# Hemoglobins in Single Chick Erythrocytes as Determined by a Differential Elution Procedure

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The switch from embryonic to adult hemoglobin (Hb) has been studied *in vivo* by a correlated cytological and electrophoretic analysis of circulating red blood cells from early, purely embryonic-Hb stages to purely adult-Hb stages including the adult chicken. It has been discovered, by using an acid buffer treatment that selectively elutes adult but not embryonic Hb from intact red blood cells, that embryonic and adult Hbs occur together in single cells, and that the switch occurs simultaneously in all cells. These results together with knowledge of the chick erythroid cell dynamics and ontogenetic titers indicate that the initiation of adult Hb synthesis occurs in the circulation in cells previously committed only to embryonic Hb synthesis.

## Introduction

In embryos of many vertebrates a number of Hbs exist that during development are replaced by different Hbs characteristic of the adult animals. Sequential synthesis of various forms of Hb has been observed in a wide variety of vertebrates [1, 2].

The Hbs of the chick embryo and the adult have been studied extensively [3 — 5]. There is a disagreement on the number of Hb types present in the chick embryo, but estimation of the number depends, among other things, on the method of analysis and on the age of the embryos examined [2]. The switch from embryonic to adult Hb begins to occur after day 5.5 of incubation; about half of the Hb is of the adult type by day 7, while only traces of embryonic Hb remain after day 11 of incubation [6].

An important aspect of the control of synthesis of embryonic (fetal) and adult Hb is whether both proteins occur together in single red cells ("co-habitation"), or reside exclusively in separate cell types. If there is co-habitation, this could come about either by turning on of adult Hb synthesis in circulating embryonic erythroid cells, or by the release of a new, intermediate (bi-typic) cell into the circulation from the hematopoietic center(s), or both. This would suggest that the mechanism for the Hb switch is based upon regulative changes in the synthesis of two different proteins in the same cell. If, on the other hand, different populations of blood cells are involved, the switch from the embryonic to the adult type might be due to the release into the circulation of new blood cells containing only adult Hb.

The question has been answered in favour of co-habitation by immunofluorescence in *Xenopus laevis* [7] and in man [8, 9], by the alkali denaturation process [10], as well as by a selective elution method in man [11], but in favor of separate cell types by immunofluorescence in *Rana catesbeiana* [12].

In the present work, the Kleihauer selective elution method was used to investigate the question of co-habitation in the chicken. This consists of incubating erythrocytes in a mildly acidic buffer which dissociates adult (human) Hb in such a manner that it allows the Hb to escape through the cell membrane leaving ghost-like cells after staining for protein with Wright's stain. In contrast, it has no effect on embryonic (human fetal) Hb, leaving embryonic erythrocytes indistinguishable from untreated controls. Chicken erythrocytes display a dramatic difference in staining intensity between embryonic and adult cells after but not before such treatment.

## Materials and Methods

### Embryos and blood collection

Embryos and adult chickens were of the White Leghorn breed. Eggs were incubated at 37.8 °C.

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Blood from embryos up to 6 days of incubation was obtained directly from the heart [13, 14], from older embryos by pricking a major extraembryonic blood vessel, and from adult birds by cardiac puncture. Sodium citrate (5%) was the anti-coagulant. The embryos were staged according to Hamburger and Hamilton [15].

### Preparation and quantitation of Hb

Citrated blood was placed in tubes containing saline (0.9% NaCl), centrifuged to remove the plasma, and further washed 3 times with saline. For lysis, 2 pellet volumes of $H_2O$ were added to the compacted cells. After freeze-thawing $2-3$ times, the stromal mass was removed by centrifugation. The Hb containing supernatant fluid was stored at $-80\,°C$ for no longer than $4-5$ days before use. The relative concentration of Hb in the various samples was determined photometrically with a Beckman DB-G spectrophotometer at a wavelength of 414 nm.

### Hb elution from cells

Blood cells from embryos at various developmental stages were washed once with saline, and were resuspended in 6 pellet volumes of saline. Blood smears were made from this dilution. The blood films were air dried and fixed for $1-2$ min in absolute ethyl alcohol. Citric acid-phosphate buffer (18 mM $C_6H_8O_7$; 16 mM $K_2HPO_4$; pH 3.6) was warmed to $36\,°C$ and the fixed smears were immersed in it for about 7 min. Saline at $36\,°C$ was used for the control slides. The slides were rinsed in a stream of tap water, dried, stained with Wright's stain for about 6 min, washed $1-2$ min with tap water, and air dried.

### Test for specificity of Hb elution method

Washed blood cells were treated with the acid buffer (cell pellet: acid buffer, 1 : 6) for 7 min, and centrifuged 2 min at 1720 R.C.F. in a clinical centrifuge. The supernatant (eluted Hb) was centrifuged for 30 min at 12,000 rpm. The presence of a tiny brownish-white pellet suggested that a few cells had lysed during the acid buffer treatment. Non-eluted Hb was prepared from the orignal cell pellet as described above. Saline treated cells were used as controls.

The percent of non-eluted Hb was determined spectrophotometrically by comparison to untreated aliquots of the same blood samples. The types of Hb eluted and non-eluted were determined by electrophoresis.

### Electrophoresis

Hbs were separated by polyacrylamide disc gel electrophoresis as described by Barker [16]. The separating, stacking, and sample gels were polymerized in order in $0.52\,cm \times 6.4\,cm$ pyrex tubes. All samples were of the same total Hb content as determined by spectrophotometry. The current was maintained at 2 ma/gel until the Hb entered the stacking gel, and then raised to 5 ma/gel. Complete separation was accomplished within $25-30$ min. Beyond this time, bands tended to lose their sharpness. The gels were photographed within 3 min without fixation or staining.

## Results

Figs $1\,A + B$ and $2\,A + B$ show that the acid buffer treated cells from both 3.5 and 4.5 day old embryos are indistinguishable from their saline treated controls. This would indicate that these cells synthesize exclusively embryonic Hb. Large round cells presumably correspond to mid polychromatic primary ("primitive") erythrocytes and constitute the predominant cell type at these stages. Small round cells which presumably correspond to the early polychromatic primary erythrocytes form the second largest cell population. Large elliptical cells which may correspond to the late polychromatic primary erythrocytes occur only occasionally [17].

Figs $3\,A + B$ show that the acid buffer treated cells from 5.8 day embryos are intermediately stained as compared to their saline treated controls. This suggests that these intermediately stained cells contain both embryonic as well as adult Hbs. Large round cells could be mature primary erythrocytes, while the smaller round cells could be the less mature late polychromatic primary erythrocytes and early polychromatic definitive erythrocytes [17].

Figs $4\,A + B$ show that the acid buffer treated cells from 7.5 day embryos display a dramatic degree of elution as compared to their controls. The large cells which are not complete ghosts could be mature primary erythrocytes which contain predominantly the adult Hb type. The remaining cell types which are complete ghosts could be cells of the definitive erythroid cell line.
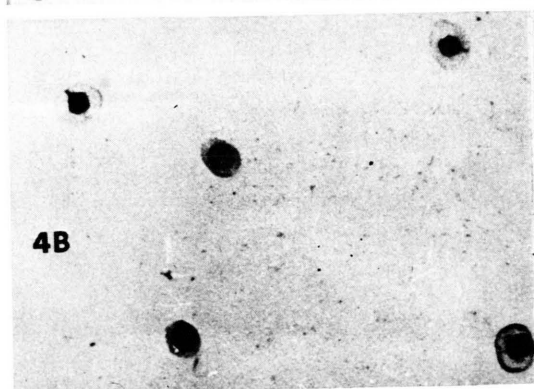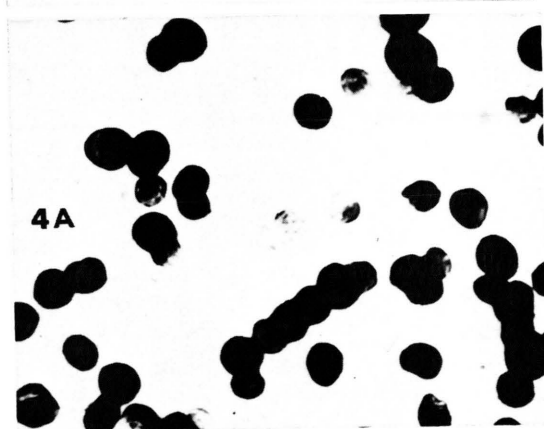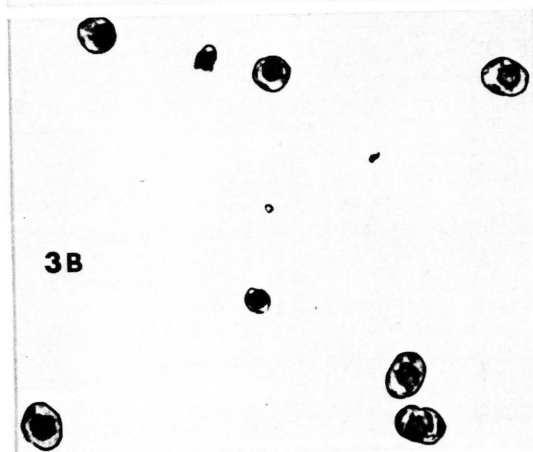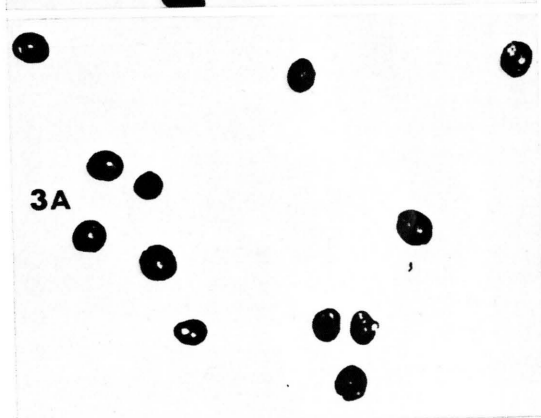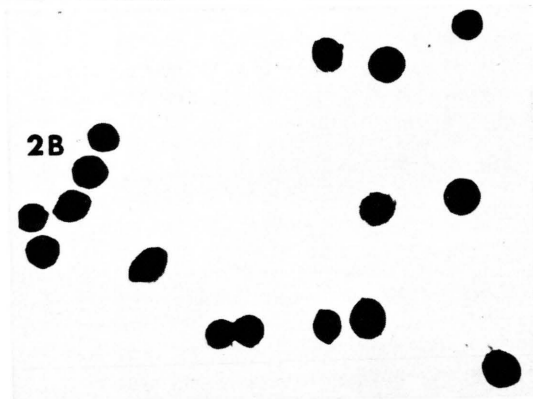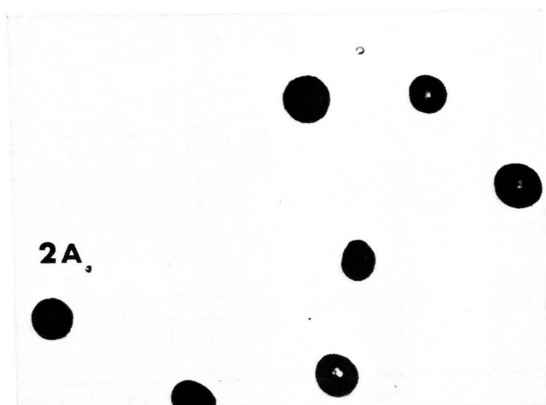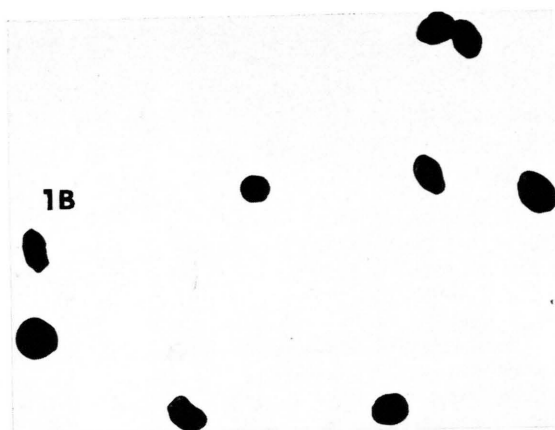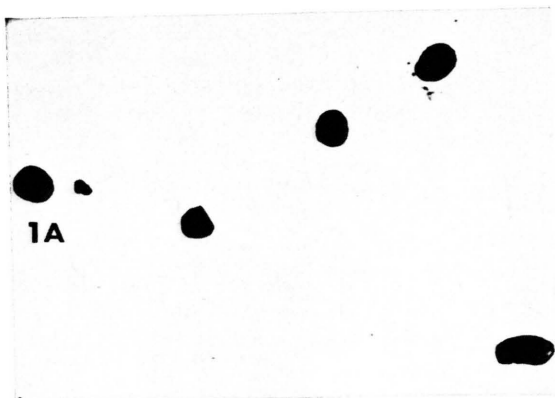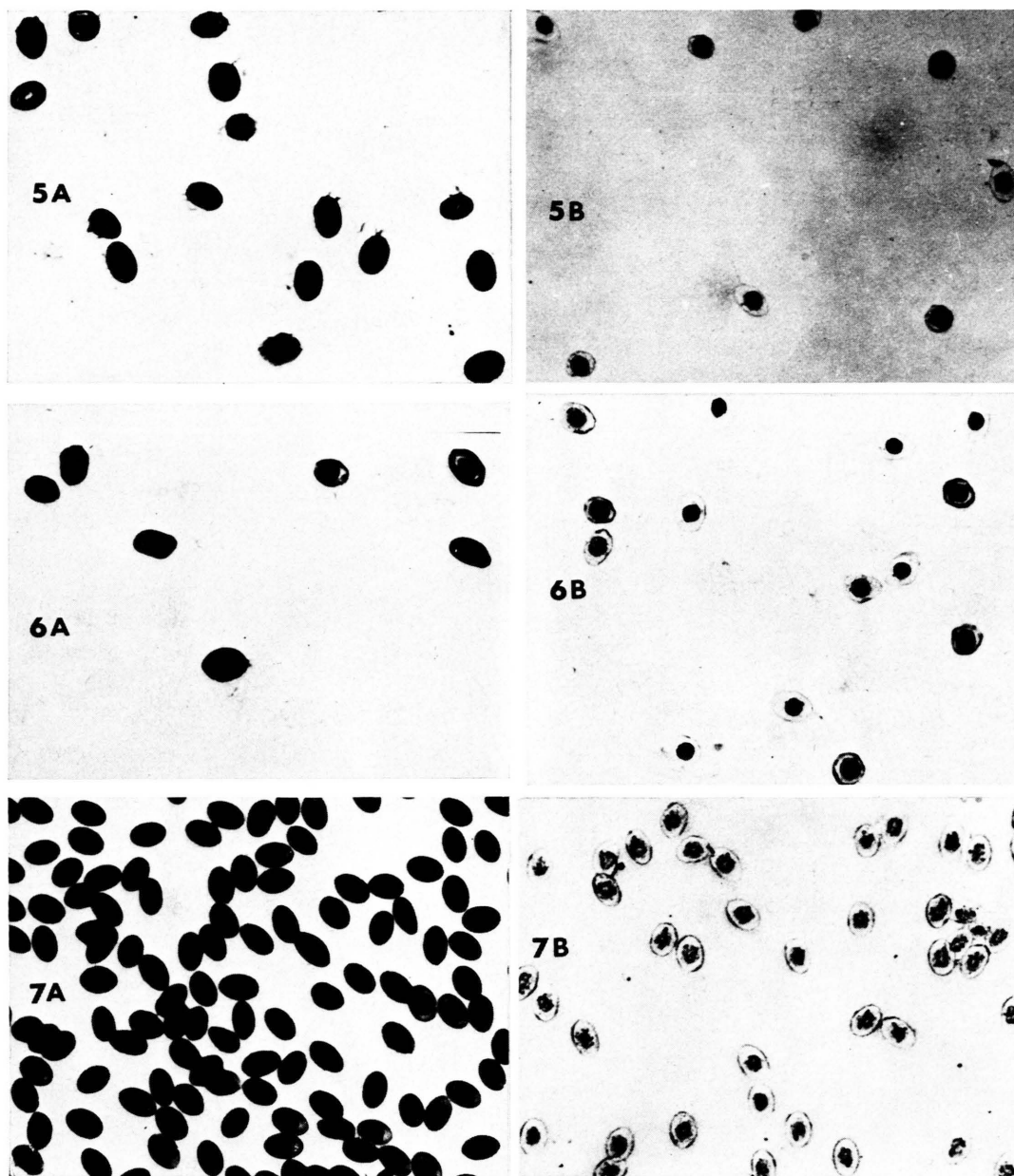
Fig. 1—7 show chick erythroid cells at 3.50 (1 A and 1 B), 4.45 (2 A and 2 B), 5.80 (3 A and 3 B), 7.50 (4 A and 4 B), 10.0 (5 A and 5 B), and 12.50 (6 A and 6 B) days of embryonic development and in the adult (7 A and 7 B). Figures A are the control groups treated with 0.9% NaCl. Figures B are the experimental groups treated with citric acid-phosphate buffer (pH 3.6). After treatment both were stained with Wright's protein stain to reveal non-eluted homoglobin. Magnification X 1050.

Figs 5 A + B show that the acid buffer treated cells from 10 day embryos display, at best, only a slightly greater decrease in staining intensity than the cells of the previous stage. The elliptical cells which constitute the greatest cell population could be late polychromatic erythrocytes of the definitive generation. The semi-elliptical (more rounded) cells could be mid polychromatic definitive erythrocytes, and the large round cells mature primary erythrocytes.

The cytological and Hb elution profiles are essentially the same at 12.5 days (Figs 6 A + B) as at 10 days (5 A + B).

The acid buffer treated cells (Fig. 7 B) from the adult chicken, in contrast to the saline treated cells (Fig. 7 A), are complete ghosts, indistinguishable from the background in stain intensity. This indicates that they contain exclusively the adult type of Hb. The typical ellipsoid cell, the mature definitive erythrocyte, is the only red cell type observed.

The major difference between embryonic and adult erythroid cells is that in the early embryonic cells the nucleus stains heavier than the nucleus of the adult cells.

Fig. 8 represents percent eluted and non-eluted erythroid cells during chick ontogeny. The acid buffer treated erythroid cells from embryos up to day 5 of development remain non-eluted, indistinguishable from their saline treated controls. By 5.8 day, about 99.5% of the acid buffer treated erythroid cells are partially eluted, intermediately stained as compared to their saline treated controls. By 7.5 day, the partially eluted cells, though eluted to a greater degree than morphologically similar red cells of even 1.5 day younger embryos, constitute the 26% of the total erythroid cell population, and are found only occasionally (1%) through day 12 of embryonic development. These cells could be late primary erythrocytes and early definitive erythrocytes as judged by morphological criteria such as cell shape, and nuclear size. 74% of the total population are complete ghosts indistinguishable from their background by 7.5 day. These are late definitive erythrocytes of which the typical ellipsoid cell is the predominant cell type by day 10, and the only cell type after day 16 of embryonic development and in adult life.
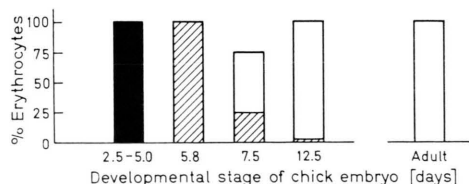


Fig. 8. Percent of non-eluted, indistinguishable from their saline treated controls (■), partially eluted, intermediately stained (▨), and completely eluted, ghost (□) erythroid cells at various stages of chick embryonic development, and in adult life. Elution procedure as described in Materials and Methods.
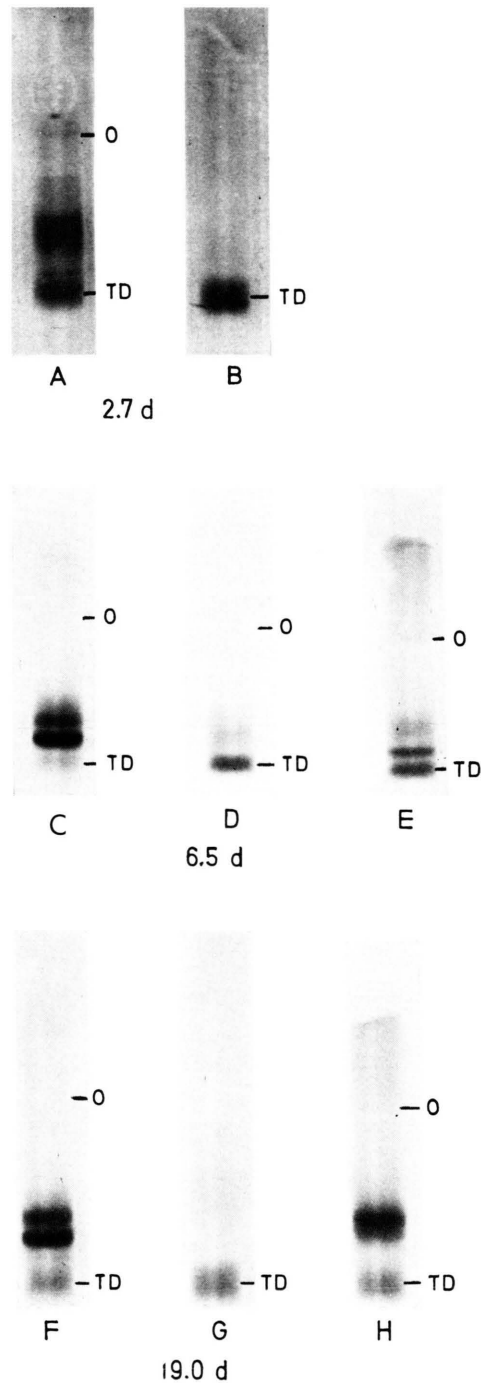


Fig. 9. Polyacrylamide gel electrophoresis of Hb from erythrocytes from various stages of development eluted (B, E, and H) or non-eluted (A, D, and G) by citric acid-phosphate buffer or from 0.9% NaCl treated control cells (C and F). The indication 2.7 day, 6.5 day, and 19.0 day refers to the developmental stage of the chick embryos from which blood was taken. O = origin; TD = tracking dye.

The co-habitation conclusion reached from the cytological study (Figs 1 – 7) depends on the assertion that adult Hb is eluted by the acid buffer, and embryonic Hb is not. To test this technical premise, electrophoresis was done of eluted (supernatant) and non-eluted (pellet-retained) Hb from cells treated with acid buffer as compared to Hb from cells treated with saline (Fig. 9).

Gels A and B (Fig. 9) represent the profiles of non-eluted and eluted Hbs, respectively, from cells of 2.7 day old embryos after acid buffer treatment. It can be seen that all the Hb at this stage is retained in the cells, confirming the non-elutability of embryonic Hb.

Gels C, D, and E (Fig. 9) show control, non-eluted, and eluted Hbs, respectively, from cells of 6.5 day old embryos. The results show that the embryonic type of Hb remained in the cells, while the new Hb type (the Hb of the switch) [13] was eluted.

Gels F, G, and H (Fig. 9) show the control, non-eluted, and eluted Hbs, respectively, from cells of 19 day old embryos. It is clear that all the Hb was eluted, leaving the cells without Hb. This confirms the complete elutability of adult Hbs.

Table I shows the percentage of Hb remaining in the cells after acid buffer elution.

In cells from 2.7 day old embryos Hb remained in the cells. It was observed that the eluted cell pellet, after cell lysis to release the uneluted Hb into the supernatant, yielded a final stromal pellet with a characteristic pinkish color instead of the typical brownish-white color of the control stromal pellet. This could suggest some form of complexing of Hb with the cell stroma as an after effect of the acid buffer treatment. This would indicate that the incomplete recovery ("non-elution") of Hb indicated by the data in Table I does not actually reflect any significant elution of embryonic Hb by the acid buffer prior to lysis. This conclusion is borne out by the low $A_{414}$ of the acid buffer supernatant (Table I).

In cells from 6.5 day old embryos 25.9% of the Hb remained in the cells, while no Hb remained in cells from 19 day old embryos.

## Discussion

The cytology and Hb electrophoresis of eluted and non-eluted (Figs 1 to 9) erythrocytes indicate that embryonic and adult Hbs occur together in single avian erythroid cells. The apparent uniformity and suddenness of the appearance of adult Hb as is shown by the presence of an intermediate degree of staining in cell types that were previously synthesizing embryonic Hb solely, together with current knowledge of chick erythroid cell dynamics and ontogenetic titers [17], eliminate the possibility of massive turnover of the circulating red cells. This would indicate that the initiation of adult Hb synthesis occurs in the circulation in cells previously committed only to embryonic Hb synthesis. That is, embryonic and adult Hbs are not cell-specific gene products characteristic of different discrete cell types or differentiation pathways.

A very interesting aspect of these results is the apparent uniformity of this response even at the earliest stages detected. Virtually no unaffected individual cells were obvious by the end of 5 days. This would seem to rule out the possibility that only certain types of preprogammed erythrocytes are capable of making the switch.

The foregoing suggests that the switch which is evident at the end of day 5 of incubation is an "on" switch, that is, it is simply the "turning on" of the adult Hb genes. The embryonic Hb genes could be undisturbed, that is, still "on" temporarily, or they could be shut off simultaneously, leaving their stable product (embryonic Hb) in the cell. Possibly pertinent to this question are the results of Fraser [18], and Zagris [19] who have shown that cells around the time of the switch synthesize more Hb than either the early embryonic or the later cells which synthesize just one kind of Hb. This scheme of genic activity is strongly supported by the result

Table I. Amount of Hb eluted from red blood cells by acid buffer treatment at different stages of development.

| Developmental stage [days] | Fraction | Total $A_{414}$ | Percent of total Hb [%] | |
|---|---|---|---|---|
| 2.7 | saline control pellet | 4.72 | 100 | |
| | acid buffer pellet | 4.11 | 87 | non-eluted |
| | acid buffer supernatant | 0.66 | 0.14 | eluted |
| 6.5 | saline control pellet | 32.80 | 100 | |
| | acid buffer pellet | 8.50 | 25.9 | non-eluted |
| | acid buffer supernatant | 23.40 | 71.3 | eluted |
| 19.0 | saline control pellet | 93.70 | 100 | |
| | acid buffer pellet | 3.10 | 3.3 | non-eluted |
| | acid buffer supernatant | 89.70 | 95.7 | eluted |

of Schalekamp *et al.* [20] who have shown that globin chains characteristic of the switch Hbs are present in blood of chick embryos even 2 days before the time of the switch.

The demonstrated applicability of the selective elution method to the avian system points to some type of physico-chemical similarity between chick and human embryonic vs. adult Hbs. This is presumably due to evolutionary conservation of the relevant parts of the proteins' structure. If so, it might be anticipated that the Kleihauer method should work on nearly any avian, reptilian or mammalian species.

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