The haemoglobins of developing duck embryos

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SUMMARY

Three haemoglobins were isolated by ion-exchange chromatography from the haemolysates of embryonic duck erythrocytes up to 8 days of development. The component globins were characterized both by electrophoresis in dissociating conditions and by finger-printing analysis. The major haemoglobin fraction E1 appears to be an embryonic tetramer since its constituent globins are different from all the others synthesized during embryonic and adult life. The two minor fractions E2 and E3 show α-type subunits that are very similar to those of the two adult haemoglobins A1 and A2 respectively. They are present all through embryonic life, as demonstrated by chromatographic analysis. For these reasons they have been considered foetal.

The two haemoglobins typical of the adult animal are found in the red cells of the embryo from 8 days of incubation. Their relative amounts change continuously during embryonic development and reach the adult value after hatching.

INTRODUCTION

Erythroid cells have been considered very helpful models in investigating mechanisms of differentiation. The main products of their biosynthetic activities are haemoglobin molecules which can be easily isolated and characterized. The correlation existing between haemoglobin types and red cell differentiation has been widely demonstrated (Ingram, 1963; Manwell, Baker & Betz, 1966; Paul, 1976). Haemoglobin types are also closely related to embryonic developmental stages (Kitchen & Brett, 1974; Bruns & Ingram, 1973). It is important to obtain further evidence characterizing the haemoglobins synthesized by the embryonic red cells at various stages of development.

Birds are suitable biological models because their embryos are completely independent of the mother and a large number of embryos at the same developmental stage can be obtained easily. Chick embryos are the most widely investigated (Manwell et al. 1966; Schalekamp, Schalekamp, Van Goor & Slingerland, 1972; Bruns & Ingram, 1973; Brown & Ingram, 1974; Cirotto, Scotto di Tella & Geraci, 1975; Schalekamp, Van Goor, Slingerland & Van Noort, 1976; Cirotto & Geraci, 1977). Most authors agree that the red cells of early chick embryos synthesize four haemoglobin types. Two of these are

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embryonic haemoglobins and the other two are foetal. At the seventh day of incubation the two adult haemoglobins appear in the haemolysate. Moreover, the relations between haemoglobin types and cell population are fairly clear: the three haemoglobin couples are synthesized in three different cell lines (Cirotto & Geraci, 1977; Shimizu, 1976; Cirotto, Panara & Geraci, 1977; Mahoney, Hyer & Chan, 1977; Chapman & Tobin, 1979).

Unfortunately such experimental evidence is available only for the chick. It is interesting however to extend similar investigations to other bird species. The investigation of embryonic haemoglobins is helpful in the study of ontogenetic processes and phylogenetic problems. For this reason we have studied the adult and embryonic haemoglobins of ducks, which are birds phylogenetically very different from chicks.

Since Peking ducks are widely used as laboratory animals, some reports are found in the literature on the isolation and characterization of the adult haemoglobins (Vandecasserie, Paul, Schnek & Leonis, 1973; Gander, Luppis, Stewart & Scherrer, 1972). On the other hand, few studies have been carried out on the embryonic haemoglobins (Borgese & Bertles, 1965; Stratil & Valenta, 1976; Borgese & Nagel, 1977).

In this work we report the characterization of the duck embryo haemoglobins and the results obtained are discussed with reference to the data concerning the chick.

MATERIALS AND METHODS

Haemoglobin preparation

Peking duck embryos at various stages of development were obtained from different poultry farms. The blood was obtained from the embryos and collected in buffered isotonic solution as described by D'Amelio & Costantino-Ceccarini (1969). The erythrocytes were washed and lysed as described in a previous paper (Cirotto et al., 1975). The clear haemolysate was dialysed on a column of Sephadex G 25 fine equilibrated with 10 mM potassium phosphate at pH 6.2 prior to the chromatographic analysis. Haemoglobins were isolated in a pure form by chromatography on a CM cellulose column (Whatman CM 52), at 4 °C, with the elution method previously described (Cirotto et al. 1975). Quantitative evaluation of the chromatographic fractions was obtained by cutting and weighing each peak of the tracings. Haemoglobin concentration was determined spectrophotometrically using ε = 11.1 \times 10^3 at 540 nm for the cyanmet derivative (Antonini, 1965).

Preparation and carboxymethylation of the globins

Haemoglobin molecules were depleted of the haems by the method of Rossi Fanelli, Antonini & Caputo (1958). Sulphydryl groups were blocked by reaction with a 10-fold molar excess of iodoacetamide in 8 M urea, 50 mM potassium phosphate pH 7.5.
The haemoglobins of developing duck embryos

Fractionation of the globin chains

Analytical separation of the globins was obtained by electrophoresis on polyacrylamide gels. The following solutions were prepared: (a) 45 g acrylamide, 0.3 g bisacrylamide in 100 ml of water; (b) 18 ml of 99% formic acid, 3.93 ml of 0.6% silver nitrate in 100 ml water; (c) 0.75 g ammonium persulfate in 100 ml water. Equal volumes of these solutions were mixed and left standing at 20 °C. The polymerization time was 2 h. The electrode solutions were 1.4 M formic acid. 60 µg of globins in 20 µl of 1.4 M formic acid containing 0.5 M urea were loaded on each gel. The gels were submitted to a pre-electrophoretic run at 4 mA/tube using methyl-green as tracing dye. Electrophoresis was carried out at 4 mA/tube. Protein bands were stained with the direct method of Malik & Berrie (1972) and the relative amounts of the globin bands were determined by scanning the gels at 650 nm on a Gilford spectrophotometer. The percentages of the globin types were obtained from the tracings as described for the haemoglobin chromatographic patterns. A quantitative separation of the globin chains was performed by chromatography on a CM cellulose column according to Gander et al. (1972). The pooled chromatographic fractions were lyophilized after dialysis against 1% formic acid.

Fingerprint analysis

Tryptic digestion of the isolated and carboxymethylated globins was carried out according to Hunt, Hunter & Munro (1969). Fingerprint analysis was carried out by the method of Ingram (1958) for the electrophoresis and by the method of Waley & Watson (1953) for the chromatography. A typical experiment has already been described (Cirotto et al. 1975). Tryptic maps were first stained with the ninhydrin reagent (Clegg, Naughton & Weatherall, 1966) and then with the specific stainings for histidine, tyrosine, arginine and tryptophane as described by Lehmann & Huntsman (1974).

RESULTS

Haemoglobin separation

In Fig. 1 are shown the chromatographic patterns of total lysates from duck embryos at 5 and 8 days of development, from hatched ducklings and from the adult. Two distinct patterns of haemoglobins are observed: one, typical of the early embryo is composed of the three fractions labelled E1, E2 and E3 according to their order of elution from the column, the other, found in the adult, is composed of two haemoglobins A1 and A2. Only the haemoglobins E1, E2 and E3 are detected in the erythrocyte haemolysates up to 8 days of incubation. The two adult haemoglobins A1 and A2 appear from 8 days of development on. It is evident from the patterns of Fig. 1 that the haemoglobin E2 of the early embryo is chromatographically indistinguishable from the minor adult haemoglobin A1. The two tetramers, however, appear to be different by electrophoretic analysis of their globins (see Fig. 2). Haemoglobin E2
Fig. 1. Elution patterns from CM cellulose columns in phosphate buffer of the total haemoglobins of 5- and 8-day-old duck embryos, of hatched ducklings and of the adult.

Fig. 2. Polyacrylamide disc gel electrophoresis in formic acid of the globins of the individual haemoglobin fractions isolated by column chromatography as shown in Fig. 1.
The haemoglobins of developing duck embryos

obtained from 5-day-old embryos differs at least in one globin from the haemoglobin A1 of the adult duck. Therefore, the inability of the chromatographic system to discriminate between these two haemoglobins makes the second peak heterogeneous from 8 days of development, when the adult haemoglobins appear, to hatching time. For this reason the globin compositions of the embryonic haemoglobins have been determined on the haemolysates of 5-day-old embryos and the characterization of the adult haemoglobins on the haemolysates of 1-year-old ducks.

In all the chromatographic profiles, in addition to the haemoglobin fractions described above, some materials appear to be eluted with the void volume of the column. Electrophoretic analysis, in denaturing conditions, of the molecules contained in this peak, demonstrated the presence of the first haemoglobin eluted from the column and other non-haem proteins.

Isolation of the globin chains

The electrophoretic analysis of the globins obtained from each chromatographic fraction shown in Fig. 1 is presented in Fig. 2. It is evident that each tetramer differs from the others in at least one globin. The three haemoglobins typical of the early embryo seem to share the fast migrating globin. A1 and A2 tetramers also show identical cathodic chains. The cathodic subunits of the haemoglobins E2 and A1, as well as those of the haemoglobins E3 and A2, appear clearly similar by electrophoretic mobility. The presence of only two protein bands in each gel confirms the purity of each chromatographic fraction. Three globin bands were observed by electrophoresis of the proteins contained in the second chromatographic peak from haemolysates of embryos older than 8 days. The globins correspond to those of the haemoglobins E2 and A1, demonstrating that they were eluted at the same time. A more detailed characterization of the structure of each globin was obtained by fingerprint analysis of tryptic peptides. For this purpose, the globin chains were isolated by chromatography on CM cellulose column in dissociating conditions as described in ‘Materials and Methods’. The chromatographic separation of the chains of the haemoglobins E1, E2 and E3 is shown in Fig. 3, while the patterns of the globins from haemoglobins A1 and A2 are shown in Fig. 4. In all cases two peaks are eluted which differ in absorbance, but have almost equal dry weights. The polypeptide chains present in each chromatographic fraction were identified by gel electrophoresis in formic acid. A typical electrophoretic pattern, shown in Fig. 4, reveals that each chromatographic peak is made of only one type of chain. Possible cross contamination does not exceed 10% of the total proteins. The molecules eluted in the band corresponding to the void volume do not give the colour reaction of the proteins.

Fingerprint analysis

The carboxymethylated globins were digested with trypsin and the peptides analysed by fingerprint. In Fig. 5 are shown the peptide maps of the more
Fig. 3. Elution patterns from CM cellulose columns of the chains constituting the haemoglobins E1, E2 and E3.

cathodic subunits of A1 and A2. The maps of the two chains show large similarities in the relative position and in the specific staining of all the spots with the exception of the dotted peptide in A2 map. A ninhydrin-stained spot in an identical position appeared also in A1 map, when the chain was pretreated with performic acid. This finding suggests that the peptide typical of A2 is an oxidized form.

Since the two maps of Fig. 5 are very similar to those of the β globins of adult chicken already reported (Cirotto, Petris, Panara & Manelli, 1974) it may be concluded that the cathodic subunits of the adult duck haemoglobins
The haemoglobins of developing duck embryos

Fig. 4. (A) Elution patterns from CM cellulose columns of the chains constituting the haemoglobins A1 and A2. (B) Disc gel electrophoresis in formic acid of globins of A2 haemoglobin isolated by chromatography on CM cellulose column.

are β-like chains. Therefore, the structural and functional differences between the two adult duck haemoglobins arise essentially from the α chains.

Figure 6 shows the fingerprints of the anodic subunits of E3 and A2 tetramers. The number, the relative position and the specific staining of the peptides are identical in the two maps, thus suggesting that the identity of the electrophoretic mobility (see Fig. 2) is due to a very similar primary structure. These two chains appear to be of the α type because their peptide maps are very similar to that of the α chain of the major adult chicken haemoglobin A2 (Cirotto et al. 1974).

Very similar peptide maps were also obtained for the anodic globins of the E2 and A1 tetramers (Fig. 7). Comparison of these peptide maps with those of the minor chicken haemoglobin chains, suggests that the former are of the α type (Cirotto et al. 1974).

In addition to the characteristics described above, the electrophoretic patterns of Fig. 2 show also identical mobilities for the β-like chains of the haemoglobins E2 and E3 and for the cathodic chain of the haemoglobin E1. Fingerprints of these globins show very similar patterns of tryptic peptides for the β-like
Fig. 5. (A) Fingerprint photographs and (B) fingerprint charts of the cathodic sub-units of A1 and A2 haemoglobins showing those peptides which have specific staining reactions for histidine (■), tyrosine (■), arginine (□), tryptophan (□).
The haemoglobins of developing duck embryos

Globins of E2 and E3 (see Fig. 8). On the contrary, the β-like chain of E1 differs from the others in the relative position of at least two peptides (Fig. 9). The difference is further emphasized by the distribution of spots positive for the specific stainings. Above all it is interesting to note that tryptophan is absent in the soluble peptides of the β-like chains of E2 and E3, whereas it is present in a nearly neutral peptide of the β-like chain of E1. The peptide map of the α-like chain of E1, shown in Fig. 9, confirms the electrophoretic data on the structural difference of this chain from the other α-like globins of the duck embryo.

Globin synthesis during embryonic life

The results of chromatographic, electrophoretic and peptide mapping analyses demonstrate the existence of an embryonic haemoglobin E1, typical of the early stages of development, which is different in both the constituent chains from all the other embryonic and adult tetramers. This molecule is detected in the haemolysates till the sixteenth day of development. The two
haemoglobins E2 and E3 found in the early embryo persist till hatching time. Both E2 and E3 haemoglobins show a globin composition typical of mammalian foetal haemoglobins, since their $\alpha$-like subunits are quite similar, and possibly identical, to the two $\alpha$-chains of the adult haemoglobins A1 and A2. At 8 days of incubation the two adult haemoglobins A1 and A2 appear in the haemolysate. If the chains showing quite similar peptide maps are assumed to be identical then six different globin chains appear to be synthesized in duck erythrocytes during embryonic life. In accordance with the labelling adopted for mammalian globins, the greek letters $\alpha$ and $\beta$ are used for the adult chains, the $\beta$-like globin of the foetal tetramers E2 and E3 is labelled $\gamma$, the constituent globins of the embryonic haemoglobin E1 are labelled $\zeta$ and $\epsilon$. On this basis globin composition

Fig. 6. Tracings of the tryptic maps of the anodic subunits of E3 and A2 haemoglobins showing those peptides which have specific staining reactions for histidine ($\square$), tyrosine ($\blacksquare$), arginine ($\blacksquare$), tryptophan ($\blacksquare$). Tryptophan residues are absent in both samples. Uncertainties in the specific staining are indicated by asterisks.
The haemoglobins of developing duck embryos

Fig. 7. Tracings of the tryptic maps of the anodic subunits of E2 and A1 haemoglobins showing those peptides which have specific staining reactions for histidine (■), tyrosine (■), arginine (■) tryptophan (■). Tryptophan residue are absent in both samples. The dotted peptide of αE2 was absent in some experiments. Uncertainties in the specific staining are indicated by asterisks.

of A1, A2, E1, E2, E3 are in the order α'2β2, α''2β2, ζ2ε2, α'2γ2, α''2γ2. The relative amounts of each globin at different stages of development are shown in Fig. 10. The percent values were obtained by quantitative evaluation of E1, A2 and E3 haemoglobins and of the electrophoretic bands of chains constituting A1–E2 chromatographic fraction.

DISCUSSION

The electrophoretic isolation of haemoglobins present in the haemolysate of early duck embryos was described some time ago by Borgese & Bertles (1965) and more recently, by Stratil & Valenta (1976) and by Borgese & Nagel.
(1977). All these authors agree in describing only one major haemoglobin fraction, classified by Borgese & Bertles as embryonic on the basis of the time over which it was present in the lysate of developing embryo and of its electrophoretic similarity to the chick embryonic haemoglobins. The chromatographic isolation of its constituent chains reported in this paper confirms that it is actually an embryonic haemoglobin since its globins differ from all the others synthesized both in embryonic and in adult life.

As demonstrated by many authors, chick haemoglobins seem to occur in pairs, each of them composed of a common β-like globin chain and two different α-like globin chains (Brown & Ingram, 1974; Cirotto & Geraci, 1977). The probable genetic process underlying the formation of these two α-like chain genes involves a duplication of an ancestor gene followed by independent
The haemoglobins of developing duck embryos

Fig. 9. Tracings of the tryptic maps of the two chains of El haemoglobin showing those peptides which have specific staining reactions for histidine (■), tyrosine (●), arginine (□), tryptophan (□). Uncertainties in the specific staining are indicated by asterisks. Spots shared by ε El, γ E2 and γ E3 are indicated by the arrows.

mutations. The duck foetal and adult haemoglobins may involve a similar scheme. The exception seems to be the embryonic haemoglobin El. However, it is likely that also in this case two distinct α-like globin structural genes are present per haploid genome, but they do not undergo any diversification. Their products are therefore chemically indistinguishable. There is substantial evidence that also in man there are two structural genes for the α chains per haploid genotype with a total of four α chains per diploid cell (Lang & Lorkin, 1976).

In duck embryo erythrocytes as in chick and in goose (Cirotto, Arangi & Panara, 1979) there are two haemoglobins that can be defined as foetal according to their globin composition and to their presence in the embryo and in the newborn duckling. Stratil & Valenta (1976) described two minor electrophoretic haemoglobin fractions in the early embryo, whereas Borgese & Bertles (1965) and Borgese & Nagel (1977) detected only one. In the case of chick
the problem concerning the identity of the foetal haemoglobins in the course of embryonic life is not yet solved. Some electrophoretic evidence suggests a sequential appearance in the embryo of different types of foetal haemoglobins (Bruns & Ingram, 1973). Nevertheless, an analysis of the primary structure of these molecules is lacking up to today. During our investigation of the haemoglobins of the embryonic duck no variation of the chromatographic elution properties of the two foetal fractions was found. For this reason we have chosen to name with the same labels E2 and E3 these two haemoglobins, independently of the developmental stage.

The persistence of foetal haemoglobins during the embryonic life seems to be lacking in a plausible physiological meaning, since they constitute too small a portion of the haemolysate. At the same time, their functional properties in the presence of organic phosphates are very similar to those of adult haemoglobins (Borgese & Nagel, 1977). It is likely that in duck, as in chick, these two haemoglobins are synthesized by a foetal red cell population (Cirotto et al. 1977).

The time of appearance of the two adult haemoglobins is delayed in ducks, in comparison with chicks, in accordance with the longer duck incubation time. Percent amounts of the adult haemoglobins change during embryonic development and reach the typical adult values only after egg hatching. This finding could be due to the different origins of the red cells of the definitive line which produce the adult haemoglobins. Before hatching, the duck definitive erythrocytes are markedly morphologically different from those of the adult, as found in chicks by Lemez (1964). It is likely then, that they differ also in the relative amounts of A1 and A2.

The results reported in this paper demonstrate the existence of strong similarities between the general picture of haemoglobins in the chick embryo and that of the duck embryo. Further studies will demonstrate the existence of a similar haemoglobin distribution in other bird species.
The authors are indebted to Professor G. Geraci for his helpful suggestions and to Mr L. Barberini for his technical assistance.

REFERENCES


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(Received 31 March 1980, revised 9 May 1980)