Re-evaluation of the presence of multiple haemoglobins during the ontogenesis of the chicken

Electrophoretic and chromatographic characterization, polypeptide composition and immunochemical properties

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SUMMARY

Haemolysates of red blood cells from embryos of several developmental stages ranging from 2 to 21 incubation days and from post-hatching chickens of various age groups were analysed by ion-exchange chromatography, agar- and starch-gel electrophoresis, immuno-electrophoresis with specific antisera and polypeptide chain electrophoresis. With these methods two adult (A_x and A_2) and six embryonic (E_1-E_6) haemoglobin types were identified. Antisera specific for the major adult haemoglobins (A_x and A_2) as well as antisera specific for the major embryonic haemoglobins (E_3, E_4) could be prepared. Throughout embryogenesis the haemoglobin types contribute in varying amounts to the total haemoglobin pattern. Three periods of haemoglobin synthesis could be recognized, the transition between these periods occurred at the 6th and 12th incubation day. The first period is characterized by the presence of two major embryonic haemoglobins (E_3 and E_4) and two minor embryonic haemoglobins (E_2 and E_5). During the second period E_3 and E_4 are largely replaced by a major adult haemoglobin (A_2) and a new embryonic haemoglobin (E_5). The third period is characterized by the appearance of a second adult haemoglobin (A_x) and a new minor embryonic haemoglobin (E_6) with a concomitant decrease of E_2 and E_5. At the time of hatching two embryonic haemoglobins (E_3 and E_6) are still present.

Besides A_x and A_2, several minor haemoglobin fractions were inconsistently found in adult chickens. Evidence has been obtained that these additional fractions are reflecting a so called minor heterogeneity or separation artifacts.

The haemoglobins A_x, A_2 and E_1-E_6 show different polypeptide chain compositions. Three embryo-specific chains could be demonstrated (β E_3 E_5, γ E_4 and δ E_6). The production of the polypeptide chains appears to be correlated with the aforementioned periods of haemoglobin synthesis.

The genetic and morphological implications of the findings are discussed.

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INTRODUCTION

During ontogenesis activation and repression of genes take place continuously, causing the process of differentiation. The factors involved are still difficult to study due to complexity of the developmental events. Emergence of new proteins is thought to be a rather direct expression of gene activity. A system in which changes in protein composition are readily demonstrable would therefore be useful as a model.

Haemoglobin maturation heterogeneity may be such a system. Embryonic and foetal haemoglobins, different from adult haemoglobins, occur in representatives of several vertebrate classes (Manwell, 1960; Ingram, 1963; Manwell, Baker, Rolansky & Foght, 1963). In a number of avian species, embryonic haemoglobins have been demonstrated: turkey and partridge (Manwell, Baker & Betz, 1966), white Peking duck (Borgese & Bertles, 1965), and house sparrow (Bush & Townsend, 1971). The presence of embryonic haemoglobins in chickens is still controversial. Some authors (Fraser, 1961, 1964, 1966; Wilt, 1962; Simons, 1966) state that the changes in haemoglobin composition as observed during ontogenesis are essentially quantitative, others (D'Amelio & Salvo, 1959, 1961; D'Amelio, 1966; Manwell et al. 1963; Manwell et al. 1966; Hashimoto & Wilt, 1966; Schürch, Godet, Nigon & Blanchet, 1968; Denmark & Washburn, 1969) report the presence of distinct embryonic haemoglobins, but do not agree about the number of haemoglobin types and the time of their appearance. There is no agreement even about the number of haemoglobins present in adult chickens (Godet, Schürch & Nigon, 1970). These discrepancies are possibly due to the fact that most authors have used only one or two different techniques. Therefore, a thorough re-analysis of the haemoglobin types present in adult and embryonic chickens seems to be necessary.

In the present study the different haemoglobin types have been characterized by agar- and starch-gel electrophoresis, by chromatography on cation and anion exchangers, by immunochemical techniques and by the analysis of the polypeptide chain composition. Furthermore, the developmental stages at which the haemoglobin types appear and disappear in the circulation have been determined.

MATERIALS AND METHODS

Preparation of the haemolysate. Blood samples were obtained from the wing vein of White Leghorn chickens of various age-groups, ranging from 1 day to 3 years post-hatching and from the vitelline vein or the heart of White Leghorn embryos at various developmental stages, ranging from 2 to 21 incubation days. Care was taken to avoid contamination with yolk, as washing of the blood cell suspension with saline does not sufficiently remove the yolk particles. For the 3- to 5-day embryonic stages the haemolysates from 300 animals were used. For the later embryonic stages haemolysates from 3–30 animals were pooled. Post-
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Hatching samples were obtained from individual chickens. The blood cells were suspended and washed three times in a tenfold volume of ice-cold saline. They were allowed to lyse for 12 h in 0.025 M-NaCl at 4 °C. After centrifugation the haemoglobins in the supernatant were converted into the CO-form. Samples were stored at 4 °C until analysed. Analyses were started within 48 h after preparation of the haemolysate, as haemoglobins are known to alter by ageing as well as by freezing and thawing (Manwell et al. 1966).

**Electrophoresis.** Agar-gel electrophoresis was carried out using 0.05 M barbiturate buffer, pH 8.6, following the method of Wieme (1959). Vertical starch-gel electrophoresis was performed according the Smithies (1959) using a discontinuous buffer system containing 0.017 M Tris, 0.07 M EDTA and 0.025 M boric acid, pH 8.9 (gel) and 0.3 M boric acid and 0.06 M NaOH, pH 8.2 (electrode vessels), or as described by Scopes (1963), who used 0.25 M sucrose, 0.0015 M citric acid and 0.020 M Tris, pH 9.1 (central part of the gel), 0.010 M boric acid and 0.06 M Tris, pH 8.6 (ends of the gel) and 0.3 M boric acid and 0.06 M NaOH, pH 8.2 (electrode vessels). All analyses were carried out in the cold room at 4 °C. In order to trace the haemoglobins, the agar- and starch-gel strips were stained by a standard peroxidative procedure, in which benzidine in acetic acid and hydrogen peroxide were used (Dessauer, 1966). Amido black or Ponceau S were used to visualize other protein components. Relative electrophoretic mobilities in agar were calculated according to Wieme (1959), using as standards 1 % human serum albumin (Behring), mobility 100, and 1 % dextran (M.w. ± 135000), mobility 0.

**Column chromatography.** Carboxymethyl (CM)-cellulose was used as cation exchanger in combination with a discontinuous buffer system. In the first elution step 0.01 M sodium phosphate buffer, pH 6.9, was used. The second step was performed with 0.02 M sodium phosphate buffer, pH 7.5, and the third with 0.02 M sodium phosphate buffer, pH 8.5, to which 0.4 M NaCl was added.

The anion exchanger diethylaminoethyl (DEAE)-Sephadex was developed with 0.05 M trihydroxymethylaminomethane (Tris) HCl buffer in a linear gradient from pH 8.6 to 7.4. All buffers were saturated with CO. The optical density (O.D.) of the effluent fractions was measured at 280, 419 and 540 nm, using a Zeiss spectrophotometer. Approximate haemoglobin concentrations were calculated from 419 nm readings. The ratio O.D. 419/280 was determined for each peak in order to locate non-haemoglobin contamination. Absorption spectra of all haemoglobin fractions were recorded.

**Polypeptide chain analysis.** Isolated lyophilized haemoglobins were submitted to vertical starch-gel electrophoresis according to (1) the method of Bucci & Fronticelli (1965) using p-chloromercuribenzoic acid (PCMB), (2) the method of Muller (1961) using pH 1.9, and (3) the method of Gilman & Smithies (1968), who used 8 M urea at pH 3.2. The latter method proved to be preferable. The haemoglobins were used as such or after conversion into haemless globins by the acetone precipitation method of Rossi-Fanelli, Antonini & Caputo (1958) or
by the method of Teale (1959) using ethyl-methyl ketone. In our hands globin preparation always caused a considerable loss of material which became insoluble. As, apart from a distinct haem zone, no difference was seen in the starch patterns obtained when either total haemoglobins or haemless globins were used, total haemoglobins were used in later experiments. Mercaptoethanol was added in all experiments in order to prevent minor fractions to arise from oxidation of SH groups (Chernoff & Petitt, 1964a).

Immunochemical techniques. Micromodifications of the double-diffusion technique of Ouchterlony (1958) and immuno-electrophoresis following the method of Scheidegger (1955) were used (Schalekamp, 1963). Antisera were prepared in rabbits according to the technique of Freund et al. (1948) with total haemolysates or purified haemoglobins and with total adult chicken serum as antigens. Absorption of antisera was performed as recommended by Boyd (1956). An adequate amount of a purified haemoglobin as calculated from microtitration readings (precipitin reaction), together with an equal amount of stromal non-haem proteins was added to the antiserum; the mixture was allowed to stand for 1 h at 37 °C and then overnight at 4 °C. After centrifugation, the supernatant was absorbed once more in the same way. The antisera were concentrated by ultrafiltration before use. In order to guarantee that the antibodies were directed against haemoglobins and not against accompanying impurities, titration experiments were performed in which increasing amounts of anti-haemolysate serum were added to fixed amounts of haemoglobin. The O.D. of the supernatant of the tubes, as measured at 419 nm, showed that haemoglobin was precipitated only by anti-haemolysate serum. The precipitation lines in the immuno-electrophoresis and Ouchterlony plates were further identified as haemoglobins by a positive benzidine reaction.

RESULTS

The presence of eight qualitatively different haemoglobins (A₁, A₂, E₁, E₂, E₃, E₄, E₅, E₆) could be established by combining several methods, including electrophoresis and polypeptide chain analysis (Fig. 1). The embryonic stages at which haemoglobins appear and disappear could be ascertained accurately with immunochemical methods (Fig. 2). The relative quantities of the haemoglobins at different developmental stages were estimated from chromatographic graphs and are presented in Fig. 3 (embryonic haemolysates) and Table 1 (post-hatching haemolysates).

Electrophoretic and chromatographic characterization of adult and embryonic haemoglobins and stromal proteins

Post-hatching haemolysates (Figs. 4, 5). Two major condensations of benzidine-positive material could be easily distinguished after agar- and starch-gel
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Fig. 1. Upper part: diagrammatic representation of the electrophoretic mobilities of the haemoglobins present in haemolysates from post-hatching and embryonic chickens in pH 9.1 starch gel. Lower part: polypeptide chains of each of the haemoglobins in pH 3.2 urea starch gel. O indicates the slot.

Abbreviations: + = anodic side; capital letters = haemoglobins; greek letters = polypeptide chains; ad = haemolysate from an adult chicken; day post-h. = day post-hatching at which the haemolysate was taken; day embryo = day of incubation at which the haemolysate was taken; anti-Hb ad = antiserum prepared to total haemolysate of an adult chicken; anti-A1 spec. = antiserum reacting exclusively with A1; anti-A2 spec. = antiserum reacting exclusively with A2, E3 and E6; anti-E3, E4 spec. = antiserum reacting exclusively with E3 and E4.

electrophoresis in the haemolysates from chickens aged 1 month and older. In starch gels an additional minor benzidine-positive condensation was found, when haemolysates from chickens aged between 1 day and 1 month were investigated. On the basis of further investigations these condensations were thought to contain two major adult haemoglobins and one persistent minor embryonic haemoglobin. They were called A1, A2 and E6 in the order of their relative electrophoretic mobilities in agar which were 25, 10 and 0 respectively. It may be noted here that all chicken haemoglobins behave more alkaline than human haemoglobin A (mobility 45).

CM-cellulose and DEAE-Sephadex chromatography also revealed two major peaks in haemolysates from chickens older than 1 month. An additional minor peak was found in haemolysates from the younger chickens when using DEAE–Sephadex chromatography. CM–cellulose chromatography in our hands
was not suitable to separate this peak. When isolated the three chromatographic peaks showed a typical CO-haemoglobin absorption spectrum and an absorbance ratio (O.D. 419/280) of ±4 which means that the main constituent was haemoglobin. All haemoglobins showed a tryptophan notch at 290 nm in their absorption spectrum. Electrophoresis identified A$_1$ and A$_2$ + E$_6$ in the first and second CM-cellulose peak and E$_6$, A$_2$ and E$_1$ in the first, second and third DEAE-peak respectively. The relative amounts of the three haemoglobins as calculated from chromatographic readings at 419 nm are given in Table 1.

Besides these three haemoglobins starch-gel electropherograms occasionally displayed two more zones in which proteins were detectable. This material could be further characterized as follows.

Additional group I proteins were situated electrophoretically more anodic than A$_1$ and contained about eight distinct lines which were benzidine-negative and were therefore considered to be non-haem stromal proteins. The most cathodic portion of this protein group was inconsistently benzidine-positive; it is suspected that ageing of the haemolysate (although maximally 48 h) might somehow have been responsible for the presence of an extra haemoglobin component in these cases. In one out of the 25 adult chickens used for this

--- = Antiserum reservoir; ● = antigen well; ← = nadir of precipitation line.

![Diagram of Fig. 2](image-url)
investigation a 'rare haemoglobin' peak was consistently found to be present in amounts of about 2% in this electrophoretic zone of stromal proteins, even in freshly prepared haemolysates.

CM-cellulose chromatography revealed the group I stromal proteins in the first elution step. The non-haemoglobin nature of the larger part of this peak was demonstrated by the ratio O.D. 419/280, which was less than 1. The peak was often slightly reddish, due to withdrawal of haemoglobins with the breakthrough volume. Degraded haemoglobin pigments may appear in this peak. The 'rare haemoglobin', when present, was also eluted in the first elution step, causing a raise in the ratio O.D. 419/280 to about 2.

After chromatography on DEAE-Sephadex the group I stromal proteins
Table 1. Relative amount of the haemoglobins in blood of post-hatching chickens

<table>
<thead>
<tr>
<th>Age of chickens</th>
<th>Haemoglobins (%)*</th>
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<tr>
<td></td>
<td>A₁</td>
</tr>
<tr>
<td>1 day</td>
<td>33.2</td>
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<tr>
<td>2 weeks</td>
<td>31.7</td>
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<tr>
<td>1 month</td>
<td>28.9</td>
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<tr>
<td>4 months</td>
<td>21.1</td>
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<tr>
<td>6 months</td>
<td>22.0</td>
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<tr>
<td>1 year</td>
<td>21.7</td>
</tr>
<tr>
<td>3 years</td>
<td>19.4</td>
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</tbody>
</table>

Each number represents the mean of at least five chromatographic samples.
* Calculated from planimetric analyses of chromatographic curves measured at 419 nm.

remained on the column, while the ‘rare haemoglobin’ appears as a shoulder behind the A₁ peak containing maximally 2% of the total amount of haemoglobin.

Additional group II proteins were situated electrophoretically between A₁ and A₂, and also contained mainly non-haem stromal proteins. Catalase activity was often found in this region. A benzidine-positive component in this group was occasionally present, but only in haemolysates which also showed benzidine positivity in the region of the group I stromal proteins; i.e. in cases of suspected ageing of the haemolysate or in the case of the one out of 25 chickens with the ‘rare haemoglobin’ in group I. This indicates the presence of more ‘rare haemoglobins’ in this chicken.

In CM-cellulose chromatography the group II stromal proteins were eluted in the third step together with A₂.

DEAE-Sephadex chromatography revealed the haem-containing component of group II, when present, as a shoulder in the A₂ peak containing maximally 18% of the total amount of haemoglobin.

Embryonic haemolysates (Figs. 6, 7). Two or three major condensations of benzidine-positive material were present in these haemolysates also. Here again the mean electrophoretic mobilities were 25, 10 and 0. However, observation of the pH 9:1 starch-gel electrophoresis patterns now permitted further subdivision. With this method seven haemoglobin fractions could be distinguished. Two of these fractions had exactly the same mobilities as A₁ and A₂ and thus seemed to represent the two major adult haemoglobins. Further analysis indicated that the A₁ fraction may also contain a major embryonic haemoglobin, E₁. This E₁ haemoglobin behaved electrophoretically and chromatographically identical to A₁, but could be distinguished with the other methods used.

The A₁–E₁ fraction and the A₂ fraction were present in increasing amounts in embryonic haemolysates from the 6th incubation day onward. The other five
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Fig. 4. Electrophoretic patterns of haemolysates from post-hatching chickens in pH 8.9 starch gels.

(A) Normal adult haemolysate and haemolysate from a 1 day old chicken, both inserted at two different concentrations. Benzidine staining.

(B) Fractions isolated from total haemolysate by CM-chromatography. The non-haem stromal proteins present in the first eluting peak and the A1 and A2 fractions from the same column are shown. Amido black staining. The duration of electrophoresis was not the same for plate A and plate B. Abbreviations: see Fig. 1.
Fig. 5. Chromatographic patterns of haemolysates from post-hatching chickens.  
(A) DEAE-Sephadex chromatogram of a haemolysate from a normal adult chicken. Optical density (o.d.) at 419 nm. 
(B) CM-cellulose chromatogram of a haemolysate from a normal adult chicken. Solid line: o.d. at 419 nm.; dotted line: o.d. at 280 nm. 
(C) DEAE-Sephadex chromatogram of a haemolysate containing the group I and group II haemoglobins. o.d. at 419 nm. 
(D) DEAE-Sephadex chromatogram of a haemolysate from a 1-day-old chicken. o.d. at 419 nm.
haemoglobin fractions were all regarded as embryo-specific. The most anodic of these fractions, a minor embryonic haemoglobin, E₂, was visible as a faint smear on the anodic side of the middle benzidine positive group up to the 18th incubation day. Furthermore two major embryonic haemoglobins, E₃ and E₄, were found in this group which were situated between E₂ and A₂. There was considerable overlap between E₃, E₄ and A₂; these haemoglobins were only observed as separate bands after long electrophoretic runs. They were most easily recognized when haemolysates with a low haemoglobin concentration from the youngest embryos (2–5 incubation days) were analysed, since no A₂ is present at these stages. Both major embryonic haemoglobins were visible in decreasing amounts until the 18th incubation day with a steep fall in concentration at the 6th incubation day. In the most cathodic benzidine-positive group, well separated from A₂, two more minor embryonic haemoglobins, E₅ and E₆, were found. E₅ was present in young embryos up to the 18th incubation day. E₆ in contrast was only detected in older embryos from the 12th incubation day onward. This haemoglobin persisted until one month post-hatching.
It was not possible to obtain a satisfactory separation of the embryonic haemoglobins using CM-cellulose chromatography. In the first elution step the larger part of the stromal proteins was eluted. In the second step the haemoglobins A1, E1, E2, E3 and E4 were eluted together, while in the third elution step the haemoglobins A2, E5 and E6, sometimes contaminated with some more E3 and E4, appeared.

DEAE-Sephadex chromatography provided good separations. Seven different haemoglobin peaks were detected in which starch-gel electrophoresis identified the haemoglobins E6, E5, A2, E4, E3, E2 and A1 and (or) E1. The relative quantities of these haemoglobins, as estimated from optical density readings at 419 nm, are changing during development (Fig. 3). Obviously the major embryonic haemoglobins are E1, E3 and E4. The minor embryonic haemoglobins E2, E5 and E6 never exceed the relative amount of 10%.

In embryos incubated for 6–10 days there is another small peak between E1 and E2; moreover, in embryos incubated 4–10 days a shoulder in the foot of the E4 peak was consistently found. These haemoglobin peaks could not be evaluated as their concentration was too low.
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When using DEAES-ephadex chromatography for preparative purposes, several difficulties were encountered. The peaks A₂, E₄, E₃ and E₂ showed a great overlap. Therefore, rechromatography was considered. However, a sample applied for the second chromatographic cycle always remained on the column for more than 80%. Apparently the preparation decomposed by ageing during the dialysing period or by the lyophilization which was performed for sampling. The denaturation of the samples could be verified with the absorption spectra of these preparations in which all peaks shifted to a longer wavelength. Similar difficulties were encountered with the first eluting peaks E₅ and E₆, which occasionally showed separation artifacts, and with the haemoglobin peaks A₁ and E₁ which showed a total overlap. As rechromatography thus seems to be impossible we had to purify the haemoglobins by selecting a haemolysate of an appropriate embryonic stage, i.e. from embryos younger than 5 incubation days (E₂, E₃ and E₄), from embryos younger than 7 incubation days (E₁), from embryos younger than 10 incubation days (E₅), from young post-hatching chickens (E₆) and from adult animals (A₁ and A₂). The absorption spectra of all haemoglobin fractions, isolated in such a way, showed a tryptophan notch at 290 nm, besides the normal haemoglobin absorption peaks.

Stromal proteins were detected in the haemolysates from all embryonic stages. After starch-gel electrophoresis seven to eight fractions were observed in the electrophoretic region of the group I proteins in post-hatching haemolysates. Proteins corresponding to the group II proteins of post-hatching haemolysates, when present, were masked by the E₂, E₃, and E₄ haemoglobins, as these haemoglobins have the same electrophoretic properties.

Polypeptide chains

Adult and embryonic haemoglobin (Figs. 8, 9). Three clearly distinguishable groups of chains became visible when total haemolysates from adult chickens were analysed with the urea pH 3.2 starch-gel method. A fourth group was found in total haemolysates from 8-day embryos, a haemolysate containing 6 out of the 8 haemoglobins found in embryonic haemolysates. The groups of chains were named α, β, γ and δ chains, according to their decreasing electrophoretic mobility from the anode. Furthermore, they received in their index the name of the haemoglobins in which they were found. The nomenclature used in this study does not imply any similarity with human globin chains. In pH 1.9 starch gels only three groups of chains were visible, corresponding to α, β + δ and γ respectively. With both methods the groups showed a subdivision. When isolated, each haemoglobin separated into two zones which was best demonstrated in the urea pH 3.2 gels. The colour intensity of the two zones, as stained with amido black, was not identical. This may have been due to a difference in affinity for the dye or to a difference in solubility of these chains in acid media rather than to a difference in molecular weight. Benzidine negativity indicated haem release in both chains even when total haemoglobin was applied.
Fig. 8. Electrophoretic patterns in urea pH 3.2 starch gel of an isolated $E_6$, $A_1$, $A_2$ fraction and of a total haemolysate from a normal adult chicken. The two $\beta$ chains show a minor difference in electrophoretic mobility and are therefore not separately visible when total haemolysate is analysed. Amido black positive zones, anodic of the $\alpha$, $\beta$ and $\gamma$ chains are sometimes visible. As these are variable in electrophoretic position and colour intensity, they were thought to be due to degradation products. Abbreviations: see Fig. 1.
Multiple haemoglobins of the chicken

Fig. 9. (A) Electrophoretic patterns in urea pH 3-2 starch gels of the polypeptide chains of total haemolysates from embryos incubated for 8 days and from adult chickens, compared with haemoglobin fractions obtained by DEAE-Sephadex chromatography of haemolysate from embryos incubated for 5 days. These fractions contain the haemoglobins E₂-E₄, E₃ and E₄ respectively.

(B) Haemoglobin fractions of another haemolysate from embryos incubated for 5 days. These fractions contain the haemoglobins E₂, E₄ and E₅. The duration of electrophoresis was not the same for plates (A) and (B). Abbreviations: see Fig. 1.

On the base of comparison of the two chains of each isolated haemoglobin with the 'total' chain pattern the presence of at least seven distinct chains was assumed. This conclusion was based mainly on the pattern in urea pH 3-2 starch gels. In pH 1-9 starch gels, seven zones could be found also. However, although all the chains showed up as circumscribed lines in urea pH 3-2 gels, one of the chains formed a smear, interfering with other chains in pH 1-9 gels. Moreover, in pH 1-9 gels the distance between some chains was small in comparison to the distances obtained in urea pH 3-2 gels. The chains belonging to one of the above-mentioned three groups showed only small differences in electrophoretic mobility. Nevertheless such small differences may be caused by a difference in primary structure as has been shown by fingerprint analyses of β₂A₁E₆ and β₂A₂E₁E₃E₄ (Muller, 1961; Saha, 1964; Moss & Thompson, 1969). Additional evidence for this view was obtained with immunochemical techniques: preliminary studies with an antiserum to a purified β₂A₂E₁E₃E₄ chain revealed that this antiserum showed specificity when the reactions with β₂A₂E₁E₃E₄ and β₂A₁E₆ preparations were compared in immuno-electrophoretic analyses.
Fig. 9A illustrates that it was possible to decide about the chain pattern of each haemoglobin, even when the haemoglobin fractions were not quite pure. For example, comparison of the E₃ and E₄ fraction indicates that E₄ contains a γ chain, whilst E₃ contains a δ chain. The corresponding faint line of a γ chain in the E₃ preparation and of a δ chain in the E₄ preparation is most likely due to contamination of the E₃ preparation with E₄ and vice versa (see also Fig. 7 A), in which case the most intensely stained chain of E₃ or E₄ will show up in the preparations of E₄ and E₃ respectively. The only remaining chain for E₄ as well as for E₃ may be identified as a β chain. In the same way the pattern of chains in E₂ may be deduced by subtraction of the E₃ chains from the pattern found for the E₂ fraction. That the γ chain present in E₂ is not identical to the γ chain found in E₄ could be demonstrated in gels in which the E₄ and the E₂ samples were run next to each other (Fig. 9 B). The sequence of the globin chains as found in urea pH 3-2 gels was from cathode to anode: α₂E₅E₆-β₂E₄; β₁E₅, β₂E₁E₃E₄-γ₁E₁E₂; γ₂E₄-δ₁E₃. In pH 1-9 gels all the β chains and the δ₁E₃ and the γ₂E₄ chain were grouped together in the fast migrating fraction. The γ₁A₁E₂ and the α₂E₅E₆ chain were situated more anodically as clearly separate spots. It may be concluded that the following chain combinations are present in the respective haemoglobins (see also Fig. 1):

**Adult haemoglobins:**

- A₁ = β₁A₁E₆, γ₁A₁E₁E₂ (major Hb),
- A₂ = α₂A₂E₅E₆, β₂A₂E₁E₃E₄ (major Hb).

**Embryonic haemoglobins:**

- E₁ = β₂A₂E₁E₃E₄, γ₁A₁E₁E₂ (major Hb),
- E₂ = β₁E₂E₅, γ₁A₁E₁E₂ (minor Hb),
- E₃ = β₁A₂E₁E₃E₄, δ₁E₃ (major Hb),
- E₄ = β₂A₂E₁E₃E₄, γ₂E₄ (minor Hb),
- E₅ = α₂A₂E₅E₆, β₂E₂E₅ (minor Hb),
- E₆ = α₂A₂E₅E₆, β₁A₁E₆ (minor Hb).

The chains β₂E₂E₅, δ₁E₃ and γ₂E₄ are exclusively embryonic, and the haemoglobins E₂, E₃, E₄ and E₅ may therefore be considered as embryo-specific. The haemoglobins E₁ and E₆ on the other hand appear to be composed of chains also available in the adult haemoglobins A₁ and A₂. These haemoglobins may be considered as hybrid forms.

The haem-containing components of group I and group II, and the slightly asymmetric foot of A₁ and A₂, when isolated by chromatography, showed minor variations in chain composition when compared to A₁ and A₂ (top) respectively. The most cathodic chain of A₁ (β₁A₁E₆) shifted slightly more to the anode, while the most anodic chain of A₂ (β₂A₂E₁E₃E₄) was slightly more cathodic in these samples. This means that these ‘haemoglobins’ are of a quite other nature than the embryonic haemoglobins which were found in the same electrophoretic zone (E₂, E₃ and E₄).

Surprisingly it was not possible to separate monomeric chains with the
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PCMB method of Bucci & Fronticelli (1965). Both haemoglobins A₁ and A₂ formed one dimeric zone, different in electrophoretic mobility from each other and from the untreated haemoglobins. Embryonic haemoglobins were not submitted to this method. The inability to form monomeric chains with PCMB was also reported by Rosemeyer & Huehns (1967) for human foetal haemoglobin and for the haemoglobins of rabbit, horse and pig, and may be due to a different location of the sulphhydryl groups in these haemoglobins when compared to human haemoglobin A.

Immunochemical characterization of adult and embryonic haemoglobins and their polypeptide chains

Post-hatching haemolysates (Fig. 10). A total haemolysate of adult chicken erythrocytes formed one main precipitation line when it was allowed to react with anti-total-adult haemolysate (anti-Hbₐd) serum in immuno-electrophoresis. When a suitable haemoglobin concentration was used, this line showed two nadirs. The electrophoretic position of these nadirs corresponded with the electrophoretic position of the sites of the highest concentration of the major adult haemoglobins A₁ and A₂. Proof that the line actually belonged to both haemoglobins and was not just the extending precipitate of one of them could be obtained by the reaction of anti-Hbₐd with chromatographically purified A₁ and A₂.

Using the Ouchterlony technique a fusion of the main part of the precipitation lines of isolated A₁ and A₂ also occurred. With this method, however, it was clear that a tiny part of the precipitates did not fuse (Fig. 10C). The formation of spurs indicated a reaction of 'partial identity' between both haemoglobins, each spur representing specificity for one or more antigenic determinants. Closer observation of the immuno-electrophoretic patterns revealed similar spurs in the zone between A₁ and A₂, but with this technique the spurs are parallel to the main precipitation line and tend to conflate with it. Especially the A₂ specific spur is only visible within a narrow range of antigen–antibody concentration ratios.

To support the immunochemical specificity of A₁ and A₂, absorption experiments were performed. Anti-Hbₐd serum was mixed with an adequate amount of A₂ and of stromal proteins, in order to remove the antibodies directed against A₂ specifically, against the common antigenic determinating groups of A₁ and A₂, and against the stromal proteins of haemolysate. The so absorbed (anti-A₁ spec.) serum reacted only with A₁ (Fig. 10A, B), thus proving the individual immunochemical specificity of A₁.

In a similar way antisera specific for A₂ could be prepared (Fig. 10A, B). This (anti-A₂ spec.) serum did not react with A₁. However, it reacted with the embryonic haemoglobin (E₆), which was found during the early post-hatching period. Absorption of the anti-A₂ spec. serum with E₆ removed all antibodies against A₂ and E₆, indicating a closer immunochemical relationship between
these two haemoglobins than between A_2 and A_1. This was also suggested by the chain composition of these haemoglobins.

The stromal proteins of group I and group II showed about seven precipitation lines when reacted with anti-Hb_ad (Fig. 10A). Some of these lines had catalase activity. None was benzidine-positive, or could be developed with either anti-A_1 spec. or anti-A_2 spec. serum (Fig. 10B). Anti-chicken serum did not develop these precipitation lines, excluding the possibility of contaminating serum proteins.

In the ‘rare haemoglobins’ of group I and group II, only haemoglobin determinants were demonstrable which were common to A_1 and A_2.

Embryonic haemolysates (Fig. 11). All embryonic haemolysates (2–21 incubation days) showed precipitation lines when they were allowed to react with anti-Hb_ad serum. Haemolysates from the youngest stages (2 incubation days) showed three precipitation lines corresponding to E_3, E_4 and E_5 (Fig. 11A). With haemolysates from older embryos these lines became confluent in one common precipitate in which E_1, E_2, A_1, A_2 and E_6 participated (Fig. 11B, C). These results indicate an immunochemical relationship between the adult and embryonic haemoglobins on the base of one or more common antigenic determinant groups. The presence of more than one line in the haemolysates from the youngest embryonic stages may be due to the presence of more than one determinant group, to which different antibodies are directed.

**Figure 10**
Immunoochemical studies.

(A) Immuno-electrophoresis. Total haemolysate at two different concentrations from a normal adult chicken (circular wells) reacting with (1) antiserum to total adult haemolysate (central longitudinal reservoir), (2) the same antiserum adequately absorbed with an isolated A_1 fraction and stromal proteins (upper longitudinal reservoir), (3) the same antiserum, here adequately absorbed with an isolated A_2 fraction, but insufficiently absorbed with stromal proteins (lower longitudinal reservoir). Photographic representation of an unstained strip.

(B) The same immuno-strip stained with benzidine reagent, no stromal proteins are visible now. The horizontal precipitation line (⊥) which extends the common precipitation line represents a reaction of double diffusion between the excess A_2, added during the absorption of the anti-A_1 spec. serum (lower longitudinal reservoir) and anti-Hb_ad (central longitudinal reservoir). Similar horizontal precipitation lines are formed with anti-A_2 spec. serum.

(C) Ouchterlony plate. Isolated A_1 (lower right well) and A_2 (upper well) reacting with anti-Hb_ad (lower left well). The spurs indicate individual immunochemical specificity of A_1 and A_2. The confluent precipitates indicate antigenic determinants common to A_1 and A_2.

(D) Ouchterlony plate. The polypeptide chains γ A_1E_1E_2 (upper left well) and β A_1E_6 (upper right well), cut out of urea pH 3·2 starch gel and inserted without further treatment, an isolated A_1 (lower left well) and A_2 (lower right well) reacting with anti-Hb_ad (central well). The precipitation lines in this plate are more diffuse than in (C), which is most probably due to the interference of urea.

Abbreviations: see Fig. 1.
Figure 11
Fig. 11. Immuno-electrophoretic patterns of haemolysates from various embryonic stages and of a haemolysate from an adult chicken (round wells), reacting with several antisera (longitudinal reservoirs). Photographs of uncoloured strips. The same strips were stained with benzidine afterwards; the precipitation lines not reacting with benzidine are stromal proteins. Abbreviations: see Fig. 1.
Fig. 12. Ouchterlony plate. The polypeptide chains $\gamma E_4$ and $\delta E_3$, cut out of urea pH 3-2 starch gel and inserted without further treatment, show a faint reaction with anti-Hb$_{ad}$, indicating antigenic determinants common to these chains and the chains of adult haemoglobins. Abbreviations: see Fig. 1.

Antisera which are specific for embryonic haemoglobins were difficult to obtain. This may be due to the presence of antigenic determinants which are common to both adult and embryonic haemoglobins and which have a relative high potency to induce antibody production. We were only successful in the preparation of some antisera containing antibodies specific for $E_3$ and $E_4$. Absorption of these antisera with total adult haemolysate left into solution only these specific antibodies (anti-$E_3$, $E_4$ spec. serum). With these antisera it could be determined that $E_3$ and $E_4$ have disappeared on the 18th incubation day (Fig. 11D, E).

Anti-$A_1$ spec. serum only reacted with $A_1$ as could be found in tests in which isolated embryonic haemoglobins were used. With this antiserum it was possible to demonstrate $A_1$ to appear on the 12th incubation day (Fig. 11A, B, C) in spite of a haemoglobin fraction with virtually the same electrophoretic and chromatographic properties which is present from the 6th incubation day onward (Fig. 3). This fraction which does not react with anti-$A_1$ spec. is considered to be an additional embryonic haemoglobin and has been designated $E_1$ in view of its electrophoretic position. Anti-$A_2$ spec. serum appeared to react with $A_2$ but also with $E_5$ and $E_6$. Due to the difference in electrophoretic mobility of $A_2$ as compared to $E_5$ and $E_6$ (mobilities 10 and 0 respectively) it was possible to determine with this antiserum the time of appearance of $A_2$ as early as the 6th incubation day. The shift of the nadir of the precipitation line was used as a criterion in these studies (Fig. 11 A, B). The results obtained with specific anti-sera are summarized in Fig. 2.
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Polypeptide chains (Figs. 10D, 12). Polypeptide chains cut from the urea pH 3.2 gels were analysed in Ouchterlony plates using anti-Hb ad. The results are of a preliminary nature, as only a very limited number of analyses could be carried out. A difficulty encountered in these studies was the interference of urea with the precipitation reaction. The polypeptide chains $\alpha A_2 E_5 E_6$, $\beta A_2 E_1 E_3 E_4$, $\beta A_1 E_6$ and $\gamma A_1 E_2 E_2$ from adult haemoglobins as well as the chains $\beta E_2 E_9$, $\gamma E_4$ and $\delta E_3$ from embryonic haemoglobins produced a precipitation line with anti-Hb ad, indicating that all chains possess a considerable part of the common antigenic haemoglobin determinants (Fig. 10D). It must be emphasized that the reaction of the embryonic chains (Fig. 12) was poorer than the reaction of the adult chains. No specific determinants could be demonstrated with anti-A$_4$ spec., anti-A$_2$ spec. or anti-E$_3$, E$_4$ spec. serum. This may be due to the fact that the specific antigenic determinants which produce the weakest lines have disappeared from the patterns because urea is present, which is known to inhibit precipitation reactions. On the other hand, it is also possible that some determining sites do not belong to a single chain, but are only present in stereometric complete haemoglobin molecules as has been reported by Ovary (1964), who prepared polypeptide chains from human haemoglobin A by counter-current distribution and found poor reactions of these chains with anti-total haemoglobin in comparison with the native haemoglobin molecule.

DISCUSSION

Technical and general considerations

Inventarization and characterization of the haemoglobins during ontogenesis

Our studies indicate a sequential synthesis of haemoglobins during the ontogenesis of the chick (Fig. 3). Three periods of haemoglobin synthesis with transitions at the 6th and 12th day of incubation could be recognized. In the earliest embryos investigated (2–5 incubation days) four haemoglobins were found which are all different from the adult haemoglobins. Two of these, E$_3$ and E$_4$, are present in relatively large quantities and are considered to be major embryonic haemoglobins. Their electrophoretic and chromatographic properties are much like those of A$_2$, but their globin chain composition is markedly different (E$_3$: $\beta A_2 E_1 E_3 E_4$, $\delta E_3$; E$_4$: $\beta A_2 E_1 E_3 E_4$, $\gamma E_4$; A$_2$: $\alpha A_2 E_5 E_6$, $\beta A_2 E_1 E_3 E_4$). Their immunochemical properties are also different from A$_2$, as could be demonstrated by two specific antisera, anti-A$_2$ spec. and anti-E$_3$, E$_4$ spec. The globin chains $\delta E_3$ and $\gamma E_4$ are embryo-specific. Two minor embryonic haemoglobins, E$_2$ and E$_5$, could be distinguished from E$_3$ and E$_4$ and from A$_1$ and A$_2$ by electrophoresis and chromatography as well as by their globin-chain composition (E$_2$: $\beta E_2 E_5$, $\gamma A_1 E_1 E_2$; E$_5$: $\alpha A_2 E_5 E_6$, $\beta E_2 E_5$). $\beta E_2 E_5$ represents an embryo-specific chain. Immunochemical specificity of these embryonic haemoglobins has not been demonstrated.

The second period of haemoglobin synthesis appears to start at the 6th
incubation day. At this time the haemoglobins A₂ and E₁ appear and increase rapidly (Fig. 3) to become the predominant haemoglobins of this period. Haemoglobin A₂ is the normal major adult haemoglobin, consisting of the chains \( \alpha A₂E₅E₆ \) and \( \beta A₂E₁E₃E₄ \). Haemoglobin E₁ is considered to be a major embryonic haemoglobin which could not be distinguished from A₁ in electrophoretic and chromatographic studies. Its immunochemical properties, however, are different from those of A₁, as could be demonstrated with specific anti-A₁ serum. Furthermore, its \( \beta \)-chain is electrophoretically somewhat different from the \( \beta \)-chain of A₁ (E₁: \( \beta A₂E₁E₃E₄ \), \( \gamma A₁E₁E₂ \); A₁: \( \beta A₁E₆ \), \( \gamma A₁E₁E₂ \)). In the same period a rapid decrease of the major embryonic haemoglobins of the first period, E₃ and E₄, occurs. The haemoglobins E₂ and E₅ remain present in about equal relative amounts.

The third developmental period appears to start around the 12th incubation day, when replacement of E₁ by A₁ and of E₅ by E₆ occurs. E₆ is a minor embryonic haemoglobin, slightly different from E₅ in electrophoretic and chromatographic properties. Furthermore, its \( \beta \)-chain is different from that of E₅ (E₆: \( \alpha A₂E₅E₆ \), \( \beta A₁E₆ \); E₅: \( \alpha A₂E₅E₆ \), \( \beta E₂E₅ \)). Immunochemical specificity of this embryonic haemoglobin has not been detected. During this period haemoglobin E₂ disappears.

After hatching the embryonic haemoglobin E₆ disappears during the first month. The time of disappearance of E₁ could not be determined exactly since the electrophoretic and chromatographic properties of E₁ are very similar to those of A₁. On the other hand, the use of anti-A₁ spec. serum enabled us to pinpoint the time of appearance of A₁ rather precisely. A further shift in the amount of A₁ and A₂ takes place in the post-hatching period.

The post-hatching haemoglobins

The presence of two major adult haemoglobins in the haemolysate of post-hatching chickens has been reported by almost all authors working on the subject (Godet, 1970, review). However, some of them emphasize the presence of additional minor haemoglobins (D’Amelio & Salvo, 1959, 1961; Buschmann, 1963; Matsuda & Takei, 1963; Schall & Turba, 1963; Alekseenko & Orekhovich, 1964; Marchis-Mouren & Lipman, 1965; D’Amelio, 1966; Hashimoto & Wilt, 1966; Simons, 1966; Godet, 1967; Schürch et al. 1968; Godet et al. 1970).

At least four fundamentally different explanations for the finding of these extra haemoglobin fractions may be considered.

(1) Separation artifacts resulting from the procedure by which the haemolysate was prepared. Freezing and thawing of the blood cells, ageing of the haemolysate, oxidation of the haemoglobins to methaemoglobin with or without the use of a ligand like cyanide have been described to induce hybrids, aggregation products and conformationally changed or degraded haemoglobin products (Manwell et al. 1963, 1966; Matsuda & Takei, 1963; Chernoff &
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Pettit, 1964b; Hammel & Bessman, 1965; Riggs, Sullivan & Agee, 1964). Moreover, the technique which is used for separating the haemoglobins may cause artificial components. Column chromatography especially is notorious in this respect, because initial pH anomalies (Huisman, Martis & Dozy, 1958; Felland & Snyder, 1968) as well as overloading of the column and withdrawal of haemoglobin with the break-through volume (Bargellesi, Callegarini & Conconi, 1969) have been described to cause artificial peaks.

(2) Separations resulting from minor heterogeneity. As discussed by Huisman (1969), the extra haemoglobins are thought to be derivates of the major haemoglobins or of their precursors. Since the primary structure is not different from those of the major haemoglobins, these derived haemoglobins may be determined by the same genetic mechanism. In humans, derived haemoglobins are shown to be already present in the circulating blood cells, especially in case of severe anaemia (Horton & Huisman, 1965). Ageing of the haemolysate, however, is known to cause an increase in the number and concentration of these derived haemoglobins which suggests that metabolic activity plays a role. For human haemoglobins, blockade of the N-terminals of one or more of the polypeptide chains is described as a cause (Holmquist & Schroeder, 1964, 1966a, b). Especially in case of an acetyl loaded N-terminal, as found in human haemoglobin F₁ (Schroeder, Cua, Matsuda & Fenninger, 1962) and also in chicken haemoglobins (Satake, Sasakawa & Maruyama, 1963; Matsuda, Maita & Nakajima, 1964; Marchis-Mouren & Lipman, 1965), loading with more complex structures seems possible (Huehns & Shooter, 1966). Complex formation of β-chain SH groups with glutathione residues, as has been found in the case of human haemoglobin A₁₀ (Huisman & Dozy, 1962; Huisman, Dozy, Horton & Nechtman, 1965), provides another possibility for chain loading.

(3) Separations resulting from genetic heterogeneity of the animals from which the blood was taken. The incidence of genetic polymorphism in avian haemoglobins seems to be rare. The avian haemoglobins are regarded as relatively conservative on the species level (Brush & Power, 1970; Saha, 1964). Only Washburn (1968a) reported two allelic co-dominant chicken types: type I possessed 2 haemoglobins comparable to our A₁ and A₂, type II showed 3 haemoglobins, one comparable to our A₂, a different ‘A₁’ and an additional haemoglobin in the region of our group II stromal proteins. This author was able to breed the heterozygote which had all these haemoglobins.

(4) Separations resulting from maturation heterogeneity. The use of chickens of different ages may introduce also extra haemoglobin fractions. Young chickens are shown to contain a minor haemoglobin, more cathodic than the adult haemoglobin A₂ (Huisman & Schillhorn van Veen, 1964; Washburn, 1968b). Following severe anaemia by repeated bleeding or by treatment with phenylhydrazine, embryonic haemoglobins may reappear in the haemolysate of older chickens (Stino & Washburn, 1970). Similarly, haemoglobin F is found in humans under several pathological conditions (Huisman, 1969).
Taking into account the above-mentioned arguments, we conclude that the minor haemoglobins which have occasionally been found by several authors and by ourselves in the region of our group I and group II stromal proteins are due to artificial separations or minor heterogeneity. The varying number and amount of these extra haemoglobins also point strongly in this direction. The haemoglobins found on the base of artificial and minor heterogeneity are believed to possess an identical primary structure as the haemoglobins A₁ and A₂, from which they are derived. They seem therefore to be irrelevant from genetic point of view. Our chicken with 'rare' haemoglobins might have been genetic polymorph, but we have not carried out breeding experiments.

Three groups of authors characterized the extra haemoglobins in chicken haemolysates more extensively. Alekseenko & Orekhovich (1964) found after freezing and thawing of their haemolysates an extra haemoglobin in the region of our group II stromal proteins. They performed amino acid analyses and found small differences between all chains. However, they mention on the base of the presence of six disulphide bonds, the possibility of glutathione binding in this haemoglobin. Unfortunately, up to now they were unable to identify glutathione in their preparations. Nevertheless we consider their haemoglobin to be most probably a derivate of the major haemoglobin A₂. Hashimoto & Wilt (1966) reported three haemoglobins in the electrophoretic zone of our component I. They performed polypeptide chain analyses and found one polypeptide chain of these haemoglobins to have a comparable electrophoretic mobility as our γ A₁E₁E₂ chain, whereas the other polypeptide chains are only slightly different from our β A₁E₆ chain. Therefore we consider these haemoglobins to be derived from haemoglobin A₁. D’Amelio (1966) found an extra haemoglobin in the region of our group II stromal proteins. This haemoglobin was reported to contain the chains of A₁ as well as of A₂. This may point to an aggregation product in their preparations.

In conclusion it may be said that until now no absolute evidence has been adduced in the literature for the existence of extra haemoglobins which are different in primary structure from the two major haemoglobins, and which are situated in the zone of our group I and group II stromal proteins. On the other hand, the major haemoglobins (A₁ and A₂) which have been found in previous studies and by us are in our opinion different in primary structure. This view is supported by the following observations: (1) the haemoglobins A₁ and A₂ are consistently present in freshly prepared haemolysates; (2) the electrophoretic and chromatographic properties of these haemoglobins are markedly different; (3) these haemoglobins have different antigenic properties; (4) the electrophoretic properties of the polypeptide chains γ A₁E₁E₂ and α A₂E₅E₆ are also markedly different.

The minor haemoglobin (E₆) which is found in the haemolysates of young chickens is considered to be due to maturation heterogeneity and is also thought to be different in primary structure from A₁ and A₂ on the base of their electro-
phoretic and chromatographic properties and their polypeptide composition. This haemoglobin, however, might be a natural hybrid of $A_1$ and $A_2$.

The embryonic haemoglobins

Our results on the molecular forms of haemoglobins in the chick at different stages of development confirm and extend the results of the authors who agree about the existence of embryonic haemoglobins and are in contrast with those authors who report only quantitative changes in haemoglobin composition during the ontogenesis. (It seems of interest to compare our data in greater detail with these pertinent previous studies.)

D'Amelio & Salvo (1959, 1961) investigated the haemoglobins of chicken embryos with the Ouchterlony technique and immuno-electrophoresis, but did not report the presence of embryo-specific antigenic determinants. After agar-electrophoresis they observed two embryonic haemoglobins or groups of haemoglobins, in the electrophoretic region of our $E_2 - E_3 - E_4$ and $E_5 - E_6$ respectively. In a later study D'Amelio (1966) reported on the presence of one embryo-specific polypeptide chain on the base of pH 1-9 starch-gel electrophoresis of haemolysates from 3 to 7 days incubated embryos. Using starch-gel electrophoresis at pH 8-6 Hashimoto & Wilt (1966) compared the fractions of haemolysates from 5 days incubated embryos with those from adult animals and described the presence of three embryonic haemoglobins which are comparable to our $E_3$, $E_4$ and $E_5$. A more extensive study has been carried out by Manwell et al. (1966) by means of starch-gel electrophoresis at pH 8-5. Their electrophoretic results are in agreement with those of Hashimoto & Wilt (1966). Both groups of authors isolated their embryonic haemoglobins from the pH 8-5 starch gels and separated the polypeptide chains in pH 1-9 starch gels. As the resolving power of pH 8-5 starch-gel electrophoresis for chicken haemoglobins is low, in comparison with DEAE-Sephadex chromatography, the peptide patterns which were obtained by both groups of authors are somewhat difficult to interpret, the more so because the patterns in pH 1-9 starch gels are less distinct than in the urea pH 3-2 starch gels used in our study. These difficulties may explain the conflicting conclusions of Hashimoto & Wilt (1966) and of Manwell et al. (1966) concerning the polypeptide chain patterns of embryonic haemoglobins. Yet these authors also conclude to the presence of three embryo-specific chains. They further report a switch-over in the synthesis of haemoglobins at the end of the 5th incubation day, when both adult haemoglobins are thought to appear. However, the fraction which was considered by these workers to be $A_1$ appeared to be different from $A_1$ in our study both in polypeptide-chain composition and immunochemically.

Qualitative changes in the haemoglobin patterns during ontogenesis have also been claimed by Schürch et al. (1968). Their poly-acrylamide-gel electrophoresis patterns indicate the presence of at least one embryonic haemoglobin. Denmark & Washburn (1969) analysed haemolysates from 4 to 21 days incubated
embryos by means of cellulose-acetate electrophoresis. Their patterns are very similar to our starch-gel electrophoresis patterns. The interpretation by these authors, however, is quite different. They report the presence of only two embryonic haemoglobins which correspond to our E₂ and (or) E₃, and E₅ and (or) E₆, respectively. Furthermore, they observed in embryonic haemolysates two fractions called M and m which they thought to represent the adult haemoglobins A₂ and A₁, but which in the present study turned out to be E₄ and (or) A₂, and E₁ and (or) A₁ respectively. Therefore, we do not agree with the conclusions of Denmark & Washburn that A₂ and A₁ are present from the 3rd and 6th day of incubation respectively.

No equivocal evidence for the presence of embryonic haemoglobins was obtained by Fraser (1961, 1964, 1966) who used electrophoresis on paper and cellulose-acetate, chromatography on CM-cellulose and polypeptide chain analyses and by Wilt (1962) who carried out Ouchterlony’s method and immunoelectrophoresis with an anti-total adult haemolysate serum. More recently, Simons (1966) was also unable to detect qualitative changes in CM-cellulose chromatographic patterns of embryonic haemolysates. Applying the elution scheme of this author (potassium phosphate, pH 6-8, in a linear gradient from 0-004 to 0-080M) we found no clear separation between the major embryonic haemoglobins E₁, E₃ and E₄ and the adult haemoglobin A₁. The haemolysates of embryos incubated for 7 days as studied by Simons (1966) showed two chromatographic peaks, I and II; peak I contained our E₁, E₃ and E₄, peak II contained A₂. Haemolysates from older embryos also showed two peaks which appeared at about the same elution volume as peak I and peak II of the haemolysates from embryos incubated for 7 days. However, from the 14th incubation day onward, A₁ was the main constituent of peak I. Thus, the lack of evidence for the occurrence of qualitative changes in the analyses of Simons (1966) may be explained by the fact that the fractions which he obtained with CM-cellulose chromatography are far from pure.

Genetic and morphological considerations

Evidence from studies on human haemoglobins indicates that each polypeptide chain is coded by at least one gene (Ingram, 1963). On the base of this view the following tentative conclusions on genetic control of haemoglobin synthesis in the chicken embryo may be put forward.

During the first period of haemopoiesis (3–5 incubation days), most of the structural haemoglobin genes involved in haemoglobin synthesis are already active. This conclusion is based on the observation that six of the seven chains found in this study are present at these stages. Quantitative data indicate that the structural genes for βA₂E₁E₃E₄, δE₃ and γE₄ transcribe at a high rate, and that the genes coding for βE₅E₆, αA₂E₅E₆ and γA₁E₁E₂ are only permitted to transcribe at a very low rate.

The second period (6–12 incubation days) starts with two events: a repression
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of the activity of the genes coding for the major embryo-specific chains \( \delta E_3 \) and \( \gamma E_4 \), and an activation (or derepression) of the genes for the adult chains \( \alpha A_2E_5E_6 \) and \( \gamma A_1E_1E_2 \). The high activity of the \( \beta A_2E_1E_3E_4 \) gene seems to be unaffected. When the chains produced at the highest rate combine, they form the new haemoglobins \( A_2 (\alpha A_2E_5E_6, \beta A_2E_1E_3E_4) \) and \( E_1 (\beta A_2E_1E_3E_4, \gamma A_1E_1E_2) \) which appear during this period. The major embryonic haemoglobins disappear, because the \( \delta E_3 \) and \( \gamma E_4 \) genes become inactivated. The \( \beta E_2E_5 \) gene seems unaffected, resulting in the persistence of the haemoglobins \( E_2 (\beta E_2E_5E_6, \gamma A_1E_1E_2) \) and \( E_5 (\alpha A_2E_5E_6, \beta E_2E_5) \).

The third period (12–21 incubation days) starts with the activation of a new gene producing the chain \( \beta A_1E_6 \). This chain was not observed before, but may have been already present in one or both of the haemoglobins which were observed in the foot of the \( A_2 \) peak and between the peaks of \( E_1 \) and \( E_2 \) after DEAE-Sephadex chromatography. These haemoglobins were not studied in detail. The \( \beta A_1E_6 \) chain apparently combines with the chains \( \alpha A_2E_5E_6 \) and \( \gamma A_1E_1E_2 \) in unequal amounts to form the newly appearing haemoglobins of this period, \( E_6 \) and \( A_1 \) respectively. At the same time the gene coding for the embryonic chain \( \beta E_2E_5 \) is switched off, resulting in the disappearance of the haemoglobins \( E_2 \) and \( E_5 \). At the time of hatching four haemoglobins are present again in the circulating blood cells of the chick: the two major adult haemoglobins \( A_1 (\beta A_1E_6, \gamma A_1E_1E_2) \) and \( A_2 (\alpha A_2E_5E_6, \beta A_2E_1E_3E_4) \) and the two minor embryonic haemoglobins \( E_1 (\beta A_2E_1E_3E_4, \gamma A_1E_1E_2) \) and \( E_6 (\beta A_2E_5E_6, \beta A_1E_6) \). These haemoglobins are combinations composed of only four different chains and may be considered as hybrid forms.

The demonstration of three clearly marked periods of haemoglobin synthesis makes the hypothesis of Hall (1934) that the different molecular forms of haemoglobin might be synthesized in different haemopoietic organs and the suggestion of Craig & Russell (1964) that these forms might be packaged in morphologically distinguishable erythrocytes most interesting. In Fig. 13 the cell strains and haemopoietic organs involved at different stages of development are represented. This figure is based upon data summarized by Romanoff (1960). The correlations between these data and our chemical findings are striking. The sudden decrease in the relative amount of the major embryonic haemoglobins \( E_3 \) and \( E_4 \) (Fig. 3) occurs when the relative number of primitive erythrocytes shows a steep fall.

The occurrence of the first erythrocytes of the definitive lines seems to correlate with the sudden appearance in the haemolysates of \( E_1 \) and \( A_2 \). Lucas & Jamroz (1961) point out the existence of several generations of definitive erythrocytes. The last definitive erythrocyte generation is thought not to appear before the end of the second incubation week, the time at which we observed the beginning of the third period in which \( E_1 \) and \( E_6 \) are replaced by \( A_1 \) and \( E_6 \) respectively. Similar correlations exist with the periods in which different haemopoietic organs are active. During the first period the yolk sac is the only
(extra-embryonic) haemopoietic organ. The second period is characterized by the onset of transient erythropoiesis in several intra-embryonic organs, e.g. liver and spleen. The third period starts when the bone marrow initiates definitive haemopoiesis. These correlations suggest a relation between the morphological and chemical differentiation of the haemopoietic system of the chicken, but additional data are needed. Especially the question whether specific haemoglobins are present in special erythrocytes and made in special haemopoietic organs should be answered. Immuno-fluorescence studies, aimed at detecting specific haemoglobins in erythrocytes from different tissue sources, will contribute to the solution of the problem under consideration.

After this discussion on the differences between the haemoglobins found, it seems justified to emphasize again our genetically interesting finding of a striking relationship between these haemoglobins. The possibility that the adult haemoglobins $A_1$ and $A_2$ and the embryonic haemoglobin $E_1$–$E_6$ might genetically be derived from one monomeric polypeptide-chain ancestor is supported by our observation that these haemoglobins have many antigenic determinants in common. This similarity in immunochemical behaviour was not due to the presence of common $\alpha$, $\beta$, $\gamma$, or $\delta$ polypeptide chains, but rather to smaller common polypeptide formations which are present in all these chains.
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