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Egg albumen polymorphisms in the fowl: The ovalbumin locus

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1. INTRODUCTION

In the first paper of this series (Lush, 1961) it was tentatively concluded that ovalbumin may exist in two genetically different forms and that the alleles which determine this difference are segregating in three of the Poultry Research Centre (P.R.C.) Brown Leghorn populations. In this paper a modified starch gel electrophoresis technique and enzymatic modification of ovalbumin have been used to study the nature of the genetic difference between the two types of ovalbumin and the causes of the electrophoretic heterogeneity within each type of ovalbumin. Some data on the mode of inheritance of the ovalbumin types are presented. Finally, the occurrence of ovalbumin in the fluid contained in right oviduct cysts is demonstrated.

2. MATERIALS AND METHODS

Poultry Research Centre (P.R.C.) Brown Leghorns were used exclusively for this work. The storage of eggs and preparation of albumen have already been described (Lush, 1964).

Starch gel electrophoresis. For some experiments the procedure described in the previous paper (Lush, 1964) was used; this is referred to as 'alkaline buffer conditions'. For other experiments a new procedure was developed, referred to as 'acid buffer conditions', in which the gel buffer contained 0·00935 M Tris (hydroxymethyl) amino methane and 0·005 M citric acid, pH 5·1; and the end-tray buffer contained 0·041 M succinic acid and 0·05 M sodium hydroxide, pH 5·0. The gel was made in a glass tray 3 mm. deep. The electrophoresis was carried out in a cold room at 5°, the gel being cooled to this temperature before the samples were inserted. A voltage gradient of 12·5 volts/cm. was maintained along the gel for about 4 hours, during which it was cooled by a fan. After electrophoresis the gel was sliced in half with a 6 in. length of razor blade (a blade of suitable thickness was obtained from the Ever-Ready Razor Co., Colquhoun Ave., Glasgow) and stained overnight in nigrosine dissolved 0·01% in a 5:5:1 mixture of water, methanol and acetic acid. To elute fractions from the gel, the section of the gel containing the wanted material was frozen to −20° and thawed. The gel was then spongy in texture and the liquid contained in it could be squeezed out.

Crystallization of ovalbumin. The procedure was based on that of Cole (1932) and differed only in that the thin portion of the albumen was used in preference to whole

albumen. This eliminated the need for any mechanical homogenization, which was thought to be unwise in view of the ease with which ovalbumin can be denatured. The first crystallization was allowed to continue for some days, after which the crystals were collected in the centrifuge and redissolved in 0·9% saline to give an approximately 1% solution of ovalbumin which was stored at $-20°$.

Dialysis was carried out at 5°. Visking dialysis tubing (18/32 in.) was cleaned by twice heating in water at 80° to 90° and subsequently soaking in several changes of water (Hughes & Klotz, 1956). During dialysis the external medium was continuously stirred.

Enzymes. Human prostatic phosphatase was extracted by the method of Schmidt (1955) from hypertrophic prostate glands collected fresh from the operating theatre and stored at $-20°$. The prostatic extract was assayed against 4-nitrophenyl phosphate (Bell & Siller, 1962) and its activity is expressed as micromoles of 4-nitrophenyl phosphate transformed by 1 ml. of extract in 1 min. at 37° in 0·2 M citrate buffer, pH 4·9.

Calf intestinal phosphatase, technical grade (Armour Pharmaceutical Co.) was dissolved in 0·9% NaCl and its activity was assessed in a similar manner to the prostatic phosphatase but using a 0·1 M $NaHCO_3$—Na_2CO_3 buffer, pH 10·08 (Bell, 1960).

Subtilisin (subtilopeptidase A), which was a gift from Dr M. Ottesen, had been prepared by the method of Guntelberg & Ottesen (1952 & 1954).

Sterilization. Some samples were sterilized by centrifugation through Oxoid membrane filters in a modified Hemmings filter (Beaumaris Instrument Co., Rosemary Lane, Beaumaris, Anglesey).

3. RESULTS

(i) *Description of the phenotypes*

Two ovalbumin phenotypes were originally described on the basis of electrophoresis in alkaline buffer conditions (Lush, 1961). With slight refinements of this technique it is possible to identify the presumptive heterozygote phenotype, but a great improvement can be achieved by electrophoresis of diluted albumen through a thin, cooled, gel at an acid pH, as described in the *Methods* section. The complexity of ovalbumin can best be appreciated by considering the results from both methods. With both methods three phenotypes can be recognized. One is identical with an equal mixture of the other two and probably represents the heterozygote. In the new nomenclature adopted in this paper the symbol for the genetic locus involved in the ovalbumin polymorphism has been changed from *I* to *Ov.*, and the three phenotypes are Ov.A, Ov.AB and Ov.B.

Plate Ia shows part of a starch gel of albumen of the three Ov. types, electrophoresed under alkaline buffer conditions, together with the five-times crystallized sample of ovalbumin obtained from Dr F. Steven and illustrated in a previous paper (Lush, 1964). Unfortunately, ovalbumin has no unusual biochemical property, such as the binding of iron by conalbumin or of haemoglobin by mammalian haptoglobin, which might serve for the identification of the ovalbumin components.

among the other egg albumen proteins in a starch gel, therefore an electrophoretic fraction was assumed to be a form of ovalbumin if it was involved in the genetic variation in that part of the electrophoretogram. On this basis it can be seen that the four presumptive ovalbumin fractions of the Ov.A albumen (fractions A3, A4, A5 and A6) are present in the five-times crystallized ovalbumin sample. Fraction 8, which showed no genetic variation and which was hardly detectable in some preparations of ovalbumin, was assumed to be a contaminant.

The first few eggs laid by some Ov.B pullets were unusual in that fractions B4 and B5 were increased in amount as compared with the normal adult pattern. This pecularity was most noticeable in the very first egg, and gradually disappeared in the course of the next 8–12 eggs. Albumen from an egg of this kind is illustrated in Plate Ia.

Plate Ib shows an acid gel of diluted samples of albumen of the three phenotypes, together with once-crystallized ovalbumins of types Ov.A and Ov.B, and a sample of ovomucoid. Under these buffer conditions the strong fractions of undiluted albumen tend to spread and distort the electrophoretic picture and so make comparisons between types difficult. On the other hand the weak fractions are lost on dilution. A dilution of 1 volume albumen to 4 volumes 0·9% NaCl was chosen as a compromise. The acid buffer conditions show that each of the homozygote albumens has two fast fractions (fractions 1 and 2) which vary their mobility in step with the ovalbumin variation. These two new fractions are present in crystallized ovalbumins and are assumed also to be forms of ovalbumin. Albumen which had been diluted in either the acid gel buffer or the acid end-tray buffer 4 hours before electrophoresis showed no increase in fractions 1 and 2. This seems to confirm that they are natural forms of ovalbumin and not artefacts of electrophoresis.

The effects of the acid buffer are to increase the difference in mobility between ovalbumin A and ovalbumin B and to separate fractions 1 and 2 from fraction 3. Unfortunately it was not found possible to separate by electrophoresis ovalbumin from ovomucoid, which comprises at least two fractions (see Plate Ib). The strongest of these lies just behind fraction A4 of the Ov.A albumen sample and is much reduced after one crystallization. Similarly the ovomucoid components of the Ov.B albumen are reduced after one crystallization. Fraction 8a is a new albumen component revealed by the acid gel conditions.

The relationship between the electrophoretic picture of the phenotypes in the two buffer systems was established as follows. The Ov.B albumen from pullet J.6231 illustrated in Plate Ia was run in an alkaline gel and segments of unstained gel containing each of the three fractions were cut out and the proteins eluted. These protein samples were then re-run in an acid gel alongside Ov.B albumen and found to correspond as shown in Plate Ia and b. Similarly the fractions of Ov.A albumen were eluted from an alkaline gel and re-run alongside diluted Ov.A albumen in an acid gel. The very faint fraction A6 was not visible in the gel shown in Plate Ib, but its position is known from experiments with more concentrated samples and has been indicated by a line. To summarize; ovalbumin A consists of six fractions numbered A1 to A6. Ovalbumin B consists of 5 fractions, numbered B1 to B5. Ovalbumin AB

is identical with an equal mixture of ovalbumin A and ovalbumin B. With the exception of fractions 5 and 6, ovalbumins A and B give substantially the same electrophoretic pattern, the difference lying in their mobilities. Both ovalbumin A and B crystallized from ammonium sulphate in the characteristic form of needles and rosettes.

(ii) *Genetical data*

The biochemical basis of the electrophoretic heterogeneity of each ovalbumin will be discussed in the next section. To explain the genetic difference it is sufficient to suppose (1) that ovalbumins A and B differ from each other in some part of the molecule which is present in all the electrophoretic fractions, (2) that this difference in structure involves a difference in charge detectable as a difference in electrophoretic mobility, and (3) that a subsidiary effect of the structural difference is a reduction of the relative amounts of fractions 5 and 6 in ovalbumin B as compared with ovalbumin A. The genetic hypothesis is that there exists an autosomal locus *Ov* which determines the structure of the part of the molecule common to all the electrophoretic fractions and at which two alleles, Ov^A and Ov^B, occur. The nomenclature to be adopted is therefore as follows.

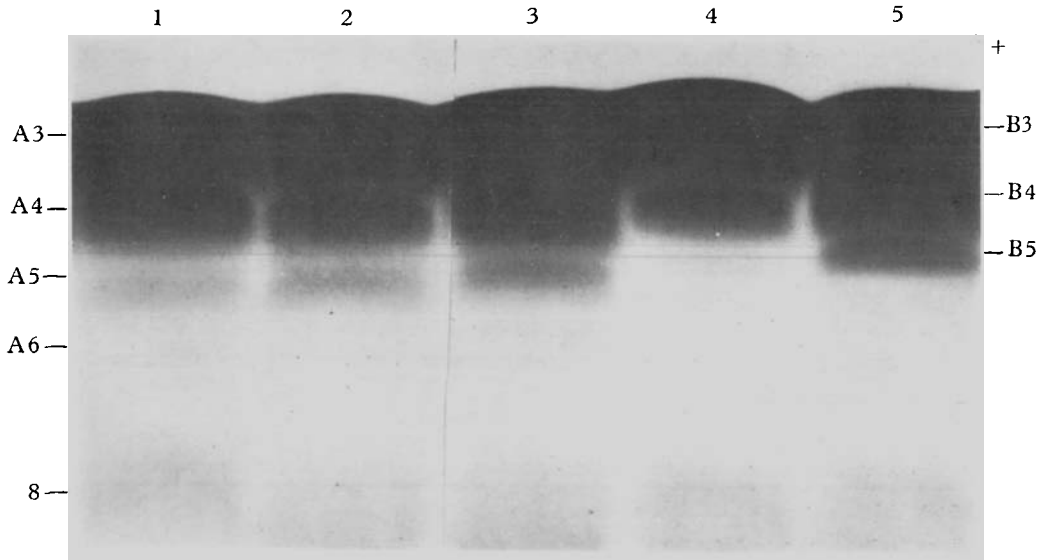
Genotype	Corresponding phenotype
Ov^A/Ov^A	Ov.A
Ov^A/Ov^B	Ov.AB
Ov^B/Ov^B	Ov.B

A large mass of breeding data produced no example which contradicted the above hypothesis, but practical difficulties made it impossible to set up critical matings. The most extensive family is shown in Table 1 and, although the numbers are small, it can be seen that the segregation data fit the hypothesis on the assumption that J.4200 was a heterozygote.

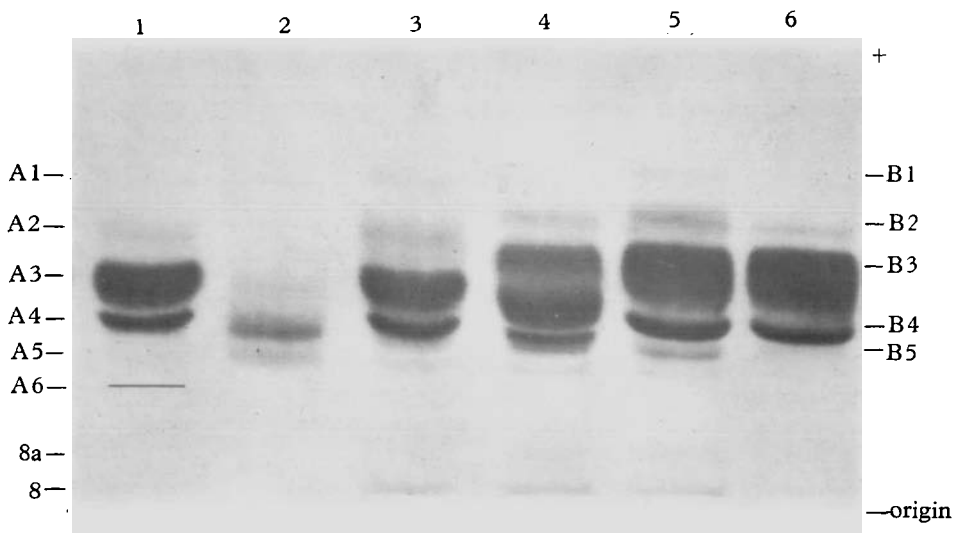
Table 1. *A family demonstrating segregation at the Ov. locus (hypothetical genotype of the sire in parenthesis)*

Sire	Phenotype of dams	Number of dams	Phenotype and number of offspring			χ^2	P
			A	AB	B		
J.4200 (Ov^A/Ov^B)	Ov.A	2	5	7	0	0·08	> 0·7
	Ov.AB	4	6	8	4	1·16	> 0·5
	Ov.B	1	0	2	0	0·50	> 0·3

With regard to the population genetics of the *Ov* locus, it is becoming clear from published data (Lush, 1961; Feeney *et al*, 1963) and unpublished work (McDermid, personal communication; Lush, unpublished) that in most commercial and scientific flocks the Ov^B allele is rare or absent. In this respect the Poultry Research Centre



a. Part of an electrophoretogram of ovalbumin and egg albumen; alkaline buffer conditions. Sample 1 was an 8% solution of five-times crystallized ovalbumin. Samples 2, 3 and 4 were egg albumens of phenotype Ov.A, Ov.AB and Ov.B respectively. Ov.AB can be distinguished from Ov.A by the virtual absence of the separation between fractions 3 and 4. Sample 5 was from the second egg laid by an Ov.B pullet, J.6231. Gel stained with naphthalene black.



b. Electrophoretogram of an acid gel to demonstrate the ovalbumin phenotypes. Sample 1 was once-crystallized ovalbumin A. Sample 2 was 1% ovomucoid. Samples 3, 4 and 5 were albumens of Ov. type A, AB and B respectively, diluted five times in normal saline. Sample 6 was once crystallized ovalbumin B.

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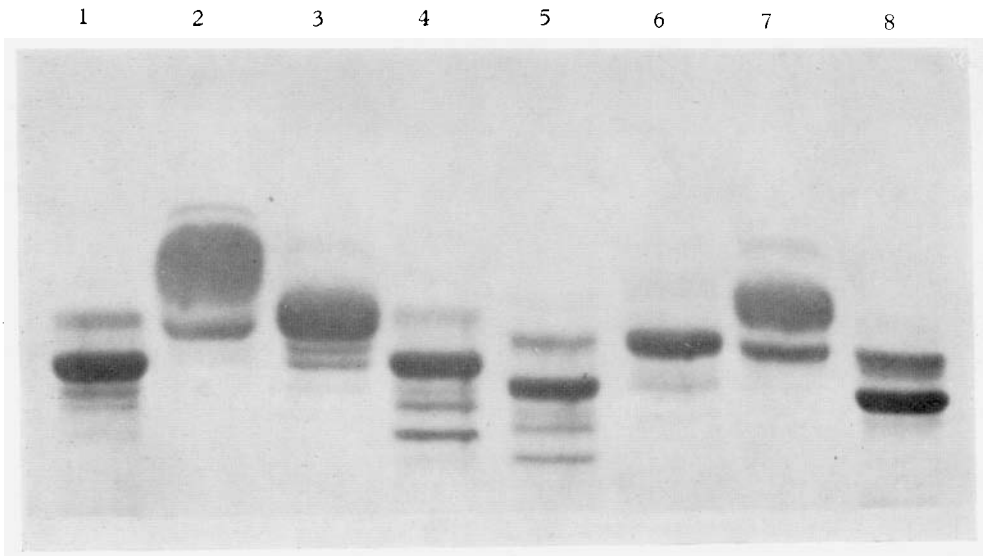
Plate II



Electrophoretogram of an acid gel to show the effects of dephosphorylation. Sample 2 was untreated ovalbumin B. Sample 3 was ovalbumin B treated with prostatic phosphatase. Sample 4 was ovalbunin B treated with both prostatic and intestinal phosphatase. Samples 7, 6 and 5 were corresponding ovalbumin A samples. Samples 1 and 8 were albumens (diluted five times), of Ov. type B and A respectively, treated with both enzymes.

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BR line, in which the Ov^B allele predominates, is unusual. As information on more flocks becomes available it will be interesting to see if the Ov^B allele is found more often in some breeds of fowl than in others. Baker & Manwell (1962) have published photographs of starch gels, showing genetic variation in ovalbumin. Their phenotypes probably correspond to those obtained in the alkaline gel conditions in the present work, but there are some apparent differences.

(iii) *Enzymatic modification of ovalbumin*

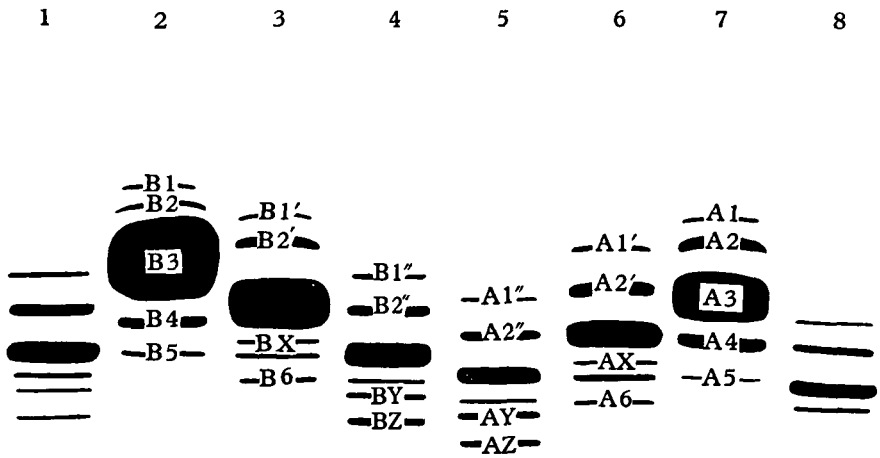
Longsworth and his collaborators (Longsworth, 1939; Longsworth, Cannan & MacInnes, 1940; MacPherson, Moore & Longsworth, 1944) analysed crystallized ovalbumin by Tiselius electrophoresis in the pH range 5–10 and demonstrated the presence of three fractions, A_1 A_2 and A_3 in order of decreasing amount and mobility. Cann (1949) showed that the degree of separation of the three fractions in the Tiselius apparatus, which influences their apparent relative amounts, depended to some extent on the ionic strength of the buffer. Analysis of 1% ovalbumin in phosphate buffer at pH 6·81, ionic strength 0·1, yielded 76·0% A_1, 18·2% A_2 and 5·8% A_3.

Perlmann (1952 & 1953), following up a suggestion by Linderstrøm-Lang & Ottesen (1949), demonstrated that the electrophoretic heterogeneity of ovalbumin in the Tiselius apparatus is due to the different phosphate contents of the electrophoretic fractions. At pH 6·8 she found the three fractions in the proportions 80–85% A_1, 15–20% A_2 and a trace of A_3. Ovalbumin which had been treated with human prostatic phosphatase gave an electrophoretic picture of 0% A_1, 94% A_2 and 6% A_3. Subsequent treatment with calf intestinal phosphatase produced an electrophoretic picture consisting of 100% A_3. Prolonged incubation (24 hours) of untreated ovalbumin with intestinal phosphatase converted it to 100% A_3. Perlmann interpreted these results and her detailed measurements of the phosphorus content and electrophoretic mobility of the three ovalbumin fractions in the following way. Ovalbumin A_1 consists of ovalbumin molecules with two separate charged phosphate radicals attached to each. Ovalbumin A_2 consists of ovalbumin molecules with one or other of the two phosphate radicals attached to each. Ovalbumin A_3 contains no phosphate. One of the phosphate radicals can be detached by prostatic phosphatase. Both phosphate radicals can be detached by intestinal phosphatase. It is not possible now to know which genetic type of ovalbumin, or mixture of types, was analysed by Perlmann and the other workers. In most of the populations sampled by Lush (1961, and unpublished work) ovalbumin A is probably the only ovalbumin present. If Perlmann's ovalbumin was also of type A, comparison of the amounts and relative mobilities of the fractions suggests the following correspondence between Perlmann's fractions and those described in this paper.

	Ovalbumin fractions		
Perlmann (1952)	A_1	A_2	A_3
Lush (this paper)	A3	A4	A5

It was decided to settle the matter by experiment.

The 1% solutions of ovalbumin of each genetic type had a pH value between 4·8 and 5·0. The acid phosphatase activity of the prostate gland extract was 299 units and the activity of the intestinal phosphatase solution used was 17·9 units. For the prostatic dephosphorylation, 0·5 ml. samples of 1% ovalbumin were incubated at 37° for 17 hours with 0·01 ml. prostate extract. After subsequent dialysis against 0·002 M $MgCl_2$ for 6 hours at 4°, this partially dephosphorylated ovalbumin was either analysed by electrophoresis or further dephosphorylated by alkaline phosphatase as follows. To each 0·5 ml. sample was added 0·03 ml. of 0·04 M NaoH, 0·01 ml. of 0·1 M $MgCl_2$, and 0·05 ml. of intestinal phosphatase solution. The mixture was incubated for 17 hours at 37° and then analysed by electrophoresis. The effect of dephosphorylation on the electrophoretic pattern of each type of ovalbumin is shown in Plate II and diagrammatically represented in text-fig. 1. Samples 2 and 7 are untreated ovalbumins of type B and A respectively. Dialysis slightly increased the mobility of all fractions, and therefore the untreated ovalbumin samples were also dialysed to facilitate their direct comparison with the dephosphorylated samples.



Text-fig. 1. Diagram of Plate II.

The main result of prostatic dephosphorylation of ovalbumin of both types is an increase in the amount of fraction 4 at the expense of fraction 3. This is in agreement with Perlmann, who found that the proportion of her fraction A_1 fell from 85% to 0% while fraction A_2 rose from 14% to 94% on prostatic dephosphorylation. However, in Plate II it can be seen that fractions 1 and 2 are also affected by the enzyme and have been replaced by two slower fractions, named 1′ and 2′. At first sight it might appear that fractions 1′ and 2′ could be remnants of fractions 2 and 3 left due to failure of the enzymic reaction to go to completion; however, the use of five times as much enzyme did not alter the picture and they have therefore been interpreted as being fractions 1 and 2 moved back by dephosphorylation in the same way as fraction 3. Another result of prostatic dephosphorylation is an increase in fraction 5,

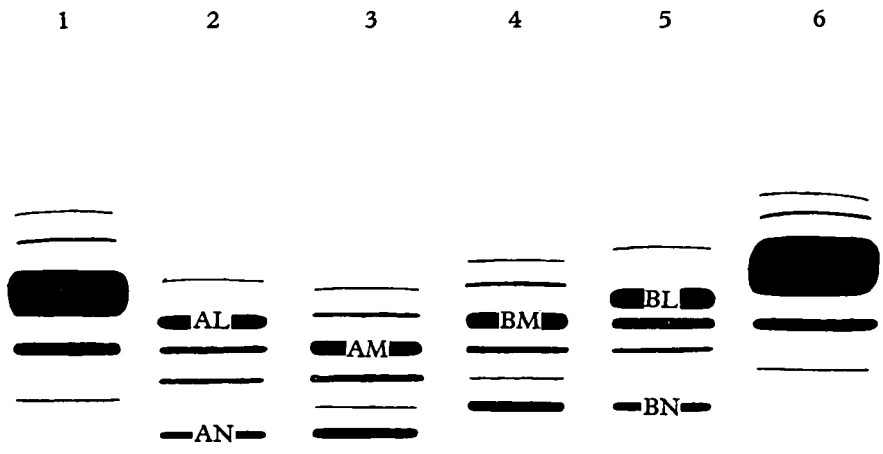
again in agreement with Perlmann who found that her fraction A_3 increased from a trace to 6%. Two other effects can be seen. The first is the appearance of a new fraction, marked as *X*. The second is the appearance of a slow fraction, which in the case of ovalbumin A is concurrent with the A6 of more concentrated untreated samples, and has therefore been labelled as fraction 6. Fraction B6 has not been seen in any untreated samples, but it appears to occupy a comparable position to A6.

When ovalbumin is treated with both phosphatases fraction 5 becomes the major fraction at the expense of fraction 4, again in substantial agreement with Perlmann. As in prostatic-treated ovalbumin, two minor fast fractions remain. These are taken to be fractions 1′ and 2′ moved back again by the second dephosphorylation and are labelled 1″ and 2″. The use of a stronger intestinal phosphatase solution results in an increased conversion of all the remaining ovalbumin fractions (1″, 2″, 5 and 6) into the new fractions *Y* and *Z*. Perlmann (1953) found that prolonged treatment of ovalbumin with intestinal phosphatase gave an electrophoretic picture consisting of 95% of her fraction A_3 and 5% of a slower fraction which she interpreted as a proteolytic degradation product. It seems likely that Perlmann's 5% fraction is subdivided by starch gel electrophoresis and appears as fractions *Y* and *Z*. Evidence in favour of the proteolytic origin of fractions *Y* and *Z* was obtained from the dephosphorylation of albumen. Albumen of each genetic type was diluted five times in normal saline and adjusted to about pH 5·0 by the addition of 1/6 volume of 0·1 N HCl to each volume of diluted albumen. The albumens were dephosphorylated in the same way as the ovalbumin samples except that a more concentrated solution of intestinal phosphatase was used (45·3 units). Since egg albumen contains not only ovomucoid, which is a trypsin inhibitor, but also ovoinhibitor (Matsushima, 1958), which inhibits trypsin and at least two other proteolytic enzymes, it was hoped that either or both of these inhibitors might inhibit any proteolytic component of the intestinal phosphatase solution whilst not interfering with the phosphatase itself. Plate II shows that this result was achieved with the Ov.A albumen but a little proteolysis remained uninhibited in the Ov.B sample. The possibility that fractions *Y* and *Z* are the products of the breakdown of ovalbumin by the growth of micro-organisms in the incubation mixture was excluded by sterilizing a mixture before incubation. Fractions *Y* and *Z* appeared in undiminished strength after sterile incubation with intestinal phosphatase.

The above results confirm that ovalbumin fractions 3, 4 and 5 correspond to Perlmann's fractions A_1, A_2 and A_3 respectively, but, whereas in the Tiselius method used by Perlmann totally dephosphorylated ovalbumin comprised one fraction only (apart from the proteolytic product), starch gel electrophoresis shows that all the electrophoretic heterogeneity has not been removed. Two faster fractions (1″ and 2″) and one slower fraction (6) still remain. The cause of this remaining heterogeneity is unknown. If one assumes that the ovalbumin in each egg is homogeneous in its amino acid composition, then its electrophoretic heterogeneity must be caused by the attachment of different charged groups to small proportions of the total population of ovalbumin molecules. While the genetic change affects the

degree of phosphorylation of ovalbumin, (except in some early Ov.B eggs), the non-phosphate heterogeneity appears to be similar in the two genetic types. From the assumption that the non-phosphate charged groups are attached at random to certain proportions of the ovalbumin molecules, both phosphorylated and non-phosphorylated, it follows that all the electrophoretic fractions of untreated ovalbumin, except the first and the last, are mixtures of more than one molecular species of ovalbumin. It would also follow that those in early Ov.B eggs which have a fraction B5 comparable in strength with A5 there should be visible a fraction B6. However, in such eggs no B6 could be detected unequivocally and therefore the subdivision of ovalbumin into its various molecular species may be more complex. No explanation was found for fraction *X*.

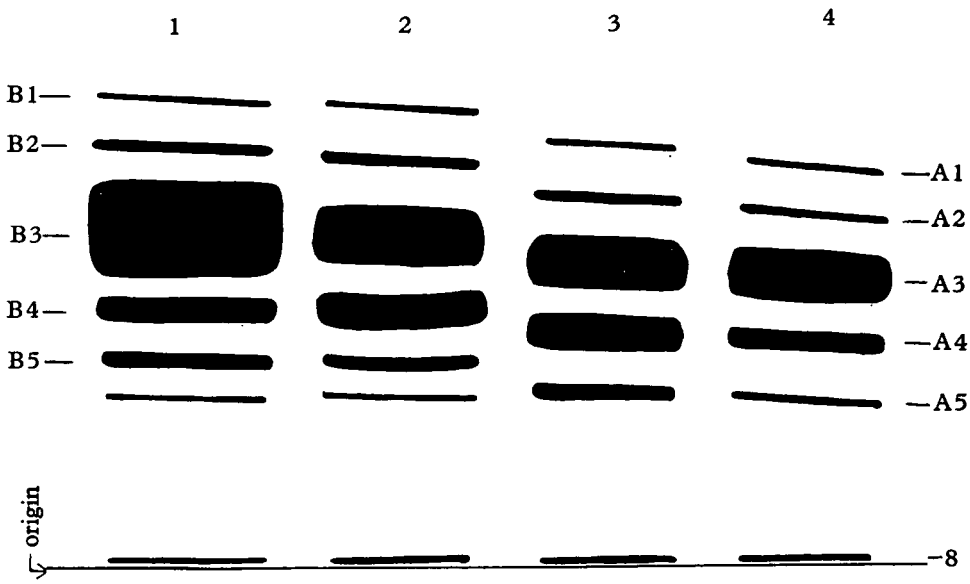
The action of the enzyme subtilisin on ovalbumin has been investigated in detail by Ottesen and his collaborators (Ottesen & Wollenberger, 1953; Ottesen, 1958). Subtilisin is a proteolytic enzyme released into the culture medium by some strains of *Bacillus subtilis*. The action of purified subtilisin on ovalbumin is to release from it seven amino-acid residues, either as one heptapeptide or as a dipeptide followed by a pentapeptide. The heptapeptide has the following sequential structure, Glu–Ala–Gly–Val–Asp | Ala–Ala and the smaller components are produced by scission at the point indicated. Perlmann (1949) showed that, while the degree of phosphorylation and the electrophoretic heterogeneity of the altered ovalbumin, which is called plakalbumin, is the same as ovalbumin, the components P_1 and P_2 have electrophoretic mobilities less than those of the corresponding ovalbumin fractions A_1 and A_2 by an amount consistent with the loss of two ionized acidic



Text-fig. 2. Diagram of an acid gel electrophoretogram to show the effect of subtilisin on ovalbumin. Sample 1 was untreated ovalbumin A. Samples 2 and 3 were ovalbumin A treated with subtilisin for 2 hours and 17 hours respectively. Samples 6, 5 and 4 were the corresponding ovalbumin B samples.

amino-acids per molecule. The genetic type of the ovalbumin which was used for this work is unknown, but if the structural basis of the electrophoretic difference between ovalbumins A and B lies within the heptapeptide that is removed by subtilisin, then the corresponding plakalbumins A and B should have the same electrophoretic mobility. If the genetic difference lies somewhere in the rest of the molecule, then the plakalbumins will differ in mobility as the ovalbumins do.

To decide between these alternatives 0·5 ml. samples of approximately 0·5% ovalbumin of each genetic type were incubated at 20° with 0·02 ml. of 0·04 N NaoH and 0·01 ml. of a 0·1% solution of subtilisin for varying periods, and then analysed



Text fig. 3. Diagram of an acid gel electrophoretogram. Samples 1 and 2 are, respectively, egg albumen (diluted five times) and undiluted cystic fluid from the same Ov.B hen. Samples 4 and 3 are the corresponding samples from an Ov.A hen. Note that the genetic difference is evident in the cystic fluid ovalbumin as in the egg-white ovalbumin.

by starch gel electrophoresis. The results are shown in text-fig. 2. The pattern after 17 hours' incubation resembles ovalbumin in that the main fraction, M, is preceded by two faint faster fractions and followed by a fairly strong fraction, N. The use of three times as much enzyme gave the same result, which was therefore taken to represent complete conversion to plakalbumin. After only 2 hours' incubation the major component was an intermediate fraction, L. Fraction L may be one of the two intermediate forms described by Ottesen or it may be plakalbumin which still retains the glutamic acid of the heptapeptide (Ottesen, 1958). The relevant point for the present work is that plakalbumins A and B differ electrophoretically to the same extent as ovalbumins A and B and therefore the genetic difference lies outside the heptapeptide removed by subtilisin.

(iv) *Cysts of the right oviduct*

In the domestic hen only the left oviduct is functional, the embryonic right oviduct usually persisting into adult life only as a small rudiment attached to the cloaca. Very occasionally this rudiment becomes greatly swollen and filled with a thin watery fluid. It was thought to be of interest to see if this abnormal secretion bears any biochemical resemblance to egg albumen. Samples of egg albumen and right oviduct cystic fluid from six hens were analysed on starch gel under the alkaline buffer conditions. A general resemblance to egg albumen was at once obvious; ovalbumin and conalbumin were present and, in some samples, a trace of lysozyme. The other components of the electrophoretic picture were faint and ill-defined. Cystic fluid from two hens, one of phenotype Ov.A and the other Ov.B, were electrophoresed in acid buffer conditions, together with samples of diluted egg albumen from the same birds. Text-fig. 3 shows that the genetic difference is expressed by the ovalbumin in the cystic fluid as in the egg albumen, although the components differ in relative amount. McBride (1962) has presented evidence that the development of the right oviduct rudiment into either a partially developed or a complete right oviduct is under genetic control.

4. DISCUSSION

While there is good evidence that the prostatic phosphatase-labile phosphate of ovalbumin is present as phospho-serine the nature of the second phosphorus bond is still uncertain (Flavin, 1954; Perlmann, 1955). Sanger & Hocquard (1962) incorporated several radioactive amino-acids, [^{14}C] mannose and ^{32}P into minced oviduct tissue from a laying hen (probably of type Ov.A) and found that dephospho-ovalbumin (fraction 5) was synthesized first, followed by diphospho-ovalbumin (fraction 3). Last to appear was monophospho-ovalbumin (fraction 4). They suggest that the dephospho-ovalbumin is formed as an intermediate in the synthesis of ovalbumin and that the two phosphates are then attached to it enzymically. The order of appearance of the fractions is curious and seems to imply that both phosphate groups are added simultaneously, or nearly so. It is clear from comparison of the two ovalbumins in an alkaline gel that, in the mature bird, ovalbumin B contains less dephospho-ovalbumin than does ovalbumin A. It seems likely, therefore, that the genetic difference lies in some part of the molecule spatially adjacent to one or both of the phosphate groups and so influences the freedom of action of the phosphorylating or dephosphorylating enzymes in the oviduct. Why the early eggs of an Ov.B hen should differ from the later eggs in this respect is not clear. Rhodes, Bennett & Feeney (1959) have described a riboflavin-binding egg albumen protein which contains 7 to 8 phosphate groups per molecule. They consider that this protein also may exist in the egg in more than one degree of phosphorylation. Apart from an acetyl group at the *N*-terminal end of the molecule (Narita & Ishii, 1962), the only known non-amino-acid component of ovalbumin is the carbohydrate moiety. This has been the subject of sustained investigation by Neuberger and his collaborators (Marks *et al.*, 1963; Fletcher *et al.*, 1963) and probably com-

prises 5 mannose and 3 *N*-acetylglucosamine moieties attached as a unit to one aspartic acid residue in each ovalbumin molecule; however, the latest work of Cunningham (Cunningham *et al.*, 1963) shows that the glycopeptide fraction from a pepsin digest of ovalbumin can be fractionated chromatographically into four fractions which differ in the relative amounts of mannose and glucosamine which they contain. This result may provide the explanation of the electrophoretic heterogeneity of dephosphorylated ovalbumin.

Ovalbumin has occupied an important place in protein chemistry for over half a century and it remains today the subject of many physical, chemical and immunological investigations. The polymorphism of ovalbumin in some populations of hens will come as no surprise to students of biochemical genetics, who have learnt that within any population which is not highly inbred it is safer to assume that a given protein is genetically heterogenous than the reverse. It remains to be seen to what extent ovalbumins A and B differ in their chemistry, but it would seem to be a prudent policy for biochemists working on the fine structure of proteins to obtain their material, so far as possible, from highly inbred lines or from individual animals and so minimize the risk of introducing into their data unrecognized variation of genetic origin.

SUMMARY

1. Ovalbumin was shown, by starch gel electrophoresis, to exist in two genetically different forms, A and B. It was suggested that these are determined by two alleles at one locus, named *Ov*.

2. Ovalbumin of each genetic type is electrophoretically heterogeneous. Dephosphorylation of each type with human prostatic phosphatase and calf intestinal phosphatase removed some of the heterogeneity, but some remained.

3. The genetic difference was shown not to reside in the fragment released from ovalbumin by the proteolytic enzyme subtilisin.

4. The genetic difference was evident in the ovalbumin present in the fluid contained in right oviduct cysts.

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