ON THE FRACTIONATION OF THE PROTEINS OF EGG YOLK

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For many years the senior author has attempted to fractionate the yolk proteins of the Atlantic salmon with each annual laying season, but without conspicuous success. The newer electrophoretic technique has now provided us with a better criterion of the success of our methods as applied to the egg yolk of the hen as well as of the salmon (1).

The general subject has been reviewed by Needham (2, 3) and by Jukes and Kay (4). Until recently the existence of three proteins had been claimed in egg yolk: (1) a lipoprotein (lipovitellin or ichthulin), (2) a pseudoglobulin (livetin or thuchtin), and (3) a mucoid (vitellomucoid). In 1946 Fevold and Lausten (5) isolated a lipoprotein from hen’s egg yolk which they named lipovitellenin. This protein differed from lipovitellin in containing a higher lipide content, 36 to 41 per cent, and in exhibiting a greater instability in contact with acetone. Mecham and Olcott (6) have isolated another phosphoprotein which they have named phosvitin. Their preparation contained 10 per cent of phosphorus and comprised 7.0 per cent of the total proteins. Shepard and Hottle (7) have attempted to purify livetin for use as an antigen. Their preparations exhibited three components on electrophoretic analysis.

Although our methods have not achieved fractions of the desired degree of homogeneity, it seems useful to publish the results at the present time. The fractionation of yolk proteins into the three components, demonstrable electrophoretically, does not appear to be readily accomplished in salmon yolk as tested electrophoretically.

EXPERIMENTAL

Unfertilized eggs, from the Atlantic salmon (Salmo salar), were obtained in early November at a local hatchery. They were washed repeatedly in 1 per cent sodium chloride at 0° to remove ovarian fluid. The eggs were placed in fine cheese-cloth, broken by pressure, and squeezed to separate yolk from casings. The yolk was centrifuged to separate as much as possible of the free lipide in the form of a deep red supernatant oil. After separation, the yolk was diluted with an equal volume of saline solution, usually 10 per cent sodium chloride, adjusted to pH 7.5. The diluted yolk was next extracted 5 to 10 times at 0° with peroxide-free ethyl ether in a
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separatory funnel, until the amount of lipide extracted in the ether layer was negligible (i.e., about 50 mg.). To this point, the procedure is essentially that of Calvery and White (8).

The proximate analysis of the original yolk was found to be as follows: water 67.2, lipide 9.1, protein 22.5, ash 1.2 per cent. In this respect the salmon egg is very similar in composition to those of the trout and sturgeon (3).

The same procedure has been applied to hen's egg yolk to obtain a solution suitable for electrophoresis.

Methods—The chemical methods used to characterize the fractions separated included determinations of total nitrogen by the Pregl micro-Kjeldahl procedure, total phosphorus by the method of Allen (9), moisture by desiccation to constant weight over phosphorus pentoxide in a "pistol" desiccator at 35°, ash by direct ignition, lipide by the method of Chargaff, Bendich, and Cohen (10).

Electrophoretic analysis was carried out in the Longsworth modification of the Tiselius apparatus. It was found that a minimum of 0.2 M sodium chloride was necessary to keep the protein in solution and the total ionic concentration of the buffer mixtures was approximately 0.3. The concentration of protein was determined by means of a dipping refractometer on the basis of a specific refractive increment of 0.002.

Fractionation—Various methods have been tried to separate the yolk proteins, especially of salmon eggs. A mixture of all proteins was prepared from the stock solution by the acetone method of Piettre (11). A snow-white powder was obtained which retained only partial solubility (20 per cent) in saline solution. Considerable denaturation must have resulted and this method was useful only in furnishing average analytical figures for the total proteins, as shown in Table I. Although we have obtained many fractions, the application of the adopted analytical criteria has not indicated separation of characteristic fractions except in some instances. We have tried separating the lipophosphoprotein by dilution with controlled hydrogen ion concentration, by dialysis, by isoelectric precipitation, by ethanol fractionation, and by means of sodium sulfate.

Lipovitellin was prepared from the stock solution by adjusting the pH to 3.5 and storing at 0° overnight. The resulting precipitate was redispersed in 10 per cent sodium chloride at pH 7.5 or in 0.5 per cent of ammonium hydroxide at pH 10. It was reprecipitated at pH 3.7 and dehydrated with anhydrous acetone or by lyophilizing. Repetition of dissolution and precipitation tended to make the material insoluble. Half saturation with ammonium sulfate of the filtrate from the initial isoelectric precipitation and adjustment of the acidity to pH 5.6 did not furnish a characteristic fraction.
Lipovitellin was also prepared by dialysis of the stock solution, essentially by the procedure of Chargaff (12). A portion of the final precipitate in the moist state was dissolved in a buffer solution as listed below and examined electrophoretically. A single boundary was obtained for hen lipovitellin after three precipitations, but there were always three boundaries in the case of the salmon yolk preparation, even after four reprecipitations.

Livetin was prepared from the stock solution by dilution with 10 volumes of water adjusted to pH 5.0. The precipitate was centrifuged but redissolved in 10 per cent sodium chloride with some difficulty. The solution was again diluted with water as before. The precipitate was washed with water until free from chloride, then with acetone (reagent), and finally with anhydrous acetone. A fine white powder was obtained. pH 5 was selected from the experience of Bernheim and Bernheim (13) with trout eggs and from our own preliminary experiments which indicated that some protein was precipitable at this point. The object was to separate possible nucleoprotein from phosphoprotein.

The supernatant fluid after the first centrifugation was half saturated with ammonium sulfate. A flocculent precipitate was obtained. This was dissolved in 10 per cent sodium chloride and diluted with water without formation of any precipitate. The solution was again half saturated with ammonium sulfate, and the precipitate recovered, washed with 50 per cent aqueous acetone until free from sulfate, and dehydrated with dry acetone. A fine, slightly brownish powder was obtained.

No further precipitate formed at full saturation with ammonium sulfate, even on further acidification to pH 2 or on heating to 100°.

An attempt was made to fractionate the stock solution of yolk proteins with ethanol, by diminishing the salt concentration to a minimum to retain the proteins in aqueous solution, and by controlling the pH and temperature of the mixture. The degree of precipitation was determined by estimating the total nitrogen per ml. in the filtrate and in the original solution. Operations were carried out in the cold room at 0° and −5°. The degree of separation was followed electrophoretically. The results are shown in Fig. 1. It is apparent that most of the protein was precipitated between 15 and 20 per cent of ethanol. By lowering the pH of the solution, precipitation became more complete at lower concentrations of ethanol.

Fractionation was also accomplished by means of sodium sulfate added to the stock solution as a 40 per cent solution. Precipitation began at about 15 per cent and at 25 to 30 per cent it had leveled off, with only 10 per cent of the total proteins still in solution.

Analysis—The composition of various preparations is listed in Table I. The results do not establish a uniform composition for lipovitellin as pre-
**PER CENT ALCOHOL**

Fig. 1. Effect of ethanol on the solubility of proteins of salmon yolk at 0° and at various pH levels.

**TABLE I**

*Analysis of Proteins from Salmon Yolk*

<table>
<thead>
<tr>
<th></th>
<th>N* per cent</th>
<th>P* per cent</th>
<th>Lipide* per cent</th>
<th>Ash per cent</th>
<th>Method of separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proteins</td>
<td>16.24</td>
<td>1.14</td>
<td>1.33</td>
<td>5.18</td>
<td>Piettre</td>
</tr>
<tr>
<td>&quot;</td>
<td>15.63</td>
<td>1.11</td>
<td>1.30</td>
<td>4.66</td>
<td>&quot;</td>
</tr>
<tr>
<td>Lipovitellin</td>
<td>14.1</td>
<td>2.11</td>
<td>9.6</td>
<td>?</td>
<td>Precipitation by dialysis</td>
</tr>
<tr>
<td>Vitellin</td>
<td>14.8</td>
<td>1.53</td>
<td></td>
<td></td>
<td>(NH₄)₂SO₄ after ethanol</td>
</tr>
<tr>
<td>Lipovitellin</td>
<td>15.26</td>
<td>1.20</td>
<td>2.90</td>
<td>4.73</td>
<td>Precipitation, pH 3.7</td>
</tr>
<tr>
<td>Vitellin</td>
<td>15.71</td>
<td>1.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipovitellin</td>
<td>16.73</td>
<td>1.32</td>
<td>1.62</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Vitellin</td>
<td>16.32</td>
<td>0.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracted</td>
<td>16.03</td>
<td>1.06</td>
<td>0.17</td>
<td>4.92</td>
<td>Acetone after ethanol</td>
</tr>
<tr>
<td>Livetin</td>
<td>14.9</td>
<td>0.10</td>
<td>0.68</td>
<td>0.74</td>
<td>Dilution, pH 5.0</td>
</tr>
<tr>
<td>Extracted</td>
<td>15.0</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculated on a moisture- and ash-free basis.

pared by different methods. Analyses of vitellin were done after extraction of the corresponding lipovitellin samples.

Compared with the values for hen lipovitellin obtained by Chargaff (12),
the salmon protein obtained by dialysis has a much lower lipide content, 9 versus 18 per cent, a higher nitrogen level, 14.1 versus 13.0 per cent, and a higher phosphorus content, 2.1 versus 1.5 per cent. The extracted residue, vitellin, agrees much more closely in nitrogen level, 16.0 versus 15.5, and in phosphorus, 1.0 versus 1.0 per cent.

The livetin fraction was obviously impure, as indicated by a content of 0.68 per cent of lipide. However, compared with the values for hen livetin of Kay and Marshall (14), we have nitrogen at 15.0 versus 15.4 and phosphorus at 0.1 versus 0.05 per cent. Needham (15) has carried out the only analysis of livetin from fish egg yolk, the dogfish, and obtained values of 12.1 per cent for nitrogen and 0.04 per cent for phosphorus.

**Nucleoprotein**—Qualitative tests on the acetone precipitate of total proteins showed the presence of pentose (Bial) and of desoxyribonucleic acid (16). Lipovitellin preparations, however, gave negative results, thus eliminating the possibility that the major protein of salmon yolk might have been a liponucleoprotein. The trace of nucleoprotein may have come from cellular material in the eggs.

**Isoelectric Point of Lipovitellin**—With material obtained by dialysis, the isoelectric point of this protein was determined from the point of maximum turbidity. A saturated solution of lipovitellin in dilute sodium chloride solution was added to a series of buffer solutions between pH 3.5 and 5.4. The degree of turbidity was measured nephelometrically and the pH electrometrically. Below pH 2.0 and above pH 10 no turbidity appeared. In two series the points of greatest turbidity occurred at pH 3.90 and 3.86.

This determination was repeated on a solution of lipovitellin in 5 per cent sodium chloride by adjusting the pH with 0.1 N hydrochloric acid in graduated centrifuge tubes. These were spun for 30 minutes at 2000 r.p.m. and the volume of solid observed. Under these conditions the isoelectric point was found to be 3.7. With hen lipovitellin by the same method, the value obtained was 3.4 to 3.5.

**Electrophoresis**—The distribution of proteins in the stock solution was first determined. A portion of this solution was diluted with sufficient buffer to make the final concentration approximately 1 per cent.

The buffer solutions employed and other conditions are shown in Table II. The solutions were made up as follows: 0.2 M sodium chloride (1.17 per cent), 0.1 M sodium diethyl barbiturate (2.062 per cent), 0.02 M diethylbarbituric acid (0.368 per cent), pH 8.5; 0.2 M sodium chloride (1.17 per cent), 0.02 M potassium dihydrogen phosphate (0.272 per cent), to which was added 0.02 M sodium hydroxide to obtain the desired pH level. The period of electrophoresis was usually 6 hours.

In all cases, three migrating boundaries were observed which were distributed in the proportions of 3.6, 8.5, and 87.9 per cent in salmon yolk, and 4.6, 10.2, and 85.1 per cent in hen yolk, as shown in Table III. No
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Further separation was noted in any buffer solution used within a period of electrophoresis of 15 hours. The ascending and descending boundaries were very similar in pattern. The similarity of the results for salmon and hen proteins is noteworthy. The electrophoretic picture does not confirm the finding of Fevold and Lausten (5) of the existence of a second lipo-

**Table II**

*Conditions of Electrophoresis for Yolk Proteins*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Buffer</th>
<th>$\gamma$</th>
<th>pH</th>
<th>Total protein</th>
<th>Time</th>
<th>Current</th>
<th>Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloride + phosphate</td>
<td>0.32</td>
<td>6.10</td>
<td>0.77</td>
<td>300</td>
<td>12.5</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>&quot; + &quot;</td>
<td>0.25</td>
<td>7.30</td>
<td>0.72</td>
<td>323</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>&quot; + &quot;</td>
<td>0.32</td>
<td>7.40</td>
<td>1.11</td>
<td>240</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>&quot; + barbiturate</td>
<td>0.32</td>
<td>7.45</td>
<td>0.64</td>
<td>315</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>&quot; + &quot;</td>
<td>0.32</td>
<td>8.50</td>
<td>2.38</td>
<td>363</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>&quot; + phosphate</td>
<td>0.32</td>
<td>8.74</td>
<td>0.97</td>
<td>300</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>&quot; + barbiturate</td>
<td>0.32</td>
<td>8.5</td>
<td>1.11</td>
<td>330</td>
<td>15</td>
<td>75</td>
</tr>
</tbody>
</table>

**Table III**

*Electrophoretic Distribution of Yolk Proteins*

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Per cent distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$</td>
</tr>
<tr>
<td>Salmon whole yolk, pH 7.3</td>
<td>1.8</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; 7.4</td>
<td>3.9</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; 8.5</td>
<td>5.0</td>
</tr>
<tr>
<td>lipovitellin by dilution</td>
<td>7.5</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; dialysis, pptd. twice</td>
<td>1.7</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; 3 times</td>
<td>0.0</td>
</tr>
<tr>
<td>&quot; supernatant fluid after ethanol at 22%</td>
<td>3.5</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; 50%</td>
<td>100</td>
</tr>
<tr>
<td>Hen whole yolk, pH 8.5</td>
<td>4.6</td>
</tr>
<tr>
<td>&quot; lipovitellin by dialysis</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Protein constituting approximately 30 per cent of the total proteins of hen’s egg yolk. We interpret the diagrams to indicate lipovitellin as the $\gamma$ peak, livetin as the $\beta$ peak, and vitellomucoid or phosvitin as the $\alpha$ peak. This requires experimental confirmation by isolation. If correct, the livetin fraction at 10 per cent of the total proteins is less than that claimed at 20 to 25 per cent by Kay and Marshall (14), and the mucoid is more than about 1 per cent, as stated by Onoe (17), or close to the value of 6.5 per cent for phosvitin (6). The multiple boundaries in the preparation of
livetin of Shepard and Hottle (7) can be explained as impurities rather than as due to the existence of several forms of this protein. The presence of both ovalbumin and conalbumin in hen's egg yolk, as claimed on immunological evidence by Marshall and Deutsch (18), is not apparent in our electrophoretic diagrams.

Typical electrophoretic diagrams are shown in Fig. 2.

It is apparent from Table III that the method of precipitation by dialysis will permit the isolation of a preparation of lipovitellin which is homogeneous in electrophoresis, if solution and reprecipitation are repeated three or four times. A single component may be isolated by means of 50 per cent of ethanol, but its identity has not been established.
SUMMARY

Several methods have been used to fractionate the yolk proteins of the eggs of the salmon and of the hen. Precipitation by dialysis was the most successful, if repeated three or four times, and yielded lipovitellin in a state of electrophoretic homogeneity.

Electrophoretic analysis revealed only three components, distributed in the proportions of 3.6, 8.5, and 87.9 per cent in salmon yolk, and 4.6, 10.2, and 85.1 per cent in hen's egg yolk. Electrophoresis was carried out in a buffer solution containing sodium chloride and barbiturate at pH 8.5, ionic strength 0.32, and a field strength of 7 to 13 volts per cm.

Lipovitellin from salmon eggs has been found to contain 9 per cent or less lipide, 14.1 per cent nitrogen, and 2.1 per cent phosphorus. Salmon vitellin contained 16.0 per cent nitrogen and 1.0 per cent phosphorus. An impure specimen of salmon livetin contained 14.9 per cent nitrogen and 0.10 per cent phosphorus. The isoelectric point of lipovitellin was found to be at pH 3.7 to 3.9 for the salmon, and at pH 3.4 to 3.5 for the hen. Lipovitellin appears to be readily denatured in contact with water at 20°.

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