CLVII. THE SECOND PROTEIN (LIVETIN) OF EGG-YOLK.

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IF the usual method is adopted for the preparation of lecitho-vitellin from hen's egg-yolk, namely, dilution of the egg-yolk with an equal volume of 10 % sodium chloride solution, thorough extraction of the mixture with ether, followed by dilution of the resulting clear aqueous layer with 15 to 20 volumes of water, it will be found that whilst the lecitho-vitellin is almost quantitatively precipitated, there is still an appreciable amount of protein-nitrogen in the filtrate [Gross, 1899, quoted by Plimmer]. Plimmer [1908] by acidifying the filtrate with acetic acid and boiling to coagulate the protein, obtained several grams of this material, which he called "livetin." He determined the nitrogen partition by the Hausmann method, and found that the figures agreed fairly closely with those obtained for vitellin, but differed from those obtained for ovalbumin. Since this work, little seems to have been done on this second protein of egg-yolk. Apart from the careful analyses of Plimmer just mentioned, we are not aware of any information as to its composition, or even as to the class of protein to which it belongs. Moreover Plimmer, although he brought evidence to show that his product was not ovalbumin, did not demonstrate clearly that the proteins of the white did not contribute to his coagulum. He put forward the suggestion that the product he separated might be closely related to vitellin—in fact, vitellin minus the phosphorus-containing fraction of the molecule.

During the course of the preparation of some pure ovovitellin we have taken the opportunity of examining our residues for this little studied substance, and are now able to present some more definite data as to its nature and properties.

Preparation of livetin.

We find that it is difficult, if not impossible with some eggs, to free the yolk quantitatively from adherent white by washing it simply in running water as did Plimmer. To be quite sure that the yolk is clean, we find it necessary to wash it in three or four changes of 0.9 % sodium chloride (to dissolve the globulin satisfactorily); and also to remove the chalazae with scissors. The yolk is further cleaned by allowing it to roll slowly down a strip of dry

calico sheeting, returning it, if necessary, to be washed again in the salt solution. It is finally dried, as far as possible, on the sheeting. In this way the white can be completely removed. Lecitho-vitellin is prepared from the clean yolks in the usual way¹, leaving invariably a considerable quantity of non-vitellin protein-nitrogen in the final filtrate. By half saturation with ammonium sulphate almost the whole of this protein may be precipitated. The filtrate from this precipitate gives only a very slight opalescence with trichloroacetic acid, thus demonstrating the absence of appreciable amounts of albumin. The precipitate is taken up with water, the concentration of ammonium sulphate brought up to 25 %, and the slight precipitate filtered off. To the clear filtrate sufficient saturated ammonium sulphate solution is added to bring the concentration of the salt to half saturation, the precipitated livetin filtered off, redissolved, and again precipitated. Solution and precipitation are repeated once more, the precipitated protein is then dissolved in the minimal quantity of water and the lipins are removed by alcohol-ether extraction at -15° [Hewitt, 1927]. The extracted protein is freed from alcohol and ether by Hewitt's method, only a small fraction undergoing denaturation during the process. The dried, extracted protein, containing ammonium sulphate, is taken up in water, filtered, reprecipitated with ammonium sulphate, dissolved in the minimal quantity of water, and the solution, adjusted to p_H 5.0, is dialysed in closed collodion sacs at 3° against repeated changes of distilled water until at least two days after the dialysate has ceased to give the faintest opalescence with barium chloride.

Although there are now only traces of salts in the sacs, the livetin is still in solution. Its behaviour is therefore that of a pseudoglobulin. In a few cases the dialysed solutions are opalescent, and may be clarified by filtration through paper pulp. On adding the clear, dialysed solution to 10 volumes of distilled water, an opalescence is produced, which disappears on the addition of dilute sodium chloride or ammonium sulphate solution. On continuing the dialysis for some weeks, a portion of the protein is precipitated.

EXAMINATION OF LIVETIN.

Qualitative and precipitation tests.

A 0.6 % solution of livetin gives a precipitate on boiling alone, or on boiling with very dilute acetic acid, a precipitate in the cold with three volumes of alcohol or acetone, with trichloroacetic, picric, tungstic and sulphosalicylic acids, with the salts of the heavy metals, and with colloidal ferric hydroxide. It is completely precipitated by half saturation with ammonium sulphate, by saturation with magnesium sulphate or sodium chloride (after 24 hours'

¹ A short dialysis of the lecitho-vitellin-NaCl solution against distilled water before precipitation of the phosphoprotein with water considerably reduces the amount of dilution necessary for its complete separation, and hence diminishes the bulk of the filtrate from which the livetin has to be precipitated.

standing in the case of the last two salts). It gives the biuret, xanthoproteic and Millon's reactions, a strong glyoxylic reaction, but a very weak, almost negative, Molisch reaction. With a 4 % solution of the protein, a positive sulphur reaction is given with sodium hydroxide and lead acetate. After hydrolysis with trypsin it gives a strong Pauly's reaction, and a definite but weak Totani's "secondary reaction" for histidine.

Quantitative analysis.

1. *Nitrogen, phosphorus and sulphur.* Livetin, prepared by adding to its solution in dilute ammonium sulphate, at the stage before alcohol-ether extraction, three volumes of alcohol and heating to boiling, gave, after washing the coagulated protein with water and alcohol, and drying at 100°, ash = 1 %; N = 15.12 %; P = 0.12 %. (Plimmer found N varying between 14.8 % and 15.2 %, and P values between 0.1 % and 0.65 % in five different specimens of livetin.)

A purer specimen after alcohol-ether extraction gave ash = 0.6 %; N = 15.35 %; P = 0.067 %, *i.e.* a much lower content of phosphorus. On our two purest specimens of livetin the N : P ratio was 1 g. N : 3.13 mg. P, and 1 g. N : 3.15 mg. P, respectively. This phosphorus is not extractable by alcohol-ether, either in the cold or on boiling. It is rather less than one-half of the amount of P per gram of N present in extracted egg-albumin [Hewitt, 1927]. Assuming that there is only one atom of P in each molecule of protein, the minimum molecular weight of livetin works out at about 64,000.

The total sulphur content of livetin is 1.80 % (Carius); this high figure agrees with the finding of a relatively large quantity of cystine in the molecule (see next paragraph). Taken in conjunction with the results shortly to be described for the ratio lecitho-vitellin : livetin in the yolk, and for the sulphur content of the former protein, it appears that about one-half of the total sulphur of the yolk is originally present as livetin.

2. *Amino-acids.* Using the method of Folin and Ciocalteu [1927] for tyrosine and tryptophan and that of Folin and Looney [1922] for cystine, the following values have been obtained for the percentage of these amino-acids in purified livetin (three specimens):

tyrosine 5.14, 5.20, 5.17; mean 5.17;

tryptophan 2.08, 2.34, 2.00; mean 2.14;

cystine 3.5, 4.3, 3.9¹; mean 3.9 (= 1.05 % cystine-sulphur).

According to analyses made at the same time, vitellin contains 5.0 % tyrosine, 1.6 % tryptophan and only 1.4 % cystine, with a total sulphur content of 0.6 %. Lecitho-vitellin contains only 0.5 % sulphur. Plimmer's suggestion of the possibility of a direct relationship between vitellin and livetin is not borne out by these results.

¹ Specimen of livetin from yolk of duck's egg.

Physical constants.

A preliminary investigation of some of the physical constants of the pure, dialysed protein has been made.

1. *The c_H for minimum solubility.* This was determined approximately by adding small portions of the protein solutions to acetate buffers, and then adding neutral alcohol till precipitation occurred. Using 2 cc. buffer, 0.3 cc. of 3.2 % livetin solution and 1 cc. alcohol, maximum precipitation occurred between p_H 4.8 and 5.2. A second experiment with the same livetin solution gave optimal precipitation at p_H 5.0. A different specimen of the protein (2.1 % solution) gave minimum solubility at p_H 4.8¹. It may therefore be concluded that the isoelectric point of livetin is in the neighbourhood of p_H 4.8–5.0. The titration curve of the purified protein, kindly determined by Dr J. R. Marrack, indicates a broad isoelectric zone in this region.

2. *The refractive index* of the aqueous solution was determined using Pulfrich's dipping refractometer and a glass mercury-vapour lamp. At 20°, four alcohol-ether-extracted specimens gave 0.00184; 0.00192; 0.00189; 0.00196; mean 0.00190, as the refractive index for 1 % protein. Pure ovalbumin in the same apparatus gave a mean value for 1 % protein of 0.00192.

3. *The optical rotation* of several specimens of the protein, prepared by slightly different methods, and in solutions of different concentrations was kindly determined for us by Dr L. F. Hewitt. The specific rotation varied considerably with the purity of the material; for pure, alcohol-ether-extracted specimens the following results were obtained:

$$[\alpha]_{5461}^{20} = (\text{to nearest degree}) - 55^\circ; - 53^\circ; - 56^\circ; - 58^\circ; \text{mean} - 55.5^\circ.$$

The pure protein appears, therefore, to have a slightly higher specific rotation than ovalbumin, and a rather lower one than serum-globulin [cf. Hewitt, 1927, Table V].

QUANTITY OF LIVETIN PRESENT IN THE YOLK.

A method for the estimation of the amount of livetin in a single yolk has been devised. The yolk is cleaned and dried as previously described, then transferred to a 50 cc. cylinder, the vitelline membrane being removed with forceps. (If absolute values are required, it is necessary to introduce obvious modifications here.) The volume is measured, an equal volume of 8 % NaCl is added, and the whole stirred vigorously till the mixture is homogeneous. 20 cc. of the diluted egg-yolk is transferred to a graduated, stoppered cylinder, and shaken with 40 cc. ether containing 2.5 % alcohol. The ether is removed after the layers have separated, and the extraction repeated thrice with 20 to 25 cc. of ether. The ether is removed as completely as possible and 4 % NaCl

¹ It is of interest to record that similar concentrations of pure ovalbumin, in these buffer solutions, required only one-fifth of this amount of alcohol to produce a turbidity; i.e. ovalbumin, though less readily precipitated by salt solutions, is much more sensitive to the presence of alcohol than is livetin.

solution added to make the volume up to 25 cc. 0.5 cc. is removed in duplicate for total N determination (micro-Kjeldahl) (*A*). 20 cc. are diluted to 200 cc. with distilled water. After standing overnight to allow the precipitated lecitho-vitellin to settle, the supernatant liquid is filtered. The protein in this filtrate is almost exclusively livetin. 20 cc. are taken in duplicate for total N determination (*B*). 80 cc. are treated with 20 cc. 25 % trichloroacetic acid, and filtered after standing for 15 minutes; total N is determined in duplicate on 40 cc. of the filtrate (*C*). The livetin in the original yolk may readily be calculated from the difference between *B* and *C*, whilst the difference between *A* and *B* gives the lecitho-vitellin content. (Livetin is assumed to contain 15 % and lecitho-vitellin 13 % N.)

By this method, the following values have been found:

Egg	Ratio lecitho-vitellin : livetin
Hen	3.83 : 1, 3.46 : 1, 3.89 : 1, 3.56 : 1, 3.62 : 1, 3.40 : 1; mean 3.63 : 1
Duck	3.96 : 1, 3.99 : 1, 3.35 : 1; mean 3.76 : 1

With the duck's egg the separation of the ether extract in the first stages of the determination is distinctly more difficult than with the hen's egg. A trace of capryl alcohol and the device of keeping the long extraction cylinder horizontal assist the separation.

It will be observed that, making allowance for certain almost inevitable experimental errors, the ratio lecitho-vitellin : livetin appears to be fairly constant from one egg to another, and possibly from one species to another. In the values given above for hens' eggs, the first four figures were from commercial "new-laid" eggs selected at random, the last two figures were from eggs 24 hours old, of pedigree Rhode Island Red parentage.

Between one-quarter and one-fifth of the protein of the yolk of the egg, therefore, is livetin, the absolute amount being, for an egg of average size, 0.6 to 0.9 g.

Is livetin a single protein?

Throughout this paper it has been tacitly assumed that the livetin of egg-yolk, prepared in the manner described, is a single individual. The method given above for determining the livetin in a single egg-yolk presupposes, in fact, that all the non-vitellin protein in egg-yolk is the same substance—livetin.

As against this assumption, it must be pointed out that (*a*) there is a small amount of protein precipitated by 25 % saturation with ammonium sulphate; (*b*) although there is only a trace of protein in the filtrate from 50 % ammonium sulphate precipitation, there appears to be no very sharp zone of precipitation between 25 and 50 % saturation in which the rest of the protein comes down quantitatively; (*c*) on long continued dialysis it appears possible to separate the livetin into two fractions, one more soluble in water than the other. So far the fractions have not been examined separately, but further work on this point is in hand.

In view of the work of Sørensen [1925] and of Linderström-Lang [1925], who have separated serum-euglobulin and pseudoglobulin and caseinogen into

fractions having different solubilities and phosphorus contents, the whole of our criteria as to the individuality of proteins may have to be revised. For the present it is justifiable to say that the preliminary examination of the non-vitellin protein of egg-yolk has revealed that by far the greater portion of it (90 % or more) behaves, by the usual criteria adopted up to now for protein characterisation, as a single individual.

SUMMARY.

1. It is confirmed that another protein beside vitellin is present in the yolk of the hen's or the duck's egg. This protein has been isolated in a fairly pure, undenatured form for the first time.

2. This substance, for which the name livetin suggested by Plimmer is retained, has, on the whole, the properties of a pseudoglobulin. It contains 5.2 % tyrosine, 2.1 % tryptophan, 3.9 % cystine, 0.05 % phosphorus, and 1.8 % sulphur. It has its isoelectric point in the neighbourhood of p_H 4.8-5.0, refractive index in 1 % aqueous solution = 0.00190 at 20°, $[\alpha]_{5461}^{20} = -55.5^\circ$. It is probably unrelated to ovalbumin or vitellin. Its high content of the indispensable amino-acids cystine and tryptophan suggests that it may have a considerable biological value.

3. A method is described for estimating the amount of this protein in a single egg-yolk. In the fresh yolk, the ratio vitellin : livetin is fairly constant from one egg to another. Between one-quarter and one-fifth of the yolk-proteins of the hen's or duck's egg is livetin.

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