Biochem. J. (1965) **96**, 372

Ovoglycoprotein, a Protein of Hen's-Egg White

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(*Received* 19 *October* 1964)

1. A description is given of the isolation of a glycoprotein from hen's-egg white; it has been called ovoglycoprotein. 2. It contains 13·6% of hexose, 13·8% of hexosamine and 3% of sialic acid. 3. Hexose occurs as mannose and galactose in the ratio 2:1, hexosamine as glucosamine and sialic acid as *N*-acetylneuraminic acid. 4. It has $S_{20,w}$ 2·47s and a minimum molecular weight, calculated from the tryptophan content, of 24400. 5. At pH 3·9 in acetate buffer, *I* 0·1, which is in the isoelectric region, it is resolved into two components, one of which moves slowly towards the anode while the other moves slowly towards the cathode.

As many as 19 different protein components can be separated by starch-gel electrophoresis of hen's-egg white (Lush, 1961). The major components have been known in purified form for some years (Warner, 1954), but many of the minor components are as yet unstudied. A riboflavine-binding protein has been isolated and characterized by Rhodes, Bennett & Feeney (1959), and more recently a preliminary note reported the isolation of a glycoprotein that has been called ovoglycoprotein (Ketterer, 1962). In the present paper an account is given of the isolation and some of the properties of this protein.

METHODS

Eggs. The eggs were obtained from various sources but were principally from White Leghorn × Black Australorp or White Leghorn × Black Orpington cross-bred hens.

Filter-paper electrophoresis. Filter-paper electrophoresis was performed in barbital buffer, pH 8·6 and *I* 0·05, on Whatman no. 3 filter paper by using the apparatus of Gordon, Gross, O'Connor & Pitt-Rivers (1952). Sharp separations were obtained after electrophoresis at 400v for 2hr. An aqueous bromophenol blue reagent was used to stain protein in general (Kunkel & Tiselius, 1951) and an Ehrlich dipping reagent (Smith, 1960*a*) was used to detect sialic acid-containing protein.

Carbohydrate analysis. Protein-bound hexose was determined by the orcinol method (Winzler, 1955) by using a standard composed of mannose and galactose in the ratio 2:1. The hexosamine content was determined by hydrolysis of the protein according to the method of Winzler (1955) and estimation of the released amino sugar by a modification of the Elson–Morgan reaction (Belcher, Nutten & Sambrook, 1954). Sialic acid was determined by the thiobarbituric acid assay (Warren, 1959). Methylpentose was estimated by the method of Dische & Shettles (1948).

Chromatographic analysis of sugar components. (*a*) Hexoses. A 25mg. sample of ovoglycoprotein was hydrolysed with 5ml. of 0·25N-H_2SO_4 at 105° in a sealed tube for 18hr. according to the procedure of Gottschalk & Ada (1956). The hydrolysate was adjusted to pH 7·0 with $Ba(OH)_2$ and passed through first Dowex 50 (H^+ form) and then Dowex 1 (formate form). The final effluent was freeze-dried and taken up in water, and portions were subjected to chromatography on Whatman no. 1 filter paper by using the solvent ethyl acetate–pyridine–water (12:5:4, by vol.) (Smith, 1960*b*). Glucose, galactose and mannose markers were run on the same chromatogram. The sugars were stained with an ammoniacal silver nitrate dipping reagent (Smith, 1960*b*).

In quantitative studies, Whatman 3MM filter paper that had been washed thoroughly with water and the ethyl acetate–pyridine–water solvent was used. The hexoses in the hydrolysate were compared with standards that had been submitted to the same chromatographic procedure to account for losses on the chromatogram. Each application of either standard solution or hydrolysate was run in duplicate so that one could provide a marker for the other. The areas of the chromatogram corresponding to the positions of hexoses were each cut into small pieces and extracted by shaking with 3ml. of water for 3hr. Portions of the extracts were estimated for reducing sugar by the method of King & Garner (1947).

(*b*) Hexosamine. The conditions for the hydrolysis of ovoglycoprotein were the same for the identification of hexosamines as for the analysis of hexoses. The neutralized hydrolysate was passed through a Dowex 1 (formate form) column, freeze-dried and taken up into solution in water. The hexosamines were not identified as such, but were oxidized to the corresponding pentoses with ninhydrin and identified chromatographically by comparison with glucosamine and galactosamine standards similarly oxidized with ninhydrin (Stoffyn & Jeanloz, 1954).

(*c*) Sialic acid. Sialic acid was released from ovoglycoprotein by hydrolysis with 0·1N-H_2SO_4 for 1hr. at 80°. The hydrolysate was neutralized with $Ba(OH)_2$ and then freeze-dried. Sialic acid was extracted from the freeze-

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dried material with 80% (v/v) methanol and chromatographed on Whatman no. 1 filter paper by using the butan-1-ol–propan-1-ol–0·1N-HCl solvent system of Svennerholm & Svennerholm (1958) to distinguish between *N*-acetyl- and *N*-glycolyl-neuraminic acid, and a butan-1-ol–acetic acid–water (4:1:5, by vol.) system to distinguish between *N*-acetyl- and *NO*-diacetyl-neuraminic acid (Whitehouse & Zilliken, 1960). *N*-Acetylneuraminic acid was used as a marker and the Ehrlich dipping reagent was used to detect the sialic acids. The presence or absence of *N*-glycolylneuraminic acid was further tested for by hydrolysing the sialic acid with N-H_2SO_4 for 1hr. at 100° and testing for free glycolate according to the method of Klenk & Uhlenbruck (1957).

N-Terminal amino acid. A 10mg. sample of ovoglycoprotein was treated with 1-fluoro-2,4-dinitrobenzene according to the method of Sanger (1945). The resulting DNP-ovoglycoprotein was hydrolysed in 6N-HCl in a sealed tube at 105° for 16hr.

The ether-soluble phase of the hydrolysate was subjected to two-dimensional chromatography by using the toluene–2-chloroethanol–pyridine–aq. ammonia solvent of Biserte & Osteux (1951) in the first dimension and 1·5M-phosphate buffer, pH6·0, in the second (Levy, 1954). The aqueous phase of the hydrolysate was examined chromatographically in a butan-1-ol–acetic acid–water (4:1:5, by vol.) system and the 2-methylbutan-2-ol–phthalate system of Blackburn & Lowther (1951) as recommended by Levy (1955).

Marker DNP-amino acids were prepared from the free amino acids according to the method of Sanger (1945).

The yield of DNP-amino acid was determined by elution of the spot with 1% (w/v) $NaHCO_3$ and determination of the optical extinction at 360mμ. Reference was made to published values for the extinction coefficients of the DNP-amino acids (Levy, 1955).

Other analyses. Nitrogen was determined by micro-Kjeldahl digestion. Tyrosine and tryptophan were estimated by the spectrophotometric method of Goodwin & Morton (1946). All analyses were expressed in terms of dry wt. of protein determined by heating the ovoglycoprotein sample to constant weight *in vacuo* at a temperature of 105°.

Moving-boundary electrophoresis. Moving-boundary electrophoresis was performed in the Perkin–Elmer model 38A apparatus. The following buffers were used: barbital buffer at pH8·7, phosphate buffer at pH6·8 and acetate buffer at pH5·0, 4·2, 4·0, 3·9 and 3·8. The ionic strength of all these buffers was 0·1, with the exception of the phosphate buffer, which had an ionic strength of 0·16. All runs were performed at 1°.

Ultracentrifugation. Ultracentrifugation analysis was performed in the Spinco model E ultracentrifuge at a speed of 58000rev./min. and a temperature of 18°. The buffer used was 5mM-tris at pH7·4 made 0·145M with respect to NaCl.

Anti-haemagglutination test. Ovoglycoprotein was tested for its ability to inhibit haemagglutination by the Lee strain of influenza virus according to the method of Gottschalk & Lind (1949).

Tests for anti-tryptic and anti-chymotryptic activities. Anti-tryptic and anti-chymotryptic activities were tested for by incubating ovoglycoprotein with the respective proteinase and determining proteolytic activity by the method of Kunitz (1947) by using a casein substrate.

EXPERIMENTAL

Isolation. Blended egg white was first fractionated with ethanol according to a procedure modified slightly from that of Forsythe & Foster (1950). Sodium chloride was added to the egg white to give a concentration of 0·15M and the pH was adjusted to 6·2 with acetic acid. Ethanol was then added to give a concentration of 20% (v/v) and the precipitate that formed was discarded. The supernatant was then adjusted to pH4·6 and more ethanol added until the concentration was 30% (v/v). A copious precipitate formed and was rejected. Finally the supernatant was made 70% (v/v) with respect to ethanol and the precipitate that formed was collected and dialysed. Material that went into solution on dialysis was separated and referred to as fraction A. On electrophoresis it was shown to contain only ovomucoid and ovoglycoprotein. The entire ethanol fractionation was conducted at −5°.

Ovoglycoprotein was more readily adsorbed on to calcium phosphate gel than was ovomucoid, and was selectively adsorbed from fraction A by titration with calcium phosphate gel (Colowick, 1955). The progress of the titration was followed by filter-paper electrophoresis, in which ovoglycoprotein moves faster than ovomucoid and is further distinguished by giving a pink colour after treatment with the Ehrlich dipping reagent.

Calcium phosphate gel was prepared according to the procedure of Keilin & Hartree (1938). To follow the titration by filter-paper electrophoresis it was necessary to concentrate fraction A to about 4% protein and to minimize dilution during the titration by concentrating the portions of gel used by giving them a brief centrifugation. At each addition of calcium phosphate gel the pH was adjusted to 5·5 with acetic acid and the suspension was stirred for 10min.

When most of the ovoglycoprotein was shown to have been adsorbed, the gel was separated by centrifugation and stirred with 1mM-disodium hydrogen phosphate for 20min., which selectively eluted such ovomucoid as had also become adsorbed. The ovoglycoprotein was then eluted with 0·2M-phosphate buffer, pH6·8. Repetition of the titration with calcium phosphate gel gave a product with a high degree of purity.

The amount of ovoglycoprotein in fraction A was determined by quantitative filter-paper electrophoresis (Kunkel & Tiselius, 1951) and found to be equivalent to approx. 1% of the total egg-white protein.

Criteria of purity. Ovoglycoprotein prepared thus moved as a single component on filter-paper electrophoresis at pH4·0 and 8·6. It also moved as a single boundary on free electrophoresis in the

Tiselius apparatus at pH 8·7, 6·8, 5·1 and 4·3. However, at pH 4·0, 3·9 and 3·8, which are in the region of the isoelectric point, two components were clearly resolved (see Fig. 1).

This preparation sedimented as a single boundary in the ultracentrifuge.

Only one *N*-terminal amino acid could be detected.

Carbohydrate analysis. Hexose and hexosamine contents were 13·6 and 13·8% respectively. The sialic acid content was 3·0%. Analysis for methylpentose showed that this type of carbohydrate moiety was absent. The total carbohydrate content was thus 29·4%. In accord with this high value the nitrogen content was found to have the relatively low value of 11·6%.

Identity of the carbohydrates. Mannose and galactose were the only hexoses present, occurring in the molar ratio 2:1. Hexosamine occurred exclusively as glucosamine.

Sialic acid occurred as *N*-acetylneuraminic acid. *NO*-Diacetylneuraminic acid was revealed neither chromatographically nor by analysis for the glycolyl substituent.

N-Terminal amino acid. The only α-DNP-amino acid that was detected in hydrolysates of DNP-ovoglycoprotein was α-DNP-threonine. The yield was 0·125 mole of α-DNP-threonine/10 000 g. of ovoglycoprotein.

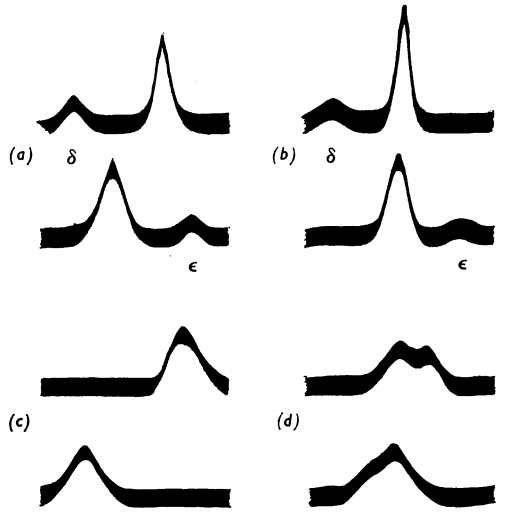

Fig. 1. Moving-boundary electrophoretic patterns of ovoglycoprotein (the ascending pattern is shown above the descending pattern in each case). (*a*) In barbital buffer, pH 8·7 and *I* 0·1, for 120 min.; (*b*) in phosphate buffer, pH 6·8 and *I* 0·16, for 120 min.; (*c*) in acetate buffer, pH 4·3 and *I* 0·1, for 180 min.; (*d*) in acetate buffer, pH 4·0 and *I* 0·1, for 150 min.

Aromatic amino acids. The tyrosine and tryptophan contents of ovoglycoprotein determined spectrophotometrically were 2·35 and 0·85% respectively. The $E^{1\%}_{280\,m\mu}$ of the protein in neutral solution was 3·8.

Solubility properties. Ovoglycoprotein is soluble in 60% (v/v) ethanol and 75% saturated ammonium sulphate at 0°, and is not precipitated by such common protein precipitants as trichloroacetic acid, phosphotungstic acid, sulphosalicylic acid and perchloric acid. In solution in water it can be heated to 100° without precipitating.

Approximate isoelectric point. In moving-boundary electrophoresis a single boundary was found in all buffers above pH 4·2. At pH 4·2 the boundary began to spread, and at pH 4·0, 3·9 and 3·8 two components clearly separated. At pH 4·0 both components moved towards the anode and at pH 3·8 both components moved towards the cathode, whereas at pH 3·9 one moved slowly towards the anode and the other moved slowly towards the cathode. Thus the isoelectric points of the two components in acetate buffer, *I* 0·1, appear to be just above and just below pH 3·9.

Sedimentation coefficient. The sedimentation coefficient corrected to water at 20°, $S_{20,w}$, was 2·47 s.

Biological activity. Ovoglycoprotein did not inhibit haemagglutination by influenza virus, had no anti-tryptic activity beyond what could be accounted for by slight ovomucoid contamination and possessed no anti-chymotryptic activity whatsoever.

DISCUSSION

The list of glycoproteins in hen's-egg white, which includes ovalbumin, ovomucoid, avidin, ovomucin and riboflavine-binding protein, has been joined by another that has been called ovoglycoprotein. Its occurrence is similar to that of the riboflavine-binding protein, accounting for about 1% of the total egg-white protein. Its chemical and physical properties are summarized in Table 1 and compared with some of those of ovomucoid, which occurs with it in fraction A. It is distinct from ovomucoid in its composition and *N*-terminal amino acid, but is similar in some of its physical properties. Its presence in certain ovomucoid preparations as a contaminant could account at least in part for the variable and non-stoicheiometric amounts of sialic acid found in these preparations (Jevons, 1960).

The value of 2:1 obtained for the mannose/galactose ratio is probably approximate. The conditions used for hydrolytic release of the hexoses are the same as those used by Gottschalk & Ada (1956) in a study of ovomucoid and some other mucoproteins. These authors report only a 70% yield of

Table 1. *Chemical composition and physical properties of ovoglycoprotein and ovomucoid*

	Ovoglycoprotein	Ovomucoid
Composition (% of dry wt.)		
Hexose	13·6	8·2*
Glucosamine	13·8	14·1*
N-Acetylneuraminic acid	3·0	
Nitrogen	11·6	
Tyrosine	2·35	
Tryptophan	0·85	
Approx. mannose/galactose ratio	2:1	3:1*
Hexosamine/hexose ratio	1:1	1·7:1*
N-Terminal amino acid	Threonine	Alanine†
Sedimentation coefficient	2·47s	2·8s‡
Molecular weight	24400	27000‡
Approx. isoelectric point	pH3·9	pH3·9‡

* Gottschalk & Ada (1956).
† Fraenkel-Conrat & Porter (1952).
‡ Fredericq & Deutsch (1949).

hexose from ovomucoid and attribute this to incomplete hydrolysis of the hexosamine–hexose link under these mild conditions. Among the mucoproteins they studied, they found that the yield of hexose decreased as the hexosamine/hexose ratio increased. On this basis the error for ovoglycoprotein with a ratio of 1:1 might be expected to be less than that for ovomucoid where the ratio is 1·7:1.

As yet no biological activity has been attributed to it. For instance, although it contains sialic acid, unlike ovomucin it does not inhibit haemagglutination by the influenza virus. It does not possess anti-tryptic activity beyond that due to trace ovomucoid contamination and has no anti-chymotryptic activity. The latter activity is sometimes found in ovomucoid preparations; but it now seems probable that its presence there is due to contamination with ovoinhibitor (Feeney, Stevens & Osuga, 1963).

Although ovoglycoprotein behaved as a single component in the ultracentrifuge, moved as a single boundary at most pH values and only one *N*-terminal amino acid could be detected, it was resolved into two components in the neighbourhood of the isoelectric point. This seems likely to be a case of micro-heterogeneity or polymorphism, for which there are precedents among other glycoproteins. For example, ovomucoid is resolved into up to five components (Bier, Duke, Gibbs & Nord, 1952; Wise, Ketterer & Hansen, 1964) and serum α_1-acid glycoprotein is resolved into seven components (Schmid, Binette, Kamiyama, Pfister & Takahashi, 1962) near their respective isoelectric points.

The minimum molecular weight determined from the tryptophan content is 24400, which is in accord with the observed sedimentation coefficient, $S_{20,w}$, of 2·47s.

Assuming a molecular weight of 24000 the yield of α-DNP-threonine in the end-group assay is only 20%. This is a very low value; however, some degree of destruction of DNP-amino acid during the acid hydrolysis of DNP-glycoproteins has been observed by Fraenkel-Conrat & Porter (1952). Acid hydrolysis of ovoglycoprotein alone produces large quantities of humin.

The author is indebted to Miss Barrett for able technical assistance, to Dr I. T. Oliver for determination of the sedimentation coefficient and to Professor N. F. Stanley for the performance of the anti-haemagglutination test. The work was supported in part by a grant from the Australian Egg Board and the Department of Primary Industry of the Government of the Commonwealth of Australia.

REFERENCES

Belcher, R., Nutten, A. J. & Sambrook, C. M. (1954). *Analyst*, **79**, 201.
Bier, M., Duke, J. A., Gibbs, R. J. & Nord, F. F. (1952). *Arch. Biochem. Biophys.* **37**, 491.
Biserte, G. & Osteux, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Colowick, S. P. (1955). In *Methods in Enzymology*, vol. 1, p. 90. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Feeney, R. E., Stevens, F. C. & Osuga, D. T. (1963). *J. biol. Chem.* **238**, 1415.
Forsythe, R. H. & Foster, J. F. (1950). *J. biol. Chem.* **184**, 385.
Fraenkel-Conrat, H. & Porter, R. R. (1952). *Biochim. biophys. Acta*, **9**, 557.
Fredericq, E. & Deutsch, H. F. (1949). *J. biol. Chem.* **181**, 499.
Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
Gordon, A. H., Gross, J., O'Connor, D. & Pitt-Rivers, R. (1952). *Nature, Lond.*, **169**, 19.
Gottschalk, A. & Ada, G. L. (1956). *Biochem. J.* **62**, 681.
Gottschalk, A. & Lind, P. E. (1949). *Brit. J. exp. Path.* **30**, 85.
Jevons, F. R. (1960). *Biochim. biophys. Acta*, **45**, 384.
Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc.* B, **124**, 397.
Ketterer, B. (1962). *Life Sci.* **5**, 163.
King, E. J. & Garner, R. J. (1947). *J. clin. Path.* **1**, 30.
Klenk, E. & Uhlenbruck, G. (1957). *Hoppe-Seyl. Z.* **307**, 266.
Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291.
Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.

Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 360.
Lush, I. E. (1961). *Nature, Lond.*, **189**, 981.
Rhodes, M. B., Bennett, N. & Feeney, R. E. (1959). *J. biol. Chem.* **234**, 2054.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Schmid, K., Binette, J. P., Kamiyama, S., Pfister, V. & Takahashi, S. (1962). *Biochemistry*, **1**, 959.
Smith, I. (1960*a*). In *Chromatographic and Electrophoretic Techniques*, vol. 1, chapter 5. Ed. by Smith, I. London: William Heinemann.
Smith, I. (1960*b*). In *Chromatographic and Electrophoretic Techniques*, vol. 1, chapter 13. Ed. by Smith, I. London: William Heinemann.
Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* **52**, 373.
Svennerholm, E. & Svennerholm, L. (1958). *Nature, Lond.*, **181**, 1154.
Warner, R. C. (1954). In *The Proteins*, vol. 2, part A, p. 435. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
Whitehouse, M. W. & Zilliken, F. (1960). *Meth. biochem. Anal.* **9**, 190.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.
Wise, R. W., Ketterer, B. & Hansen, I. A. (1964). *Comp. Biochem. Physiol.* **12**, 439.