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ONTOGENY OF HAMSTER HAEMOGLOBINS DETERMINED BY ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL

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SUMMARY

Haemoglobin ontogeny in an inbred strain of the golden hamster was determined from 12 days in gestation to adulthood. Haemoglobins, separated by isoelectric focusing in polyacrylamide gel in a linear pH gradient (7·0 to 8·0), were quantified by optical density scanning (420 nm) of the unstained gels. Three species (*adult* haemoglobins) increase in proportion, one (15 % at 12 days gestation) becomes dominant (85 % in adults), and two (nearly absent at 12 days gestation) increase and exist as minor species in adults. Two species (*foetal* haemoglobins) decrease rapidly, one (37 % at 12 days gestation) to trace levels, the other (24 % at 12 days gestation) to a persistent 3 % in adults.

Isoelectric-focused haemoglobins were eluted individually and re-identified by isoelectric focusing, disk-gel electrophoresis, and vertical gel electrophoresis. Gel exclusion studies ruled out the possibility that any one haemoglobin species is a polymer of another.

Haemoglobin proportions determined by the technique used here, isoelectric focusing, are very reproducible, both from sample to sample and from hamster to hamster of any specific age. The presence of a foetal haemoglobin fraction persisting at significant levels into adulthood suggests that this small laboratory animal is a suitable model for studies on foetal haemoglobin synthesis.

INTRODUCTION

Basic mechanisms of haemoglobin ontogeny carry broad biological interest, for they represent an intricate balance of forces influencing the types and amounts of specific proteins synthesized during a period of animal growth and development when these types and amounts are changing radically. Common usage employs *embryo* to describe an organism in the early stages of growth and differentiation, particularly during the formation of fundamental tissues and primitive organ systems. An antenatal vertebrate after the embryonic phase is called a *foetus*. During the development of many animal species from embryo to adult, synthesis of certain haemoglobins ceases and relative proportions of others undergo major shifts. Haemoglobin types present at greatest concentration during these 3 general stages of animal development are appropriately designated *embryonic*, *foetal* and *adult*. Transitions from one type to the next are usually not abrupt. Synthesis of embryonic haemoglobins can extend into foetal life, and foetal haemoglobins are often found postnatally but in rapidly decreasing

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proportions as the animal matures (summarized by Bertles, 1970; Huehns & Beaven, 1971; and Lorkin, 1973). The function and synthesis of foetal haemoglobin are currently prominent research problems. Primates and ruminants as animal models for foetal haemoglobin studies present difficulties of expense and size. Hence a small animal, inexpensive, easily bred, and carrying a foetal haemoglobin into adulthood might accelerate research progress.

Descriptions of the ontogeny of proteins are only as accurate as the techniques used for isolation and assay of the proteins under study. We present in this report the ontogeny of hamster haemoglobin, determined by the separatory technique of isoelectric focusing in polyacrylamide gel, and we provide evidence for the existence of a hamster foetal haemoglobin persisting throughout adult life.

MATERIALS AND METHODS

All hamsters were of the inbred strain LHC/Lak (Lakeview Hamster Colony, Newfield, New Jersey, U.S.A.). Blood was collected from adult hamsters by heart puncture into syringes wet with an isotonic solution of NaCl containing EDTA as anticoagulant (50 mg/ml). Blood was expressed from foetal and newborn hamsters into an isotonic solution of NaCl containing EDTA (1 mg/ml).

Blood samples were equilibrated with carbon monoxide (CO) immediately after collection. Without exception, subsequent procedures were carried out at ice-bath temperature and all buffers and wash solutions were equilibrated with CO immediately before use. Erythrocytes were washed 5 times with aqueous 0·15 M NaCl and lysed by vigorous agitation after addition of 3 vol. of distilled water and 0·4 vol. of toluene. Lysates were cleared of all debris by centrifugation at 105000*g* for 1 h at 4 °C and subsequent filtration through one layer of Schleicher and Schuell no. 595 filter paper. After re-equilibration with CO, haemoglobin solutions were stored at 4 °C. Haemoglobin components in haemolysates were separated by the technique of isoelectric focusing. The apparatus used was similar to that described by Wellner (1971). Carrier ampholytes were obtained from LKB Instruments Inc., Rockville, Maryland, U.S.A. The gel composition was similar to that described by Wrigley (1968): 6 % bisacrylamide, 2 % carrier ampholytes (pH 7·0 to 8·0), 0·05 % ammonium persulphate, and 0·05 % *N-N-N'-N'*-tetramethylethylenediamine. Glass tubes (internal diameter 6 mm; 250 mm long) previously coated internally with Canalco column coating (Canalco Inc., Rockville, Maryland) were filled to within 10 mm of the top with gel solution and overlaid with 2 mm of distilled water to secure a flat surface. Horizontal (not tilted) bands of isoelectric-focused proteins depend on the flatness of this surface. After gel formation (approximately 30 min at room temperature), a current of 1 mA per gel tube was established to remove traces of persulphate known to produce artifactual haemoglobin bands (Brewer, 1967). The amount of haemoglobin applied per gel varied, depending on the ultimate use of the gel. For example, 50 μg were adequate for photometric quantitation, 200 μg for benzidine stain and protein stain, and 400–800 μg for non-stained preparations when searching for minor haemoglobin bands. Haemoglobin samples were applied to the flat surface of the gel in 50 μl of an aqueous solution of 15 % sucrose (w/v). A protective layer of an aqueous solution of 5 % sucrose (w/v) was layered on top of the haemoglobin so that haemoglobin never came into contact with the cathodic solution (0·5 % monoethanolamine), thus preventing denaturation. The anodic solution was aqueous 0·2 % H_2SO_4.

Isoelectric focusing was performed at 4 °C for 16 h. To prevent overheating, voltage was increased gradually over a period of 30 min to a final value of 400 V. Current never exceeded 1 mA per gel tube. Quantification of the various haemoglobins was accomplished by optical density scanning (Densicord, Photovolt Corp., New York) of unstained preparations at 420 nm. Gels were stained for protein with bromphenol blue (Catsimpoolas, 1968), and for haemoglobin with benzidine dihydrochloride.

In preliminary experiments designed to determine if the possible presence of non-haem protein made any contribution to optical density scanning at 420 nm, non-haem protein was

removed from selected samples through use of CM-Sephadex C-50 ion exchange resin (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). No difference was found in haemoglobin proportions determined on these isoelectric-focused gels whether or not non-haem protein had been previously removed.

Polymers of haemoglobin molecules known to form in solutions of frog and turtle haemoglobins (Riggs, Bolling & Agee, 1964; Riggs, 1965) can erroneously indicate the presence of extra species of haemoglobin. As haemoglobin polymerization can be prevented by addition of reagents inhibiting formation of disulphide bonds, the following technique was employed to determine whether any of the bands in isoelectric-focused preparations of hamster haemoglobin were indeed polymers. Haemolysates were prepared as described above and diluted with distilled water to a haemoglobin concentration of 2·5 g/100 ml. To one aliquot was added an equal volume of 0·2 M sodium phosphate buffer (pH 7·0); to another, an equal volume of the same buffer containing iodoacetamide at 6·84 mg/ml, corresponding to a ratio of approximately 10 mol of iodoacetamide per mol of haemoglobin. Both preparations were stored for 24 h at 4 °C under CO, dialysed for 24 h against 0·1 M sodium phosphate buffer (pH 7·0) and stored at 4 °C. As iodoacetamide does not dissociate a dimer already formed, but mercaptoethanol does (Riggs, 1965), 2 vol. of hamster haemoglobin at a concentration of 0·54 g/100 ml in sodium phosphate buffer (pH 7·0) were mixed under a CO atmosphere with 1 vol. of 1·5 M mercaptoethanol in the same buffer. This provides a 50-fold molar excess of mercaptoethanol over haemoglobin. To test for polymerization in the above 2 preparations, they were passed through columns of Sephadex G-75 and G-100 (Pharmacia Fine Chemicals). Exclusion limits of the Sephadex columns were first confirmed by passage of substances of known molecular weight. The same columns were used throughout all determinations.

Disk-gel electrophoresis of haemoglobin was carried out as described by Davis (1964).

RESULTS

In the genus *Mesocricetus*, birth occurs precisely 16 days after conception, terminating gestation comparatively early in foetal development. The earliest stage at which haemoglobin in quantities adequate for our techniques could be obtained was 12 days after conception, although of course haemoglobin synthesis proceeds in younger embryos. At this point, embryos measured 10 mm (crown–rump) and 20 embryos provided approximately 0·1 ml of packed erythrocytes. Fig. 4 (p. 686) provides a visual demonstration of chronologic changes occurring in electrofocused preparations of hamster haemoglobins during ontogeny. Haemoglobin proportions determined by this technique were very reproducible, as were proportions from hamster to hamster of any specific age. Bands on gel cylinders stained with benzidine (for haem protein) or with bromphenol blue (for protein) were identical in position to those visible in unstained gels, with the exception that protein staining revealed several non-haem protein bands, at the anodic end of the gel, with isoelectric points between 5·0 and 5·5. As observed by us, 11 haemoglobin bands are found in the 12-day foetus, numbered from the cathodic end. Six bands (1, 2, 4, 8, 10 and 11), clearly visible at 12 days in gestation, decrease with time and disappear by the day of birth or immediately after (Fig. 4). Bands 3 and 6 (Hb F_I and Hb F_{II}) comprising 37 % and 24 % respectively of total haemoglobin at 12 days gestation (Fig. 1, F_I, F_{II}), gradually decrease to 10 and 7 %, respectively, by the day of birth. At 12 days after birth Hb F_I has decreased to less than 1 %: it can be seen by benzidine stain when large amounts of haemoglobin (1200 μg/gel) are electrofocused. Hb F_{II} decreases slowly and persists in the adult at a level of approximately 3 % of total haemoglobin.

Disk-gel electrophoresis demonstrated the same bands, but with considerably

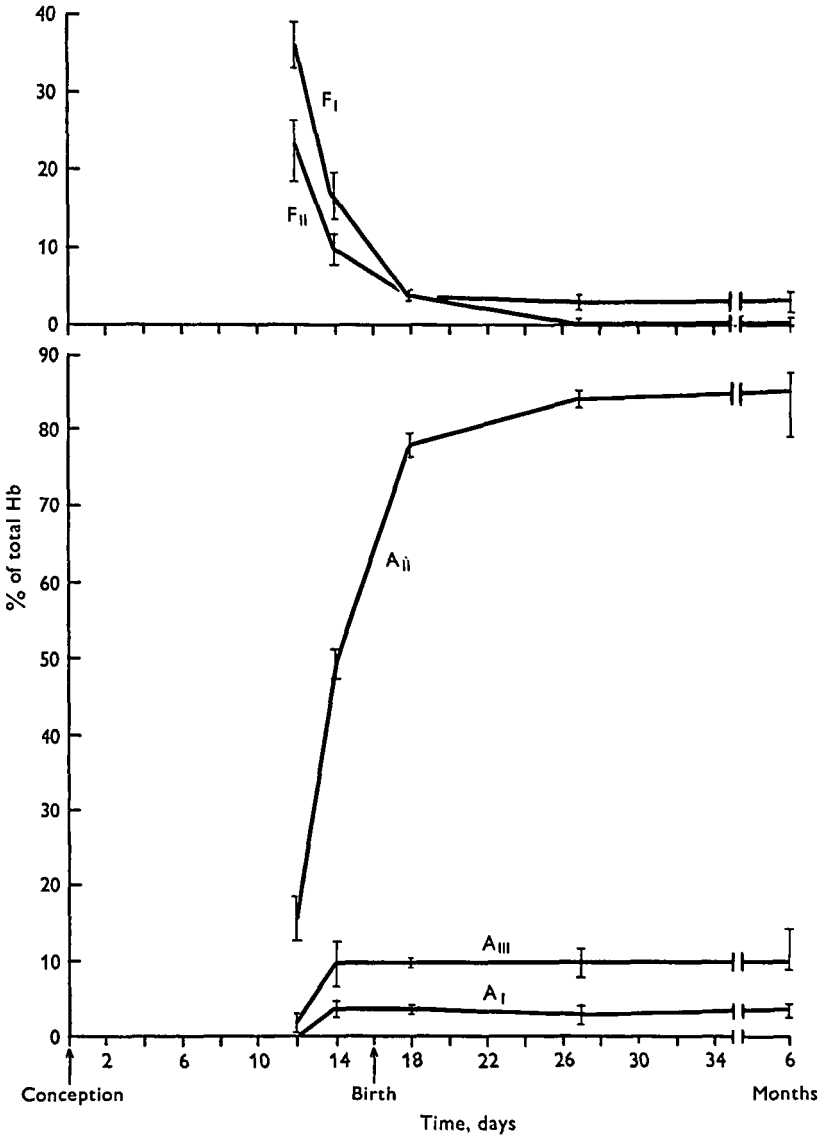

Fig. 1. Ontogenetic changes in proportions of hamster haemoglobins. Relative proportions remained constant after approximately 28 days from conception. Each point represents the full range of triplicate determinations on blood from either 10 individual hamsters, or on pooled blood from the foetuses of 3 pregnant hamsters on 10 separate occasions.

poorer resolution. A representative correlation of haemoglobins in the electrofocused gel with their isoelectric points, and confirmation of the linearity of the pH gradient in the gels, is shown in Fig. 2. In order to assay reproducibility of pH gradients from gel to gel, 21 isoelectric-focused gels were sliced into 1-cm segments and ampholytes were eluted into distilled water. Overall reproducibility was $\pm 0{\cdot}07$ (1 S.D.) pH units. Although under conditions of these experiments pH values at room temperature ran

approximately 0·2 units higher than those at focusing temperature, the convention has been to express isoelectric points at room temperature pH (Wellner, 1971; Drysdale, Righetti & Bunn, 1971). They are expressed this way in this report.

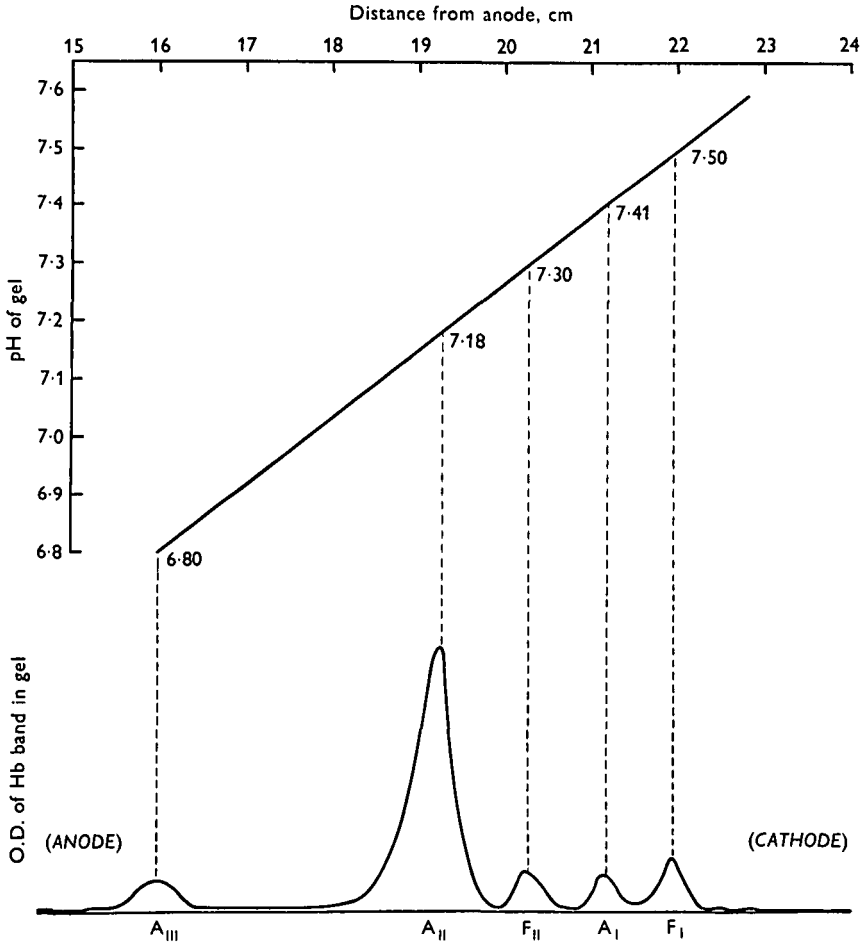

Fig. 2. Optical density scan (420 nm) of hamster haemoglobins (at day of birth) separated by isoelectric focusing in polyacrylamide gel. The haemoglobins migrate to positions in the linear portion of the pH gradient. Haemoglobin peaks are matched here with their isoelectric points measured directly in the gel.

As turtle, frog and mouse haemoglobins polymerize (Riggs *et al.* 1964; Riggs, 1965) it was necessary to examine hamster haemoglobins for this possible explanation of multiple fractions. Representative haemolysates used in determination of haemoglobin proportions were passed through columns of Sephadex G-75 or G-100. All haemoglobin eluted from these columns at a position identical to that of the eluted position of albumin (mol. wt. 60000). No haemoglobin was excluded by either resin so haemoglobin polymerization did not occur under our experimental conditions We did

note, however, that haemoglobin solutions stored for longer than 48 h at 4 °C demonstrated a splitting of the Hb A_{II} band of isoelectric-focused preparations, but that the presence of iodoacetamide or mercaptoethanol prevented this phenomenon. Testing of these haemolysates stored in the cold in the absence of iodoacetamide or mercaptoethanol demonstrated the presence of a haemoglobin species excluded by the Sephadex G-100 resin. Hence storage of hamster haemoglobin without special precautions may be expected to yield polymerization.

Eluates from bands designated as F_I, F_{II}, A_I, A_{II} and A_{III} were dialysed against 0·01 M sodium phosphate buffer (pH 7·4) for 72 h, and spectra were determined (600–240 nm) on a Cary 14 recording spectrophotometer. All spectra were characteristic of carbonmonoxyhaemoglobin. Eluted individual haemoglobins from isoelectric-focused gels, after concentration, were individually subjected again to isoelectric focusing: the behaviour of each was identical to that in the parent gel.

DISCUSSION

Delineation of the ontogeny of hamster haemoglobin presented here has been accomplished by isoelectric focusing, a technique that provides clean separation of multiple haemoglobin species (Fig. 4). Fig. 2 demonstrates the reproducibility both of the method and of hamster haemoglobin ontogeny, for each point of analysis is the full *range* of triplicate determinations on haemoglobins from either 10 individual postnatal hamsters, or pooled foetal blood from 3 pregnant hamsters on 10 separate occasions.

The technical advantage of isoelectric focusing over other methods of electrophoresis lies in the fact that individual protein species seek their isoelectric point, and that each protein 'band' in the cylindrical gel tends to narrow, rather than to diffuse, as electrofocusing proceeds. Slight differences in charge on protein molecules, insufficient to yield differences in electrophoretic mobility, may produce enough differences in isoelectric point to permit separation by isoelectric focusing if the pH range in the polyacrylamide gel is properly selected (Fig. 2).

At the twelfth day of gestation the haemoglobins designated by us F_I and F_{II} make approximately 60 % of total haemoglobin present, whereas the adult hamster's major species, A_{II}, is at the 15 % level. The foetal-adult switch in haemoglobin proportions is accomplished in the next 14 days. During that time 2 minor fractions appear (A_I and A_{III}), and A_{II} finally achieves a stable proportion of approximately 85 %. Haemoglobin F_{II} decreases to a level of approximately 3 % and remains at that level throughout hamster life. F_I decreases to trace levels only.

The disappearance of bands 1, 2, 4, 8, 10 and 11 by the day of birth corresponds to the disappearance of large erythrocytes, some nucleated, from the circulation. These cells are morphologically consistent with yolk-sac erythrocytes: hence haemoglobins 1, 2, 4, 8, 10 and 11 can be defined as embryonic. Limitations on amounts of haemoglobin obtainable from hamster embryos prior to day 12 in gestation prevented acquisition of further information on these embryonic haemoglobins.

Results of other studies on hamster haemoglobins vary from ours in ways probably

explicable on the basis of techniques used to separate the various haemoglobins. Schematics of these separations are depicted in Fig. 3. A represents our electrofocused preparations, B is the pattern obtained by Yasukochi (1970) on starch gel electrophoresis, and C is the pattern obtained by Davies & Bull (1971) on starch gel electrophoresis. Adult minor A_I (our terminology) is absent from B whereas foetal fraction

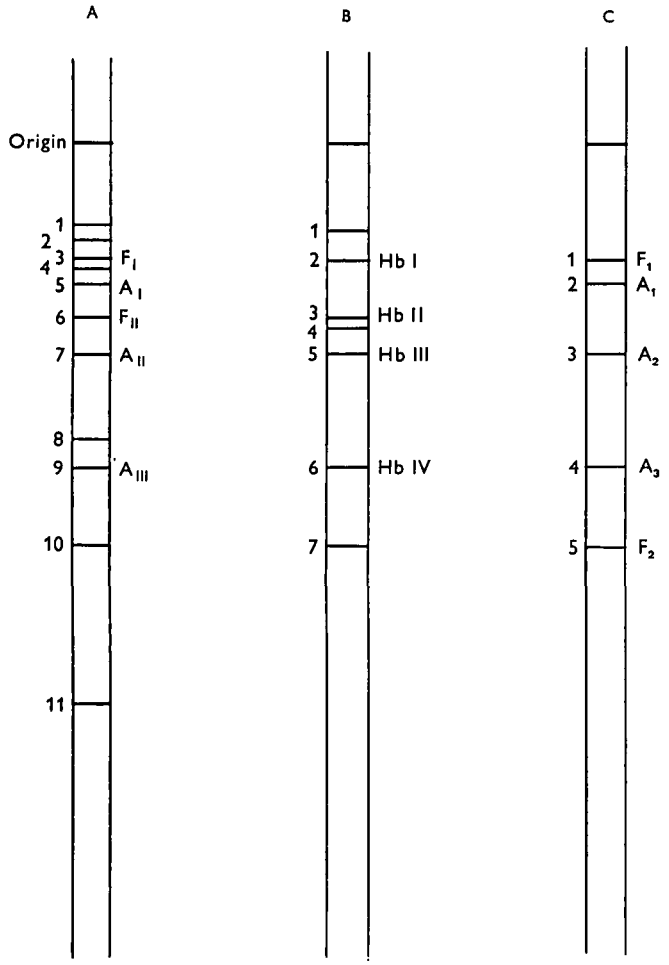

Fig. 3. Comparison of hamster haemoglobins found by us and by other investigators. A, this report (isoelectric focusing); B, Yasukochi (1970) (starch gel electrophoresis); C, Davies & Bull (1971) (starch gel electrophoresis). Designations given by individual investigators to haemoglobin bands are shown to the right of each diagram. Haemoglobin F_2 in C, corresponding to our band 10 in A, is probably an embryonic haemoglobin.

F_{II} is absent from C. In general, the other adult haemoglobins match closely in position. Fraction F_2 in C corresponds to our band 10 and is probably an embryonic haemoglobin.

Yasukochi (1970) reported results of amino acid analysis of individual globin chains from the 4 haemoglobin species he was able to identify. His work suggests that, in our

terminology, F_I, A_{II} and A_{III} share a common α chain and F_{II} and A_{II} share a common β chain. A problem arises in interpreting Yasukochi's analyses of F_I and F_{II}, for he did not find A_I: hence it is likely that A_I was hidden in either F_I or F_{II}, thereby altering amino acid proportions. Globin chain composition is presently under study in our laboratory.

Animals whose erythroid tissue has been studied in search of mechanisms of control of haemoglobin synthesis include frog (Maniatis & Ingram, 1971), toad (Jurd & Maclean, 1970), mouse (Djaldetti, Chui, Marks & Rifkind, 1970), sheep (Adamson & Stamatoyannopoulos, 1973), goat (Barker, Last, Adams, Nienhuis & Anderson, 1973), duck (Bertles & Borgese, 1968) and chicken (Kabat & Attardi, 1967). The advantage of frog and mouse is their size. However, an objection to frog or mouse serving as a model for human foetal haemoglobin synthesis lies in the fact that frog tadpole and adult haemoglobins, and mouse embryonic and adult haemoglobins, are synthesized in separate cell lines, whereas human foetal and adult haemoglobins coexist in individual erythrocytes. Toad deserves further study, for the work of Jurd & Maclean (1970) suggests that toad tadpole and adult haemoglobins occur in one cell line. Sheep and goat demonstrate an erythropoietin-dependent switch from an adult haemoglobin species to an ontogenetically earlier one, but their size places limits on their availability for experimentation. Duck (Borgese & Bertles, 1965) and chicken (Shimizu, 1972) appear to have foetal haemoglobin fractions, but numerous dissimilarities between the avian species and other vertebrates detract from their usefulness.

Work reported here suggests that the hamster is a suitable animal model for studies on control of haemoglobin synthesis. Morphologic homogeneity of adult hamster erythrocytes makes unlikely the possibility of more than one erythroid cell line but does not provide information on possible clonal distribution of the various haemoglobins. Logical next steps are to characterize the individual globin chains, and to determine whether hamster adult and foetal haemoglobins coexist in individual erythrocytes.

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REFERENCES

ADAMSON, J. W. & STAMATOYANNOPOULOS, G. (1973). Activation of haemoglobin C synthesis in sheep marrow culture. *Science, N.Y.* **180**, 310–312.

BARKER, J. E., LAST, J. A., ADAMS, S. L., NIENHUIS, A. W. & ANDERSON, W. F. (1973). Hemoglobin switching in sheep and goats: erythropoietin-dependent synthesis of hemoglobin C in goat bone-marrow cultures. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1739–1743.

BERTLES, J. F. (1970). The occurrence and significance of fetal hemoglobins. In *Regulation of Hematopoiesis*, vol. 1 (ed. A. S. Gordon), pp. 731–764. New York: Appleton-Century-Crofts.

BERTLES, J. F. & BORGESE, T. A. (1968). Disproportional synthesis of the adult duck's two hemoglobins during acute anemia. *J. clin. Invest.* **47**, 679–689.

BORGESE, T. A. & BERTLES, J. F. (1965). Hemoglobin heterogeneity: embryonic hemoglobin in the duckling and its disappearance in the adult. *Science, N.Y.* **148**, 509–511.

BREWER, J. M. (1967). Artifacts produced in disc electrophoresis by ammonium persulfate. *Science, N.Y.* **156**, 256–257.

CATSIMPOOLAS, N. (1968). Micro isoelectric focusing on polyacrylamide gel columns. *Analyt. Biochem.* **26**, 480–482.

DAVIES, J. & BULL, G. M. (1971). Stimulation of synthesis of foetal haemoglobin in adult hamsters. *Trans. R. Soc. trop. Med. Hyg.* **65**, 78–81.

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 321–349.

DJALDETTI, M., CHUI, D., MARKS, P. A. & RIFKIND, R. A. (1970). Erythroid cell development in fetal mice; stabilization of the hemoglobin synthetic capacity. *J. molec. Biol.* **50**, 345–358.

DRYSDALE, J. W., RIGHETTI, P. & BUNN, H. F. (1971). The separation of human and animal hemoglobins by isoelectric focusing in polyacrylamide gel. *Biochim. biophys. Acta* **229**, 42–50.

HUEHNS, E. R. & BEAVEN, G. H. (1971). Developmental changes in human haemoglobins. In *Clinics in Developmental Medicine*, no. 37 (ed. P. Benson), pp. 175–203. Philadelphia: Lippincott.

JURD, R. D. & MACLEAN, N. (1970). An immunofluorescent study of the haemoglobins in metamorphosing *Xenopus laevis*. *J. Embryol. exp. Morph.* **23**, 299–309.

KABAT, D. & ATTARDI, G. (1967). Synthesis of chicken hemoglobins during erythrocyte differentiation. *Biochim. biophys. Acta* **138**, 382–399.

LORKIN, P. A. (1973). Fetal and embryonic haemoglobins. *J. med. Genetics* **10**, 50–64.

MANIATIS, G. M. & INGRAM, V. M. (1971). Erythropoiesis during amphibian metamorphosis. *J. Cell Biol.* **49**, 380–389.

RIGGS, A. (1965). Hemoglobin polymerization in mice. *Science, N.Y.* **147**, 621–623.

RIGGS, A., BOLLING, S. & AGEE, R. J. (1964). Polymerization of frog and turtle hemoglobins. *Proc. natn. Acad. Sci. U.S.A.* **51**, 1127–1134.

SHIMIZU, K. (1972). Ontogeny of chicken hemoglobin. I. Electrophoretic study of the heterogeneity of hemoglobin in development. *Development, Growth Different.* **14**, 43–55.

WELLNER, D. (1971). Electrofocusing in gels. *Analyt. Chem.* **43**, 59A–65A.

WRIGLEY, C. (1968). Gel electrofocusing – a technique for analyzing multiple protein samples by isoelectric focusing. *J. Chromat.* **36**, 362–365.

YASUKOCHI, Y. (1970). Multiple hemoglobins in the golden hamster. *Biochim. biophys. Acta* **221**, 1–8.

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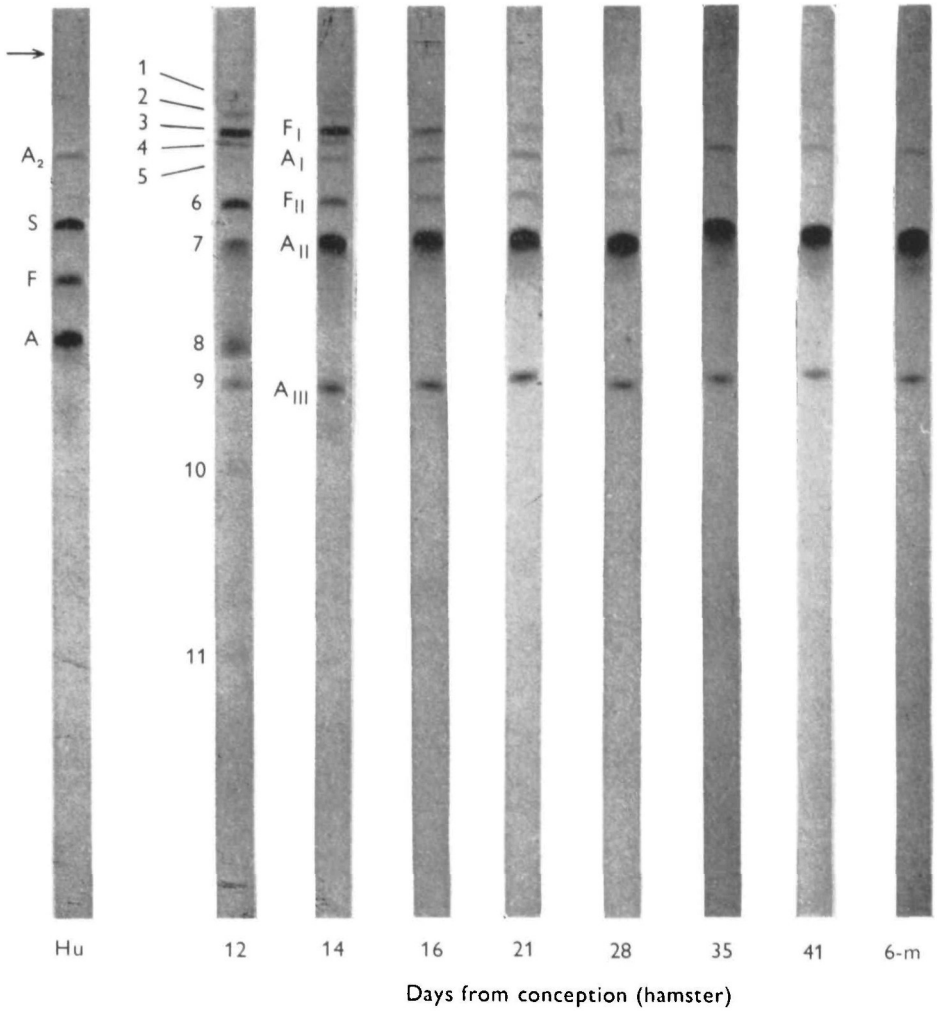

Fig. 4. Visual representation of ontogenetic development of hamster haemoglobins. Each strip is a photograph of an unstained electrofocused polyacrylamide gel cylinder; 400 μg of total haemoglobin were applied at each origin (arrow). For comparative purposes an electrofocused mixture of human haemoglobins (Hu) is shown at the left. Bands 1, 2, 4, 8, 10 and 11 are presumably embryonic haemoglobins. Of the 2 foetal fractions F_{II} persists throughout adult life at approximately 3 % of total haemoglobin present. A_{II} becomes the dominant adult haemoglobin (6-m, 6-month-old animal).