Genet. Res., Camb. (1964), **5**, *pp.* 39–49
With 4 *plates and* 3 *text-figures*
Printed in Great Britain

Egg albumen polymorphisms in the Fowl: loci *II* and *III*

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(*Received* 3 *May* 1963)

1. INTRODUCTION

In a recent examination of sixteen small populations of domestic fowls, Lush (1961) reported the occurrence and distribution of polymorphisms in three of the egg albumen proteins. Starch gel electrophoresis enabled the albumen proteins to be separated into about nineteen fractions, and in each of three regions of the electrophoretogram (I, II and III) a protein was found which was genetically variable in its electrophoretic mobility. The polymorphism in what was then described as region I of the electrophoretic picture can now be ascribed with certainty to genetic variation in the ovalbumin molecule and will be the subject of another paper. Crosses to establish the genetical basis of the polymorphism in regions II and III have been completed and are reported here.

The relationship of the polymorphic proteins to the known components of egg albumen, and to its physical structure, has also been investigated. Evidence is accumulating for the widespread occurrence of egg albumen polymorphisms, and data from other flocks are discussed. Finally, the literature on the electrophoresis of egg albumen is reviewed in the light of the new knowledge of its genetical variation.

2. MATERIALS AND METHODS

The Brown Leghorn strains at the Poultry Research Centre (P.R.C.) were used for the genetical analysis of the egg albumen types. Some hens were fertilized by natural matings and others by artificial insemination.

Eggs were stored at 3° to 5° C. as soon as possible after collection, and used within two months thereafter. Storage had no effect on the electrophoretic analysis. For routine typing the whole albumen was decanted on to a beaker covered with washed butter-muslin and the thin albumen which drained through was used for analysis. Samples of albumen can be stored at −20° C. for several months. To obtain separate specimens from the thick albumen and the inner and outer thin albumen of an egg, the following procedure was adopted.

The egg was carefully broken into a petri dish and the outer thin albumen removed. The thick albumen was then cut with scissors and the inner thin albumen which flowed out was collected. The thick albumen was blotted to remove adhering thin

and then sampled for electrophoresis by placing on its surface a small rectangle of filter paper. When this was saturated it was placed in a starch gel as described below.

Starch gel electrophoresis. Starch gels (Connaught Laboratories starch, hydrolysed for gel electrophoresis) were prepared according to Smithies (1955). The Poulik (1957) discontinuous buffer system, slightly modified (Lush, 1961), was used both for one-dimensional gel electrophoresis and for the gel phase of the two-dimensional technique. The gels were kept overnight at room temperature before use the following morning. A rectangle of Whatman No. 3 filter paper was immersed in the protein solution to be analysed, the unabsorbed excess removed from the surfaces, and the rectangle inserted into a slit in the gel made with a piece of razor blade cut to the same width. Sample slits were separated from one another by a short length of unbroken gel and were placed 3 cm. from the edge of the cathode wick. A stabilized d.c. voltage gradient of 6 to 7 V./cm. was established along the gels which were run horizontally in a cool room (below 18° C.) for 8 to 10 hours. The gels were sliced with a thin wire and the central surfaces stained with nigrosine or naphthalene black.

Region III is easier to type than region II and occasionally, because of a distorted electrophoretogram or the diffuseness of fractions 9 and 10, it was necessary to re-run a sample before it could definitely be typed with regard to region II. The amount of each stained fraction, which was assessed subjectively, could be affected by several sources of variation, and appropriate controls were run to gain an impression of their relative importance. For example, a sample from one egg was analysed on several different gels; several eggs from one bird were analysed together on the same gel; and eggs from several birds of the same type were analysed together on one gel.

Two-dimensional electrophoresis. When egg albumen was electrophoresed on a narrow (6 mm.) strip of paper the tendency of the ovalbumin fraction to trail at the edges of the strip led to a final pattern in the starch gel which was not as clear as could be wished. The two-dimensional technique of Poulik & Smithies (1958) was therefore modified as follows. Strips of Whatman No. 17 paper, 30 mm. wide, were used. With a scalpel, two parallel cuts 6 mm. apart were made along the centre of each strip through almost the whole thickness of the paper. 0·12 ml. of the sample was applied across the whole width of each 30 mm. strip and electrophoresis carried out in a hanging strip apparatus of the type described by Flynn & de Mayo (1951) using Oxoid (Owen) barbitone acetate buffer (sodium acetate hydrate, 6·5 g./l.; sodium barbitone, 8·87 g./l.; barbitone 1·13 g./l.); pH 8·6, ionic strength, 0·1. The voltage was applied for 16 to 18 hours, after which the central 6 mm. strip was gently separated from the rest with sharp scissors and the section containing the proteins was lightly sprayed with gel buffer and inserted in a starch gel.

Pervaporation was carried out in a cold room (5° C.). Visking dialysis tubing (18/32 in.) was cleaned by twice heating in a water bath at 80° to 90° C. and subsequently soaking in several changes of water (Hughes & Klotz, 1956). A dialysis bag filled with the protein solution was hung in a current of air from a fan. In this way the volume of a sample of egg albumen could be halved overnight.

Authentic protein samples

Lysozyme was obtained from Armour & Co. Avidin was a gift from Dr N. M. Green, St. Mary's Hospital Medical School, London. Conalbumin (Fe-complex), which was a gift from Dr J. Williams, Department of Biochemistry, University of Cambridge, had been prepared by the method of Warner & Weber (1951). Ovomucoid was prepared by Dr W. M. McIndoe by the method of Fredericq & Deutsch (1949).

3. RESULTS AND DISCUSSION

(i) *Genetical hypotheses and methodology*

A simple Mendelian explanation of the genetics of the proteins in regions II and III has already been proposed and can be briefly restated as follows. There exists an autosomal locus, referred to as locus *II*, which is responsible for the synthesis of an egg albumen protein which can be seen in region II (as defined by Lush, 1961) of the starch gel electrophoretogram of albumen from all hens so far examined. This protein exists in two forms, A and B, of which A travels slightly faster than B during electrophoresis at an alkaline pH. Each of these two variants of the region II protein is determined by a corresponding allele at locus *II*. In albumen from homozygous hens, only one region II protein is present and in the hererozygote both can be seen, but in smaller amount than when each is present alone.

An identical relationship exists between the two forms of a protein which can be seen in region III and an autosomal locus *III*, except that in the III.A homozygote a small amount of the III.B protein appears to persist (however, see section on *two-dimensional electrophoresis* below). The electrophoretic relationship between all the variants is shown diagrammatically in Text-fig. 1.

The nomenclature adopted for the phenotypes and corresponding genotypes is as follows.

Phenotype	Corresponding genotype
II.A	II^A/II^A
II.AB	II^A/II^B
II.B	II^B/II^B
III.A	III^A/III^A
III.AB	III^A/III^B
III.B	III^B/III^B

The sex-limited nature of the egg albumen phenotypes makes it obviously impossible to type the male birds directly and placed some constraints on the number and nature of the matings that could be set up with the available material. For the same reason, it will be necessary to describe in some detail the considerations which led to the choice of sires.

Compared with a locus which is expressed in both sexes, the structure of sex-limited inheritance provides fewer opportunities for a rigorous test of a Mendelian hypothesis. With a locus whose expression is limited to the female, the male genotype can be defined only in terms of its genetic background and behaviour. It is

desirable, therefore, to have information from at least one independent source on each cock before it is used as a sire in a cross. Information on the genotype of a cock can be obtained from the phenotypes of his dam and his female sibs and offspring. In the present investigation, information of this kind was gained from some already existing families in the P.R.C. lines. In the matings specially set up to test the genetics of the albumen types, an effort was made to use each male in more than one mating.

The data for each locus will be described separately.

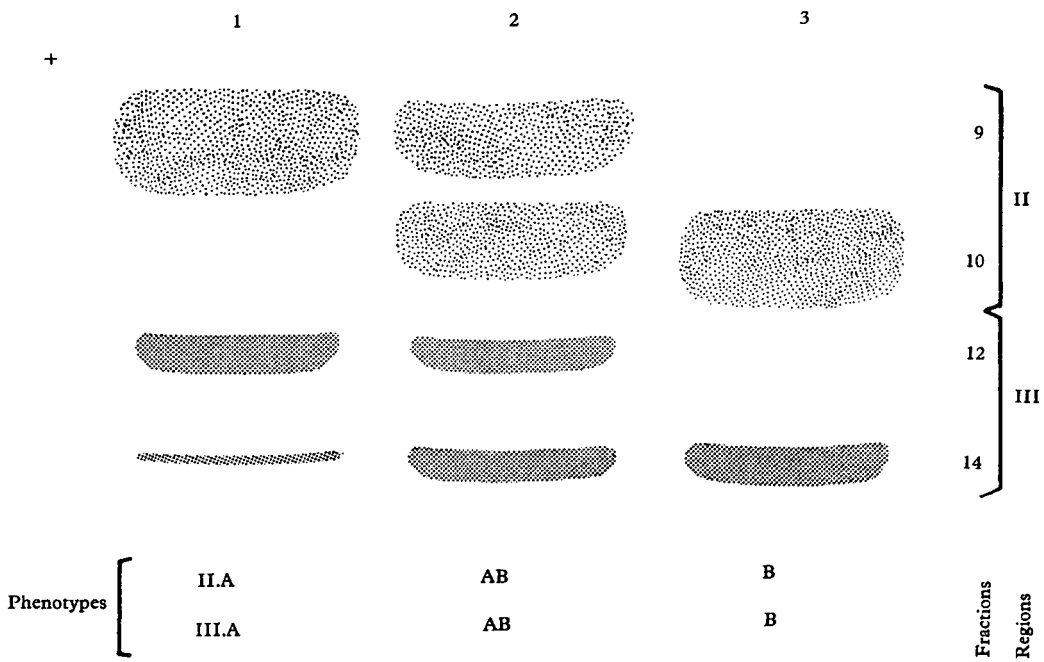

Fig. 1. Diagram of part of an electrophoretogram of three albumen samples of different region II and region III phenotypes.

(ii) *Segregation at locus* II

Sires of each genotype were chosen as follows:

(*a*) *Sires of genotype* II^A/II^A.

The published results of work on the P.R.C. Brown Leghorn Flock (Lush, 1961) show that only hens in the Breeding Line (BR hereafter) displayed variation in region II. All the hens typed in the other lines have the phenotype II.A. Large numbers of hens extending over several generations have been tested from the Red, Intensity and Large Egg lines (RD, IN, and LE hereafter) and their crosses. Consideration of their family relationships has led to the conclusion that in 1959 the allele II^B was absent from these three lines. Cocks from these lines have therefore been used as sires of genotypes II^A/II^A in the appropriate crosses.

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(*b*) *Sires of genotype* II^A/II^B

The pedigree of a family found in the BR line is shown in Text-fig. 2. This was taken as evidence that cock G3799 had the genotype II^A/II^A and his two sons, H203 and H204, were heterozygotes. Both sons were used as sires on this assumption.

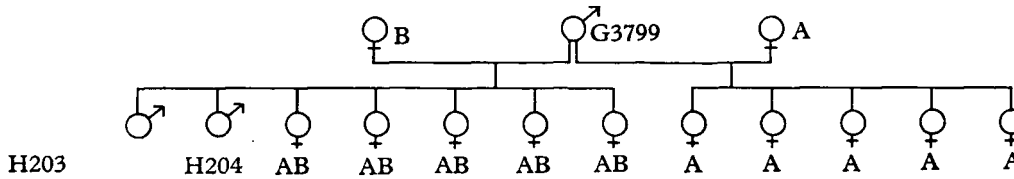

Fig. 2. A pedigree found in the BR line. The phenotype symbols (A, AB, B) refer to region II.

(*c*) *Sires of genotype* II^B/II^B

No male of this genotype was found.

The results of matings used to test the Mendelian hypothesis of the inheritance of locus *II* are set out in Table 1. The experimental results do not differ significantly from the theoretical ratios.

Table 1. *Locus* II *segregation data*

Phenotype of dam	Sire	Presumed genotype of sire	Numbers and phenotypes of female offspring		
			A	AB	B
II.AB	H203 and H204	II^A/II^B	7	10	6
A	H204	II^A/II^B	3	3	0
AB	RD, IN and LE cocks	II^A/II^A	10	10	0
B	IN and LE cocks	II^A/II^A	0	6	0

(iii) *Segregation at locus* III

(*a*) *Sires of genotypes* III^A/III^A *and* III^A/III^B

The pedigree of a family found in the RD line is shown in Text-fig. 3. This was taken as evidence that G4509 and H146 were both of genotype III^A/III^A and that H2118 was a heterozygote, III^A/III^B. H146 and H2118 were used as sires accordingly.

(*b*) *Sires of genotype* III^B/III^B

All tested hens in lines IN, LE, and BR have been III.B. On inspection of their family relationships it was concluded that in 1959 the III^A allele was absent from these three lines, and cocks from these sources were used as III^B/III^B sires.

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The experimental results from matings segregating at locus *III* are set out in Table 2. No significant deviation from the expected ratios was found.

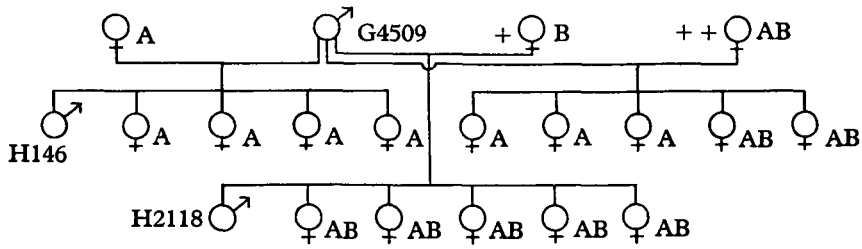

Fig. 3. A pedigree found in the RD line. The phenotype symbols refer to region III.
\+ This family represents three dams and their offspring.
\+ + This family represents two dams and their offspring.

Table 2. *Locus* III *segregation data*

Phenotype of dam	Sire	Presumed genotype of sire	Numbers and phenotypes of offspring		
			A	AB	B
III.A	H146	III^A/III^A	4	0	0
AB	H2118	III^A/III^B	4	5	1
A	H2118	III^A/III^B	2	2	0
AB	H146	III^A/III^A	0	4	0
AB	BR, LE and IN cocks	III^B/III^B	0	9	5
B	H2118	III^A/III^B	0	4	8
A	BR, LE and IN cocks	III^B/III^B	0	13	0
B	H146	III^A/III^A	0	14	0

(iv) *Two-dimensional electrophoresis*

Poulik & Smithies (1958) showed that a fuller demonstration of the complexity of human plasma proteins could be achieved by electrophoresis of a sample first on filter paper and then in starch gel than by electrophoresis in starch gel alone.

If egg albumen fractions 12 and 14 are the products of alternative alleles at locus *III*, then fraction 14 should be absent from the albumen of a III^A/III^A hen; yet it seemed to persist. This apparent anomaly might be due to the presence of an unrelated protein which happened to have the same electrophoretic mobility in starch gel as fraction 14. To test this possibility, albumen samples of the three region III phenotypes were concentrated by pervaporation and subjected to two-dimensional electrophoresis. Plate I shows the pattern obtained with the type III.A sample. The position occupied by fraction 14 when a sample of type III.AB or III.B albumen is analysed is indicated by the interrupted line. Nothing can be seen in this position with III.A albumen but it all samples of whatever type a small component marked X is present which has about the same mobility in starch gel. This is probably the

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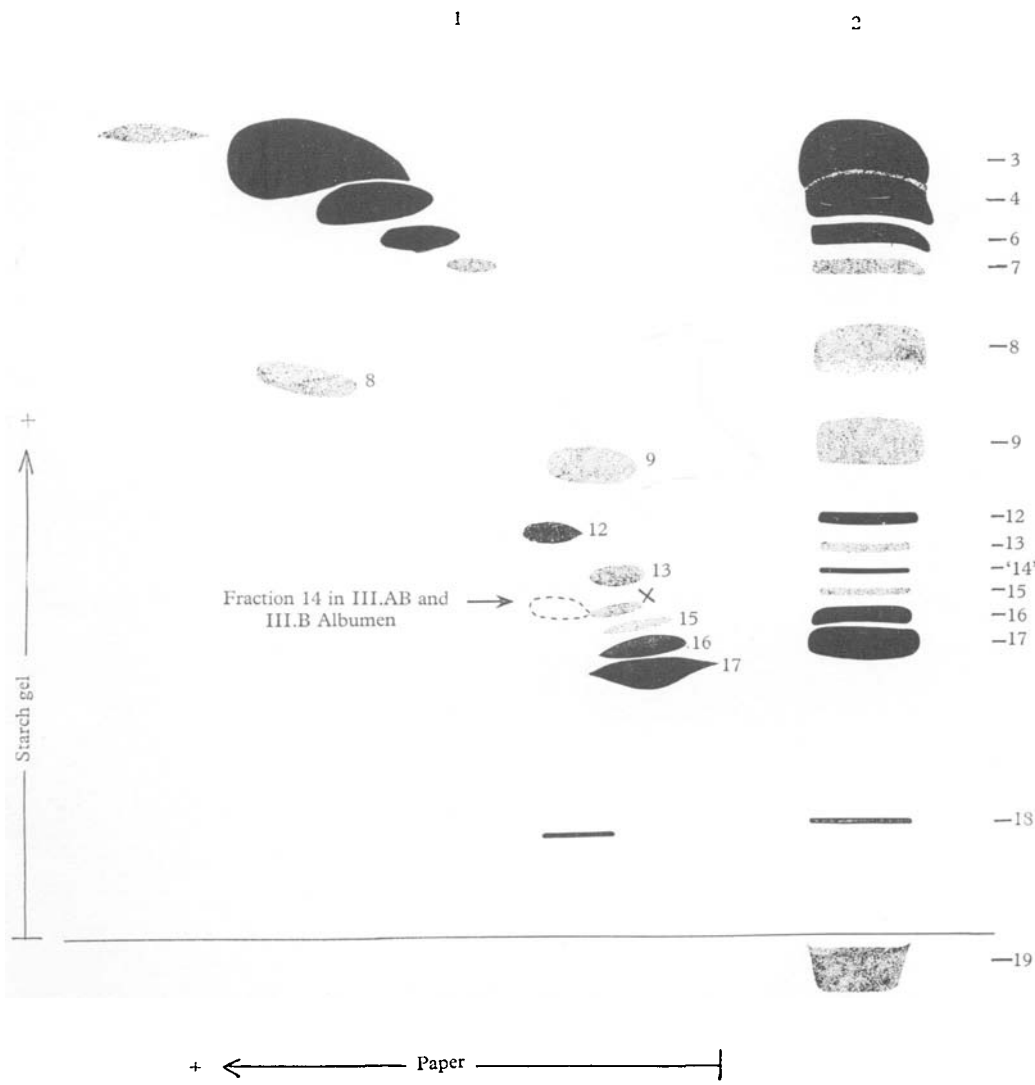

Plate 1. Diagram of two-dimensional electrophoresis of type III.A egg albumen. Sample 1 was run on paper and then in starch gel. The section of paper containing lysozyme was not included. Sample 2 was untreated albumen run in the same gel.

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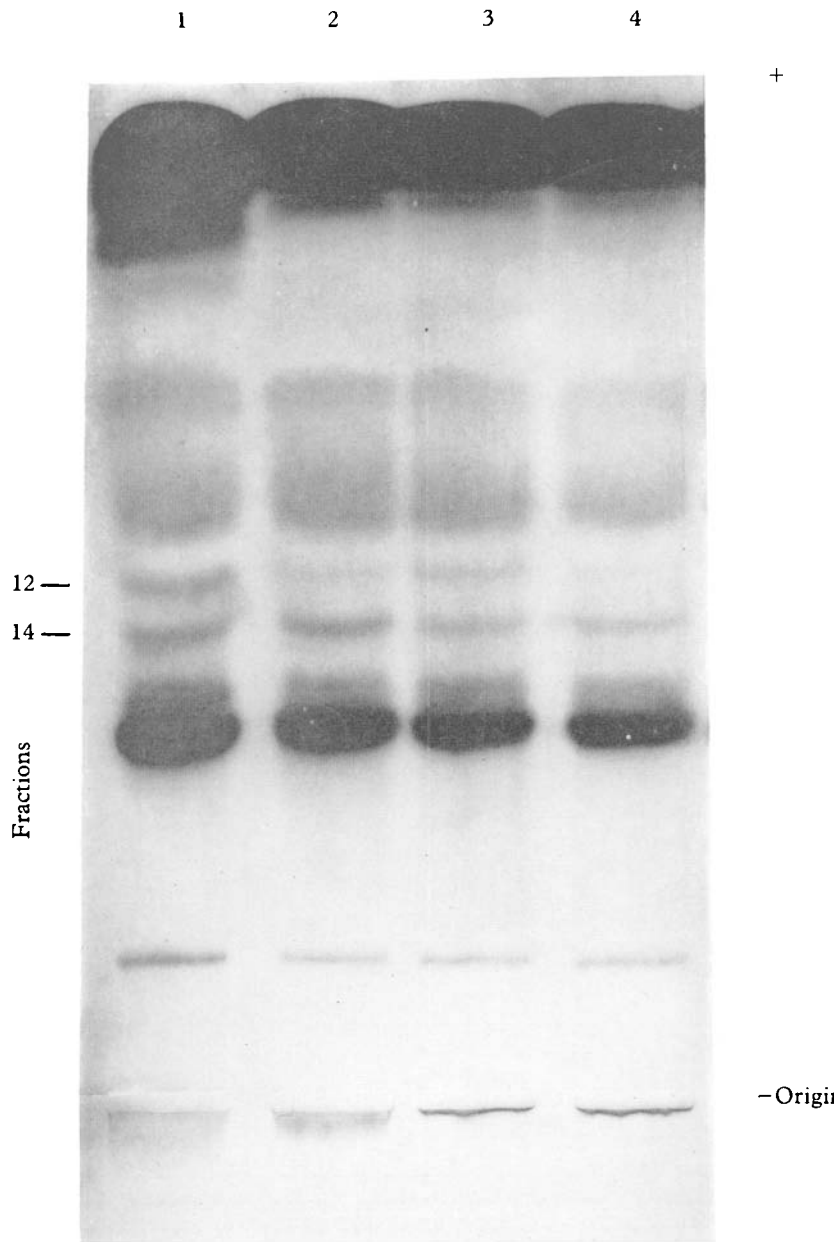

Sample 1 was normal III.AB albumen. Samples 2, 3 and 4 were from three eggs laid by Hen F3230. Note the variation in the intensity of fraction 12 relative to fraction 14.

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component which is inseparable from fraction 14 by one-dimensional starch gel electrophoresis. Thus fraction 14 is absent in albumen in type III.A.

(v) *Two hens with an anomalous phenotype*

F3230. This bird, from the IN line, was mentioned briefly in the first paper of this series (Lush, 1961). It was hatched in June 1957 and its egg albumen was first typed in June 1960 when twelve of its eggs were used in a control experiment to check that temporary egg storage at 4° C. has no ill effect on the subsequent electrophoretic picture. All twelve eggs were of type III.B, as expected from an IN hen. Another egg from the same hen was tested in September 1960 and was found, with some surprise, to be of type III.AB. Thenceforward, every egg (twenty-nine in number) laid by this bird was analysed, and it became clear that it was laying eggs in which the strength of fraction 12 varied unpredictably from complete absence (type III.B) to a strength equal to fraction 14 (type III.AB). Plate II illustrates this variation.

J2600. This bird, an IN × BR cross, was hatched in March 1960. It was mated with an IN cock and its five offspring were of albumen type III.B, as expected. However, when an egg from J2600 itself was tested in March 1962 it appeared to be a typical III.AB. The five subsequent eggs were III.B with a faint but definite fraction 12.

An explanation of this curious phenomenon has proved elusive. Both F3230 and J2600 were almost certainly genotypically III^B/III^B. Since the onset of the abnormality both hens laid very erratically and their eggs presented other unusual features. The eggshells were thin and sometimes absent. The albumen of each egg was reduced in amount and composed of a very watery thin and a very viscous, cloudy thick. All the electrophoretic fractions were reduced in concentration, as judged by eye from the electrophoretogram. Two examples are known of a specific alteration of the electrophoretic behaviour of a protein by an enzyme of bacterial origin. A proteinase (subtilisin) from *Bacillus subtilis* converts ovalbumin to plakalbumin, which has a lower mobility (Perlmann, 1949). A bacterial enzyme (probably neuraminidase) was shown by Lowe & McDougall (1961) to alter the electrophoretic pattern of the serum transferrins of the Red Deer in contaminated samples which had been stored at 1° C. It seems reasonable to suggest that the alteration in the phenotype of these two hens may have been due to an oviducal infection with a micro-organism producing an enzyme acting preferentially on fraction 14 and converting it into something of the same electrophoretic mobility as fraction 12, but why the process should stop half-way remains unexplained. The effect could not be reproduced by incubating a small amount of abnormal albumen with normal III.B albumen. Attempts to culture an organism from the abnormal albumen were unsuccessful.

Both hens were killed in May 1963. F3230 was found to have an adeno-carcinoma, probably arising from the Wolffian body remnant at the hilus of the ovary, with metastases to the lung and wall of the intestine. J2600 had an adeno-carcinoma, probably of oviduct origin, with metastases to ovary, duodenal serosa and the wall of the gut.

(vi) *Comparison of the different egg albumen layers*

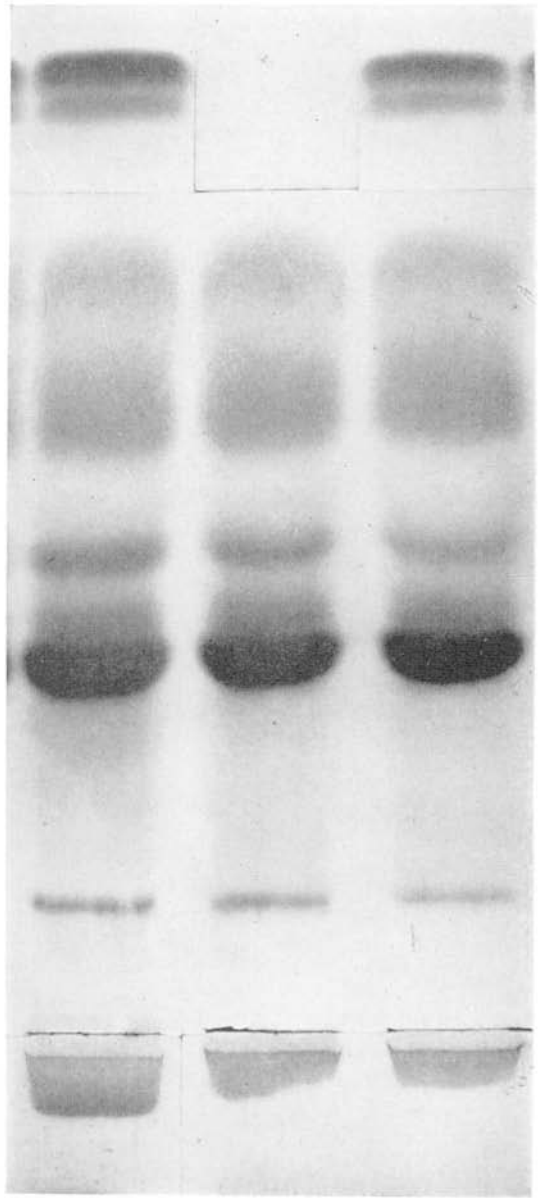
The albumen of a hen's egg is not a structureless liquid, but consists of three main layers (Romanoff & Romanoff, 1949). The outer layer of liquid ('thin') albumen surrounds a bag of mucilaginous ('thick') albumen which itself contains an inner layer of thin albumen. The three layers may readily be separated in a fresh egg (see Methods), and it has been shown that when they are compared with respect to refractive index and specific content of solid matter* (Romanoff & Sullivan, 1937) and density (Frampton & Romanoff, 1947), each of these measurements rises as one proceeds inwards from the outer thin via the thick to the inner thin.

Most electrophoretic studies on egg albumen have been done with samples from an homogenate of whole albumen, but Frampton & Romanoff (1947), who analysed samples from each layer by Tiselius electrophoresis, stated that 'no major protein components in any one layer of egg albumen may be identified with any major protein in either of the other two layers'. Forsythe & Foster (1949) came to the opposite conclusion. It was decided to re-investigate this point. Samples were obtained from the albumen layers of fresh eggs (< 4 hours old) from several different hens. The three samples from each egg were run on the same gel. In order to compare the ovalbumin more accurately, samples from inner and outer thin albumen were diluted fifteen times with 0·9% NaCl and run side by side on a gel. Plate III shows the result of a typical experiment. It can be seen that most of the electrophoretic fractions show an increase in concentration (as judged by staining) in the same direction as the increase in refractive index, total solids, and density. There was no apparent qualitative difference between the proteins of the three albumen layers.

(vii) *Identification of electrophoretic fractions*

Some of the egg albumen proteins of the fowl have been objects of research by biochemists for many years, and it was therefore of interest to see if the genetically polymorphic proteins could be identified with any of the well-characterized ones. In view of the ill-defined nature of ovomucin no attempt was made to locate it on the electrophoretogram. Brooks & Hale (1961) have described ovomucin as a 'weak elastic gel interpenetrated by a system of microscopic elastic fibres', in which case it would not be able to enter the starch gel. Plate IV is a diagram of a gel on which have been run samples of the remaining five well-known egg albumen proteins, alongside a sample of whole albumen of phenotype I.A, II.A, III.AB. The ovalbumin (five times crystallized by Dr F. Steven according to the method of Cole (1932)) can be seen to be of type A and to comprise fractions 3, 4, 6, 7 and 8. This will be discussed in detail in a later paper. Ovomucoid forms a diffusely staining band of material overlapping ovalbumin, along with a minor, faster, component. Ogden, Morton, Gilmour & McDermid (1962) have recently found a polymorphism in conalbumin within a flock of Light Sussex fowls. This iron-binding albumen protein is of added interest since Williams (1962) has conclusively shown that it occurs in a

* Defined as the percentage weight remaining after drying to constant weight in a vacuum oven at 80° C.



Electrophoretogram of samples from the three albumen layers of one egg. The ovalbumin region of the gel has been replaced by the corresponding section from a gel of inner and outer thin albumen diluted × 15. It was not possible to dilute the thick albumen. An increase in intensity from right to left can be seen in several fractions.

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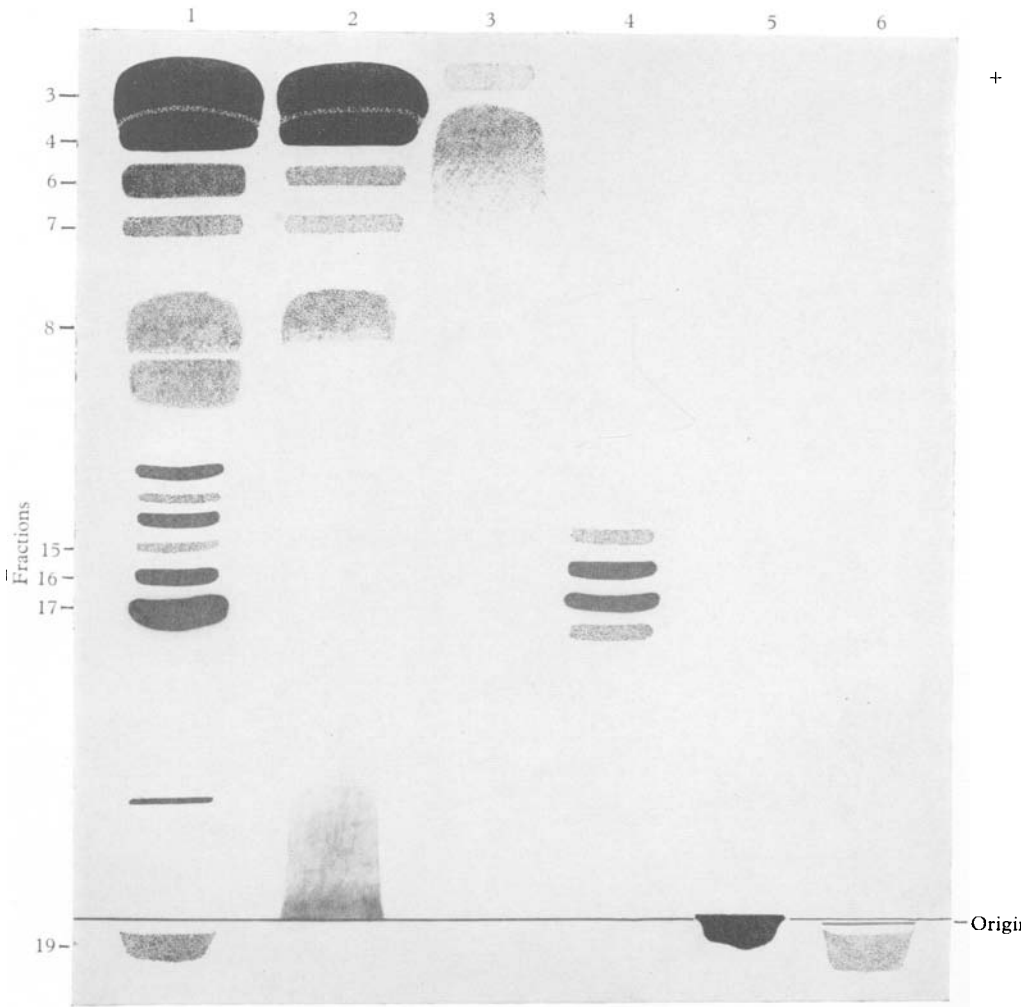

Diagram of electrophoretogram of egg albumen and individual albumen proteins. Sample 1 was untreated egg albumen. Sample 2 was 5 × crystallized ovalbumin, 6% in normal saline. Sample 3 was 3% ovomucoid in gel buffer. Sample 4 was 1·5% conalbumin in normal saline. Sample 5 was 2·5% avidin in normal saline. Sample 6 was 0·5% lysozyme in normal saline.

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slightly different form in the blood plasma; thus the polymorphism can be followed in samples from either medium and the males can be typed phenotypically. Plate IV shows a sample of the type B conalbumin of Ogden *et al.* (1962). It can be seen to comprise fractions 15, 16 and 17 and Williams (1962) has confirmed this with regard to fractions 16 and 17 by autoradiography of electrophoretograms of conalbumin labelled with ^{59}Fe. The slowest fraction in the sample illustrated in Plate IV is probably an artefact. All the eggs used in the work reported here contained only conalbumin B, with the exception of some from a flock described in the next section. Avidin migrates a small distance into the gel in both directions. Lysozyme moves towards the cathode. Rhodes, Bennett & Feeney (1959) discovered a riboflavin-binding protein (M.W. about 35,000) which is present in egg white at a concentration of 0·9 mg. per ml., and which has a higher electrophoretic mobility at pH 7 to 9 than any other previously separated constitutent of egg white. Baker & Manwell (1962) reported that this protein travels just ahead of ovalbumin in starch gel.

From the above data it is safe to conclude that the riboflavin-binding protein is not located in regions II or III and that the polymorphic proteins have yet to be characterized biochemically.

(viii) *Occurrence of the polymorphisms in other flocks*

The frequency of the different egg albumen phenotypes in samples from five populations outwith the P.R.C. was reported in the first paper of this series (Lush, 1961). Through the kindness of Dr I. S. Robertson I have now been able to sample five populations of Light Sussex hens maintained at the Royal (Dick) Veterinary College, Edinburgh. Segregation was found at the transferrin (*Tf*) locus described by Ogden *et al.* (1962), as well as at loci *II* and *III*.

Cochrane & Annau (1962) have found egg albumen polymorphism in a Canadian flock of White Rock-Leghorn crossbred hens. They used the buffer system of Smithies (1955) and it is not possible to compare their published figure directly with that of Lush (1961). However, the Canadian workers analysed samples sent from the author's laboratory and confirmed that they are dealing with the same region II and II polymorphisms already described. Baker & Manwell (1962) have also found the region II and III polymorphisms in several American flocks. The relationship between the nomenclatures adopted by the Canadian and American workers and that of Lush (1961) is summarized in Table 3.

Table 3. *Relationship between published nomenclatures for electrophoretic fractions of egg albumen*

	Lush (1961)	Cochrane & Annau (1962)	Baker & Manwell (1962)
Electrophoretic fractions	9	2	G_3A
	10	3	G_3B
	12	4	G_2C
	14	5	G_2D

(ix) *General remarks*

Apart from those already mentioned, about fifteen papers on the electrophoresis of egg albumen have appeared in the last twenty years. With one or two exceptions a constant feature throughout this work has been the blending of albumen from the eggs of several individual hens followed by the electrophoretic analysis of the mixture. It is probably this procedure, as well as the incomplete electrophoretic separation characteristic of the Tiselius, agar and paper techniques, which has caused the polymorphism of egg albumen proteins to be overlooked hitherto.

Apart from immunoelectrophoresis, the only other technique with a power to resolve complex protein mixtures comparable with that of starch gel electrophoresis is chromatography on ion exchange materials. Egg albumen has recently been chromatographed on CM-cellulose (Rhodes, Azari & Feeney, 1958) and on DEAE-cellulose (Mandeles, 1960). These authors demonstrated from three to five unidentified minor components, some of which probably correspond to the polymorphic proteins described in this paper.

Taking the work of Ingram (reviewed in Ingram, 1961) on the human haemoglobins as a guide, one could suggest that the demonstrable electrophoretic difference between the products of the two alleles at each locus described in this paper is due to a small difference in the amino-acid composition of these proteins. However this may be, it should be remembered that at any pH a large proportion of random amino-acid substitutions would involve no change of electrical charge, and would therefore be undetectable by electrophoretic screening. Thus within a population of hens of phenotype II.A, other undetected region II polymorphisms may exist. In this connection it is of interest to note that McIndoe (1962) was able to demonstrate a polymorphism in fowl serum albumin only when the electrophoresis was carried out below pH 8·0.

SUMMARY

The genetics of two polymorphic loci in the fowl was studied. Each affects the electrophoretic mobility of a different egg albumen protein, and at each locus two alleles were shown to segregate in a Mendelian manner.

The polymorphic proteins are not any of the well-characterized egg albumen proteins, viz. ovalbumin, ovomucoid, ovomucin, flavoprotein, avidin and lysozyme.

In contrast to all others, two hens were found to have an anomalous, variable, phenotype. A tentative explanation of this was offered.

This work was carried out during the tenure of a British Egg Marketing Board Studentship and, latterly, an Animal Health Trust Wellcome Fellowship. My thanks are due to Dr A. W. Greenwood and Dr T. C. Carter and the Agricultural Research Council for laboratory facilities, and to Dr D. J. Bell, Dr. J. S. S. Blyth and Dr W. M. McIndoe for many helpful suggestions.

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