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Filter Paper Electrophoresis of Serum Proteins of the Domestic Fowl.* (20642)

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(Introduced by C. P. Leblond.)
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Filter paper electrophoresis(1,2) of avian serum proteins has demonstrated (a) the separation of albumin, 2 α-globulins, a β-globulin, and a γ-globulin from the sera of cockerels and immature pullets; and (b) the appearance of a presumptive lipovitellin (PLV) fraction in the sera of laying hens or estrogenized immature pullets(3). It has further been shown(3) that electrophoresis in a veronal buffer made up with 20% methanol results in the separation of a new slow-moving fraction from the sera of estrogenized immature pullets or laying hens. This fraction is not present in comparable electropherograms of sera of immature pullets; it is poorer in lipid than the PLV fraction and rich in phosphorus. For convenience this fraction has been designated presumptive phosphoprotein (PP).

The following additional observations are reported in the present communication: (a) the presence of a third α-globulin fraction on electropherograms of fowl sera; (b) the application of the Feulgen plasmalogen reaction to the electropherograms; (c) the double nature of the PP fraction; and (d) the results of studies using P^{32} labeled inorganic phosphate.

Methods. Electrophoresis was performed as described previously(3) with the exception that the apparatus was kept in a cold room at 6° to 7°C. At this lower temperature it was necessary to raise the potential to 200 volts in order to obtain 8 to 9 ma. with filter paper 17 inches in width and 16 inches in length and run in methanolic buffer. This lower temperature has given closer reproducibility. It is specially recommended if methanolic buffer is being used.

Experimental results. The third α-globulin was first observed when making quantitative determinations of the protein fractions by eluting them from the electropherograms with 1% NaCl and applying Weichselbaum's biuret method(4) to the eluates. The positions of the fractions were established in the first place by rapidly staining a strip of the paper with Brom Phenol Blue. It was subsequently found that this α_3-globulin may be seen well on papers stained for at least 30 minutes in a saturated solution of Naphthalene Black in 60% methanol and then washed in methanol containing 10% glacial acetic acid. It is less readily seen on papers stained with Brom Phenol Blue because it seems that this dyestuff, when associated with α_3-globulin, is relatively easily washed off the papers. However, papers stained with Brom Phenol Blue may subsequently be restained with Naphthalene Black. We have in this way successfully restained older electropherograms on which the original Brom Phenol Blue had faded quite badly. The α_3-globu-

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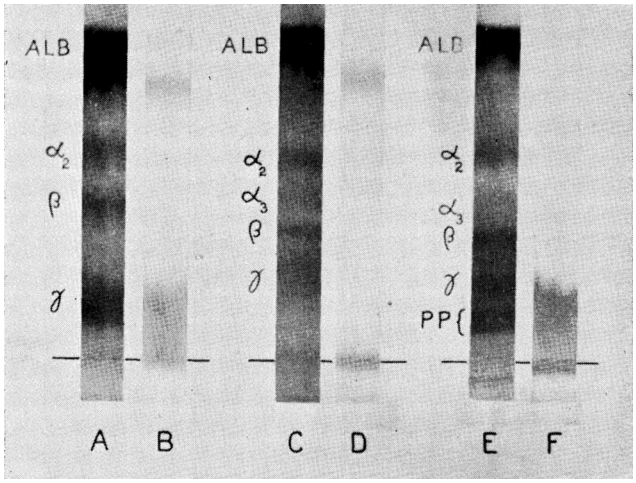

FIG. 1. Electropherograms of human serum (A and B), immature pullet serum (C and D) and serum from estrogenized immature pullet (E and F). Buffer: veronal in 20% methanol, pH 8.6. Papers A, C and E stained with Naphthalene Black; papers B, D and F stained with Oil Red O. Horizontal line marks point of application. Note lipid slightly behind albumin in immature pullet (cp. C and D) and absence of this lipid in the estrogenized pullet (cp. E and F). Note α_3-globulin fraction in C and E, but not in A. Two PP bands are clearly defined in the serum of the estrogenized pullet (E), while lipid has migrated as far as the leading edge of the γ-globulin. Sample of human serum was taken from a subject who had not fasted, hence high lipid content.

lin has been found on electropherograms whether run in aqueous or methanolic buffer. The α_3-globulin is well shown in Fig. 1C and 1E. Both these electropherograms displayed faint narrow α_1-globulin bands situated approximately one-third of the way from the trailing edge of the albumin zone to the leading edge of the α_2 zone, but these α_1 bands were too faint for satisfactory reproduction in Fig. 1.

Staining with Oil Red O(5) and osmic acid (3) has demonstrated the presence of 2 lipid fractions on electropherograms of human sera and sera of immature pullets. The first fraction moved in association with the albumin or somewhat more slowly(5,3). This slower movement is pronounced in 20% $^{v}/_{v}$ methanolic buffer as compared with aqueous buffer (Fig. 1B). Maurer(6) has shown that lipid phosphorus moves in closer association with the albumin the greater the proportion of serum that is included in the buffer. In our experience, electropherograms of sera from estrogenized immature pullets or laying hens have not displayed any lipid staining in the albumin region or behind this region despite the accompanying lipemia (Fig. 1, comparison of D and F). This has been the case whether the buffer were aqueous or methanolic. The second lipid fraction remained at the origin when sera from immature pullets were run in aqueous or methanolic veronal buffer (Fig. 1D). Post-absorptive human sera displayed lipid staining similar to that of unestrogenized immature pullets. However, in the case of a human serum from a non-fasting patient, the second lipid fraction tended to spread between the origin and a position just ahead of the γ-globulin whether the buffer used were aqueous or methanolic (Fig. 1B). These observations agree with those of Kunkel and Slater(7) on electropherograms of human sera run in aqueous veronal buffer. Electropherograms of sera of estrogenized immature pullets or laying hens displayed a great increase of the second lipid component. It has been shown that lipid staining of electropherograms of such sera run in aqueous buffer is associated with a "presumptive lipovitellin" (PLV) component(3). When such sera were run in methanolic buffer, the electropherograms stained with Oil Red O over a zone of practically the same extent as when run in aqueous buffer (Fig. 1F). Specially

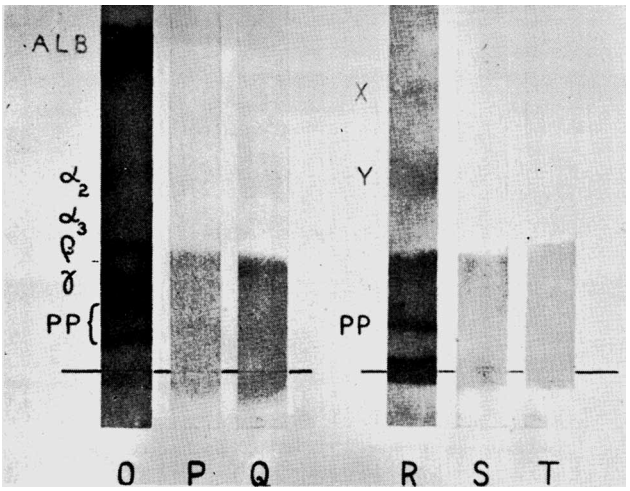

FIG. 2. Electropherograms of sera from estrogenized immature pullet (O, P and Q) and of egg yolk diluted with 1.0% NaCl (R, S and T). Buffer: veronal in 20% methanol, pH 8.6. Papers O and R stained with Naphthalene Black and P and S with Oil Red O. The Feulgen plasmalogen reaction was applied to papers Q and T. Lipid in the serum sample has moved as far as the trailing edge of the β globulin; the two PP fractions are well resolved, and the Feulgen reaction in general corresponds closely with the zone stained with Oil Red O. The yolk electropherograms have stained heavily at the point of application both with Naphthalene Black and with Oil Red O, and display a stronger plasmalogen reaction at the same position. The yolk pattern shows only one definite PP zone. Note the presence of two minor yolk protein fractions (X and Y).

intense staining in the PP region was not apparent when the birds had received 1 mg ODB (estradiol benzoate) intramuscularly on each of 3 successive days. However, when twice this amount of ODB had been given, the PP zones were associated with relatively more intense staining by Oil Red O.

The Feulgen plasmalogen reaction was applied as follows: The paper was dried at 25°C, immersed in 0.05 M mercuric chloride for one minute, washed in water, and then immersed in fuchsin-sulphurous acid reagent. (0.35 g fuchsin and 1.9 g sodium metabisulphite added to 100 ml of 0.15 N HCl and left to stand overnight. Next morning the mixture is shaken to dissolve the fuchsin, decolorized with "Norite" and filtered for use). The reaction (Fig. 1 O,P,Q) gave results corresponding qualitatively to the results obtained by staining with Oil Red O. However, the Feulgen reaction was slightly more intense in zones corresponding in position to the PP zones even when the pullets had received only 2 mg ODB on each of 3 successive days. Duplicate papers extracted with ethanol-ethyl ether (3:1) containing 5% trichloracetic acid, followed by extraction with chloroform-ethanol (1:1) no longer gave the plasmalogen reaction. These observations proved that the acetal phospholipid content of the serum of the fowl is increased in laying hens or estrogenized pullets as compared with control immature pullets. The presence of plasmalogen in egg yolk was first noted by Stepp, Feulgen, and Voit(8). Feulgen and Bersin(9) later estimated that egg phosphatides only contained approximately 0.1% plasmalogen as against 10 to 12% in muscle phosphatides. The amount of plasmalogen in the phospholipid of avian sera is presumably low; Raney *et al.*(10) did not mention its presence in fowl sera. Electropherograms of human sera also gave a faint Feulgen plasmalogen reaction in the regions that stained with Oil Red O. The plasmalogen reaction has a methodological advantage over staining with Oil Red O in so far as it is a reaction rather than a staining procedure. The reported presence of desoxyribonucleic acid in avian sera(11) prompted attempts to demonstrate a positive Feulgen nucleal stain on papers freed from lipid by extraction with alcohol-ether free from tri-

chloracetic acid. These attempts have not been successful, but this may be related to the minute concentrations of DNAP recorded for serum.

A feature of the PP fraction separable on electropherograms prepared with sera of estrogenized pullets or laying hens with methanolic buffer was that this fraction appeared as 2 distinct bands, although these were close together (Fig. 1E). The double nature of the PP fraction tends to be obscured in more heavily estrogenized birds because of "tailing" associated with high lipid content of the sera. The double nature of the PP band was, therefore, partially obscured in electropherograms of sera of estrogenized pullets previously figured by us(3).

The serum of estrogenized pullets was diluted with an equal volume of water, and the resultant precipitate of serum "lipovitellin" was then taken up in 1.0% saline and subjected to electrophoresis in methanolic buffer. The electropherogram displayed a doublet of bands corresponding in mobility to the PP bands of a parallel electropherogram of the original serum run on the same paper.

An electropherogram of egg yolk diluted with 1.0% NaCl and run in methanolic buffer is shown in Fig. 2R, S, and T. The yolk for dilution was carefully withdrawn by syringe from washed intact yolks in order to obviate contamination by egg-white proteins. The origin showed heavy staining with Naphthalene Black and a distinct band (marked PP in Fig. 2R) which probably corresponds to one of the PP bands of Fig. 2O. The dark and wider band in advance of this (Fig. 2R) may correspond to the other PP band of Fig. 2O. The electropherogram of the diluted yolk also showed plainly the presence of a component corresponding in position to the α_2-globulin of serum (marked Y) and of another fainter component corresponding in position to α_1-globulin (marked X).

It seemed possible that the heavy deposit of lipoprotein at the point of application (Fig. 2R and S) might be associated with the physical structure of the yolk emulsion. Accordingly, yolk samples (2 ml) were suspended in 1.0% NaCl and centrifuged. The supernatant was submitted to electrophoresis

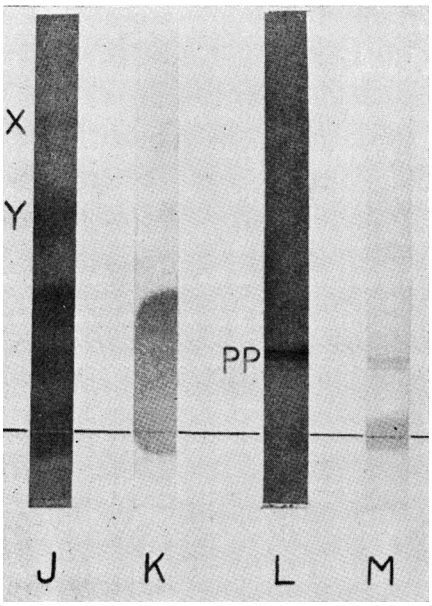

FIG. 3. Electropherograms of supernatant from centrifuging a dispersion of egg yolk in 1% NaCl (J and K) and of a solution prepared by dissolving the precipitate in 10% NaCl and then making up to the original volume with 10% NaCl (L and M). Buffer: veronal in 20% methanol, pH 8.6. Papers J and L stained with Naphthalene Black. Papers K and M stained with Oil Red O.

using methanolic buffer (Fig. 3J and K). The precipitate of lipoprotein was dissolved in 10% NaCl. This solution was then diluted with 10.0% NaCl to the same total volume as the volume of the original suspension in 1.0% NaCl. Filter paper electrophoresis (methanolic buffer) gave the results shown in Fig. 3L and M. The supernatant obviously contained a lipoprotein fraction which occupied a wide zone on the paper, and also contained the X and Y fractions. The precipitate showed a double protein band containing some lipid and corresponding to the PP fractions of sera of estrogenized pullets. There was marked retention of lipid at the point of application but no marked retention there of protein, as had been the case when the simple suspension of egg yolk was examined (Fig. 2R and S). The proteins on paper J represented approximately 1.8 times as much protein as was present on paper L. The relation of these fractions to the lecithovitellin and livetin fractions described in the literature calls for further study.

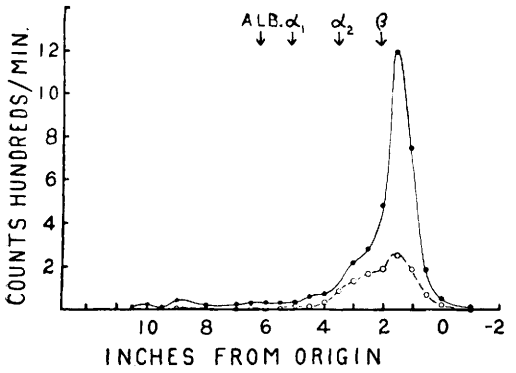

FIG. 4. Distribution of P^{32} on electropherogram of serum of immature pullet (8 wk of age) which had received 8 daily injections of 1 mg ODB. Orthophosphate labeled with P^{32} (63 μc total activity) injected subcutaneously on morning after last ODB injection. Blood drawn 12 hr after injection of radioactive phosphate. Papers scanned through ¼ in. slit under Geiger-Mueller counter. Buffer: aqueous veronal, pH 8.6; ionic strength = 0.05. Full line and dots = total counts; broken line and circles = counts after extraction of lipid.

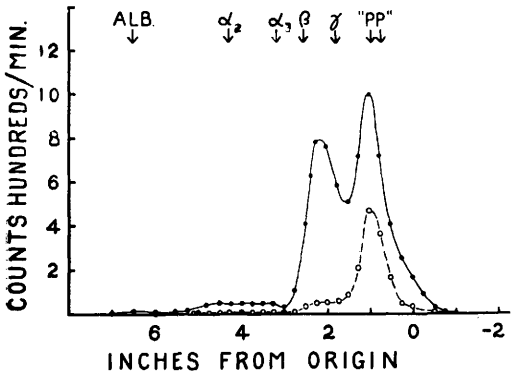

FIG. 5. Distribution of P^{32} on electropherogram of serum of immature pullet (8 wk of age) which had received 3 daily injections of 2 mg ODB. Orthophosphate labeled with P^{32} (68 μc total activity) injected subcutaneously on morning after last ODB injection. Blood drawn 11 hr after injection of radioactive phosphate. Papers scanned through ¼ in. slit under Geiger-Mueller counter. Buffer: 20% methanolic veronal, pH 8.6. Full line and dots = total counts; broken line and circles = counts after extraction of lipid.

Studies using subcutaneous injections of inorganic phosphate labeled with P^{32} gave electropherograms (aqueous buffer) with high counts in the PLV region. Extraction of lipid from these papers as described above and recounting gave considerably lower counts in the PLV region (Fig. 4). When these experiments were repeated using methanolic buffer as previously reported, the new slow-moving band (PP fraction) was associated with a high count (Fig. 5). In a previous report(3) it was mentioned that the counts of the PP band were not appreciably reduced by extraction of lipids, although the counts in the heavy lipid staining region ahead of this were reduced. These earlier experiments, however, were conducted at room temperature. In later work at 6° to 7°C a proportion of the lipid did remain in association with the PP fraction, as may be seen clearly from Fig. 5. The resolution of the counting arrangement was not adequate to determine whether or not the PP zone counted as a double zone.

Discussion. The relation of the results of filter paper electrophoresis of avian sera to the results secured by a number of workers (12-16) by the classical Tiselius apparatus is not at this stage clear. It is possible, however, that further elucidation of the lipids and lipoproteins of avian sera may be facilitated by comparisons of results obtained by both technics. Clegg and Hein(16) have recently described results obtained by subjecting avian sera to the classical electrophoresis in borate buffer of pH 8.6. They distinguished 6 components in sera from untreated chickens and 7 components in sera from estrogenized chickens. The following very tentative correlations between the components described by Clegg and Hein and those here described may be suggested.

Component of Clegg and Hein(16) Control chicken	Component as presently described Immature pullet
2	Albumin
3	α_1 globulin (and lipid ?)
4	α_2 "
?	α_3 "
5	β "
6	γ "

The enormous increase in component 5 as a result of estrogen treatment might then be equated with the fraction which we have designated PLV(3). We have not been able to observe on paper electropherograms any fraction corresponding exactly to component 1 of Clegg and Hein(16), whether the buffer were veronal, borate or diethanolamine. (The latter buffer yields excellent separations but has the disadvantage of interfering with

the biuret and Kjeldahl methods of determining protein). In conclusion it should be stressed again that the foregoing correlations of the results by the 2 methods are purely tentative.

Summary. 1. An albumin and 5 globulin fractions have been observed on electropherograms of sera of sexually immature pullets. The albumin fraction of such sera was associated with lipid, but lipid was not observed in association with the albumin of sera of estrogenized pullets. 2. Zones of the electropherograms of sera of pullets that stained with Oil Red O also gave the Feulgen plasmalogen reaction. The Feulgen reaction was stronger with sera of estrogenized birds. 3. Electropherograms of yolk dispersed in saline were prepared using 20% methanolic veronal buffer. A presumptive phosphoprotein fraction was distinguishable on these papers; it resembled closely the presumptive phosphoprotein fraction of sera of laying hens or estrogenized immature pullets. The presumptive phosphoprotein zones were doubled for both yolk and sera. 4. Studies of electropherograms of estrogenized immature pullets that had received injections of phosphate labeled with P^{32} showed that a large moiety of the phospholipid moved ahead of the phosphoprotein fraction when methanolic buffer had been used to separate this PP fraction, although a part of the phospholipid did remain in association with the PP fraction.

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