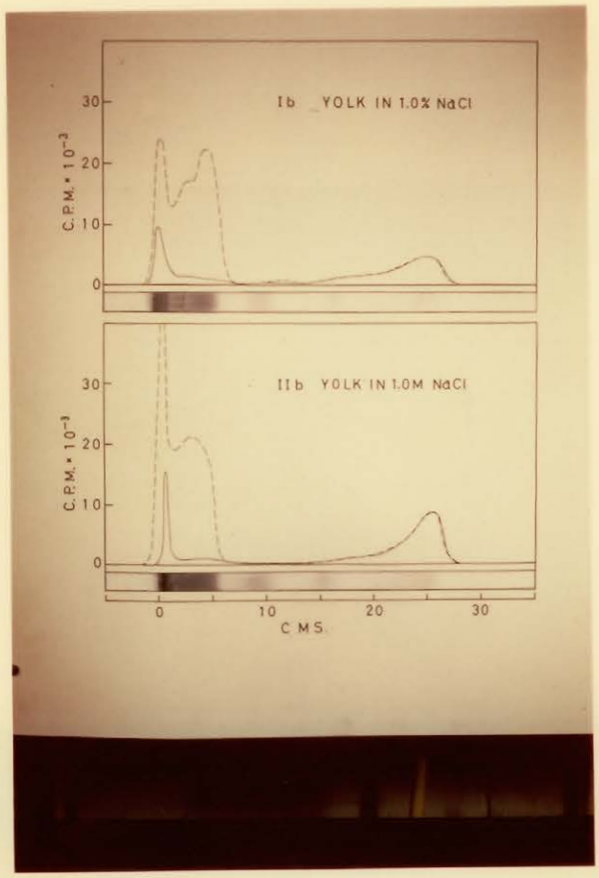

Ib YOLK IN 1.0% NaCl
IIb YOLK IN 1.0M NaCl
C.P.M. * 10⁻³
30
20
10
0
0
10
20
30
CMS.

FIGURE 21

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Centrifugal Fractions of Whole Yolk Sample A Pretreated with Complexone.

(IIIb) Granule material dispersed in 1.0 M sodium chloride solution.

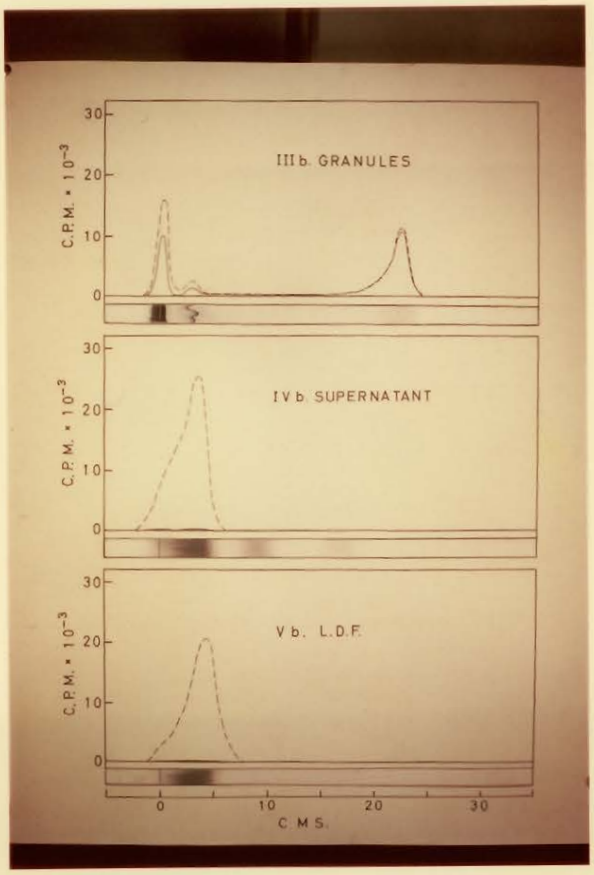
(IVb) Supernatant from segregation of granule material.

(Vb) Low density fraction dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.


III b. GRANULES
IV b. SUPERNATANT
V b. L.D.F.
C.P.M. × 10⁻³
30
20
10
0
0
10
20
30
C.M.S.

zone (Part I, section 2.7.5.4.). Comparable treatment of a dispersion of whole egg yolk in 1.0% sodium chloride solution (Figure 20, Ib) similarly increased the migration of protein-bound P^{32} from the lipoprotein regions, especially the P-1 zone, to the 'fast' phosvitin region, but still left an appreciable amount of protein-bound P^{32} in the P-2 region. In addition, the pretreatment with Complexone resulted in the appearance of a sharply-defined narrow line of protein staining between the P-2 zone and the most strongly-staining region of the P-1 zone (see Figure 20, Ib). This was associated with a small peak of total P^{32} and a perceptible minor peak of protein-bound P^{32}.

The electropherograms of Complexone-treated dispersion of egg yolk in 1.0 M sodium chloride solution (Figure 20, IIb) displayed the same trends as those shown by the dispersion of egg yolk in 1.0% sodium chloride solution, except that there was no indication of the narrow sharply-defined protein zone between the P-1 and P-2 zones. The addition of Complexone increased the amount of protein-bound P^{32} in the 'fast' phosvitin zone as compared to electropherograms run in the absence of Complexone (see Figure 16, IIa).

Pretreatment of the dispersion of granule material

in 1.0 M sodium chloride solution with Complexone (Figure 21, IIIb) appeared to increase slightly the proportion of protein-bound P^{32} that migrated from the P-2 region, but part of this protein-bound P^{32} was associated with the sharply-defined narrow line of protein staining material in advance of the P-2 zone, which corresponded with the similar narrow zone mentioned above. At the present time no adequate explanation is available for this effect of Complexone treatment, but this point calls for further investigation.

The results for the supernatant and the LDF preparations (Figure 21, IVb and Vb) showed that the pretreatment with Complexone did not sensibly affect the paper electrophoretic behavior of these fractions, which were entirely similar to those of the corresponding fractions from yolk not treated with Complexone. The same was true of the subnatant from the separation of LDF (fraction VIb), which it was not considered necessary to show diagrammatically.

Similar electrophoretic runs as the foregoing were performed on preparations I, II, and III of yolk sample B following pretreatment with Complexone. The results, presented diagrammatically in Figure 22 and in Figure 23, were in close agreement with the results of the

FIGURE 22

Distribution of Total P^{32} and of Protein-bound P^{32} on Electropherograms of Dispersions of Whole Yolk Sample B Pretreated with Complexone.

(I'b) Whole yolk dispersed in 1.0% sodium chloride solution.

(II'b) Whole yolk dispersed in 1.0 M sodium chloride solution.

Strips stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.

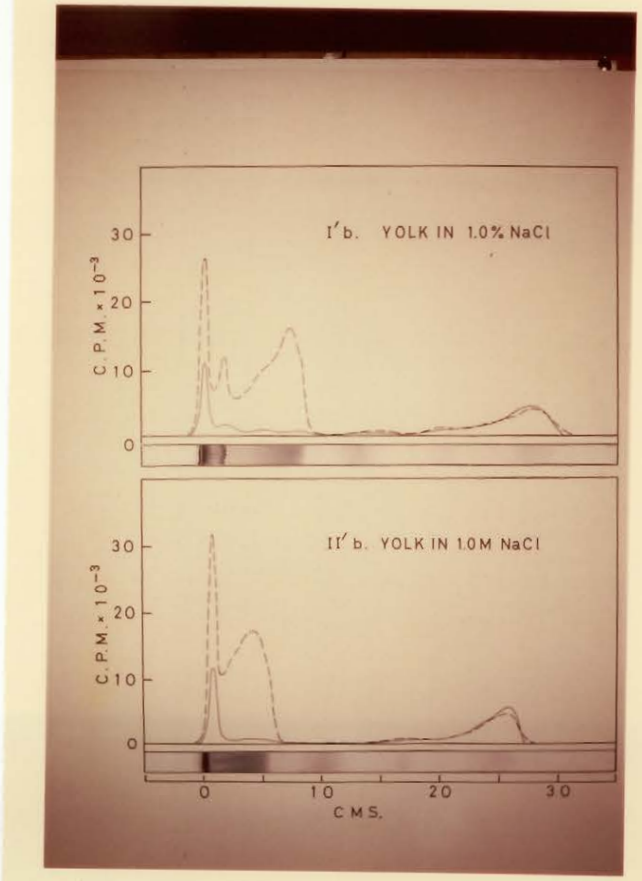

I′b. YOLK IN 1.0% NaCl
II′b. YOLK IN 1.0M NaCl
C.P.M. × 10⁻³
30
20
10
0
0
10
20
30
CMS.

FIGURE 23

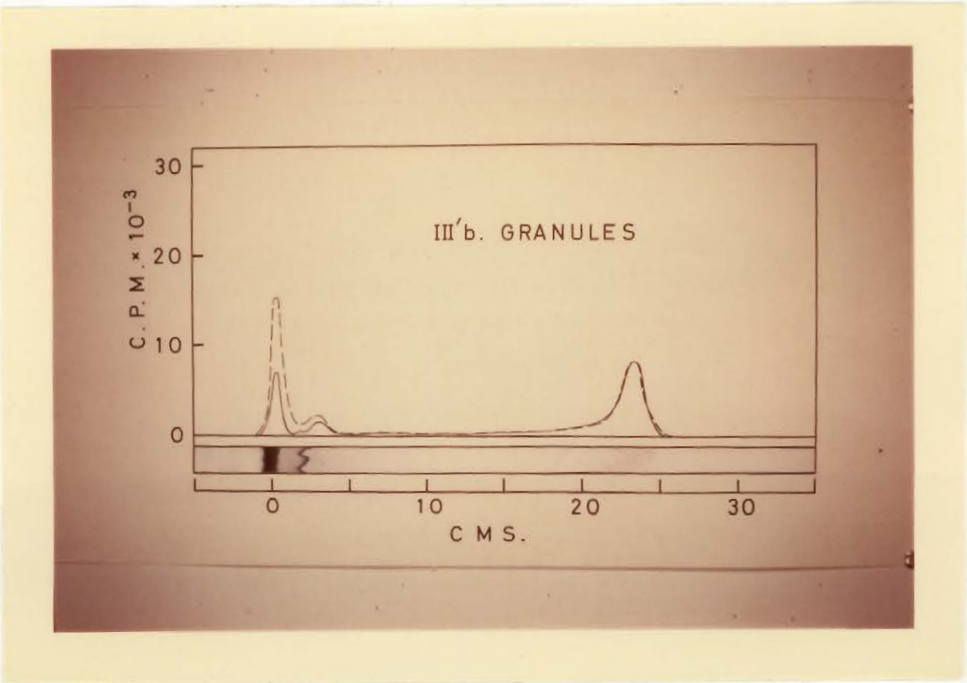
Distribution of Total P^{32} and of Protein-bound P^{32} on an Electropherogram of a Dispersion of a Centrifugal Fraction of Whole Yolk Sample B Pre-treated with Complexone.

(III'b) Granule material dispersed in 1.0 M sodium chloride solution.

Strip stained with Amidoschwarz.

Broken line = total P^{32}.

Solid line = protein-bound P^{32}.


III'b. GRANULES
C.P.M. × 10⁻³
30
20
10
0
0
10
20
30
C M S.

corresponding preparations of yolk sample A, apart from a slightly lower general level of radioactivity and similar descriptions apply. Electrophoreses of the supernatant, LDF, and subnatant preparations (IV, V, and VI) of yolk sample B treated with Complexone were omitted, since the electropherograms of similar preparations of yolk sample A pretreated with Complexone showed the same protein staining, protein mobility and P^{32} distribution as did those of the corresponding untreated preparations.

2.8.5.4. Quantitative Results for Total P^{32} and for Protein-bound P^{32} on Paper Electropherograms of Various Preparations of Egg Yolk.

The results for the total P^{32} and the protein-bound P^{32} on electropherograms of the various preparations of yolk samples A and B are presented in Table VII. The values for total P^{32} showed reasonably good agreement as between the dispersion of whole yolk in 1.0% sodium chloride solution or in 1.0 M sodium chloride solution without or with Complexone.

Protein-bound P^{32} accounted for slightly more than one-third of the total P^{32} of the dispersions of whole egg yolk in saline in the absence of Complexone and slightly less than one-third of the total P^{32} of the

TABLE VII

TOTAL P^{32} AND PROTEIN-BOUND P^{32} ON ELECTROPHEROGRAMS OF VARIOUS PREPARATIONS OF EGG YOLK SAMPLES A AND B. RADIOACTIVITY CALCULATED TO THE BASIS OF C.P.M. x 10^{-4} PER ML. EGG YOLK.

Preparation		Without Complexone			With Complexone		
		Sample A	Sample B	Average	Sample A	Sample B	Average
I Whole yolk in 1.0% sodium chloride solution	Total	231.4	201.2	216.3	235.3	195.3	215.3
	Protein-bound	89.2	69.0	79.1	74.6	65.8	70.2
	Protein-bound / Total x 100	38.5	34.3	36.6	31.7	33.7	32.6
II Whole yolk in 1.0 M sodium chloride solution	Total	227.5	190.7	209.1	276.6	179.6	228.1
	Protein-bound	75.1	68.6	71.8	85.3	59.0	72.1
	Protein-bound / Total x 100	33.0	35.9	34.3	30.8	32.8	31.6
III Granule material	Total	96.4	70.7	83.5	89.7	62.8	76.2
	Protein-bound	85.3	59.2	72.3	68.8	50.1	59.4
	Protein-bound / Total x 100	88.4	83.9	86.6	76.7	79.9	78.0
IV Supernatant from segregation of granule material	Total	124.3	126.9	125.6	141.1	-	141.1*
	Protein-bound	1.5	2.5	2.0	1.5	-	1.5*
	Protein-bound / Total x 100	1.2	2.0	1.6	1.1	-	1.1
V LDF	Total	107.8	94.7	101.2	115.8	-	115.8*
	Protein-bound	1.6	1.3	1.4	1.4	-	1.4*
	Protein-bound / Total x 100	1.5	1.3	1.4	1.2	-	1.2
VI Subnatant from separation of LDF	Total	1.8	1.8	1.8	2.0	-	2.0*
	Protein-bound	0.0	0.0	0.0	0.0	-	0.0*
	Protein-bound / Total x 100	0.0	0.0	0.0	0.0	-	0.0

* Data for sample A only.

corresponding preparations in the presence of Complexone.

The protein-bound P^{32} recovered in the granule material (preparation III) accounted for over 90% of the protein-bound P^{32} of the whole yolk dispersions in the absence of Complexone. This observation was in relatively good agreement with the corresponding recovery of protein-bound phosphorus as reported in Table VI. The recovery of protein-bound P^{32} in the granule material in the presence of Complexone appeared to be distinctly less, i.e., about 84%. This observation suggests that treatment with Complexone may have resulted in the liberation of some P^{32} from the granule material in forms that migrated right off the electropherograms.

Protein-bound P^{32} accounted for an average of 86.6% of the total P^{32} of the granule material but for an average of only 78.0% of the total P^{32} of the granule material treated with Complexone. This observation affords some support for the suggestion that Complexone treatment may result in the liberation of P^{32} from the granule material in forms that migrate off the electropherograms.

Comparison of the data for preparations IV and V showed that the total P^{32} recovered from the LDF accounted for an average of 81% of the total P^{32} of the supernatant

in the absence of Complexone and for an average of 82% in the presence of Complexone. Similar considerations apply to the corresponding recoveries of protein-bound P^{32}. The recoveries of P^{32} in preparation VI did not account for the differences. Part at least of the difference arose from experimental loss (see discussion of data for Table VI, section 2.8.5.1.) and there was no reason in this instance to suspect loss of P^{32} by liberation in forms that migrated off the electropherograms.

An interesting observation was made on the electropherograms of subsample VI'a (the subnatant from separation of LDF) of yolk sample B. Two different aliquots were taken from this preparation for electrophoresis. Both electropherograms revealed a very fast P^{32} zone. This zone had a mobility similar to that of the serum 'zone A' described in Part I, section 2.7.6. of this thesis. The quantity of P^{32} in this zone was of the order of 3,000 c.p.m. per ml. egg yolk (3,367 c.p.m. per ml. for one electropherogram and 3,134 c.p.m. per ml. for the other electropherogram). This fast yolk P^{32} zone was not observed on any of the other electropherograms of the egg yolk preparations studied, including the subnatant from separation of LDF (subsample VIa) of yolk sample A. This zone was observed only on unextracted electropherograms, hence the material of this

zone was extracted by the reagents used for extracting lipid. This zone did not stain with Amidoschwarz or with Oil Red O. It was neither a protein nor a lipid but it was not identified further.

2.8.5.5. Quantitative Results for Distribution of Protein-bound P^{32} Between Certain Paper Electrophoretic Zones.

Average results for the distribution of protein-bound P^{32} between zones P-2, P-1 and PT and for the specific activities of the protein-bound phosphorus of these zones are presented in Table VIII.

The average specific activities of the protein-bound phosphorus in the various zones were sufficiently uniform to warrant the conclusion that the distributions of protein-bound P^{32} and of protein-bound phosphorus were substantially identical. Three divergently lower values for specific activity were associated with zones that contained relatively small absolute amounts of phosphorus, hence these lower values might well be due to analytical error in the determinations of phosphorus. Consequently, the differences of these three values from the overall average specific activity of 4.5×10^{-5} c.p.m. per mgm. phosphorus were considered not to constitute evidence of a real difference

TABLE VIII

DISTRIBUTION OF TOTAL PROTEIN-BOUND PHOSPHORUS AND P^{32} AMONG THE MAJOR PHOSPHOPROTEIN-CONTAINING ZONES OF PAPER ELECTROPHEROGRAMS OF EGG YOLK PREPARATIONS. AVERAGE VALUES FOR YOLK SAMPLES A AND B ALL EXPRESSED PER ML. ORIGINAL YOLK. VALUES IN BRACKETS REPRESENT P^{32} OR PHOSPHORUS EXPRESSED AS PERCENTAGES OF RELEVANT VALUES.

Preparation		Without Complexone				With Complexone				
		P-2	P-1	PT	Sum	P-2	P-?	P-1	PT	Sum
Yolk in 1.0% sodium chloride solution	P^{32}, c.p.m. x 10^{-4}	31.4	27.8	9.2	68.4	15.6	4.2	4.5	34.5	58.8
	P^{32}, % of sum	(46)	(41)	(13)	(100)	(26)	(7)	(8)	(59)	(100)
	P, mgm.	0.65	0.62	0.18	1.45	0.35	0.11	0.15	0.84	1.45
	P, % of sum	(45)	(43)	(12)	(100)	(24)	(8)	(10)	(58)	(100)
	Spec. act., c.p.m. x 10^{-5} per mgm. P	4.8	4.5	5.1	4.7	4.5	3.8	3.0	4.1	4.1
Yolk in 1.0 M sodium chloride solution	P^{32}, c.p.m. x 10^{-4}	22.7	5.4	33.2	61.3	16.7	-	4.0	40.0	60.7
	P^{32}, % of sum	(37)	(9)	(54)	(100)	(27)	-	(7)	(65)	(100)
	P, mgm.	0.53	0.20	0.80	1.53	0.35	-	0.16	0.85	1.36
	P, % of sum	(35)	(13)	(52)	(100)	(26)	-	(12)	(62)	(100)
	Spec. act., c.p.m. x 10^{-5} per mgm. P	4.3	2.7	4.2	4.0	4.8	-	2.5	4.7	4.5
Granule material in 1.0 M sodium chloride solution	P^{32}, c.p.m. x 10^{-4}	20.2	-	39.3	59.5	12.6	2.7	-	33.6	48.9
	P^{32}, % of sum	(34)	-	(66)	(100)	(26)	(6)	-	(69)	(100)
	P, mgm.	0.45	-	0.72	1.17	0.28	0.06	-	0.80	1.14
	P, % of sum	(38)	-	(62)	(100)	(25)	(5)	-	(70)	(100)
	Spec. act., c.p.m. x 10^{-5} per mgm. P	4.5	-	5.5	5.1	4.5	4.3	-	4.2	4.3

in specific activity of the phosphorus of these minor zones.

The presence of Complexone or the use of 1.0 M sodium chloride solution as the dispersion medium increased the proportion of the total protein-bound P^{32} that migrated from zones P-2 and P-1 to zone PT. As a consequence, the proportion of total protein-bound P^{32} in the PT zone increased from about 13% to between 50% and 60%. However, one-quarter to one-third of the protein-bound P^{32} remained in the P-2 zone. The main effect of Complexone or of 1.0 M sodium chloride solution was to move protein-bound P^{32} from the P-1 zone to the PT zone. The presence of Complexone in the yolk dispersion in 1.0 M sodium chloride solution increased slightly the proportion of protein-bound P^{32} in the PT zone with a concomitant decrease of protein-bound P^{32} in the P-2 zone as compared to the dispersion of yolk in 1.0 M sodium chloride solution in the absence of Complexone.

The presence of Complexone in the dispersion of granule material in 1.0 M sodium chloride solution had much the same quantitative effect as it had on the dispersion of yolk in 1.0 M sodium chloride solution, except for the appearance of about 6% of the protein-bound P^{32} in the narrow extra zone (P-?) on the electropherograms

of the Complexone-treated material.

2.8.6. The Proportions of Protein Nitrogen and Protein Phosphorus in Zones P-1 and P-2 of Paper Electropherograms of Egg Yolk and of Ultracentrifugal Fractions of Egg Yolk.

The P-1 and P-2 zones of alcohol-ether extracted paper electropherograms of the various preparations of yolk samples A and B were analyzed for nitrogen and for phosphorus. The results are presented in Table IX.

The results for the yolk dispersion in 1.0% sodium chloride solution showed a somewhat greater recovery of protein nitrogen in zone P-1 than reported previously (section 2.8.3.), but the proportion of protein nitrogen recovered in zone P-2 was similar to that found previously, as were the results for protein-bound phosphorus. Consequently, the ratio protein nitrogen:protein phosphorus for zone P-1 was higher than the value reported previously although the result further confirmed that zone P-1 as resolved in veronal buffer contains phosvitin. The results also demonstrated the effect of Complexone and of 1.0 M sodium chloride solution in leading to greater migration of presumptive phosvitin phosphorus from both P-1 and P-2 zones with a consequent raising of the relevant ratios of

TABLE IX

PROPORTIONS OF PROTEIN NITROGEN AND OF PROTEIN PHOSPHORUS IN ZONES P-1 AND P-2 OF PAPER ELECTROPHEROGRAMS OF EGG YOLK AND ULTRACENTRIFUGAL FRACTIONS OF EGG YOLK IN THE ABSENCE AND IN THE PRESENCE OF COMPLEXONE. AVERAGE VALUES FOR SAMPLES A AND B. THE NUMBERS IN BRACKETS INDICATE THE NUMBER OF ELECTROPHEROGRAMS ANALYZED.

Preparation		Without Complexone		With Complexone	
		Zone P-1	Zone P-2	Zone P-1	Zone P-2
Yolk in 1.0% sodium chloride solution	Protein N, mgm. per ml. yolk	10.74 (10)	9.91 (10)	7.49 (4)	8.33 (4)
	Protein N, % of total	41.0	37.8	28.6	31.8
	Protein P, mgm. per ml. yolk	0.46 (10)	0.62 (10)	0.10 (4)	0.31 (4)
	Ratio, protein N:protein P	23.3	16.0	74.9	26.9
Yolk in 1.0 M sodium chloride solution	Protein N, mgm. per ml. yolk	6.92 (8)	12.70 (8)	6.79 (6)	12.23 (6)
	Protein N, % of total	26.4	48.5	25.9	46.7
	Protein P, mgm. per ml. yolk	0.13 (8)	0.43 (8)	0.11 (6)	0.26 (6)
	Ratio, protein N:protein P	43.2	29.9	61.7	47.0
Granule material in 1.0 M sodium chloride solution	Protein N, mgm. per ml. yolk		9.84 (6)		8.88 (6)
	Protein N, % of total		37.6		33.9
	Protein P, mgm. per ml. yolk		0.37 (6)		0.25 (6)
	Ratio, protein N:protein P		26.6		35.5
Supernatant from segregation of granule material	Protein N, mgm. per ml. yolk	6.69 (3)			
	Protein N, % of total	25.5			
	Protein P, mgm. per ml. yolk	0.13 (3)			
	Ratio, protein N:protein P	51.6			
LDF	Protein N, mgm. per ml. yolk	4.40 (6)			
	Protein N, % of total	16.8			
	Protein P, mgm. per ml. yolk	0.08 (6)			
	Ratio, protein N:protein P	55.0			

protein nitrogen:protein phosphorus. The results for protein nitrogen of the yolk dispersion in 1.0 M sodium chloride solution showed a somewhat different distribution of protein nitrogen between zones P-1 and P-2 than did the yolk dispersion in 1.0% sodium chloride solution. The quantity of protein nitrogen in zone P-2 was considerably increased. From the results of the dispersion of the granule material it would appear that zone P-2 on the electropherograms of the yolk dispersion in 1.0 M sodium chloride solution contained protein material other than the lipovitellins and phosvitin. A comparison of the results for protein nitrogen in zone P-1 of the various preparations suggested that if the LDF is lipovitellenin, then the P-1 zone of the other preparations was contaminated with other materials (presumably γ-livetin and phosvitin). The quantitative results described above provide further confirmation of the effects of Complexone and of 1.0 M sodium chloride in liberating phosvitin from complexes with the materials of zones P-1 and P-2 so that it can migrate freely on paper electrophoresis. The results for protein nitrogen also offer further evidence that zones P-1 and P-2 contained materials other than lipovitellenin and lipovitellin.

2.8.7. The Effects of Increasing the Ionic Strength of the Veronal Buffer on the Mobilities of Egg Yolk Proteins.

Bernardi and Cook (1960a) used a veronal buffer pH 9.0, μ = 0.3 (containing 0.25 M sodium chloride) for their free electrophoretic studies of the high density fraction of egg yolk. They stated that the high ionic strength was necessary to maintain the lipoproteins in solution. Bernardi and Cook (1960a) were able to separate electrophoretically the two lipoproteins (α- and β-lipovitellin) by the use of this high ionic strength buffer. The use of veronal buffer pH 8.6, μ = 0.05, had permitted the resolution of a single lipovitellin zone (P-2) on the paper electropherograms described previously in this thesis. Accordingly, the effects of increasing the ionic strength of the veronal buffer pH 8.6 were studied. Veronal buffers of ionic strengths 0.050, 0.062, 0.075, 0.100, 0.150, 0.200, and 0.300 were prepared by the addition of appropriate quantities of sodium chloride. Dispersions of egg yolk in 1.0% sodium chloride solution and in 1.0 M sodium chloride solution were used for electrophoresis. The results were as follows:

(a) Paper electropherograms of the dispersion of yolk in 1.0% sodium chloride solution.

$\mu = 0.050$. The results were entirely similar to those described above.

$\mu = 0.062$. The usual five zones (X,Y,Z,P-1 and P-2) plus two faintly-staining phosvitin zones were observed. The mobilities of all the zones except zone P-2 were somewhat less than those observed when the electrophoresis was done in veronal buffer, $\mu = 0.050$.

$\mu = 0.075$. The mobilities of all the zones except zone P-2 were greatly reduced. A γ-livetin (Z) zone was not observed presumably as a consequence of the lesser mobilities. There were still indications of two phosvitin zones.

$\mu = 0.100$. A γ-livetin zone was not observed and the mobilities of the zones were decreased further, with the exception of zone P-2. A single phosvitin zone was observed. Some of the material of zone P-2 had migrated away from the line of application.

$\mu = 0.150$. The electropherograms showed a phosvitin zone, one livetin zone and zones P-1 and P-2 which were not so well separated as at lower ionic strengths. The P-2 zone had moved slightly from the line of application.

$\mu = 0.200$. A phosvitin zone and one general protein zone were observed on the electropherograms. The leading portion of the protein zone evidently represented livetins since it did not contain lipid-staining material. All of the protein material, as shown by Amidoschwarz staining, had moved away from the line of application.

$\mu = 0.300$. All of the material had moved away from the line of application. A phosvitin zone and one general protein zone with associated lipid staining was resolved.

(b) Paper electropherograms of the dispersion of yolk in 1.0 M sodium chloride solution.

Similar descriptions apply for the electropherograms of the dispersion of yolk in 1.0 M sodium chloride solution when run in buffers of similar ionic strengths, but with the addition that a narrow line of deeply-staining protein material was present at lower ionic strengths in about the position of zone P-? (section 2.8.5.3.).

The foregoing results demonstrated that it was possible to move the material of the P-2 zone from the line of application by the use of veronal buffers of sufficiently high ionic strengths. The high ionic

strength buffers decreased the mobilities of the livetins so that they were not separated from the lipoproteins. Since the proteins migrated as a single zone when the ionic strength was high enough to cause the P-2 material to move from the line of application, it was impossible to observe whether any separation of the lipovitellins had occurred. Hence, it would be desirable to separate the granule material from the whole yolk by a preliminary ultracentrifugation before electrophoresis in order to determine whether paper electrophoresis in the high ionic strength buffers might separate the lipovitellins.

2.9. General Discussion.

The experiments described above have shown that the main protein-staining component of zone P-1 is the LDF (or 'lipovitellenin'); that zone P-1, under normal conditions of electrophoresis in veronal buffer, includes some phosphoprotein, presumably largely phosvitin, plus most of the γ-livetin fraction. Similarly, zone P-2, as ordinarily resolved, comprises all of the lipovitellin fraction plus some phosvitin, but it seems unlikely that zone P-2 includes any significant proportion of the γ-livetin fraction. The movement of zone P-2 was slight, if any, on paper electropherograms of dispersions of yolk

in 1.0% sodium chloride solution. The movement of zone P-2 appeared more appreciable for yolk dispersions in 1.0 M sodium chloride solutions, but this may be ascribed to local temporary solution of the lipovitellins due to a relatively high initial ionic strength at the line of application. Confirmation of this surmise was provided by the results of paper electropherograms run in veronal buffers of high ionic strength.

The results reported above also suggest that, in order to obtain a P-1 zone free from phosvitin, it is necessary to make a preliminary ultracentrifugal separation of granule material from a dispersion of yolk in water or in 0.16 M sodium chloride solution. The LDF may subsequently be separated and then dispersed in 1.0 M sodium chloride solution for electrophoresis if desired. Indeed, a simple preliminary centrifugal separation of the granule material might prove advantageous in routine examination of egg yolks by paper electrophoretic methods. However, while the granule material may be segregated readily by preliminary ultracentrifugation, its resolution by paper electrophoresis calls for further study, especially in relation to the possible further resolution of the lipovitellins.

The precise relationships of the materials of the P-1 and P-2 zones resolved on paper electropherograms in veronal

buffer to the yolk X_1-phosphoprotein and yolk X_2-lipoglycoprotein fractions described by Urist et al. (1958) and by Schjeide and Urist (1959) are not completely clear. Schjeide and Urist (1959) have stated that the terms 'X_1' and 'X_2' were used as a device to describe egg granule components resolved on the basis of density, and they have suggested that the phosvitin of Mecham and Olcott (1949) is a relatively pure preparation of centrifugally-resolved yolk X_1-phosphoprotein, a suggestion which might be taken to indicate that they regard their yolk X_1-phosphoprotein as an impure phosvitin. Later, Schjeide and Urist (1960) state that they used the term X_1-phosphoprotein in place of phosvitin and the term X_2-lipoglycoprotein in place of lipovitellin. Ultracentrifugation of whole yolk by itself results in the segregation of all the phosphoprotein and of all the lipovitellins as an insoluble fraction (Schmidt et. al., 1956; Burley and Cook, 1961). It is difficult to resist the view that the natural form of occurrence of phosvitin in the yolk is as an insoluble complex with either the lipovitellins, or with other cations present, or with both. The phosvitin can be dissociated from the natural complex or association by dispersing it in 1.0 M sodium chloride solution, and much of the phosvitin can then be separated from the lipovitellins by paper electrophoresis as shown above. It is possible that this

separation may correspond in some degree to the ultra-centrifugal separation of the constituents designated X_1-phosphoprotein and X_2-lipoglycoprotein by Schjeide and Urist (1959). It seems that the natural phosvitin-lipovitellin complex may be liable to more or less dissociation when dispersed in almost any solvent except perhaps isotonic saline. Once the natural complex in the granule material has been dissolved, it may be difficult to decide whether differences between X_1-phosphoprotein and X_2-lipoglycoprotein as centrifugally resolved from such solutions on the one hand, and purified phosvitin and lipovitellin preparations on the other hand, are a consequence of denaturation or a consequence of interactions between phosvitin and lipovitellins. In this thesis the granule material separated by centrifugation has been regarded as consisting almost entirely of the lipovitellins plus the phosvitin of the yolk. If ultra-centrifugation for the segregation of the insoluble granule material is unduly prolonged, then the segregate may be more or less contaminated through ultracentrifugal sedimentation of γ-livetin, but this may be minimized by restricting the time of the initial segregation of the granule material.

The rising fraction, LDF, may be obtained substantially

free from phosvitin provided the initial segregation of the granule material is made in a dispersion of low salt content, i.e., by using water or 0.16 M sodium chloride solution. The livetins remain in the subnatant.

It should be noted that the lipovitellins of egg yolk may be typical of a group of lipoproteins of relatively low lipid:protein ratio which may be regarded as macromolecules; whereas the LDF, or lipovitellenin, of egg yolk may be typical of another group of proteins of relatively high lipid:protein ratio and which are more properly regarded as microemulsions (Cook and Martin, 1962). Such microemulsions are likely to be heterogeneous, and the appearance presented by zone P-1 on paper electropherograms of whole egg yolk and of the LDF zone on paper electropherograms thereof is in accordance with such a view. If the lipoprotein material prepared by McIndoe (1959b) was lipovitellenin, then his results would support further this view because he found that, in the analytical ultracentrifuge, the lipoprotein material rose rapidly at specific gravity 1.006 and tended to separate into two fractions.

3. SUMMARY

Six protein zones have been distinguished on paper electropherograms of saline suspensions of egg yolk run in aqueous veronal buffer. The six protein zones have been identified provisionally, and in order of decreasing mobility under the conditions used, as phosvitin, α-livetin, β-livetin, γ-livetin, lipoprotein P-1 (a lipovitellenin complex), and lipoprotein P-2 (a lipovitellin complex). Evidence is submitted that the foregoing two major lipoprotein zones from egg yolk are closely similar to the two lipoprotein zones from sera of laying hens or estrogenized pullets.

Staining for ester-linked protein phosphorus (ELPP) gave positive reactions with both the main lipoprotein zones (P-1 and P-2) of paper electropherograms of egg yolk in certain buffers, including veronal buffer pH 8.6. Dispersion of the yolk in 1.0 M sodium chloride solution or treatment with complexing agents (Complexone, veronal-citrate buffer) resulted in the appearance of a greater proportion of the ELPP in the phosvitin (PT) zone. The distribution of the livetin zones suggested that the lipoprotein zone P-1 overlapped the greater part of the γ-livetin zone. This suggestion was supported by the results of immunoelectrophoretic experiments.

Yolks in which the phosphorus had been labelled with P^{32} were separated by ultracentrifugation into (a) granule material; (b) low density fraction (LDF); and (c) the soluble livetin fraction. Paper electrophoretic studies of whole egg yolk and of these preparations showed (a) that the P-1 zone of electropherograms of whole egg yolk in veronal buffer comprised all of the LDF plus most of the γ-livetin plus a proportion of the phosvitin that depended on the strength of the salt solution used to disperse the yolk and the absence or presence of complexing agents; and (b) that the P-2 zone comprised the lipovitellin fraction together with some phosvitin. Dispersions of the yolk in 1.0 M sodium chloride solution, pretreatment of the yolk dispersion with ethylenediaminetetraacetate (Complexone), or use of veronal-citrate buffer led to the appearance of relatively high proportions of the total protein-bound phosphorus and P^{32} in the 'fast' phosvitin (PT) zone.

Nitrogen and phosphorus analyses of the lipoprotein zones, P-1 and P-2, of paper electropherograms of egg yolk confirmed that these zones included some phosphoprotein material (presumably phosvitin) when the electrophoresis was done in veronal buffer, since similar protein nitrogen:protein phosphorus ratios were calculated for the

two zones. The effects of dispersing yolk in 1.0 M sodium chloride solution and of treatment with Complexone were confirmed by similar analyses of the appropriate electropherograms because the ratio protein nitrogen:protein phosphorus increased with increase of the amount of phosvitin that migrated from the P-1 and P-2 zones to the phosvitin zone. The nitrogen value for the P-2 zone of paper electropherograms of yolk dispersion in 1.0 M sodium chloride solution was higher than the value obtained for the P-2 zone of electropherograms of yolk dispersed in 1.0% sodium chloride solution or of granule material dispersed in 1.0 M sodium chloride solution.

The material of the P-2 zone of paper electropherograms of yolk was relatively immobile when veronal buffer, $\mu = 0.05$ was used. Use of high ionic strength veronal buffers resulted in movement of this material from the line of application, but at the same time the mobilities of the other protein fractions were decreased to such an extent that no separation of other protein zones occurred.

CLAIMS TO ORIGINAL RESEARCH

1. The development of techniques whereby two major lipoprotein zones on paper electropherograms of sera from laying or estrogenized fowl may be resolved consistently in aqueous veronal buffer.

2. The correlation of two previously recognized and one newly observed protein zones on paper electropherograms of egg yolk with α-, β-, and γ-livetins.

3. The observation of a new zone, or zones, on paper electropherograms of egg yolk and their recognition as due to phosvitin.

4. The demonstration that a phosvitin zone may be distinguished on paper electropherograms of a solution of the crude phospholipophosphoprotein precipitate obtained by dilution of serum of estrogenized fowl.

5. The first application of staining reactions for ester-linked protein phosphorus to the detection of phosphoprotein zones on paper electropherograms of serum and egg yolk.

6. The demonstration that the movement of phosvitin on paper electropherograms of avian sera or egg

yolk in various buffers may be followed qualitatively by staining for ester-linked protein phosphorus; and the demonstration thereby that the movement of phosvitin is greatly affected by the presence or the absence of complexing agents in the buffer.

7. The elucidation of the movement of protein-bound and phospholipid phosphorus on paper electropherograms of avian sera and egg yolk by use of serum and yolk components labelled with P^{32}.

8. The demonstration, by combined ultracentrifugal and paper electrophoretic techniques, and in conjunction with the application of P^{32}-labelling of the yolk, (a) that zone P-2, as ordinarily resolved in veronal buffer, corresponds to the lipovitellins plus some of the phosvitin; and (b) that zone P-1, as ordinarily resolved in veronal buffer, corresponds to the lipovitellenin plus part of the γ-livetin fraction plus part of the phosvitin; the foregoing findings being supported by quantitative observations on the nitrogen, phosphorus and P^{32} contents of the various centrifugal and electrophoretic fractions examined. (The author does not claim originality

in respect of the immunoelectrophoretic evidence, which was obtained by Miss Chi-Ching Mok).

9. The provision of further evidence, based on staining for ester-linked protein phosphorus, that the pre-albumin components of embryonic fowl sera are not phosphoproteins.

10. Paper electropherograms of serum from an estrogenized pullet were run in veronal-citrate buffer, the phosphorus of the bird having been labelled with P^{32}. A moiety of the serum P^{32} equivalent to one-third of the total serum P^{32} was observed to run as a zone well in advance of the phosvitin zone. The phosphorus compound responsible was not inorganic phosphate, and it was removed from the paper by the reagents used for lipid extraction. It stained neither with Amidoschwarz nor with Oil Red O nor with ninhydrin, but gave a strong reaction for ELPP. It was not, therefore, a protein or a lipid but it was not further identified.

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