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Serum Proteins and the Livetins of Hen's-Egg Yolk

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The water-soluble proteins of egg yolk comprise the livetin fraction (Plimmer, 1908). Many studies have shown that the fraction is heterogeneous. In moving-boundary electrophoresis there are three main components (Shepard & Hottle, 1949) which were called α -, β - and γ -livetins by Martin, Vandegaer & Cook (1957), and three main fractions were also found by paper electrophoresis (McCully, Maw & Common, 1959) and by ammonium sulphate fractionation (Gorini & Lanzavecchia, 1955). Chromatography on diethylaminoethylcellulose, on the other hand, gave ten components (Mandeles, 1960). Onoe (1936) isolated a glycoprotein (vitellomucoid) from the livetin fraction and emphasized its distinction from ovomucoid. Schjeide & Urist (1959) concluded from studies with the ultracentrifuge that the egg-white protein, ovalbumin, is present in livetin in large amounts (45% of the total yolk protein), although the immunological

studies of Marshall & Deutsch (1951) showed only traces (0.2% of the total livetin protein). Some enzymes have also been found in the livetin fraction (Lineweaver, Morris, Kline & Bean, 1948).

Jukes & Kay (1932a) first noted an immunological similarity between serum globulin and livetin, which they thought to be a single protein, and suggested that livetin might be 'merely a translocated blood constituent' (Jukes & Kay, 1932b). Later immunological studies by Schechtman (1947), Knight & Schechtman (1954) and Nace (1953) supported this view; it had also been proposed by Shepard & Hottle (1949), who found the electrophoretic mobilities of the livetins to be similar to those of serum fractions.

The experiments described below were carried out primarily to characterize and identify the three bands of protein observed in paper electrophoretograms of livetin. The methods of immunoelectro-

phoresis, starch-gel electrophoresis and comparison of peptide patterns were used, and the results suggest that all the livetin proteins correspond to serum proteins. The most abundant livetin components were identified as serum albumin, low-molecular-weight α_2 -glycoprotein, transferrin and γ -globulin. Ovalbumin was not detected in livetin. This paper describes the identification of albumin, glycoprotein and γ -globulin.

EXPERIMENTAL

Materials

Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. Trypsin was obtained from Novo Terapeutisk Laboratorium, Copenhagen, Denmark, and chymotrypsin from Worthington Biochemical Corp., Freehold, N.J., U.S.A. 'Starch-hydrolysed' was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Buffers

The following buffers were used in paper electrophoresis: succinate, pH 5.4 (0.2M-succinic acid adjusted to pH 5.4 with 0.2N-NaOH); phosphate, pH 6.5 (0.023M-KH₂PO₄, 0.0105M-Na₂HPO₄); barbitone, pH 8.6 (0.1M-sodium diethylbarbiturate, 0.02M-diethylbarbituric acid); glycine, pH 10.0 (0.2M-glycine adjusted to pH 10.0 with 0.2N-NaOH).

Preparation of egg proteins

These were prepared either from commercial hen's eggs or from eggs of White Leghorn hens kept by the School of Agriculture, Cambridge University. In some experiments duck eggs were used.

Whole livetin. This was prepared by the method used by Martin *et al.* (1957). Care was taken to avoid contamination of the yolk with egg white. The yolks were rolled on Whatman 3 MM filter paper to remove adherent egg white and the vitelline membrane was then punctured.

α - and β -Livetin. These were prepared by paper electrophoresis. Freeze-dried livetin was dissolved in barbitone buffer, pH 8.6, and applied across the middle of a full sheet of Whatman 3 MM chromatography paper (1.5 mg. of protein/cm.). Electrophoresis was carried out in a hanging-strip apparatus (Durrum type) at 4° with barbitone buffer. A potential gradient of about 4 v/cm. was applied for 18 hr. Guide strips were stained with bromophenol blue. The bands corresponding to α - and β -livetin were eluted with water, dialysed against water overnight and freeze-dried.

γ -Livetin. This was prepared by the method of Martin & Cook (1958). Yolk was mixed with 4 vol. of 16% saturated (NH₄)₂SO₄ and centrifuged at 100 000g for 3 hr. γ -Livetin was precipitated by dialysis of the clear lower layer against 40% saturated (NH₄)₂SO₄ and twice reprecipitated by dialysis against 34% saturated (NH₄)₂SO₄. It was then dissolved in water, extracted once with diethyl ether at room temperature, dialysed against water and freeze-dried.

Vitellogenin. As far as possible the method of Onoe (1936) was followed. Yolk was mixed with 1 vol. of 8% NaCl. Lipid was extracted with ether, instead of ethanol which caused complete precipitation of proteins. The

aqueous solution was dialysed overnight against tap water and the precipitated lipovitellin centrifuged down. Solid (NH₄)₂SO₄ was added to give 50% saturation and the mixture filtered. The filtrate was dialysed against tap water and then against distilled water, and then reduced to a small volume in a rotary evaporator. It was boiled for 5 min. and filtered, and the opalescent filtrate was treated with 5 vol. of ethanol at room temperature. The precipitate was centrifuged down and dissolved in a small volume of water. It was dialysed for several hours against water adjusted to pH 8 with NH₃. The solution was then freeze-dried.

Duck ovalbumin. This was prepared by the method of Sørensen & Høytrup (1917).

Preparation of serum proteins

White Leghorn cocks kept by the School of Agriculture, Cambridge University, were bled from the wing vein. The blood was allowed to clot and the serum stored at -15°. In the experiments on serum albumin and α -livetin, serum and eggs were obtained from the same White Leghorn hen. Serum albumin was also prepared from duck blood.

Serum albumin. Serum was dialysed overnight against water and the precipitate filtered off. Saturated (NH₄)₂SO₄ (1 vol.) was added to the filtrate which was then refiltered. The filtrate was again dialysed against water and freeze-dried. This material was subjected to electrophoresis on paper with barbitone buffer. The albumin band was eluted with water, dialysed overnight against water and freeze-dried.

γ -Globulin. The rivanol procedure of Horesji & Smetana (1956) was used. Rivanol (3.5 vol. of aq. 0.4%) was slowly added to serum that had been adjusted to pH 9.0 with NaOH. The precipitate was filtered off and rivanol was removed from the filtrate with charcoal (Norit SX special). γ -Globulin was then precipitated from the solution by the addition of solid (NH₄)₂SO₄ to give 35% saturation according to the nomogram of Dixon (1953). The precipitate was dissolved in water, dialysed against water overnight and freeze-dried.

Starch-gel electrophoresis

Horizontal starch-gel electrophoresis (Smithies, 1955) was employed with the discontinuous buffer system of Poulik (1957). Protein samples were applied as 1% solutions on rectangular pieces of Whatman 3 MM paper. Serum samples were applied in the same way. Electrophoresis was carried out for 4-5 hr. at room temperature with a potential gradient of 7 v/cm. across the gels. After slicing, the gels were stained with Amido Schwarz 10B or with Nigrosine.

Antisera

(i) Anti-cock serum and anti-hen serum were produced by injecting rabbits subcutaneously with 0.5 ml. of cock or hen serum daily for 1 week. The injections were repeated 2 weeks later and the rabbits were bled after a further interval of 7 days.

(ii) Anti-livetin serum was produced by daily injection of 1 ml. of 2% livetin in 0.9% NaCl into rabbits. Two courses of injections were given.

(iii) Anti-hen-globulin serum was a gift from Dr E. S. Orland. It had been produced by immunizing rabbits with globulins precipitated from hen serum with 17% Na₂SO₄ and twice reprecipitated by 15% Na₂SO₄.

(iv) Anti-specific-precipitate serum was also given by Dr E. S. Orlans. Rabbits had been immunized with the specific precipitate formed by mixing immune-hen serum with its homologous antigen (namely bovine serum albumin). The precipitate had been well washed before use to remove hen serum proteins other than antibody.

(v) Rabbit anti-ovalbumin serum was a gift from Professor J. R. Marrack.

All antisera were stored at -15° .

Immunoelectrophoresis

Micro-immunoelectrophoresis according to Scheidegger (1955) was carried out in 1.5% agar gels [buffer (g./l.): sodium tetraborate, 10.9; succinic acid, 5.52; sodium azide, 1]. Electrophoresis was carried out for 45–75 min. according to the migration of a sample of hen serum albumin stained with Evans Blue. Precipitin lines were allowed to develop for 2 days in a humid atmosphere at room temperature. After washing in 0.9% NaCl to remove soluble proteins the gels were dried at 37° and stained with Amido Schwarz 10B or with Nigrosine.

Agar diffusion was carried out in 1.5% agar gels, according to Ouchterlony (1949). The gels were developed for 2 days at room temperature.

Anti-cock serum was absorbed with livetin. Livetin (10 mg.) was added to 1 ml. of anti-cock serum which was kept at 37° for 1 hr. and then left overnight at 4° . The precipitate was spun off and the supernatant used in immunoelectrophoresis. Anti-livetin serum was similarly absorbed with hen serum (0.2 ml./ml. of antiserum). Anti-hen-globulin serum was successively absorbed with increasing amounts of livetin (6.6, 16.6 and 27.8 mg./ml.) and the removal of antibodies was observed by immunoelectrophoresis after each absorption.

Peptide patterns

Protein samples were first oxidized with performic acid (Hirs, 1956). Excess of performic acid was removed directly by freeze-drying without dilution. The sample was then suspended in water and the freeze-drying repeated. Digestions were performed with trypsin at 37° in 0.05M-ammonium acetate buffer, pH 9.0, with an enzyme:substrate ratio of 1:20 (w/v). Serum albumin and α -livetin samples were digested for 8 hr. The digests were dried *in vacuo* over P_2O_5 and then dissolved in water. A volume equivalent to 3 mg. of protein was applied to a sheet of Whatman 3 MM paper for two-dimensional separation by electrophoresis and chromatography. For one-dimensional electrophoretic peptide patterns, 1 mg. of protein was applied to 1.5 cm. of starting line.

Electrophoresis was performed under toluene with pyridine-acetate buffers, pH 3.5 and 6.4, and a potential gradient of 40 v/cm. Descending chromatograms were run for 18 hr. with butan-1-ol-acetic acid-water (3:1:1, by vol.). Peptide spots were revealed by dipping the papers in 0.1% (w/v) ninhydrin in acetone and specific stains were applied to reveal peptides containing arginine, tyrosine and histidine (Jepson & Smith, 1953).

Carbohydrate analysis

Estimations of protein-bound hexose were made by the orcinol- H_2SO_4 reaction according to Schönenberger, Kellner, Südhoff & Haupt (1958) against a standard solu-

tion containing equal parts by weight of galactose and mannose. Protein-bound hexosamine was determined by the Elson-Morgan reaction (Schultze, Schmidtberger & Haupt, 1958) against a standard solution of glucosamine hydrochloride. Protein-bound sialic acid was released by heating in 0.1N- H_2SO_4 at 98° for 1 hr. Protein was then precipitated with 10% (w/v) trichloroacetic acid and sialic acid in the supernatant was estimated by the resorcinol-HCl method of Svennerholm (1957). A preparation of crude livetin, which was kindly assayed by Dr G. W. Cook against *N*-acetylneuraminic acid and found to contain 1% of sialic acid, was used as a secondary standard.

Moving-boundary electrophoresis, sedimentation and diffusion

Moving-boundary electrophoresis was done in the Tiselius apparatus (Perkin-Elmer Corp., Conn., U.S.A., model 38) at 4° with barbitone buffer, pH 8.6 (Deutsch & Goodloe, 1945). Hen serum was diluted with buffer (2 vol.) and the mixture dialysed against buffer overnight at 4° before electrophoresis. Electrophoresis of β -livetin was carried out with a 1% solution in barbitone buffer.

Sedimentation patterns were obtained with the model E analytical Spinco ultracentrifuge. Protein samples were dissolved in 0.1M-NaCl at about 1%.

The Tiselius apparatus was used in the diffusion experiment.

RESULTS

Immunoelectrophoresis with anti-cock serum and anti-hen serum. Cock or hen sera developed with the homologous antisera gave the pattern of precipitin lines shown in Fig. 1. The lines were identified by comparison with the results of Kaminski & Durieux (1956). Prealbumin could be demonstrated occasionally in both cock and hen sera. Other lines corresponded to albumin, α_1 - and α_2 -globulin, transferrin or conalbumin, and γ -globulin. Anti-hen serum gave a much weaker α_1 -globulin line than did anti-cock serum but the γ -globulin arc was sometimes doubled when anti-hen serum was used (γ_a in Fig. 1).

Fig. 1. Immunoelectrophoresis of cock serum (S) (dilution 1:8). A scheme showing the precipitin arcs obtained with rabbit anti-hen and anti-cock sera. In this and other Figures the substances are identified as follows: α_1 , α_1 -globulin; α_2 , α_2 -globulin; β , β -globulin; γ , γ -globulin.

Livetin developed with anti-cock serum or anti-hen serum (Fig. 2) gave four precipitin lines corresponding to albumin, α_2 -globulin (weak for anti-hen serum), transferrin or conalbumin, and γ -globulin. Anti-cock serum absorbed with livetin gave no reaction with cock serum.

Immunoelectrophoresis with anti-livetin serum. Cock or hen sera developed with this serum showed albumin, α_1 -, α_2 - and β -globulin, transferrin or conalbumin, and γ -globulin. Livetin showed albumin, α_1 - and α_2 -globulin, transferrin or conalbumin, and γ -globulin (Fig. 3). Absorption of this serum with hen serum removed its ability to react with livetin. Relative to the other lines the α_2 -globulin line was much longer in livetin than in serum, suggesting that this protein is relatively more abundant in livetin.

Immunoelectrophoresis with anti-hen-globulin serum. Hen serum developed with this serum showed a weak albumin, two α_1 -globulins, a strong α_2 -globulin and a strong double γ -globulin (Fig. 4). The double γ -globulin line was noted occasionally by Kaminski & Durieux (1956). The serum did not react with transferrin. Livetin showed a weak albumin, a weak α_2 -globulin and a strong double γ -globulin. Absorption of the serum with livetin did not completely remove its ability to react with serum. There remained the slower α_1 -globulin,

apparently undiminished, and a faint line in the γ -globulin region.

Immunoelectrophoresis with anti-specific-precipitate serum. Both livetin and hen serum gave a single elongated arc in the position of γ -globulin (Fig. 5).

Experiments with anti-ovalbumin serum. Immunoelectrophoresis of hen's-egg white (diluted 1:64 with 0.9 % sodium chloride) with this serum showed only one precipitin line corresponding to ovalbumin. In agar-diffusion tests by the Ouchterlony method, the serum gave no reaction with conalbumin [prepared as described by Williams (1962)] or with undiluted cock serum, but reacted with undiluted sera from laying hens. The precipitin lines formed with egg white and laying-hen serum made a smooth continuous line (Fig. 6).

Anti-ovalbumin serum reacted with a preparation of duck ovalbumin and a spur between the lines given by hen's-egg white and duck ovalbumin showed that the duck protein had precipitated only part of the anti-ovalbumin antibodies (reaction of partial identity). The antiserum gave no reaction with either hen- or duck-livetin preparations (Fig. 6).

Fig. 2. Immunoelectrophoresis of livetin (L) (1% soln.). A diagram of the precipitin arcs obtained with anti-cock serum.

Fig. 3. Immunoelectrophoresis of livetin (L) and serum (S). A diagram of the precipitin arcs obtained with anti-livetin serum.

Fig. 4. Immunoelectrophoresis of livetin (L) and serum (S). A diagram of the precipitin arcs obtained with anti-hen-globulin serum. In the upper antiserum trough was placed anti-hen-globulin serum which had been absorbed with a total of 27.8 mg. of livetin/ml. of antiserum.

Fig. 5. Immunoelectrophoresis of livetin (L) (1% soln.) and hen serum (S) (1:4 dilution). Diagram of the arcs obtained with anti-specific-precipitate serum.

Paper electrophoresis. At pH 5.4, 6.5, 8.6 and 10.0 livetin showed three bands which moved towards the anode and which were identified as α -, β - and γ -livetins (McCully *et al.* 1959). Compared with serum, the livetin fractions had the mobilities of albumin, α_2 -globulin and γ -globulin respectively (Fig. 7). In some cases the slowest band was partly resolved into two components. Knight & Schechtman (1954) obtained resolution of the slowest band

Fig. 6. Agar-gel diffusion plate. Rabbit anti-ovalbumin serum was placed in the centre well. Well 1 contained hen livetin (2% soln.); well 2 contained undiluted laying-hen serum; well 3 contained hen's-egg white (1:64 dilution); well 4 contained duck ovalbumin (0.1% soln.); well 5 contained cock serum; well 6 contained duck livetin (2% soln.).

Fig. 7. Paper electrophoresis of livetin and of cock serum. Barbitone buffer, pH 8.6, I 0.1. Electrophoresis was carried out for 18 hr.

into β - and γ -globulin components. The bands were eluted from the electrophoretograms and identified by immunoelectrophoresis: α -livetins gave the albumin line, β -livetins the α_2 -globulin line, and γ -livetins the transferrin (or conalbumin) and γ -globulin lines.

Serum albumin and α -livetins. Serum and eggs were obtained from the same bird and serum albumin and α -livetins isolated. They gave single symmetrical peaks in the ultracentrifuge with uncorrected sedimentation coefficients of 4.1 and 4.4s respectively for approximately 1% solutions. In starch-gel electrophoresis at pH 8.6, α -livetins and serum albumin gave sharp bands which had identical mobilities. Trypsin digests gave indistinguishable peptide patterns when subjected to electrophoresis at pH 6.4 and chromatography (Fig. 8). In addition to the correspondence of the ninhydrin spots there was identity of the peptides containing arginine, tyrosine or histidine. The peptide patterns were repeated on several occasions with eggs from four closely inbred lines of White Leghorn hens. The peptide pattern was reproducible and showed no differences between the four lines. A comparison was also made of duck serum albumin and α -livetins from duck eggs (Fig. 9). The patterns were closely similar to one another but quite different from those given by the hen proteins.

β -Livetin. Moving-boundary electrophoresis of β -livetins and of laying-hen serum in barbitone buffer, pH 8.6, gave the following mobilities ($\text{cm}^2/\text{v}/\text{sec}.$; descending boundaries): serum albumin, 7.03×10^{-5} ; α_1 -globulin, 6.44×10^{-5} ; α_2 -globulin, 4.49×10^{-5} ; β -globulin, 3.12×10^{-5} ; γ -globulin, 2.34×10^{-5} . β -Livetin moved as a symmetrical peak with a mobility ($\text{cm}^2/\text{v}/\text{sec}.$) of 4.88×10^{-5} , confirming its electrophoretic identification as an α_2 -globulin.

In starch-gel electrophoresis at pH 8.6, β -livetins gave four bands (Fig. 10). The main band which was rather broad moved in the albumin region. The other bands were narrower and less intense and moved with smaller mobilities.

In the ultracentrifuge a 0.8% solution of β -livetins gave a single symmetrical peak with a sedimentation coefficient ($S_{20,w}$) of 1.9s. The diffusion coefficient was 7.0×10^{-7} and, assuming $\bar{v} = 0.75$, a molecular weight of 37 000 was obtained.

A strongly positive Molisch reaction was obtained, and carbohydrate analysis showed 7.0% of hexose, 3.7% of hexosamine and 0.8% of sialic acid.

β -Livetin was not precipitated from 0.5% solution by 0.6M-perchloric acid or by boiling in water, but was precipitated by 0.2M-sulphosalicylic acid or by 5% (w/v) trichloroacetic acid.

A preparation of Onoe's (1936) vitellomucoid migrated in paper electrophoresis at pH 8.6 as a single band with the same mobility as β -livetin. Similarly, in starch-gel electrophoresis at pH 8.6, the proteins gave identical patterns. The carbohydrate analysis of vitellomucoid was similar to that of β -livetin: 7.8 % of hexose, 5.5 % of hexosamine and 2.0 % of sialic acid. Sulphosalicylic acid (0.2M) caused a 0.5 % solution of vitellomucoid to become turbid but did not give the heavy precipitate obtained with β -livetin.

γ -Livetin and γ -globulin. Whole livetin was treated with ammonium sulphate at 37 % saturation. Immuno-electrophoresis with anti-hen serum

and anti-hen-globulin serum showed the precipitate to contain all the γ -globulin and some of the α_2 -globulin and albumin. Most of the albumin and all of the transferrin were in the supernatant. Fig. 11 shows starch-gel electrophoresis (pH 8.6) patterns of γ -livetin and of γ -globulin: γ -globulin gave a diffuse band moving towards the anode; in the γ -livetin preparation most of the protein was present in a similar diffuse band whose mobility was slightly greater than that of the serum γ -globulin. Some sharp fast-moving bands were also present in the γ -livetin preparation. The two preparations showed immunological identity against anti-specific-precipitate serum (Fig. 12).

Fig. 8. Tryptic peptide patterns of hen-serum albumin (A) and α -livetin (B). The electrophoresis was carried out at pH 6.5 for 105 min. In addition to the ninhydrin-positive spots, the areas labelled *a* contained arginine, areas labelled *h* contained histidine and those labelled *t* contained tyrosine.

Fig. 9. Tracings of tryptic peptide patterns of duck serum albumin (A) and duck α -livetin (B). The electrophoresis was carried out at pH 6.5 for 105 min. Corresponding peptide spots are indicated by the numbers.

DISCUSSION

The above results suggest that the four main components of livetin (albumin, α_2 -glycoprotein, transferrin and γ -globulin) are identical with proteins which regularly occur in cock and hen sera. The removal of all anti-livetin antibodies from anti-livetin serum by absorption with serum shows that all the livetin antigens are present in serum. The removal of all anti-serum antibodies from anti-cock serum by absorption with livetin would suggest, similarly, that all serum antigens are present in livetin. Anti-hen-globulin serum, on the other hand, did not lose all its anti-serum antibodies when treated with livetin, so that some serum proteins may be absent from livetin or present in low concentrations, and it is likely that anti-cock serum and anti-hen-globulin serum do not contain antibodies to the same α_1 -globulins.

Martin *et al.* (1957) decided that, although the tyrosin:tryptophan ratios and the molecular weights of serum albumin and α -livetin are very similar, the proteins are not identical because α -livetin had the solubility properties of a pseudoglobulin (namely precipitation by 50% saturated ammonium sulphate). In the present studies it was found that, although some α -livetin was precipitated at low concentrations of ammonium sulphate, most of it was not precipitated by 50% saturation, and the immunological results and peptide patterns suggest that serum albumin and α -livetin are probably identical proteins.

Jukes (1933) found that livetin contained 4% of

carbohydrate by the orcinol method. It now seems likely that this may be attributed to the presence of large amounts of β -livetin which contains 7% of hexose. Martin *et al.* (1957) showed that β -livetin is distinct from ovalbumin although their molecular weights are similar. The results given here show that β -livetin corresponds immunologically to a serum protein and may be considered an α_2 -glycoprotein from its electrophoretic mobility and carbohydrate content. The behaviour of β -livetin in starch-gel electrophoresis shows it to be heterogeneous, although it appeared homogeneous by ultracentrifuging and by electrophoresis on paper and in the Tiselius apparatus. It appears to be similar to the α_2 -glycoproteins with a sedimentation coefficient of 3s of human serum which have been described recently (Schmid & Bürgi, 1961; Bürgi & Schmid, 1961), although the sialic acid content of β -livetin appears to be lower than that of the human serum proteins. Thus the proteins precipitated by Ba^{2+} ions were found by Schmid & Bürgi (1961) to contain 6% of hexose, 5% of hexosamine and 5% of sialic acid; the more soluble proteins precipitated by Zn^{2+} ions contained 7% of hexose, 4% of hexosamine and 7% of sialic acid (Bürgi & Schmid, 1961).

Vitellomucoid, the glycoprotein isolated from livetin by Onoe (1936), appears to be an α_2 -glycoprotein with the same electrophoretic mobility on paper and starch gel as β -livetin, although the hexosamine and sialic acid contents of a single preparation of vitellomucoid were higher than those for β -livetin. Like β -livetin vitellomucoid was heterogeneous in starch-gel electrophoresis.

The immunoelectrophoretic results given here indicate the presence in livetin of either transferrin or the closely related protein conalbumin. The isolation of this protein and its identification as transferrin are described in the next paper (Williams, 1962).

Martin *et al.* (1957) found that the electrophoretically slowest component of livetin (γ -livetin) was precipitated by 37% saturated ammonium sulphate, and Martin & Cook (1958) noted that in its molecular weight (150 000), lability, heterogeneity and tendency to undergo aggregation γ -livetin resembled serum γ -globulin. The immunological and starch-gel results given here indicate that the γ -livetin of Martin & Cook (1958) consists principally of γ -globulin. The cause

of the greater electrophoretic mobility of the yolk protein as compared with serum γ -globulin, however, is not clear.

As Knight & Schechtman (1954) pointed out, such data do not prove that livetin proteins are derived from serum: indeed Abdel-Samie, Broda & Kellner (1960) have shown that chick-mesenchyme cells and human-HeLa-tumour cells are capable of synthesizing serum proteins *in vitro*. Nevertheless, it appears more likely that the livetin proteins are transmitted across the follicle and the vitelline membrane from the serum than that they are synthesized by the follicle cells or by the ovum itself. The passage of antibody into the yolk is well known and Knight & Schechtman (1954) found that foreign serum proteins, other than antibody molecules, injected into the hen also pass into the livetin. Similarly, Patterson (1961) has demonstrated the transmission of labelled chick serum proteins into the yolk.

Fig. 10. Diagram of the starch-gel electrophoresis of β -livetin and hen serum. Electrophoresis was carried out at pH 8.6 for 4 hr. Prealbumin, albumin, transferrin and γ -globulin bands in the serum are labelled.

Fig. 11. Starch-gel electrophoresis of serum γ -globulin and γ -livetin. Electrophoresis was carried out at pH 8.6 for 4 hr.

Fig. 12. Agar-diffusion plate. The centre well contained anti-specific-precipitate serum: wells 1 and 4 contained serum γ -globulin (1% and 0.1% respectively): wells 2 and 5 contained γ -livetins (1% and 0.1% respectively): wells 3 and 6 were empty.

Shepard & Hottle (1949) calculated the relative proportions of the livetins from the areas of the three main peaks obtained with the Tiselius apparatus. α -Livetin comprised 17.3, β -livetins 32.7 and γ -livetins 50.0% of the total protein. By the same method Gorini & Lanzavecchia (1955) obtained the values 18.7, 44.1 and 32.4%. Shepard & Hottle (1949) found that in hen plasma the peak whose mobility corresponded to that of α -livetins comprised 53.3% of the total protein, the peaks whose mobilities were similar to that of β -livetins together comprised 18.3% of the total protein, and the slow peaks whose mobilities corresponded to that of γ -livetins comprised 28.3% of the total protein. Similarly, from the immunoelectrophoretic and paper electrophoretic results given here it appears that the relative proportions of the proteins occurring in livetins are not the same as in serum (Figs. 3 and 7). Thus α_2 -glycoprotein is much more abundant in livetins, relative to albumin, than in serum, and this may indicate selectivity in the uptake of serum proteins by the ovum. Knight & Schechtman (1954) found no evidence of such selectivity with bovine serum albumin and bovine γ -globulin. Indeed, since a variety of materials (including antibodies and other proteins, Sudan III, arsenic and virus) enter the yolk most readily at the time of its maximum growth rate, Schechtman (1956) concluded that transmission might be determined more by the physiological state of the ovum

or follicle than by the chemical nature or molecular size of the substances concerned. Patterson (1961), on the other hand, found that labelled chick γ -globulin was concentrated by the yolk to a greater extent than labelled chick albumin. Clearly, it would be interesting to have more information on the relative rates of transmission of different proteins.

In amphibia and insects similar processes appear to occur. Thus Glass (1959) observed the presence of serum-like materials in frog oocytes by immunofluorescence techniques and Telfer (1961) has demonstrated elegantly the passage of serum proteins into the oocytes of saturniid moths.

The conclusion by Schjeide & Urist (1959) that ovalbumin occurs in livetins to the extent of 45% of the total yolk protein appears to be based solely on ultracentrifuge patterns, and it does not seem likely that this method is adequate by itself to identify proteins. Marshall & Deutsch (1951), with anti-ovalbumin serum, found only traces of ovalbumin in cock serum, non-laying-hen serum and livetins, where it formed 0.2% of the livetins protein. In the sera of laying hens and chick embryos a larger amount was present. Kaminski & Durieux (1956), with immunoelectrophoresis and agar-diffusion tests, found small amounts of ovalbumin in laying-hen serum and in chick-embryo serum, but none was found in sera of cocks or non-laying hens. The present experiments with anti-ovalbumin serum confirm the absence of ovalbumin from cock serum and its presence in laying-hen serum. Neither hen nor duck livetins reacted with the antiserum which was free from antibodies to conalbumin. The finding of small amounts of ovalbumin in cock serum and livetins by Marshall & Deutsch (1951) may possibly have been due to the presence of anti-conalbumin antibodies in the antiserum. Such antibodies would be capable of reacting with the transferrin present in serum and livetins.

SUMMARY

1. The protein constituents of the livetins fraction of hen's-egg yolk have been studied. Livetin proteins have been identified with serum proteins by means of immunoelectrophoresis, starch-gel electrophoresis and comparison of peptide patterns.
2. α -Livetin has been identified as serum albumin.
3. β -Livetin has been identified as an α_2 -glycoprotein with a sedimentation coefficient of 2.9s.
4. γ -Livetin has been identified as serum γ -globulin.
5. Immunoelectrophoresis indicated the presence of transferrin in livetins.
6. Ovalbumin was not detected in livetins.

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A Comparison of Conalbumin and Transferrin in the Domestic Fowl

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It is well known that transferrin, the iron-binding β -globulin of serum, is very similar to conalbumin, the iron-binding protein of avian-egg white. One molecule of either protein readily combines with two atoms of iron and two molecules of carbon dioxide to give a pink complex with an absorption maximum at 460–470 $m\mu$ (Schade, Reinhart & Levy, 1949; Warner & Weber, 1951). In both cases the iron-protein complexes are more resistant to thermal denaturation and to proteolytic degradation than are the iron-free proteins (Azari & Feeney, 1958). Conalbumin and transferrin are also similar in their molecular weights.

Bain & Deutsch (1948) obtained a value of 85 000 for conalbumin by sedimentation and diffusion studies, and iron-binding studies by Warner & Weber (1951) gave a value of 76 600. Laurell & Ingelman (1947) obtained a molecular weight of 88 000 for pig transferrin by sedimentation and diffusion studies, and 90 000 was obtained for human transferrin by Koechlin (1952) with iron-binding and light-scattering methods and by Oncley, Scatchard & Brown (1947) by osmotic and sedimentation measurements.

The iron-binding protein of fowl serum has also been found to be similar to conalbumin. Hektoen