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Biologia dello sviluppo. — *Comparison between the erythrocyte morphological changes and the different hemoglobin species found in developing chick embryo*(*). Nota(**) di CARLO CIROTTO, HARRY MANELLI, VINCENZO D'AMELIO e ANNA PETRIS, presentata dal Socio P. PASQUINI.

RIASSUNTO. — Durante lo sviluppo embrionale del pollo si distinguono, nella circolazione sanguigna, almeno due tipi di eritrociti: quelli della serie primitiva e quelli della serie definitiva, diversi per caratteristiche morfologiche e per tempo di comparsa. Anche i tipi di emoglobine presenti nelle due serie mostrano marcate differenze nel comportamento cromatografico ed elettroforetico. A 5 giorni di incubazione, quando la quasi totalità degli eritrociti appartiene ancora alla serie primitiva, l'emolizzato presenta 4 componenti emoglobiniche, diverse, o per proprietà cromatografiche o per proprietà elettroforetiche, dalle due emoglobine del pulcino alla schiusa, i cui globuli rossi appartengono tutti alla serie definitiva.

It is known that chick embryo hemolysate shows an oxygen affinity which is higher than the adult chicken hemolysate (Hall, 1935; Manwell *et al.*, 1963). This affinity decreases progressively during incubation and reaches the adult values at about 60 days after hatching (Hall, 1935). These results were interpreted by Hall as indicating the existence of a specific embryonic hemoglobin, which is gradually replaced by an adult hemoglobin.

Further experiments demonstrated that both embryo and adult hemolysate contain multiple hemoglobin components (Fraser *et al.*, 1972; Schalekamp *et al.*, 1972; Hashimoto *et al.*, 1926), and at least one type of hemoglobin of the early embryo is absent in the late embryo and in the adult chicken (D'Amelio *et al.*, 1961; Manwell *et al.*, 1966). However, there are some disagreements in literature on the number of embryonic hemoglobins and their structural and functional properties (Wilt, 1967; Schalekamp *et al.*, 1972; Fraser *et al.*, 1972).

The erythrocyte population shows remarkable morphological changes during embryonic development. There are, at least, two subsequent cell lines or " generations ": the primitive strain and the definitive one (Lucas *et al.*, 1961; Romanoff, 1960), which have different morphological characteristics.

We have developed a chromatographic procedure for the analysis of the various types of hemoglobins which appear during chick embryo development. Such an analysis permits to correlate the changes of the hemoglobin components with the morphological changes of the erythrocyte population.

(*) Most of this work was carried out at the Institute of Histology and Embryology (Faculty of Sciences), University of Perugia, during winter-spring 1971.

(**) Pervenuta all'Accademia il 24 settembre 1973.

Materials and Methods

Preparation of hemoglobin samples.

Erythrocytes from chick embryos at different stages of development were isolated as described by D'Amelio *et al.* (1969). Blood of new hatched chick was obtained by decapitation and collected in cold 3,5 % sodium citrate pH 7.0. The blood cells were washed several times in 10 volumes of .9 % NaCl, and then lysed in 3.3 mM $MgCl_2$. The suspension was centrifuged at 10,000 g for 15 minutes and the clear supernatant was isolated. All the preparation steps were done at 4° C.

Chromatographic and electrophoretic analysis.

Chromatography was carried out on a .8 × 12 cm CM cellulose column (Whatman CM 52) with a linear gradient of pH and salt concentration obtained mixing 5.1 of 10 mM potassium phosphate pH 6.2 with .5 l of 20 mM potassium phosphate pH 8. About 50 mg of proteins were loaded on the column. The hemoglobin concentration was determined spectrophotometrically as described by Antonini (1965). After each chromatographic analysis the hem oxidation state was checked spectrophotometrically.

The electrophoretic analysis of the hemoglobins was carried out on cellulose acetate strips (Millipore) equilibrated with 50 mM veronal buffer pH 8.6, at about 1 mA/strip for 20 minutes.

It has been reported that chicken hemoglobins undergo misleading modifications with the ageing of the sample (Manwell *et al.*, 1966; Godet *et al.*, 1970). For this reason, all the analyses were carried out within 24 hours after blood collection.

Blood smears.

Blood smears were stained with May-Grünwald-Giemsa. In order to obtain the statistical distribution of primitive and definitive red cells in the blood of the studied embryonic stages, at least 1000 cells were counted.

Results

The erythrocytes of the primitive strain are predominant in the blood of chick embryo from the second day of incubation. On the fourth day the immature cells of the definitive strain appear; these cells reach the highest percentage between the sixth and the seventh incubation day (Romanoff, 1960; Lucas *et al.*, 1961). Fig. 1 (Plate I) shows a typical blood smear of a 5-day embryo. About 90 % of the erythrocytes belongs to the primitive strain; the immature cells of the definitive strain are the remaining 10 %.

The 5-day embryo hemolysate, when analysed by CM cellulose column chromatography, shows the elution pattern of fig. 2. The hemoglobin peaks # 1 and # 2 are prominent, and account for about 39 and 41 per cent, respectively, of the total hemoglobin. Each of the two minor fractions (# 3 and # 5 on the figure eluted at higher pH and salt concentration) is about 10 per cent of total hemoglobins.

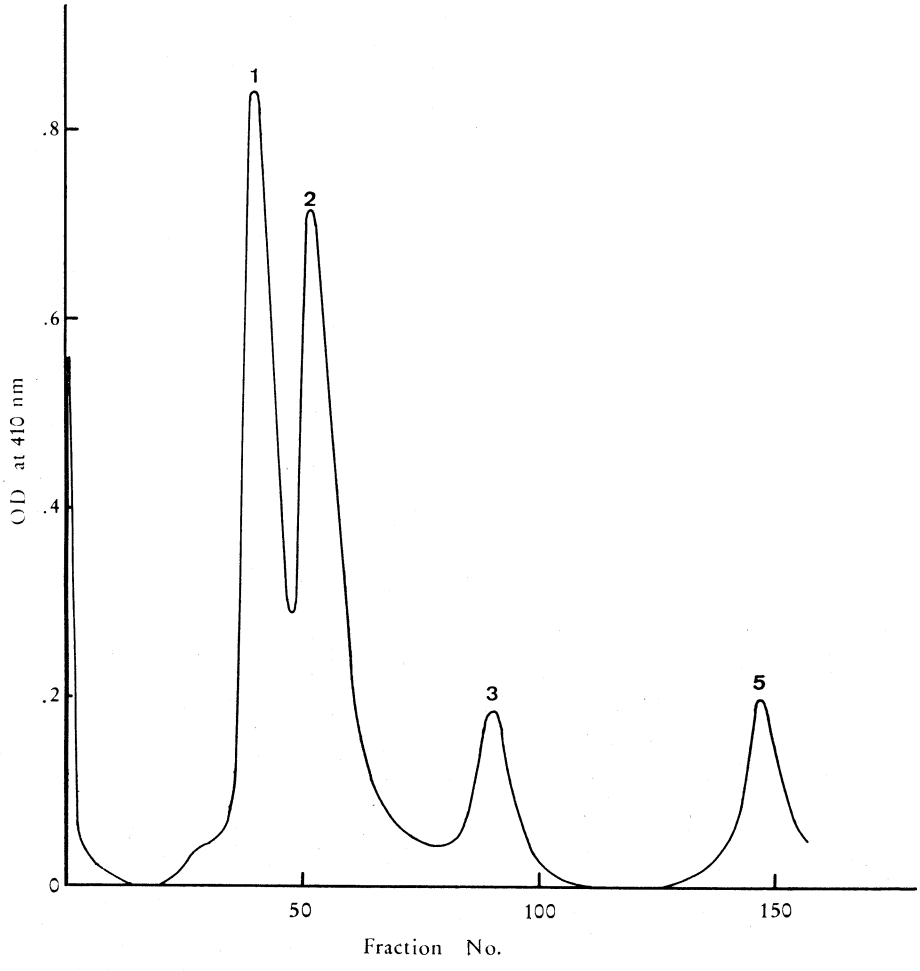


Fig. 2. – Elution patterns of 5-day embryo hemoglobins from a CM cellulose column chromatography with a linear gradient of pH and salt concentration. The four fractions are typical of the early embryo, and are absent in the new hatched and adult chicken.

At 7 days of incubation, most of red cells belong to the definitive strain, and constitute 85 per cent of the erythrocyte population (fig. 3, Plate I). The elution pattern of 7-day embryo hemolysate is shown in fig. 4. A new peak, (# 4 on the figure) is present. The percentage amount of the hemoglobin of fraction 2 does not change considerably. This last fraction, when

analysed by electrophoresis on cellulose acetate strips, shows two protein bands when stained with Amido-Black, corresponding to the coloured bands of the hemoglobins.

Fig. 5 summarizes the electrophoretic patterns of hemoglobins of peak 2 from the hemolysate of 5- and 7-day embryos, and of new hatched chick. The chromatographic peaks # 2 of both 5-day embryos and hatched chick

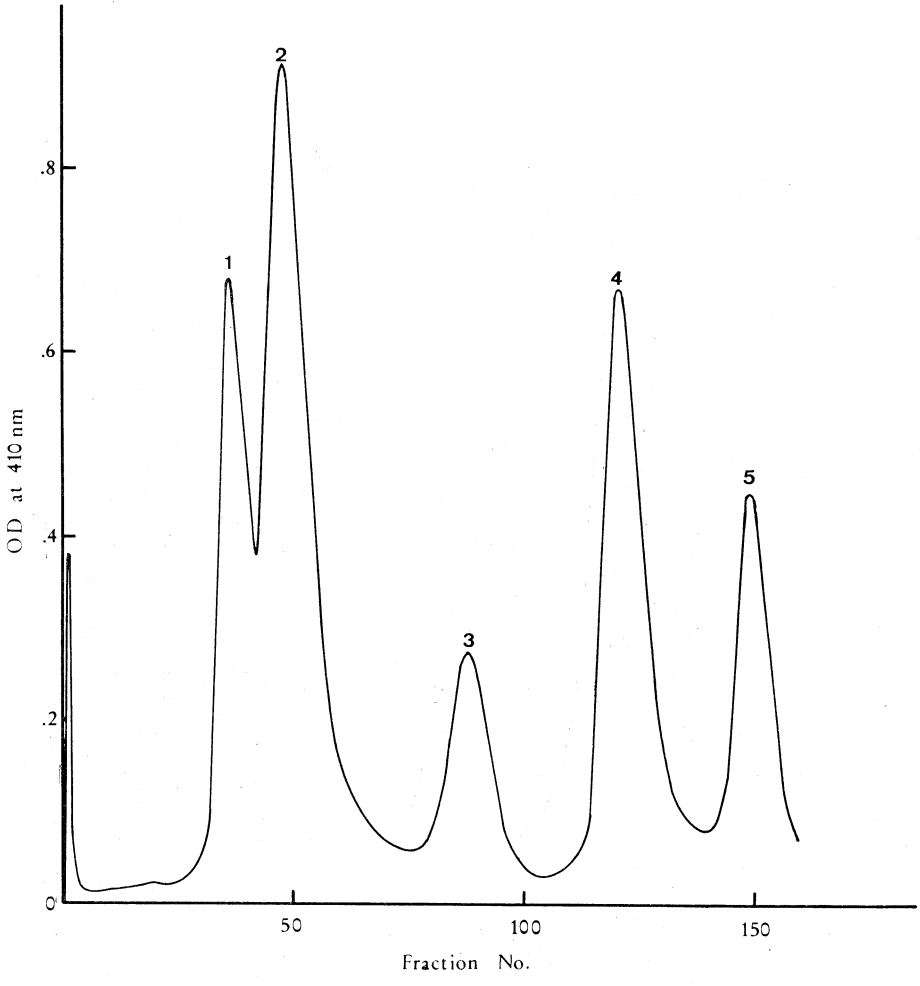


Fig. 4. – Elution pattern of 7-day embryo hemoglobins: same experimental conditions as in fig. 2. At this stage two new types of hemoglobins appear: the one is present in the peak # 2, the other constitutes the new peak # 4.

are homogeneous and contain different types of hemoglobins, which are both present in the peak # 2 of the 7-day embryos hemolysate.

The chromatographic pattern of the hemolysate of the chick at hatching consists of the two peaks # 2 and # 4 (fig. 6). At this stage all the erythrocytes belong to the definitive strain (fig. 7, Plate I). Peak # 2, when

analysed by electrophoresis, gives a single protein band (fig. 5); this peak is the same as the one which is present in the chromatographic pattern of adult chicken hemoglobins and constitutes the minor hemoglobin fraction.

The absorption spectra of each single fraction are consistently those of oxyhemoglobin.

DISCUSSION

The results of our experiments show that several types of hemoglobin are present in chick erythrocytes. This multiplicity is complicated by the changes in elution profiles that occur during embryonic development.

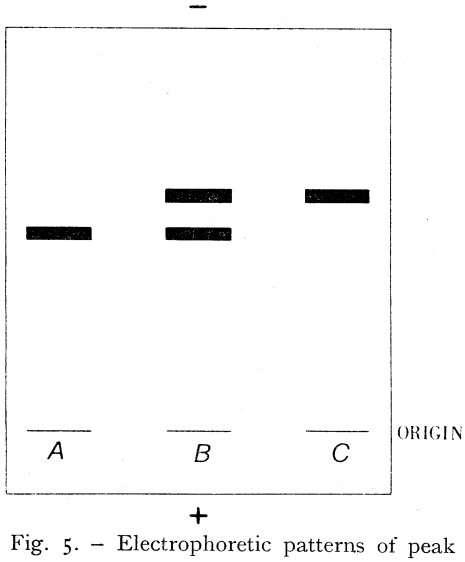


Fig. 5. – Electrophoretic patterns of peak #2 of 5-(A) and 7-day (B) embryos anf of new hatched chick (C).

In the 5-day embryos most of the erythrocytes belong to the primitive strain, and four hemoglobins are detectable by column chromatography (fig. 2). These risults are in agreement with the data of Schalekamp *et al.* (1972), who found four chromatographically different hemoglobins in the 5-day embryo. Neither starch gel nor acrylamide disc gel electrophoretic analysis permit to distinguish more than three hemoglobin types (Hashimoto *et al.*, 1966; Manwell *et al.*, 1966; Bruns *et al.*, 1973).

In the 7-day embryo erythrocytes are present both the four hemoglobins typical of the early embryo and the two hemoglobins typical of the hatched and adult chicken. By comparing the elution patterns (figg. 2, 4, 6) with the blood smears at different stages of development (figg. 1, 3, 7, Plate I), it is possible to correlate the appearance of adult hemoglobins with the great increase of the erythrocytes of the definitive strain in the 7-day embryo.

These results suggest that the two lines of erythrocytes contain different hemoglobin species. In fact, the four hemoglobins present in the erythrocytes of the primitive strain are different from those present in the red cells of the definitive strain, either in their chromatographic or electrophoretic properties. Therefore, our experiments demonstrate that the differences between the hemoglobins found in the primitive strain and those found in the definitive one, are qualitative, contrary to what was observed by Fraser (1964), who found the same electrophoretic pattern both for the primitive and the definitive strain hemoglobins. However the chromatographic analysis is much more discriminative than the electrophoretic one.

Manelli (1963), Raunich *et al.* (1966) have suggested the existence of a second generation of erythroid cells, which appear at about 5 days of incubation. However our data do not support the hypothesis that these cells synthetize types of hemoglobin which differ from those of the primitive and

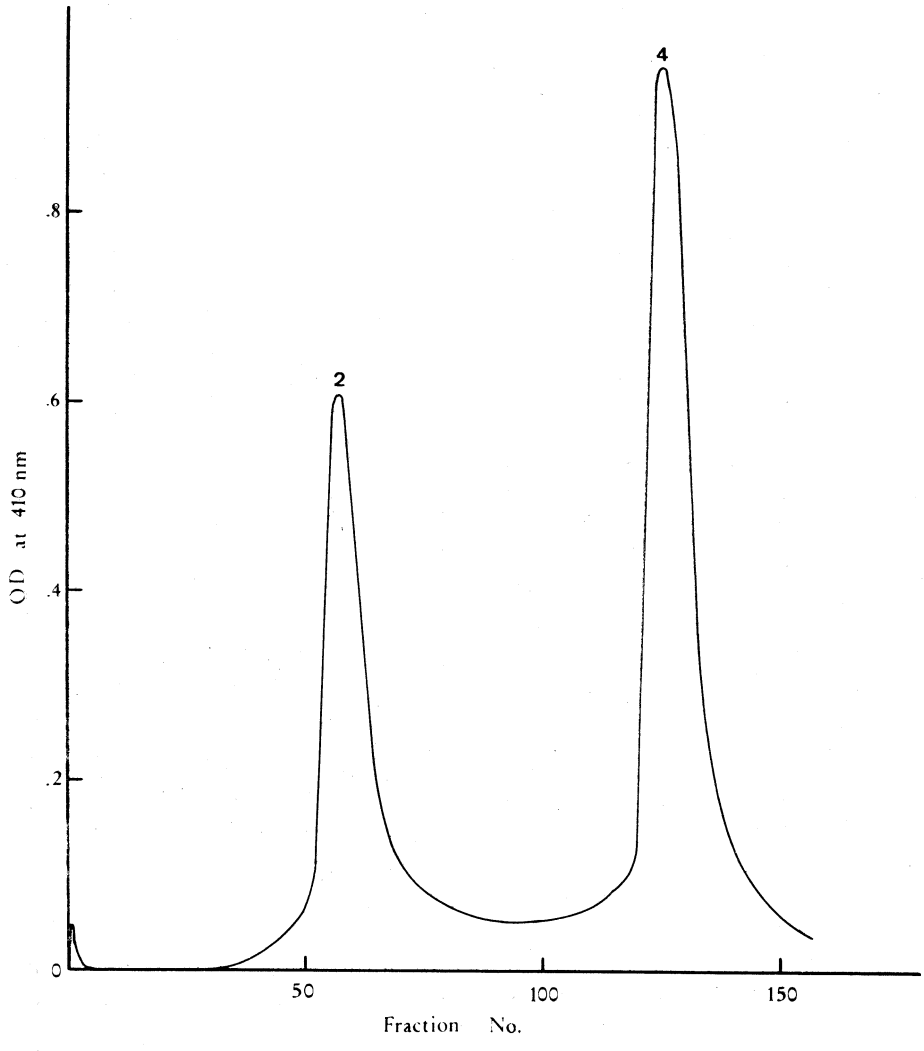
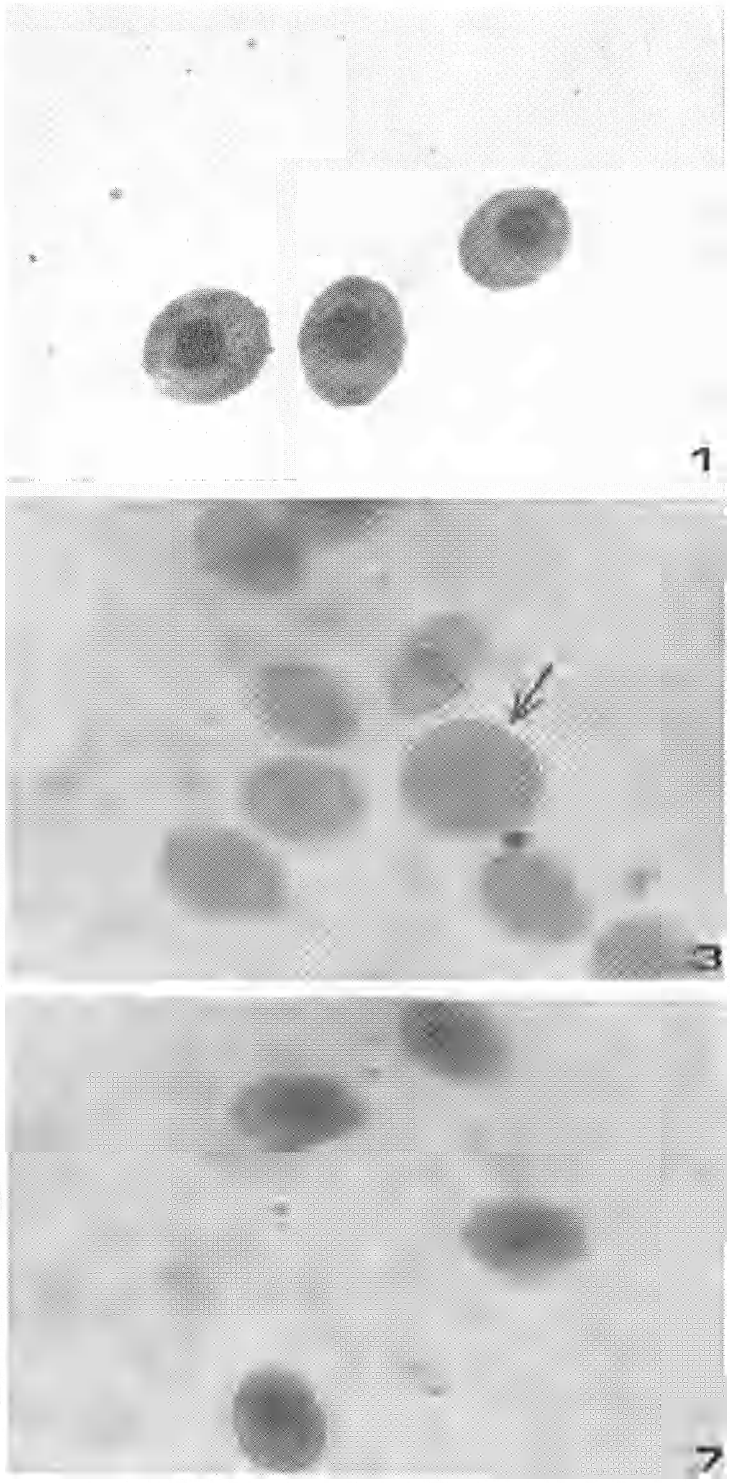


Fig. 6. – Elution pattern of new hatched chick: same experimental conditions as in fig. 2. The two fractions are the same as those typical of the adult chicken.

definitive strain: in fact, the chromatographic patterns of the hemolysate of 3-, 4-, 5- and 6-day old embryos do not show any significant difference.

In all the elution patterns presented here, a peak eluted at the beginning of the gradient is consistently present. The magnitude of this peak changes during development, and its ratio O.D. 410/280 nm is less than 1. These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3
7

results are in agreement with the data of Schalekamp *et al.* (1972), who assumed that the red colour of the peak is due to " withdrawal of hemoglobins with the breakthrough volume ".

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EXPLANATION OF PLATE I

Fig. 1. – Blood smear of 5-day embryo. May-Grünwald-Giemsa stain. 1000×. About 90% of the erythrocytes belong to the primitive strain, and appear as large elements, almost circular in outline, with a round granular nucleus.

Fig. 3. – Blood smear of 7-day embryo. May-Grünwald-Giemsa stain. 1000×. The definitive strain erythrocytes appear as oval cells; the nucleus of these cells maintains its round and granular aspect. The arrow indicates an erythrocyte of the primitive strain.

Fig. 7. – Blood smear of new hatched chick. May-Grünwald-Giemsa stain. 1000×. All the erythrocytes belong to the definitive strain; the cells are oblong, with an oval compact nucleus.