HAEMOGLOBIN SYNTHESIS IN FUSED CELLS

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SUMMARY

When primitive erythroid cells from 5-day-old chick embryos are exposed to inactivated Sendai virus they do not undergo haemolysis but fuse with other cells by the normal process of cytoplasmic coalescence. In this way cells actively engaged in the synthesis of haemoglobin may be fused with others that are not. In heterokaryons formed by the fusion of such erythroid cells with cells from established mouse or hamster lines, haemoglobin synthesis initially continues at a high level, but then declines and ceases altogether within a period of about 60 h. This decline affects the synthesis of both haem and globin and reflects the activity of a specific regulatory mechanism, for under these conditions other chick proteins continue to be synthesized. The haemoglobin synthesized in the heterokaryons is entirely chick, and not mouse or hamster, haemoglobin.

INTRODUCTION

When adult hen erythrocytes or the erythrocytes of late (12–15 day) chick embryos are treated with inactivated Sendai virus they undergo haemolysis to produce erythrocyte ghosts. Fusion of such ghosts with tissue culture cells results in the introduction of the erythrocyte nuclei into the cytoplasm of the recipient cell with only a minimal contribution of erythrocyte cytoplasm (Schneeberger & Harris, 1966). The erythrocyte nuclei undergo reactivation in the cytoplasm of the tissue culture cell, resume the synthesis of RNA and DNA (Harris, Watkins, Ford & Schoefl, 1966), and determine the synthesis of chick specific proteins (Harris, Sidebottom, Grace & Bramwell, 1969b; Harris & Cook, 1969); they do not, however, induce the synthesis of haemoglobin (Cook, 1970). On the other hand a more primitive series of erythrocytes from early (3–5 day old) chick embryos do not undergo lysis in the presence of Sendai virus and may thus be fused with other cells by the normal process of cytoplasmic coalescence (Cook, 1970; Harris, 1970). These immature erythrocytes synthesize haemoglobin. When they are fused with other cells, the synthesis of haemoglobin, as measured by the incorporation of $^{59}$Fe, continues in the fused cell, but at a progressively reduced rate. By the fifth day after fusion, haemoglobin synthesis cannot any longer be detected (Cook, 1970). In the present experiments the synthesis of haemoglobin in fused cells of this kind was examined more closely. The incorporation of both $^{59}$Fe and $[3H]$-leucine was monitored and the haemoglobin synthesized purified by appropriate separative procedures.
MATERIALS AND METHODS

Cell lines and cell fusion

5-day-old chick embryo red cells were obtained by breaking eggs onto a layer of phosphate-buffered saline in a Petri dish so that the intact embryo floated on top of the yolk. The embryo was then cut in half and allowed to bleed, the blood being collected with a Pasteur pipette.

The A9 and MSWBS cell lines have been previously described (Harris et al. 1969a). WG3 is a pseudo-diploid established line of Chinese hamster cells (Westerveld, 1971). The techniques of cell culture were essentially those described by Harris et al. (1969b). The A9, WG3, or MSWBS cells were irradiated with a gamma source before fusion, as described in the foregoing paper.

The cells were fused together by means of inactivated Sendai virus as described by Harris et al. (1966).

Incubation procedures

The heterokaryons were incubated in 4- or 20-oz. (114- or 570-ml) medical flat bottles in Eagle's medium (Eagle, 1959), containing 10% foetal calf serum but without added leucine. The cells were labelled with 2 μCi/ml of 59Fe (1-10 mCi/mg of iron) and/or 4 μCi/ml of 1-10 mCi/mg of iron and/or 4 μCi/ml of [3H]-leucine (17 Ci/mmol) (Radiochemical Centre, Amersham, Bucks.).

After the heterokaryons had adhered to the floor of the bottles, unfused red blood cells were removed from the monolayers by washing the cultures 10 times with small volumes of phosphate-buffered saline at pH 7.3. Control experiments have shown that all unfused red blood cells are removed by this procedure (Cook, 1970). Cells were harvested by trypsinization, spun down, washed 3 times in phosphate-buffered saline and counted in a haemocytometer. They were then spun down again, resuspended in 0.5 ml of the appropriate buffer, and stored at −70 °C.

Separation of haemoglobin

Acid-butanone method. Cells labelled with 59Fe were washed and resuspended in 0.5 ml of 0.1 M Tris 0.01M EDTA buffer, pH 7.5, and lysed by freezing and thawing 3 times. 0.2 ml of 1 N hydrochloric acid was then added to the cell extracts followed by 1 ml of ethyl methyl ketone at 4 °C. All subsequent operations were carried out at 4 °C. The mixture was mixed thoroughly and the 2 phases were then separated by centrifugation at 500 g for 5 min. 0.1-ml samples of the butanone phase were then pipetted onto 2-cm GF/C fibre disks (Whatman) and the butanone evaporated by heating at 37 °C for 1 h. The radioactivity in each disk was then determined by liquid scintillation counting.

Carboxymethyl cellulose chromatography. The cells in 0.5 ml of 0.01 M sodium phosphate buffer, pH 6.9, were lysed by freezing and thawing 3 times. Cell debris was removed by centrifugation at 500 g for 5 min. Additional particulate material was removed by centrifugation at 105,000 g for 2 h. 0.2 ml of the 105,000 g supernatant was mixed with 1 mg of chicken haemoglobin carrier and chromatographed on 10 x 0.8 cm carboxymethyl cellulose columns (CM 32 Whatman). A linear gradient of 0.01 M to 0.2 M sodium phosphate pH 6.9 was used, and 1-ml fractions collected. The optical density of individual fractions was measured at 280, 414 and 540 nm and 0.5-ml samples of the fractions were assayed for radioactivity.

Sephadex G-100 chromatography. The cells were resuspended in 0.5 ml of 0.1 M Tris 0.01M EDTA buffer, pH 7.5, and the soluble protein fraction prepared as for carboxymethyl cellulose chromatography. Columns were eluted with Tris-EDTA buffer, pH 7.5.

Separation of globin. Globin was prepared from haemoglobin solutions by precipitation with acid acetone. The concentration of protein in the haemoglobin solution was made approximately 0.5% (w/v) with bovine serum albumin. Protein was then precipitated from 1 ml of solution by the addition of 20 ml of acid acetone (20 ml conc. hydrochloric acid in 1 l. of acetone) at −20 °C (Ostertag et al. 1972).

Polyacrylamide gel electrophoresis. Precipitated globins were redissolved in 1/100 gel buffer containing 0.1 M 2-mercaptoethanol and 8 M urea. Globins were electrophoresed on 9-cm 11 % acrylamide gels containing 6 M urea in the buffer system of Reisfield, Lewis & Williams (1962). Samples were run at 65 V for 16–20 h.
Haemoglobin synthesis in fused cells

Scintillation counting. Scintillation fluid contained 15 g of 2,5-diphenyl oxazole and 0.25 g of 1,4-di-2-(5-phenyl oxazolyl)-benzene in 2.5 l. of toluene. When aqueous samples were counted, a scintillation fluid/TritonX100 (7:6) mixture was used (Falvey & Staehelin, 1973).

RESULTS

Rate of haemoglobin synthesis

Figs. 1–4 show the results of carboxymethyl cellulose chromatography of the soluble protein fraction from 5-day-old chick embryo erythrocytes and A9/5-day-old chick embryo erythrocyte heterokaryons, exposed to [3H]leucine. Adult hen haemoglobin separates into two major components (Matsuda & Takei, 1963) and 5-day-old chick embryo haemoglobin into one major and one minor component. The minor 5-day-old chick embryo haemoglobin probably corresponds to adult hen haemoglobin All (Davis, 1974).

![Elution profile from a carboxymethyl cellulose column of tritium-labelled 5-day-old chick embryo haemoglobins.](image)

Fig. 1. Elution profile from a carboxymethyl cellulose column of tritium-labelled 5-day-old chick embryo haemoglobins. 2 x 10^6 5-day-old chick embryo erythroid cells were incubated with [3H]leucine (2 μCi/ml) for 5 h. Haemoglobin was prepared from the soluble proteins of the cell lysate by Sephadex G-100 chromatography and precipitation with 45% ammonium sulphate at 4 °C. ▲, cpm x 10^-3; ○, O.D, at 280 nm; ●, phosphate concentration.

Fig. 9 shows the results of polyacrylamide gel electrophoresis of globins from various sources. The faster migrating mouse globin band coincides with band 3 of 5-day-old chick embryo globin. This band is only partially resolved from the Chinese hamster globin band.

The level of haemoglobin synthesis in heterokaryons at various times after fusion was measured by determining the amount of 59Fe incorporated into haem (Fig. 5A and B) and of [3H]leucine incorporated into haemoglobin (Fig. 5B and C). The level of haemoglobin synthesis was high immediately after fusion, but declined exponentially and ceased altogether after about 60 h. Fig. 6 shows the level of haemoglobin synthesis in a range of different populations of heterokaryons containing varying numbers of fused red cells.
Fig. 2. Carboxymethyl cellulose chromatography of the 105,000 g supernatant from the lysate of Ao/5-day-old chick embryo erythrocyte heterokaryons, with adult hen haemoglobin as carrier. The adult hen haemoglobins are numbered after Matsuda & Takei (1963). Heterokaryons were labelled with [3H]leucine (4 μCi/ml) from 0 to 9 h after fusion. The heterokaryon population contained 180 chick erythrocyte nuclei per 100 cells. ●, cpm x 10⁻³; O, O.D. at 280 nm; ×, O.D. at 414 nm; ▲, phosphate concentration.

Species of haemoglobin synthesized

Although it seemed likely that the haemoglobin synthesized in the heterokaryons was chick haemoglobin, the possibility remained that some of the haemoglobin formed might have been specified by genes from the other parent cell. This was tested by examining the haemoglobin synthesized in 2 sorts of heterokaryons: those made with mouse Ao cells and those made with Chinese hamster WG3 cells.

Ao cells were derived originally from a C3H strain mouse (Littlefield, 1963; Earle, 1943). If these cells still possess haemoglobin genes they would be expected to produce the haemoglobins characteristic of this strain (Gilman & Smithies, 1968). It was found that with the electrophoretic system used here, the 2 haemoglobin bands characteristic of C3H mice were seen in both the mature and the mid-term mouse embryo, although their relative proportions differed (Davis, 1974).

Fig. 7 shows the results of electrophoresis of globins purified from the soluble protein fraction of Ao/5-day-old chick embryo erythrocyte heterokaryons labelled with [3H]leucine for 22 h. The optical density trace shows the position of carrier chick and mouse globins. The bulk of the material in the first third of the gel is bovine serum albumin. In the region of the gel occupied by the slower mouse globin, only background labelling was seen. In the region occupied by the faster mouse globin the radioactivity present could be accounted for by the label in the fastest chick globin.
WG3 cells were derived from a non-inbred Chinese hamster (Westerveld, 1971), so that the possibility of genetic polymorphism exists. Blood samples from a number of adult hamsters and from mid-term hamster embryos none the less all showed a single haemoglobin band on CMC chromatography and on electrophoresis at pH 9.5 (Davis, 1974). They also showed a single globin band on electrophoresis at pH 4.3. Although separation of this band from 5-day-old chick embryo globin band 3 was not complete, it was found that hamster haemoglobin eluted with the major 5-day-old chick embryo haemoglobin on carboxymethyl cellulose columns. By pooling only the major peak fractions, chick globin band 3 could thus be eliminated, so that any hamster globin present in the heterokaryons could be detected more easily.

Fig. 8A shows the result of carboxymethyl cellulose chromatography of an extract from WG3/5-day-old chick embryo erythrocyte heterokaryons. Fig. 8B shows the result of polyacrylamide gel electrophoresis of material from region A of the carboxymethyl cellulose gradient. Only background radioactivity is seen in the area occupied by hamster globin. Fig. 8C shows the result of electrophoresis of material from region B. Radioactivity associated with chick globin band 3 is visible.
Fig. 5. Haemoglobin synthesis in A9/5-day-old chick embryo erythrocyte heterokaryons. Heterokaryons were labelled for 9 h with $^{59}$Fe (2 μCi/ml) and/or $[^3]$H]leucine (4 μCi/ml), incorporation of $[^3]$H]leucine into haemoglobin. The heterokaryons contained 30 chick red cell nuclei per 100 cells. A, incorporation of $[^3]$H]leucine into haemoglobin, O and •, respectively. The heterokaryons contained 60 chick red cell nuclei per 100 cells. O and ●, incorporation of $^{59}$Fe and $[^3]$H]leucine into haemoglobin. The heterokaryons contained 180 chick red cell nuclei per 100 cells.

Fig. 6. Incorporation of $^{59}$Fe into haemoglobin in different populations of MSWBS/5-day-old chick embryo erythrocyte heterokaryons. Cells were labelled with $^{59}$Fe (2 μCi/ml) for 9 h. O, MSWBS/5-day-old chick embryo erythrocyte heterokaryons (433 chick nuclei/100 cells); O, △, x, □, heterokaryons with 386, 196, 108 and 52 chick nuclei/100 cells, respectively.

DISCUSSION

Cook (1970) previously observed that when primitive erythroid cells from 5-day-old chick embryos were fused with A9 cells, haemoglobin synthesis, as measured by the incorporation of iron, persisted in the heterokaryons at a high level for a few hours and then progressively declined. In the present experiments, this observation has been confirmed, and it has further been shown that the decline involves not only the incorporation of iron into haem, but also the incorporation of amino acid into
globin. The progressive decline and eventual cessation of haemoglobin synthesis reflects the operation of specific regulatory mechanisms, for it has been shown that under these conditions other chick-specific proteins continue to be synthesized, and their synthesis can even be initiated (Cook, 1970; Harris, 1970; Deák, Sidebottom & Harris, 1972).

The mechanisms by which haemoglobin synthesis is suppressed while the synthesis of several other chick proteins continues is not clear. The primary effect would seem to involve the synthesis of globin, rather than haem, for the decline in haemoglobin synthesis in the heterokaryon is not altered by the addition of haemin to the culture medium (Davis, 1974). The haemoglobin synthesized is entirely chick haemoglobin, so that one might conclude that fusion with chick erythroid cells, themselves active in haemoglobin synthesis, does not induce the expression of haemoglobin genes in the mouse or hamster cell, on the assumption that these cell lines still contain haemoglobin genes. The most likely explanation of the result is that the cytoplasm of the erythroid cells introduces an active haemoglobin-synthesizing system into the heterokaryons and that this system continues to operate for a short period, but then decays. This decay would be explained most simply by assuming that the messenger ribonucleic acids responsible for haemoglobin synthesis are slowly degraded and not replaced by the continued transcription of the haemoglobin genes in the chick nucleus.

It is of interest that the level of haemoglobin synthesis in the heterokaryon is not determined simply by the number of erythroid cells fused with the tissue culture cells.
Fig. 8. Analysis of globins synthesized in WG3/5-day-old chick embryo erythrocyte heterokaryons. A, carboxymethyl cellulose chromatography of a mixture of the 105 000 g supernatants from lysates of WG3/5-day-old chick embryo erythrocyte heterokaryons, 5-day-old chick embryo erythrocytes, and Chinese hamster erythrocytes. The heterokaryons were labelled for 19 h with [3H]leucine (2 µCi/ml). The heterokaryon population had 440 chick red cell nuclei per 100 cells. •, cpm x 10^-3; X, O.D. at 280 nm; ○, O.D. at 414 nm. Pooled fractions from regions A and B of the gradient were mixed with carrier albumin and concentrated to 1 ml in an ultrafiltration cell. The buffer strength was reduced to 0.001 M sodium phosphate at pH 6.9. Globin was precipitated with acid acetone and analysed on pH 4.3 polyacrylamide gels. B, optical density (-----) and radioactivity (●) profile of region A from the carboxymethyl cellulose column shown in A. C, optical density (-----) and radioactivity (●) profile of region B from the carboxymethyl cellulose column shown in A.
As the input of erythroid cells in the fused cell population rises, the initial level of haemoglobin synthesis in the heterokaryon increases disproportionately. The mechanism for this stimulation of haemoglobin synthesis with higher inputs of erythroid cells is at present completely obscure.

REFERENCES


(Received 11 January 1975)
Fig. 9. Polycrylamide gel electrophoresis of various globins. 1, 5-day-old chick embryo; 2, adult mouse; 3, adult Chinese hamster; 4, adult mouse and 5-day-old chick embryo; 5, adult Chinese hamster and 5-day-old chick embryo.