Ontogeny of haemoglobin in the chicken

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INTRODUCTION

Study of an individual protein type such as haemoglobin during development provides a convenient way to study the embryological expression of a small number of genes. Knowledge of the quaternary structure of the protein is necessary because a number of proteins have been found to be made up of more than one type of polypeptide chain, and each polypeptide chain with a different amino acid sequence is coded from a distinct gene (cistron). For example, human foetal haemoglobin is \(\alpha_2\gamma_2\); human adult haemoglobin is \(\alpha_2\beta_2\). Both chemical and genetical studies indicate that the \(\alpha\) chains of both haemoglobins are coded from the same cistron, whereas the embryological change involves a 'switch-over' from the \(\gamma\)- to the \(\beta\)-chain cistron (reviewed by Ingram, 1963).

Embryonic, larval or foetal haemoglobin, distinct from adult haemoglobin, occurs in representatives of several vertebrate classes (reviewed by Manwell, 1960, 1963). However, as the following summary of the literature shows, the presence of a distinct embryonic haemoglobin in the chicken is controversial.

Fraser (1961) and Wilt (1962) claim that there are basically only two haemoglobins in the domestic fowl and that during ontogeny there is only a change in relative amounts of the two haemoglobins, although Fraser (1964) has recently reported finding a third haemoglobin appearing 2 weeks before hatching and continuing to occur in the adult chicken. Among the criteria used by Fraser (1964) is that one of the haemoglobins has a higher content of methionine than the others, whereas other workers report the absence of this amino acid from chicken haemoglobin, whether it is resolved into two (van der Helm & Huisman, 1958) or three (Alekseenko & Orekhovich, 1964) components. Huisman and colleagues (Huisman, Van Veen, Dozy & Nechtman, 1964; Huisman & Van Veen, 1964) state that similar haemoglobins are found in embryonic and adult chickens, although they examined no material earlier than 14 days of incubation and they did report the existence of a trace basic haemoglobin component in late embryos.

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(foetuses) and newly hatched chicks which does not occur in adult chickens. They find that the differences in the oxygen affinity of late embryo and adult chicken haemoglobins, such as those reported by Hall (1934), are due to differences in the concentration of phosphate ion, which also influences the electrophoretic behaviour of the minor (anodal) chicken haemoglobin component.

However, D'Amelio & Salvo (1961) found two distinct embryonic chicken haemoglobins present only in the first few days of incubation; by from 9 to 11 days of incubation they observed that the embryonic haemoglobins were largely replaced by the adult type of haemoglobins characteristic of foetuses and adults. Using starch-gel electrophoresis, Manwell, Baker, Roslansky & Foght (1963) obtained results very similar to those of the Italian workers. Up to 6 days of incubation chicken embryos of three different breeds contain a major and a minor haemoglobin, both different from the major and minor haemoglobin of late foetuses, chicks and adult chickens. Thus, the 'switchover' from embryonic haemoglobins to adult haemoglobins is very rapid in the chicken; by 7 days of incubation approximately half of the haemoglobin is of the adult type. Two other independent biochemical criteria for the distinctness of embryonic and adult chicken haemoglobins have been supplied (Manwell, Baker, Roslansky & Foght, 1963): (a) tryptic peptide patterns ('fingerprints') of the haemoglobins from 5-day embryos and from adult chickens differ by several peptides; (b) oxygen equilibria of 5-day embryo and adult haemoglobins, dialysed against the same phosphate buffers (thus avoiding the influence of any possible phosphate ion difference, as reported by Huisman et al. (1964), are very different. Recently Deuchar & Dryland (1964) found two distinct embryonic chicken haemoglobins, using cellulose acetate electrophoresis, and Borghese & Bertles (1965) have found a similar ontogenetic change for the haemoglobins of the duck.

It is clear that some of the controversy arises from the failure to sample embryos sufficiently early (e.g. Huisman & Van Veen, 1964; Huisman et al. 1964). However, that does not explain the results of Fraser (1961, 1964) or Wilt (1962). Fraser (1964) mentions using the methaemoglobin cyanide derivative, often preparing the haemoglobin by freezing and thawing. It is known that there are differences in the electrophoretic mobility of various haemoglobin derivatives (Chernoff & Pettit, 1964a) and that freezing and thawing can blur the electrophoretic resolution of haemoglobin (Manwell, Baker & Childers, 1963; Huntsman et al. 1964); in addition, in the presence of appropriate ions, freezing and thawing can result in hybridization of different types of lactate dehydrogenases (Kaplan, 1964) or haemoglobins (Manwell, unpublished studies).

Accordingly, in the present paper we report the results of additional studies on embryonic and adult chicken haemoglobin, including studies on chemical modification, polypeptide chain composition, electrophoresis in additional buffer systems, and measurements on oxygen-binding properties of purified and dialysed haemoglobins. Also, as the erythrocyte represents an easily isolated
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‘pure’ cell type, studies have been made on electrophoretic properties of several erythrocyte enzymes to see if the haemoglobin ‘switchover’ is paralleled by other ontogenetic changes in red blood cell proteins.

MATERIAL AND METHODS

Haemoglobin preparation, vertical starch-gel electrophoresis, and oxygen equilibrium determination are as described elsewhere (Manwell, 1963; Manwell, Baker, Roslansky & Foght, 1963). All haemoglobin preparations were kept in the carbon monoxide form during purification and electrophoresis, except in specific electrophoretic comparisons of oxyhaemoglobin, methaemoglobin and methaemoglobin cyanide, or where the sample was destined for oxygen equilibrium studies. It was necessary to pool 5-day embryos to provide enough haemoglobin for determination of the oxygen equilibrium under reasonable conditions of haemoglobin concentration (2–5 %); however, no individual embryonic or adult variation has been observed in screening on an individual basis a total of 98 adults and 78 5-day embryos, plus 70 embryos of other ages and 18 newly hatched chicks; the breeds and strains surveyed are: New Hampshire, Columbian, New Hampshire × Columbian, White Leghorn, Silkie, Light Sussex type (Thornbers’ ‘strain 6’) Thornber 404 Hybrids, and Thornber 606 Hybrids. Nevertheless, as an additional precaution, in polypeptide-chain studies no haemoglobin samples were pooled. The electrophoretic buffers used in comparison of embryonic and adult haemoglobins, as well as in surveying for individual variation, included: Smithies’ (1959) standard borate (gel pH 8-4–8-5); tris-EDTA-borate (gel pH 8-6–8-8); barbiturate (gel pH 8-4) (see Baker, 1964, for details); phosphate (ionic strength = 0-02, gel pH values of 6-0, 6-7 and 7-3–7-5); and Ferguson & Wallace’s (1961) lithium-containing modification of Poulik’s discontinuous tris-citrate-borate system (gel pH 8-0). The borate and tris-EDTA-borate buffers provide the best resolution of the multiple embryonic and adult chicken haemoglobins, although the phosphate systems are also useful, especially as they sharpen the minor (more acidic) adult haemoglobin component; the Ferguson & Wallace (1961) buffer tends to make the resolution of the multiple haemoglobins indistinct although it provides excellent resolution of erythrocyte esterases and lactate dehydrogenases.

After separation of the multiple haemoglobins of 5-day embryos and adult chickens the individual components were cut out from the starch gels with a scalpel blade and transferred into the sample slots of an acid (pH 1-8–2-0) gel for chain separation. In studies with urea gels it is necessary to remove the haem from the globin by the standard acid-acetone technique (Chernoff & Pettit, 1964b); however, whether haemoglobin or globin components were used at low pH, the results are identical. It should be emphasized that the use of low pH starch gels, using HCl, formic acid, lactic acid, acetic acid, or propionic acid, or some combination of two or three of these, provides resolution of the
polypeptide chain types of various vertebrate and invertebrate haemoglobins better than the ion-exchange chromatographic methods usually employed in separating mammalian haemoglobin $\alpha$ and $\beta$ chains (Muller, 1960; Dozy, Reynolds, Still & Huisman, 1964; Elzinga, 1964; Efremov & Braend, 1965; Manwell, 1966; and unpublished studies on multiple haemoglobins of fishes, elasmobranchs, lampreys, annelids, sea-cucumbers and chironomids). Electrophoretic methods of chain separation also appear superior in studies on the heterogeneity of H and L chains of antibodies (Cohen & Porter, 1964). In studies on the chain types of chicken haemoglobins an acid buffer made by dilution of 4 ml concentrated HCl and 14 ml conc. (85 %) formic acid to 1 l. had proved especially useful; a similar HCl-formate buffer was employed in separation of polypeptide chains of bullfrog haemoglobin (Elzinga, 1964). In addition, acid gels were run in pairs, one of the pair containing in addition 2 or 4 ml mercaptoethanol/l., both to check on possible disulphide groups holding chains together and to prevent minor zones arising from oxidation of —SH groups (Chemoff & Pettit, 1964b). Such acid gels are poured between plates of glass and run at 0 °C with fan cooling to avoid overheating during electrophoresis. That chicken haemoglobin resembles other haemoglobins and dissociates into individual polypeptide chains at a pH below 3 has been established by both analytical ultracentrifugation and surface monomolecular spreading techniques (Sasakawa et al. 1963). Another check on polypeptide-chain dissociation and interchange with chicken haemoglobin has been provided by repeating the molecular hybridization procedure of Itano & Robinson (1960) with mixtures of adult human and adult chicken haemoglobins.

Standard histochemical methods for localization of $\alpha$-naphthyl acetate esterases, lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) on starch gels after electrophoresis were employed (Burstone, 1962; see also Manwell, 1966).

RESULTS

Electrophoresis of haemoglobin

Typical electrophoretic resolutions of multiple embryonic and adult chicken haemoglobin from individuals of different ages are shown in Plate 1. It is obvious that there is a developmental change in haemoglobin type, although, as previously emphasized by D’Amelio & Salvo (1961) and by Manwell, Baker, Roslansky & Foght (1963), the ‘switchover’ from embryonic to adult-type haemoglobin takes place very quickly. Synthesis of adult haemoglobin begins by the sixth day of incubation, 15 days before hatching and by from 7 to 8 days of incubation 50 % of the haemoglobin is of the adult type. By the time of hatching nearly all of the haemoglobin is of the adult type. The most obvious haemoglobin zones are:

1) Three- to five-day embryos have two easily separated haemoglobin zones: major embryonic haemoglobin, which with longer electrophoretic times
Ontogeny of haemoglobin in the chicken. The same sixteen samples from sixteen different embryos, chicks and chickens have been placed in corresponding positions in a pH 8.5 borate gel (upper half of figure) and a pH 7.0 phosphate gel (lower half of figure).
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can be readily resolved into two components, and minor embryonic haemoglobin. The former zone makes up approximately 80% of the haemoglobin in 3- to 5-day embryos. Under some conditions the minor embryonic haemoglobin can also be fractionated into two components. All of these multiple haemoglobins are different in their electrophoretic mobility from any of those in adult haemoglobin samples.

(2) Adult chickens also have two easily separated haemoglobin zones: major adult haemoglobin, comprising 80% of the total, and minor adult haemoglobin. There is a tendency for both of these adult zones to subdivide, but never as clearly as the separation of the major embryonic haemoglobin into two components.

The haemoglobin of late embryos and foetuses behaves electrophoretically as if it were essentially a mixture of embryonic and adult haemoglobins in different proportions. However, there are zones in low concentration that are distinct from either the adult or the 3- to 5-day-embryo haemoglobins. One of these is a minor zone migrating between the minor adult haemoglobin and the major embryonic haemoglobin, and is most conspicuous in phosphate gels (see Plate 1); this zone disappears before hatching. A second transitional zone is a trace haemoglobin present in foetuses and chicks; it is visible in the borate-gel photograph of the 8-day chick haemolysate in Plate 1. This trace zone is never more than 5% of the total and behaves consistently in all buffer systems as if it possessed a slightly more alkaline isoelectric point than the most basic of all the other haemoglobin zones, the minor embryonic haemoglobin. The nature of these minor and trace foetal and juvenile haemoglobins will be dealt with in another publication. The remainder of this paper is concerned with the nature of the four principal haemoglobin zones, two embryonic and two adult.

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**Plate 2**

A. Electrophoresis at pH 2 of globins from the isolated multiple haemoglobins of embryonic, foetal and adult chickens. From left to right the eight samples are: (1) Hb zone from an 8-day embryo in the same electrophoretic position as the major adult haemoglobin (A$_2$F$_2$); (2) major adult haemoglobin (A$_2$F$_2$); (3) anodal fringe of the major adult haemoglobin (A$_2$F$_2$); (4) minor adult haemoglobin (A$_2$E$_2$); (5) minor haemoglobin zone from an 8-day embryo in the same electrophoretic position as the minor adult haemoglobin (A$_2$E$_2$); (6) major embryonic haemoglobin, anodal component (B$_2$C$_2$); (7) major embryonic haemoglobin, cathodal component (A$_2$C$_2$); (8) minor embryonic haemoglobin (D$_2$F$_2$).

B. Molecular hybridization of human and chicken haemoglobin. From left to right the samples are as follows. (1) Mixture of human haemoglobin (Hb A) and chicken adult haemoglobin, each of which had been dialysed separately against pH 4-7 buffer, followed by dialysis against pH 7-5 buffer. Note that no new haemoglobin zones are formed. (2) and (5) Mixture of human haemoglobin (Hb A) and adult chicken haemoglobin which have been dialysed together against first pH 4-7 buffer and then pH 7-5 buffer. (3) Adult chicken haemoglobin which has been dialysed first against pH 4-7 buffer and then against pH 7-5 buffer. Note the loss of the minor adult chicken haemoglobin. (4) Adult chicken haemoglobin which had been dialysed only against pH 7-5 buffer. The hybrid haemoglobin zones appear only when the two haemoglobins, human and chicken, are together at pH 4-7.
If the haemoglobin of 5-day embryos and adult chickens is studied as oxyhaemoglobin, carbonmonoxyhaemoglobin, methaemoglobin, or methaemoglobin cyanide, the differences between adult and embryonic haemoglobins remain. In agreement with Chernoff & Pettit (1964a) the different derivatives are found to have different electrophoretic mobilities; this is most conspicuous for methaemoglobin, although a very small but consistent difference in electrophoretic mobility exists even between oxyhaemoglobin and carbonmonoxyhaemoglobin, as might be expected on the basis of slight differences in protein configuration associated with combination with different ligands (Manwell, 1960, 1964).

**Electrophoresis of globins and polypeptide chain composition**

A typical electrophoretic separation of the polypeptide chains of isolated embryonic, foetal, and adult chicken haemoglobin components is shown in Plate 2A. On the basis of electrophoretic mobility six different kinds of polypeptide chains can be distinguished. Addition of mercaptoethanol has no conspicuous effect on chain resolution and, thus, —S—S— bonds do not contribute to the quaternary structure of embryonic or adult chicken haemoglobins, in agreement with studies on other vertebrate haemoglobins (Dozy et al. 1964; Elzinga, 1964; Efremov & Braend, 1965; Manwell, 1966, and unpublished experiments).

The polypeptide chain composition of the major and minor adult and embryonic haemoglobins is summarized diagrammatically in Text-fig. 1. The polypeptide chains are arbitrarily given Roman letters, A to F, solely on the basis of electrophoretic mobility; Greek letters are not used at present to avoid confusion with α, β and γ chains of human adult and foetal haemoglobin. As it has been established that adult chicken haemoglobins have the typical vertebrate haemoglobin molecular weight of 66,000 (Huisman et al. 1964; Sasakawa et al. 1963), it will be assumed that there are four polypeptide chains per molecule, two of one kind and two of the other, as in most other vertebrate haemoglobins.

The minor and major adult chicken haemoglobins are A₂E₂ and A₂F₂, in agreement with Saha & Ghosh (1960), who suggest that the major and minor haemoglobins have one polypeptide chain type in common. When these adult haemoglobin components become apparent in electrophoresis of haemolysates from 7- and 8-day embryos, the polypeptide chains also correspond to those of the adult haemoglobin (see Plate 2A). In agreement with Huisman and associates (Huisman et al. 1964; Huisman & Van Veen, 1964), the electrophoretic differences in the minor anodal haemoglobin of foetuses and adults are eliminated in phosphate buffers and, more significant of identity of the haemoglobins, the polypeptide chains have identical electrophoretic behaviour.

Thus, the polypeptide chain studies show that the ontogeny of chicken haemoglobin resembles that of human haemoglobin; certain polypeptide chain types are present throughout ontogeny (human α chain, chicken A and F chains),
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while others are 'switched' (human $\gamma$ chain in foetuses, $\beta$ chain in adults; chicken B, C and D chains in embryos, replaced by E chains in foetuses and adults).

![Diagram](image)

Text-fig. 1. Diagrammatic representation of the resolution of 5-day embryonic and adult chicken haemoglobin, first into multiple haemoglobin components by electrophoresis at pH 8.5, and then into polypeptide chains by electrophoresis of the isolated globins at pH 2.

**Polypeptide chain hybridization**

A possible explanation of claims for the presence of adult chicken haemoglobin in the earliest embryos (Wilt, 1962; Fraser, 1961, 1964) is as follows. Two of the embryonic haemoglobins have an adult haemoglobin polypeptide chain type—the major embryonic haemoglobin cathodal component is $A_2C_2$ and the minor embryonic haemoglobin is $D_2F_2$. It is conceivable that any manipulation in haemoglobin preparation which would facilitate polypeptide chain exchange would allow recombination to form $D_2C_2$, a new haemoglobin
component, and $A_2F_2$, the major adult haemoglobin component. Attempts at polypeptide chain recombination of various chicken haemoglobin components have not been entirely successful, for only the major adult chicken haemoglobin is not denatured after the usual molecular hybridization technique (Itano & Robinson, 1960) of dialysis against pH 4.7 buffer. However, the major adult chicken haemoglobin can be hybridized with various adult human haemoglobins, including Hb A (Plate 2B). Thus, there is evidence that polypeptide chain exchange can occur for at least one of the chicken haemoglobins, as well as an indication of a homology between human $\alpha$ and $\beta$ chains and chicken $F$ and $A$ chains.

Text-fig. 2. Bohr effect for the oxygen equilibrium of haemoglobin from 5-day embryos and adult chickens. The haemoglobins have been purified by ammonium sulphate fractionation but have not been separated into multiple components, and have been dialysed exhaustively against potassium phosphate buffers of different pH values but constant ionic strength (ionic strength = 0.30), $P_{50}$ is the oxygen partial pressure at which the haemoglobin is exactly half saturated with oxygen, i.e. a ‘dissociation equilibrium constant’ for the combination of haemoglobin with oxygen. Two to five per cent haemoglobin solutions at 24–26 °C.

**Oxygen equilibrium of embryonic and adult haemoglobins**

The possession of some common chains between adult and embryonic chicken haemoglobins raises an interesting question with regard to the oxygen equilibrium. Even taking the precaution of repeated dialysis against common buffers, as suggested by Allen, Wyman & Smith (1953) in comparison of human adult and foetal haemoglobin (see also Manwell, 1964), early embryonic chicken
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haemoglobin has a much higher oxygen affinity and lower Bohr effect than adult chicken haemoglobin (Text-fig. 2). Riggs (1965) has indicated that because chicken embryo haemoglobin has a higher oxygen affinity it does not imply that it has a smaller Bohr effect as reported in previous studies (Manwell, Baker, Roslansky & Foght, 1963). However, inspection of the overall oxygen equilibrium constant, \( p_{50} \), as a function of pH, as shown in Text-fig. 2 of the present paper, indicates that chicken embryonic haemoglobin does have a significantly smaller Bohr effect. Using the typical measure of Bohr effect magnitude (Manwell, 1964; Riggs, 1965),

\[
\phi = \frac{\partial \log p_{50}}{\partial \mathrm{pH}},
\]
evaluated over the approximately physiological significant range of pH 7.0-7.5, in analysis of the data in Text-fig. 2, we obtain \( \phi = -0.67 \) for chicken adult haemoglobin and \( \phi = -0.40 \) for chicken embryonic haemoglobin; Riggs (1965) has considered \( \phi \) values closer than these as significantly different in studies on the Bohr effect of various mammalian haemoglobins. In fact, the data on chicken embryonic and adult haemoglobins are consonant with suggestions by Riggs & Herner (1962) that the \( \beta \)-chain type determines the bulk of the oxygen equilibrium properties in an \( \alpha_2 \beta_2 \) haemoglobin molecule, for the major differences between the embryonic and adult haemoglobins reside in those polypeptide chain types of relatively more rapid electrophoretic mobility, which Efremov & Braend (1965) suggest to be \( \beta \) chains. From a physiological standpoint it is not surprising that early chicken embryos have a haemoglobin with a much higher oxygen affinity and lower Bohr effect, for this embryonic haemoglobin predominates in that phase of development prior to vascularization of the surface of the egg and the air sac.

Electrophoresis of erythrocyte enzymes in embryonic, foetal, juvenile and adult chickens

The 'switchover' in haemoglobin raises the question of whether or not there are changes in erythrocyte enzymes at the same time. Although up to six \( \alpha \)-naphthyl acetate esterase zones are observed in electrophoresis of chicken erythrocyte lysates (see also general avian data in Baker, Manwell, Labisky & Harper, 1966), these zones are the same whether 3-day embryos or adult chickens are used. There are some minor quantitative changes in activity of the different esterase zones, but there are no unique embryonic or adult erythrocyte esterases. Similarly, the pattern of lactate dehydrogenase remains essentially unchanged; using the terminology of Kaplan's (1964) group, the \( H_4 \) LDH isozyme, along with traces of the \( H_3M \) and \( H_2M_2 \) isozymes, occurs in embryonic, foetal, juvenile and adult chicken red blood cells, although a 'switchover' from \( H \) to \( M \) chains occurs in liver and muscle towards the end of chicken incubation, as reported by others (Cahn, Kaplan, Levine & Zwilling, 1962;
Two malate dehydrogenase zones are observed after electrophoresis of haemolysates; again there is no major change during development in the erythrocytes, although in agreement with Conklin & Nebel (1965) there are ontogenetic changes in MDH in other tissues. Embryonic and foetal erythrocytes do have a higher activity for the more acidic MDH erythrocyte isozyme but this isozyme is usually also present in adult erythrocytes.

DISCUSSION

The distinctness of embryonic and adult chicken haemoglobins

The electrophoretic studies on embryonic and adult chicken haemoglobins confirm and extend the studies by D'Amelio & Salvo (1961), Manwell, Baker, Roslansky & Foght (1963), Deuchar & Dryland (1964) and Borghese & Bertles (1965). Adult and embryonic chicken haemoglobins are readily differentiated by electrophoresis in higher-resolution zone systems (cellulose acetate, starch gel or acrylamide), in a variety of buffer systems, and whether the haemoglobin is combined with oxygen or carbon monoxide, or oxidized to methaemoglobin with or without additional combination with a low-spin ligand such as cyanide. It is difficult to explain satisfactorily the results of Wilt (1962) and Fraser (1961, 1964). The use of antibodies (Wilt, 1962), although successful in D'Amelio & Salvo's (1961) studies, to differentiate between adult and embryonic haemoglobins might fail in the case where the antibody was formed against the A or F chains common to embryonic and adult haemoglobins; for example, antibodies formed against the F chain or the A chain might not differentiate the major adult haemoglobin, A2F2, from either the cathodal component of the major embryonic haemoglobin, A2C2, or the minor embryonic haemoglobin, D2F2. Resolving power of the techniques employed by Wilt (1962) and Fraser (1961, 1964) might also provide a problem; examination of the column chromatographic data in Fraser (1961) suggests that the two haemoglobin components from early embryos are eluted at slightly different positions from the two haemoglobin components of adults—and in exactly the order predictable from isoelectric points estimated from the starch-gel electrophoretic results in the present paper. Yet another possibility involves preparative procedure; the embryonic haemoglobins and the minor adult haemoglobin are more unstable than the major adult haemoglobin; theoretically any treatment favouring polypeptide chain exchange among the embryonic haemoglobins could generate the adult haemoglobin, A2F2. Freezing and thawing of embryonic haemoglobin does result in additional haemoglobin zones, one of which is in the same position as the major adult haemoglobin, and in a loss of sharpness in electrophoretic resolution. The molecular hybridization experiments indicate that at least the major adult chicken haemoglobin will exchange some polypeptide chains with another haemoglobin as different as human Hb A or Hb S. Survey on an
individual basis of the different breeds and strains of chickens mentioned in the ‘materials and methods’ failed to find any individual embryonic or adult variation in haemoglobin types. Thus, it is possible that Fraser (1961, 1964) and Wilt (1962) employed chickens lacking in distinct embryonic haemoglobins; if so, the condition does not exist among any of the English or American flocks of chickens examined by us—though great variation exists in the presence and absence, as well as relative gene frequencies, of genetic variants for other biochemical systems in different groups of chickens (Baker & Manwell, 1962; Baker, 1964).

**Implications of the ontogeny of chicken haemoglobins to analysis of genetic control systems**

The genetic implications of these data on common and distinct polypeptide chains of embryonic and adult haemoglobin must be considered carefully. It is tempting to postulate that each distinct chain, A, B, C, D, E and F, is coded by a different cistron, as are the α, β, γ and δ chains of human haemoglobins. However, two possibilities must be kept in mind:

1. **Some of the polypeptide chains considered to be identical on the basis of electrophoretic mobility may be in fact different in amino acid sequence.** Electrophoretic methods have provided the most accurate resolution of polypeptide chain types of various haemoglobins (Chernoff & Pettit, 1964; Elzinga, 1964; Efremov & Braend, 1965; Manwell, 1966) and of antibodies (Cohen & Porter, 1964); in addition, the superior resolution of electrophoresis in starch gel is largely responsible for the development of the isozyme concept in biochemistry. Nevertheless, the possibility of additional chain types cannot be ruled out, although it will not invalidate the distinctness of embryonic and adult chicken haemoglobins. Amino acid sequence studies, a formidable task, will be required to settle this question. However, one other possible approach would be to find genetic polymorphism or close species differences involving, for example, the A chain of the minor adult haemoglobin, but not the A chain of the major adult haemoglobin or the cathodal component of the major embryonic haemoglobin. So far a search for such differences has been unsuccessful.

2. **Polypeptide chains with different electrophoretic mobilities may be coded from the same cistron.** It is known that some haemoglobin heterogeneity involves —SH group modification (Sullivan & Riggs, 1964; Riggs, Sullivan & Agee, 1964; Riggs, 1965; Manwell, unpublished studies on fish and shark haemoglobins); however, the failure of mercaptoethanol when added either to alkaline or to acid starch gels to alter the heterogeneity or polypeptide chain type of chicken haemoglobins eliminates this possibility. Two proteins with different electrophoretic mobility but coded from the same gene are the serum transferrin and egg-white conalbumin of birds (Williams, 1962; Baker et al. 1966); the amino acid sequence of these two proteins, judging from ‘fingerprint’ studies, is identical, but different carbohydrate moieties are attached. So far
carbohydrate residues have not been found covalently attached to any haemoglobin, but this point has not been sufficiently explored with modern methods of biochemical analysis. Another type of chemical modification involves acetylation of the N-terminal group. It is known that the N-terminal amino acid of one of the polypeptide chain types of adult chicken haemoglobin is acetylated (Satake, Sasakawa & Maruyama, 1963; Marchis-Mouren & Lipmann, 1965; Matsuda, Maekawa & Otsubo, 1965). However, that adult chicken haemoglobin is not simply embryonic haemoglobin with the N-terminals acetylated is indicated by the existence of a number of tryptic peptides unique to either embryonic or adult haemoglobin (Manwell, Baker, Roslansky & Foght, 1963). This does not eliminate the possibility that some of the difference between adult and embryonic haemoglobins involves presence or absence of acetylated N-terminal groups. Mixtures of embryonic and adult chicken crude haemolysates that have stood for several days retain the electrophoretic distinctness of adult and embryonic haemoglobin components, except for an extra minor haemoglobin zone which probably represents a polypeptide chain hybrid. This observation suggests that the distinct haemoglobins of embryo and adult are not readily interconverted by enzyme systems, either those involved in N-terminal acetylation (Marchis-Mouren & Lipmann, 1965) or intracellular proteases, present in either embryonic or adult erythrocytes.

Thus, it can be concluded that there are distinct embryonic haemoglobins in the chicken and that progression from embryonic to adult haemoglobin takes place in a manner resembling human haemoglobin differentiation where some chain types are 'switched' and others are not. The difference between chicken and human haemoglobin ontogeny is that more chain types appear to be involved in the chicken and that the 'switchover' occurs much earlier in development. The precision of the genetic control system involved in ontogeny of chicken haemoglobin is shown by the fact that erythrocyte esterases, lactate dehydrogenase and malate dehydrogenase undergo no more than moderate changes in relative amounts, rather than qualitative changes paralleling those for the haemoglobins.

The change from embryonic to adult type of haemoglobin in 6- to 7-day chick embryos appears to correspond to a change in the type of erythrocyte and site of erythropoiesis (see haematological data in Lucas & Jamroz, 1961). Whether there is a complete separation of embryonic and adult haemoglobins into different cells throughout chicken ontogeny cannot be answered from the present data. It also remains unknown as to why, if from the beginning of haemoglobin production embryos can synthesize both adult A and F chains, these are not in the same molecule until after the time of the 'switchover' at day 6 of incubation. It may be that: (a) A and F chains of adults and embryos are different in some subtle way, (b) that A and F chains are in different cells in early embryos but not in later ones or adults, (c) that A and F chains are in the same cell in early embryos but preferentially combine with the unique
embryonic haemoglobin chains (B, C and D), or (d) that A and F chains are in the same cell but because of some trick in biosynthesis never get the opportunity to combine with each other until after day 6. Whatever the explanation, the presence of purely embryonic haemoglobin during the first few days of development ensures that the embryo has a haemoglobin type with a very high oxygen affinity and small Bohr effect when compared with that of the adult.

The specificity of the haemoglobin ‘switchover’ is also shown by comparing the time for the change of haemoglobin type in different species of birds. Adult haemoglobin begins to appear at the end of day 5 or the beginning of day 6 in all of the chicken types enumerated earlier; however, in a few mixed Bantam chickens the ‘switchover’ took place 12–24 h later, even when they were incubated in the same oven and at the same time as several of the other breeds. The chukar partridge *Alectoris graeca* resembles the chicken in that the ‘switchover’ is apparent by the sixth day of incubation. In the turkey the ‘switchover’ occurs around day 8 of incubation; and the duck—both White Pekin studied by Manwell & Baker (unpublished) and that used by Borghese & Bertles (1965)—is the same, or perhaps one day later. The red-wing blackbird, on the other hand, has a similar haemoglobin electrophoretic profile at from 2 to 14 days of incubation, having both embryonic and adult haemoglobins from the beginning of ontogeny (Manwell, Baker, Roslansky & Foght, 1963). The existence of such species differences suggests that different control genes may well be manipulating the time and type of haemoglobin ‘switchover’, such as has been postulated to account for the ‘High Hb F’ condition and the various thalassaemias in man (Ingram, 1963). A variety of other variations on the pattern of haemoglobin differentiation exist and are reviewed by us elsewhere (Manwell, 1963, 1964; Manwell & Baker, 1966).

**SUMMARY**

1. The controversy as to whether haemoglobin embryology in the chicken involves qualitative or merely quantitative changes is settled by the observation of consistent electrophoretic differences between the haemoglobins of early embryos and the haemoglobins of foetuses, chicks and adult chickens.

2. Studies on polypeptide chain composition indicate that the multiple haemoglobins of 3- to 5-day incubation embryos are $A_2C_2$, $B_2C_2$ and $D_2F_2$. The major and minor haemoglobins of foetuses and adult chickens are $A_2F_2$ and $A_2E_2$. Thus, although embryos and adults have no haemoglobins in common, they appear to have two kinds of polypeptide chains in common, A and F. Embryos have three unique polypeptide chain types and adults have one, on the basis of differences in electrophoretic mobility.

3. Some degree of polypeptide chain interchange occurs with at least one of the chicken haemoglobins in that one of the adult haemoglobins will hybridize with human adult haemoglobin, $\alpha_2\beta_2$. 
4. Although two of the three 3- to 5-day chick embryonic haemoglobin components have one of their polypeptide chain types in common with the major adult haemoglobin, the oxygen equilibria and Bohr effect of adult and early embryonic haemoglobin are very different. Chick embryo haemoglobin has an extremely high oxygen affinity and a Bohr effect only two-thirds that of the adult.

5. The haemoglobin 'switchover' at the end of day 5 of incubation is not paralleled by changes in erythrocyte esterases, lactate dehydrogenase or malate dehydrogenase isoenzymes.

Résumé

Ontogénie de l'hémoglobine chez le poulet

1. La controverse sur le fait de savoir si la genèse de l'hémoglobine chez le poulet implique des modifications qualitatives ou simplement quantitatives est réglée par l'observation de différences électrophorétiques nettes entre les hémoglobines de jeunes embryons et celles d'embryons âgés, de poulets et d'individus adultes.

2. Des recherches sur la composition des chaînes polypeptidiques indiquent que les hémoglobines multiples du 3ème au 5ème jour d'incubation sont $A_2C_2$, $B_2C_2$ et $D_2F_2$. Les hémoglobines majeure et mineure des embryons âgés et des adultes sont $A_2F_2$ et $A_2E_2$. Ainsi, bien que les embryons et les adultes n'aient pas d'hémoglobines communes, ils ont en fait deux chaînes polypeptidiques en commun, $A$ et $F$. Les embryons ont trois types de chaînes polypeptidiques qui leur sont propres, et les adultes un seul, en se basant sur les différences de mobilité électrophorétique.

3. Des échanges entre chaînes polypeptidiques se produisent à un certain degré avec au moins une des hémoglobines de poulet, en ce que la principale hémoglobine adulte, $A_2F_2$, s'hybridera avec l'hémoglobine humaine adulte, $\alpha_2\beta_2$.

4. Bien que deux des trois composants de l'hémoglobine embryonnaire de poulet de 3 à 5 jours aient un de leurs types de chaînes polypeptidiques en commun avec la principale hémoglobine adulte, l'équilibre de l'oxygène et l'effet Bohr des hémoglobines embryonnaire, jeune et adulte sont très différents. L'hémoglobine embryonnaire de poulet a une affinité très élevée pour l'oxygène et l'effet Bohr n'est que les deux tiers de celui de l'adulte.

5. Le renouvellement des hémoglobines à la fin du 5ème jour d'incubation n'est pas accompagné de modifications des estéras érythrocytaires, isozymes des déshydrogénases lactique ou malique.
Ontogeny of haemoglobin in the chicken

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