The minor haemoglobins of primitive and definitive erythrocytes of the chicken embryo. Evidence for haemoglobin L

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Summary

A new minor haemoglobin, L, was isolated from the haemolysates of chicken embryos more than 7 days old. Electrophoresis in denaturing conditions and tryptic peptide maps of the globins show that the β-like globin of HbL is identical to that of the minor haemoglobin H(β11) while the α-like globin is very similar to that of the adult haemoglobin D (αB). HbL completes the description of the map of the minor chicken haemoglobins during embryonic development. In early embryos two minor haemoglobins, M and E, are produced which have the same β-like globin (ε) and differ in their α-like globins (αB and αA, respectively). The same two α-like globins will make up the minor haemoglobins of the late embryo, L and H, which differ from HbM and HbE on account of their β-like globin (β11).

The native tetramers L and M are hard to distinguish from each other. However the constituent ε globin can be easily separated from β11 by electrophoresis on polyacrylamide gel in formic acid. With this method we found that the switch of the minor haemoglobins in the blood of chicken embryos starts at the 7th incubation day.

The two red cell populations, primitive and definitive, present in the blood of 7-day-old embryos were separated on an albumin gradient and their minor haemoglobins analysed. The haemoglobin couple M/E was found in the primitive erythroid cells whereas the L/H couple was found in the definitive ones. The disappearance of the early haemoglobin couple and its substitution by the late one during embryonic development correlates with the replacement of erythroid lines in the blood.

Key words: haemoglobin, chick embryo, erythrocytes.

Introduction

Haemolysates of chicken embryos show different haemoglobin patterns during development depending on the various differentiation stages and the different respiratory needs. There is widespread agreement among researchers about the structural characteristics and the production time of the major haemoglobin fractions. The haemolysates of early embryos contain two major haemoglobins named P and P' whose globin composition is α2β2 and α2β2 (Bruns & Ingram, 1973a; Brown & Ingram, 1974; Cirotto, Scotto di Tellia & Geraci, 1975; Schalekamp & Van Goor, 1984). Their primary structure is well known (Chapman, Tobin & Hood, 1980; Chapman et al. 1981).

At about 6 days of development, the two haemoglobins D and A, with globin composition αDβ2 and αAβ2, begin to appear in the embryo haemolysates (Bruns & Ingram, 1973a; Cirotto et al. 1975). They constitute the two major haemoglobin fractions of the embryos from the 7–8th day of development and persist for the whole adult life (Moss & Hamilton, 1974). The primary structure of these two haemoglobins is also well known (Takei et al. 1975; Matsuda, Takei, Wu & Shiozawa, 1971; Matsuda, Maita, Mizuno & Ota, 1973; Vandecasselere, Paul, Schnek & Leonis, 1975; Knochel et al. 1982).

Although the description of the major haemoglobins is complete the same cannot be said for the minor ones. Different authors report different results. Only recently has isolation and characterization of some of the minor fractions contributed to the
partial solution of this problem. Two minor haemoglobins, M and E, have been isolated from the blood of embryos of up to 6 days development (Brown & Ingram, 1974; Schalekamp, Schalekamp, Van Goor & Slingerland, 1972; Cirotto et al. 1975). The subunit composition of HbM has been investigated by various methods (Keane, Abbott, Brown & Ingram, 1974) and more recently by determining the primary structure of its globin chains (Chapman, Hood & Tobin, 1982a). All the data indicate that the globin composition of HbM is $\alpha^2\beta^2\epsilon^2$ where $\alpha^D$ has the same structure as the $\alpha$-like globin of adult HbD and $\epsilon$ is a $\beta$-like globin different from both the adult $\beta$ and all the other $\beta$-like embryo globins.

Similar investigations were carried out on the other minor haemoglobin E. The primary structure of its constituent globins was determined (Chapman, Hood & Tobin, 1982b). The amino acid sequence of the $\alpha$-like globin is the same as that reported by Knochel et al. (1982) for the adult $\alpha^A$. The $\beta$-like subunit has a primary structure identical to the $\epsilon$ globin of HbM.

There is no such clear description in the literature on the minor haemoglobins of late embryo. Some authors describe a haemoglobin (H) typical of the late embryos and of the newly hatched chicks (Godet, 1974; Bruns & Ingram, 1973a,b; Moss & Hamilton, 1974). Its globin composition is of $\alpha^2\beta^1\epsilon^1$ type, the $\alpha$-like globin being the same as adult HbA and the $\beta$-like globin differing from both the adult $\beta$ and the other $\beta$-like globins of the early embryos (Moss & Hamilton, 1974). Other authors, using different experimental approaches, have described two minor haemoglobin fractions of late embryo which have been characterized only in part (Cirotto et al. 1975).

Many investigations have been carried out to define the localization of the major haemoglobins P, P', D and A in the primitive and definitive erythrocyte populations of chicken embryo. All the authors agree that HbP and HbP' are present in the primitive red cells while HbD and HbA are absent (Shimizu, 1976; Chapman & Tobin, 1979; Schalekamp & Van Goor, 1984) and that HbD and HbA are present in the definitive red cells while HbP and HbP' are absent (Cirotto et al. 1975; Beaufain, 1985) or present only in small quantities (Chapman & Tobin, 1979; Schalekamp & Van Goor, 1984). As regards the erythrocyte localization of the minor haemoglobins, it has been shown that HbM and HbE are contained in a subpopulation of the primitive erythrocyte line (Cirotto, Panara & Geraci, 1977).

Our aim was to examine in more detail the chicken minor haemoglobins at various stages of embryonic development, paying special attention to the late embryos, giving as complete a description as possible of their distinctive properties and their relations with the erythrocyte populations.

Materials and methods

Preparation and isolation of haemoglobins
Fertilized eggs of different chick strains were purchased from Cl.C. ZOO (Perugia). Erythroid cells were obtained from the embryos at various stages of development as previously described (Cirotto et al. 1975), washed several times in 7 mm-phosphate buffer pH 7-4 containing 0-9 % NaCl (PBS) and lysed in H$_2$O–CCl$_4$ (1:5:0.5 V/V) (Bruns & Ingram, 1973a). The ghosts were centrifuged off and the clear supernatant used directly for electrofocusing analysis or dialysed against 10 mm-phosphate buffer pH 6-2 for chromatographic separation. Isoelectric focusing on 4 % polyacrylamide gels in the pH gradient 7-9 was carried out as described by Drysdale, Righetti & Bunn (1971). Protein bands were stained with Coomassie Brilliant Blue by the Malik & Berrie (1972) method. Their densitometric analyses were performed on a Varian 634 gel scanner at $\lambda = 650$ nm. In some experiments, as soon as focusing was completed, the gels were frozen and sections of 2 mm were cut with a gel cutter. To elute the haemoglobin fractions the slices were collected separately, vortexed and allowed to stand overnight in 0-5 ml 50 mm-phosphate buffer pH 7-0.

Chromatographic isolation of the haemoglobins was performed on Whatman CM 52 cellulose columns (1-8x20cm). Elution was done with a pH and salt concentration linear gradient obtained from a gradient mixer containing 0-51 of 10 mm-potassium phosphate buffer, pH 6-2 in the mixing chamber and 0-51 of 20 mm-potassium phosphate buffer, pH 8-0 in the reservoir (Cirotto et al. 1975).

Haemoglobin concentration was determined spectrophotically using $e = 11-1 \times 10^3$ at 540 nm for the cyan-met derivative (Antonini, 1965). Quantitative evaluation of the chromatographic fractions was obtained by cutting and weighing each peak of the tracings.

Separation of globin chains
Haemoglobin molecules were depleted of the haem groups by the Rossi Fanelli, Antonini & Caputo (1958) method. The analytical separation of the globins was obtained by electrophoresis on polyacrylamide gels and the quantitative separation was performed by chromatography of CM cellulose columns.

Electrophoresis on polyacrylamide gel in formic acid was carried out as reported in a previous paper (Cirotto, Arangi & Panara, 1980). In a typical procedure, 100 ml of the polymerization mixture contained 15 g acrylamide, 0-1 g bisacrylamide, 6 ml of 99 % formic acid, 0-25 g ammonium persulfate and 7-8 mg silver nitrate. Both pre-electrophoresis and electrophoresis were carried out at 4 mA per tube, using methyl green as tracing dye. Protein bands were stained with Coomassie brilliant blue as described by Malik & Berrie (1972).

For the quantitative separation of the globin chains, two different chromatographic techniques were used. The globins constituting HbD, HbM and HbL were separated by the Gander, Luppis, Stewart & Scherrer (1972) method. About 80 mg of globins were loaded on a CM cellulose column (2-5x10 cm) equilibrated with 15 mm-acetate buf-
fer pH 5.4, 8 M-urea, 50 mM-2-mercaptoethanol and were eluted by means of a linear gradient of acetate concentration ranging from 15 mM to 0.3 M. pH 5.4 in 8 M-urea, 50 mM-2-mercaptoethanol.

The \( \alpha \) and \( \beta \) globins of HbH were separated on CM cellulose columns (2.5x10 cm) using a linear gradient of formic acid (0.1 M to 1.5 M). In both cases, the chromatographic fractions were lyophilized after dialysis against 1% formic acid and their purity tested by gel electrophoresis.

Fingerprint analysis

Sulphydryl groups of isolated globins were blocked by reaction with a 10-fold molar excess of iodoacetamide in 8 M-urea, 50 mM-potassium phosphate pH 7.5.

Tryptic digestion of the carboxymethylated globins was according to Hunt, Hunter & Munro (1969). Fingerprint analysis was carried out by the Ingram method for the electrophoresis (Ingram, 1958) and by the Waley & Watson (1953) method for the chromatography. Tryptic maps were first stained with ninhydrin and then with the specific stainings for histidine, tyrosine, arginine and tryptophan as described by Lehmann & Huntsman (1974).

Separation of erythrocyte populations

Primitive and definitive erythroid cells from 7-day-old embryos were separated on 12 ml gradients of bovine serum albumin (BSA) 2-5% in PBS. The cells \( (2x10^6) \) were suspended in 0.2 ml of 2% BSA, layered on the gradient and left standing for 14 h at unit gravity at 4°C. The lower cell band contains the primitive erythrocytes, the upper band contains the definitive erythrocytes. The homogeneity of cell bands was judged microscopically. Only pure fractions were pooled (Fucci, Vitale, Cirotto & Geraci, 1987).

Results

In chicken embryos of less than 6 days of development, the chromatographic profile of haemoglobins obtained by CM cellulose columns does not differ qualitatively from that shown in Fig. 1A which refers to the haemolysate of 5-day-old embryos. The elution pattern is quite similar to that previously reported by Brown & Ingram (1974) and Cirotto et al. (1975). The major haemoglobins P and P' are eluted first, followed by the two minor haemoglobins M and E. Under the chromatographic profile, a gel of isoelectric focusing is shown (Fig. 1B) on which the 5-day embryo haemoglobins were separated. The two major haemoglobins P and P' and the two minor ones M and E are evident.

In Fig. 2, the chromatography and isoelectric focusing patterns of 15-day-old embryos are shown. Two major and two minor haemoglobins are evident. The two major ones differ in their chromatographic properties and in their isoelectric points from the early major haemoglobins P and P'. They are HbD and HbA, characteristic of the mature embryo and adult chick. The two minor haemoglobin fractions do not differ to any great extent from HbM and HbE of early embryos. From literature, it is known that at this developmental stage the last haemoglobin to be eluted by cation-exchange chromatography is HbH (Moss & Hamilton, 1974) which can be distinguished from HbE only by electrophoresis in a definite pH (Bruns & Ingram, 1973b). For these reasons and because of the data reported further on in this paper, the last chromatographic fraction has been identified as HbH. The other minor fraction, eluted from the column between HbD and HbA has been named

Fig. 1. (A) Elution profile of 5-day-old chicken embryo haemoglobins from a CM cellulose column. (B) The haemoglobins of 5-day-old chicken embryos after isoelectric focusing separation. Top of the polyacrylamide gel column: pH 9, bottom: pH 7.
HbL (Late haemoglobin) to distinguish it from HbM of the early embryo. The quantitative evaluation of minor haemoglobins from chromatographic patterns gives the same values as those obtained from isoelectric focusing profiles: HbM 10%, HbE 5%, HbL 4% and HbH 5%.

Electrophoresis on polyacrylamide gel in formic acid proved useful for separating the constituent globin chains of the minor haemoglobins of chicken embryo. Fig. 3 shows the electrophoretic patterns of the globins from HbE, HbH, HbM and HbL. As already demonstrated (Cirotto et al. 1980), this electrophoretic technique gives good separation of the α-type globins from the β-type ones. The latter have a more cathodic migration than the former. Comparing the bands of the two gels on which the globins of HbE and HbH were loaded confirmed the results obtained by other investigations. The two haemoglobins differ from each other on account of the β-like subunit. Electrophoretic migration of the β-like subunit of HbH is more cathodic than the corresponding globin of HbE. The electrophoretic mobility of the two α-type globins is instead identical in both cases. The globins of HbM and HbL behave in the same electrophoretic manner. The migration of the HbL β-like globin is more cathodic than that of the HbM and the two α globins cannot be distinguished. Comparing on gel the positions of all the β-like globins it clearly appears that the β globins of HbE and HbH are equal to those of HbH and HbL respectively. Instead, the mobility of the α-like globins of HbE and HbH are different from the corresponding α-like globins of HbM and HbL.

These electrophoretic results agree perfectly with data in literature which show that the α-like globin of HbE and HbH is very similar to the αA globin of the adult (Chapman et al. 1982b; Moss & Hamilton, 1974) and the α-like globin of HbM is identical to the αH of the adult (Chapman et al. 1982a).

HbL is the only one for which the composition of the subunit is not adequately described. From our electrophoretic analysis, HbL appears to be composed of an α-type globin which is exactly the same as αD and by a β-type globin which is exactly the same as βH. For more detailed knowledge about the subunit...
composition of HbL, we did fingerprint analysis of the tryptic peptides of each globin chain.

Fig. 4 shows the fingerprinting of the β-like globin of HbL and, as reference, those of β^H obtained from HbH and of ε obtained from HbM. The β^L map, as regards number of peptides, their position on the paper sheet and distribution of peptides positive for specific amino acid stainings, is very similar to β^H but is clearly different from ε. Comparison of the fingerprints of the α-like globins obtained from HbD and HbL shows that the two maps are very similar (Fig. 5).

These data, together with those previously reported in literature, show that during development the early haemoglobins M and E, with a globin composition α^E2 and α^E2, are replaced by the haemoglobin couple L/H with a globin composition α^H2 and α^H2. These differ from the minor haemoglobins M and E of the early embryo on account of the β-type subunit.

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**Fig. 4.** Fingerprint photographs of β^L (A), β^H (B) and ε (C) globins. Beside each photograph a chart shows those peptides positive for histidine (●), tyrosine (●), arginine (●) and tryptophan (●). The dotted peptide of β^H was absent in some experiments.
With formic acid electrophoresis, the time course for the substitution of the haemoglobin couple M/E by L/H during embryonic development can be followed. Fig. 6 shows the electrophoretic patterns of the globin chains present in M/L and E/H chromatographic peaks obtained from embryos at various stages of development. The band patterns show that replacement of the ε globin by the βH one begins at 7 days of embryonic development. From 7 days to about 14 days the minor chromatographic peak eluted after HbD contains both HbM and HbL and the last peak contains both HbE and HbH. After the 14th day of incubation the two peaks are homogeneous and composed solely of the two molecular forms HbL and HbH respectively. The substitution time in the haemolysate of HbM and HbE by HbL and HbH is the same as the substitution time of primitive erythrocytes by the definitive ones in the circulating blood (Romanoff, 1960). This suggests that the two haemoglobin couples are localized in the two different cell populations. This hypothesis was confirmed by the results of the experiments below.

On the albumin gradient prepared as described in Materials and methods, the primitive red cells of 7-day embryos were easily separated from the definitive erythrocytes. The haemoglobins of the two erythrocyte types were isolated by electrofocusing on polyacrylamide gel. The two minor haemoglobins were then collected separately and their constituent globin chains analysed by electrophoresis on polyacrylamide gel in formic acid. The results are shown in Fig. 7. Fig. 7A shows three gels; in the control gel, the globin components of the M/L fraction isolated from the haemolysate of 7-day embryos are analysed. One α-like globin and two different β-like globins are
Discussion

The data reported in this paper fully confirm the previous observations about the globin composition of the minor haemoglobins M, E and H of chicken embryo (Chapman et al. 1982a, b; Moss & Hamilton, 1974). They also describe a new minor haemoglobin, named L, which is typical of the late embryo. The electrophoretic analysis of its globins and, more so, the analysis of the tryptic peptides of each globin show that HbL is made up of an α-like globin very similar to αD and a β-like globin identical to βH.

HbL completes the minor haemoglobin map of the late embryo, showing it is very similar to that of the early embryo. In both cases, in fact, the haemoglobins are present in couples constituted by the two adult α-like subunits, αD and αA, and by a characterizing β-like globin. It has been demonstrated that αD and αA are synthesized from the very beginning of embryonic development (Fucci, Cirotto, Tomei & Geraci, 1983). Their production continues for the whole life and, combining with different β-like globins, they constitute the various haemoglobin couples. Therefore, in the early embryo, αD and αA joined to the ε globin chain form the haemoglobin couple M/E. In the late embryo, the same α-like globins, joined to the β, form the couple of adult haemoglobins D/A. If, besides this couple of major haemoglobins, there was only one minor haemoglobin produced, HbH (till now the only one described) it would mean that either βH is incapable of binding αD or that HbH is confined to red cells where the αD globin is not produced. Neither of these hypotheses seems tenable. The existence of HbL, therefore, introduces a rational order in the chicken embryo haemoglobin map. The two globins αD and αA, being synthesized at all ages, combine with different β-like globins and lead to the formation of different haemoglobin couples.

The period of embryonic life during which alternation of the haemoglobin couples is most evident is about 6–7 days. At this age, the minor couple HbM/HbE begins to be substituted by the HbL/HbH couple and the major couple HbP/HbP′ by the HbD/HbA couple. From the data presented here about the cytological localization of the minor haemoglobins, it seems that HbM/HbE is confined to the primitive erythrocyte population and HbL/HbH to the definitive one. It cannot be said, on the basis of our electrophoretic data alone, that there is complete compartmentalization of the two haemoglobin couples in the two erythrocyte populations. In fact, formic acid electrophoresis is not able to evidence bands of β-like globin quantitatively less than 1 % of the total proteins so any eventual reciprocal contamination at less than this value would not be evidenced.

The debate about cytologic compartmentalization of the major haemoglobin couples P/P′ and D/A of chicken embryo as an index of a special switch mechanism, is very topical in literature. The data of Chapman & Tobin (1979) on the presence of small
The second hypothesis seems more probable and is difficult to think that the synthesis of HbL/HbH takes place only when the red cells are completely mature. The existence of couples of α-like globins leads to the constitution of couples of tetramers sharing the β-like subunit.

Quantities of HbP and HbP' (about 5%) in erythrocytes of the definitive line, indicating a ‘molecular’ mechanism of haemoglobin switch, have not been confirmed either by Beaupain (1985) who has even used the same immunological method, or by Fucci et al. (1987) who have used different techniques. The results reported here about the minor haemoglobin couples rule out reciprocal contamination of more than 1% therefore suggesting a compartmentalization of the two haemoglobin couples in the two different erythrocyte populations.

If the parallel between the major and minor haemoglobin couples is valid for the cytological localization, it is not so for the switch time. The first appearance of HbA and HbD in the haemolsate, in fact, is observed about 24 h before the appearance of HbL and HbH (Schalekamp et al. 1972; Cirotto et al. 1975). This fact could be interpreted in two different ways: (1) in the red cells of the definitive line the synthesis of the HbL/HbH couple takes place after the synthesis of HbD/HbA, (2) the HbD/HbA and HbL/HbH couples are contained in different definitive erythrocyte subpopulations which enter the circulation at different times. The first of these two explanations is the most difficult to accept since the definitive red cells, when released into the blood stream are already rich in haemoglobin and at an advanced stage of maturation (Romanoff, 1960). It is difficult to think that the synthesis of HbL/HbH takes place only when the red cells are completely mature. The second hypothesis seems more probable and is also supported by what is known about the cytological localization of the minor couple HbM/HbE of the early embryo. It has in fact been demonstrated that HbM and HbE are contained in a subpopulation of the primitive line which has been clearly evidenced immunologically (Cirotto et al. 1977). Therefore, it is plausible that, in the mature embryo also, HbL and HbH are localized in a definitive subpopulation of red cells, released into circulation at 7 incubation days.

Keeping in mind all the data given in this investigation about the globin composition of minor haemoglobins, the times of their appearance and disappearance and the results reported by other authors about major haemoglobins, the scheme of Fig. 8 is proposed as a summning up.

This scheme is fundamentally that given by Brown & Ingram (1974) with the addition of HbL and the indications about the cytological localization of the various major and minor haemoglobins.

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References


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