HAEMOGLOBIN SYNTHESIS DURING EMBRYONIC DEVELOPMENT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
GENEESKUNDE AAN DE ERASMUS UNIVERSITEIT ROTTERDAM,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. B. LEIJNSE
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 2 NOVEMBER 1977,
DES NAMIDDAGS TE 4.15 UUR PRECIES

DOOR

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GEBOREN TE BATAVIA

1977
BRONDER-OFFSET B.V. – ROTTERDAM

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CONTENTS

Proefschriften vinden meestal een zeer kleine lezerskring van wetenschappelijk "ingewijden". Dit geldt temeer, wanneer het eigenlijke werk al eerder in de vorm van publikaties is verschenen. De "buitenwacht", die meestal meer belangstelling toont, kan het boekje over het algemeen niet lezen vanwege het specialistisch jargon dat gebruikt wordt. Ik heb daarom geprobeerd het onderwerp waarover dit proefschrift handelt in een ook voor hen begrijpelijke taal in te leiden. De inhoud van dit voorwoord loopt gedeeltelijk parallel met de introduction.

Al van oudsher hebben ontwikkelingsbiologen zich bezig gehouden met de vraag hoe het mogelijk is, dat uit één enkele bevruchte eicel een heel wezen ontstaat, dat is opgebouwd uit een groot aantal weefsels, elk met een veelvoud aan celtypes. Wij weten tegenwoordig, dat de blauwdruk volgens welke een organisme zich ontwikkelt, is te vinden in de erfsubstantie, die zich in de kern van elke cel bevindt. Echter de erfsubstantie van de oorspronkelijke moedercel verschilt chemisch niet van die van de uiteindelijk er van afstammende gespecialiseerde weefselcellen. Hoe komt het nu, dat tijdens de ontwikkeling telkens andere delen van de erfsubstantie werkzaam zijn. Om deze vraag te kunnen beantwoorden is het nodig te weten, hoe het actief zijn van een deel van de erfsubstantie - een gen - kan worden afgelezen en dus herkend. Het ontstaan van een nieuw kenmerk, zoals b.v. het verschijnen van oogblazen, is een heel complex gebeuren, waaraan veel genen te pas komen. Een dergelijk verschijnsel weerspiegelt wel het actief worden van genen, maar is door zijn complexiteit niet zonder meer voor onderzoek vatbaar. Wanneer een gen - een stukje DNA - actief wordt, betekent dit, dat er een complementair molecuul - een stukje RNA - wordt gevormd. Dit RNA maakt zich los uit de kern en brengt, als een stempel, zijn boodschap over op bepaalde elementen uit de cel. Deze elementen voegen zich samen en produceren dan een eiwitmolecuul, zoals dit werd gedicteerd - gecodeerd - vanuit de kern. De gevormde eiwitten kunnen structureel of functioneel zijn, d.w.z. zij kunnen bijdragen tot de vorm en zo tot de functie

van de cel, zoals b.v. het langwerpige contractiele spiereiwit myosine en de doorzichtige ooglenseiwitten, of wel het zijn eiwitten die zorgen dat de cel kan leven, zoals b.v. enzymen die nodig zijn voor de stofwisseling van de cellen. Elk eiwit in het lichaam wordt gecodeerd door bepaalde specifieke genen, zodat omgekeerd gesteld kan worden, dat het verschijnen van nieuwe eiwitten tijdens de ontwikkeling een teken is van het actief worden van de daarvoor coderende genen. Bestudering van de eiwitsamenstelling van jonge zich ontwikkelende wezens - embryonen - kan ons dus iets vertellen over de werking van de erfsubstantie tijdens die ontwikkeling.

Niet ieder eiwit is even geschikt om te volgen. Vele eiwitten komen in alle cellen en in alle stadia van de ontwikkeling voor. Interessant zijn de z.g. orgaan-specifieke eiwitten: eiwitten, die alleen in bepaalde weefsels voorkomen en waarvan de aanwezigheid dus gebonden is aan het verschijnen van een bepaalde morfologische structuur. Via de bestudering van zo'n eiwit kan het ontstaan van een nieuw orgaan gekoppeld worden aan het actief worden van een bepaald gen. De lensontwikkeling en het verschijnen van verschillende lenseiwitten zijn een voorbeeld van een dergelijk onderzoek. Vele organen zijn opgebouwd uit meerdere celtypes, waaronder cellen, die in alle weefsels voorkomen, zoals de cellen van bloedvaten, zenuwen en bindweefsel. Wanneer dergelijke organen worden geanalyseerd, worden zeer veel verschillende eiwitten waargenomen, het percentage orgaan-specifieke eiwitten is meestal erg laag. Bij ons eigen onderzoek hebben wij gezocht naar een weefsel dat zoveel mogelijk uit één soort cellen bestaat en dat een hoog percentage van een orgaanspecifiek eiwit bevat.

Bloed is een weefsel, dat voor meer dan 90% bestaat uit rode bloedcellen - erythrocyten -. In uitgerijpte toestand bevatten deze cellen vnl. één bepaald soort eiwit: de rode kleurstof (haemoglobine). Haemoglobine is een eiwit, dat chemisch goed is bestudeerd. We weten dat het is opgebouwd uit vier eiwitketens (globines). Elk globine draagt een haem molecuul, waarin het ijzer zit. De vier globines - twee aan twee gelijk - zijn zodanig in elkaar gevoegd, dat een geometrische figuur ontstaat met een grotvormige opening er in. In deze opening wordt een zuurstof molecuul opgenomen en vervoerd van de

longen naar de weefsels. De functie van het haemoglobine molecuul - het zuurstof transport - is afhankelijk van deze bijzondere geometrische vorm, die in stand wordt gehouden door bepaalde bindingen tussen de verschillende delen van het molecuul. De plaatsing van deze bindingen en dus ook de structuur van het bijbehorende stuk molecuul zijn essentieel voor het goed functioneren er van. Tijdens de evolutie zijn weliswaar meerdere haemoglobinetypes gevormd, maar deze lijken allemaal chemisch zeer veel op elkaar. Een niet functionerend haemoglobine selecteert zichzelf uit, omdat het een niet levensvatbaar individu doet ontstaan. Deze eigenschap van de haemoglobines heeft gemaakt, dat veel erfelijkheidsonderzoekingen met behulp van dit eiwit zijn gedaan en dit op zijn beurt is er weer de oorzaak van dat over de codering van haemoglobine meer bekend is dan van de meeste andere eiwitten. Zo weten wij, dat elk globine gecodeerd wordt door een specifiek DNA-stuk (globine-gen). Een bepaald globine-gen komt slechts 1 tot 4 maal voor in de erfsubstantie. Het niet goed functioneren van zo'n gen in een volwassen individu veroorzaakt de vorming van te weinig of afwijkende haemoglobines, met als gevolg ziektes als thalassemie. Behalve voor de bestudering van de mechanismes, die tijdens de embryonale ontwikkeling een rol spelen, is het dus ook interessant de haemoglobine samenstelling te bestuderen in verband met bepaalde aangeboren afwijkingen in het bloed. Het actief en inactief worden van de haemoglobine genen wordt tegenwoordig ook nog bestudeerd bij sommige vormen van kwaadaardige tumoren bij dieren, waarbij cellen gevonden worden, die in weefselkweken kunnen worden gedwongen tot haemoglobine vorming, door behandeling met bepaalde chemicaliën. Een bijdrage tot bovengenoemde onderzoekingen wordt eveneens geleverd door waarnemingen zoals wij die doen,over de haemoglobine synthese bij zich ontwikkelende embryonen, omdat hier natuurlijke "switches" in haemoglobine-gen activiteit worden geobserveerd.

Embryonen zijn zeer klein. Onderzoekingen ermee kunnen altijd maar gebruik maken van zeer kleine hoeveelheden materiaal. Hoewel de technieken voor eiwit analysering steeds gevoeliger en specifieker worden is het toch nog steeds nodig, dat materiaal van meerdere embryonen wordt "gepooled". Daarom is het nodig te kunnen beschikken

over genoeg embryonen van een bepaalde leeftijd. Het meest gemakkelijk is dit te verwezenlijken met kippe-embryonen, die met honderden tegelijk in een broedstoof zijn te kweken. Er zijn nog meer voordelen verbonden aan het werken met kippe-embryonen. Eén er van is dat de bloedcellen van kippen - i.t.t. die van vele andere dieren en de mens - hun celkern gedurende de hele levenscyclus behouden, waardoor het in de toekomst mogelijk wordt het haemoglobine patroon terug te vervolgen tot op de ervoor coderende DNA- en RNA moleculen. Een tweede voordeel is dat bloedcellen, die vroeg tijdens de embryonale ontwikkeling worden gevormd - primitieve bloedcellen - morfologisch verschillen van cellen, die later in de ontwikkeling verschijnen - definitieve bloedcellen -, dit eveneens i.t.t. de overeenkomstige celtypes van vele andere dieren en de mens. Dit maakt het ons mogelijk à vue vast te stellen in welk celtype de gevonden haemoglobines voorkomen en ook of het mogelijk is dat twee verschillende haemoglobines - b.v. één van een embryonaal en één van een volwassen type - in dezelfde cel worden gevormd. Een voorbeeld van een dergelijk onderzoek ziet u in fig. 4 in de introduction van dit proefschrift, waar de primitieve (grote ronde) en definitieve (kleine ovale) bloedcellen uit het bloed van 12 dagen bebroede embryonen zijn gekleurd met behulp van stoffen die specifiek reageren met embryonaal haemoglobine. Het is duidelijk dat alleen de primitieve cellen het embryonale haemoglobine bevatten.

Nadat voor kippe-embryonen een aantal technieken waren ontwikkeld, die een nauwkeurige haemoglobine analyse mogelijk leken te maken, hebben wij ook muize-embryonen in ons onderzoek betrokken. Dit gebeurde o.a. omdat de eerder genoemde haemoglobine vormende tumorcellen groeien bij muizen en verder onderzoek ermee werd bemoeilijkt door onvoldoende kennis omtrent de ijzer- en haem houdende eiwitten in haemolysaten, afkomstig van muizen en tumorcellen.

De onderzoekingen, die wij verrichtten in de afgelopen jaren zijn gepubliceerd in drie artikelen, die in dit proefschrift als appendix aanwezig zijn. Een kort Nederlands overzicht van de resultaten wordt in de samenvatting gegeven.

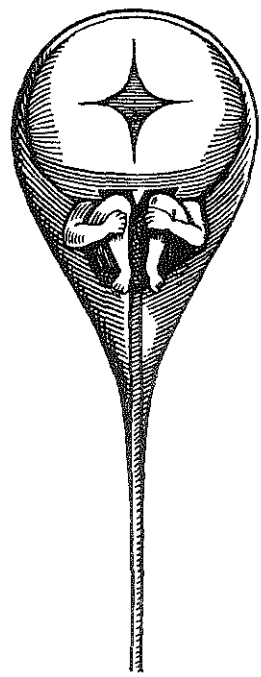

Fig. 1. Homunculus as drawn by Hartsoeker in 1694. Reproduced from Bellairs (1971).

INTRODUCTION

The development of a complete organism from one single cell is a fascinating phenomenon, that has attracted attention from earliest times. However analysis of the mechanism underlying this process has proved difficult. This thesis forms a new attempt. In the introduction, a few lines along which earlier and present-day embryologists have successfully or unsuccessfully tried to elucidate some of these mechanisms are described. Our own work on haemoglobin synthesis during ontogeny is discussed in relation to the literature.

Some basic concepts in embryology, historic overview.

Description of the historical development of embryology (Needham, 1950; Waddington, 1966; Trinkaus, 1969; Bellairs, 1971; Hamburgh, 1971; Brachet, 1974; Graham and Wareing, 1976) indicates that embryology was already a lively science in the 17th and 18th centuries. At that time two contrasting conceptions of embryonic development were debated. One, the theory of preformation, regarded the development of an organism only as growth: a complete miniature organism was thought to be already present in the spermatozoon, according to the so-called homunculists, or in the ovum, according to the school of ovists (fig. 1). The opponents advocated the principle of epigenesis, in which development was thought to be caused by the appearance of structures and functions by progressive differentiation of an in principle unstructured fertilized egg. Epigenists seemed to win the battle in the 19th century, when microscopy showed, that no miniature organism was present in spermatozoon or oocyte. This made embryologists interested in the how's and why's of the increasing complexity of developing organisms. Again two camps arose. The old preformation idea revived in a group of scientists led by Roux (1883). They propagated the theory of mosaic development, in which regions of the embryos - presumptive areas - are thought to give rise to the development of certain circumscript parts of the organism; the development of the entire embryo being the sum of development of the individual parts.

This theory was based on experiments of Roux with frog eggs. He damaged one of the cells in the two blastomere stage - when the egg is divided into two cells - and noted that the other cell was then only able to give rise to a half-embryo. A regulative theory was introduced by another group, led by Driesch. They proposed that after the first cell divisions a developing egg is capable to generate complete embryos not only from the total egg, but also from the isolated blastomeres. The autonomy of the individual blastomeres leading to this possible adaptation was called entelechy, which Driesch interpreted as a non-spatial agent pursuing wholeness of organisms. This view was based on experiments (1891), in which the cells of sea-urchin eggs were separated in the two and later also in the four blastomere stage. The isolated cells were then shown to be able to form complete larvae. As Roux's particular experiment turned out to be exceptional: the amphibian blastomeres are not always mosaic, the regulation theory of Driesch prevailed. Later in development, however, the adaptive properties or regulative abilities of the embryonic tissue, are gradually lost. This results in eventual determination, i.e. the embryonic tissue becomes fixed in an unchangeable developmental pathway. Originally pluripotent tissue becomes unipotent or, in still other embryological jargon, the competence of the tissue diminishes.

As determined cells can only differentiate according to one pathway, the mosaic theory regains validity after determination. The time of determination is different in various species. Some ascidian and amphibian eggs are determined already very early and follow from then on mosaic development, whereas other species retain regulative abilities until the end of gastrulation, the process whereby the organism becomes organized into three circumscript layers. This fact may have caused the diverging results of experimentators in both camps.

Cells which are determined gain eventually a circumscript phenotype, following so-called self-differentiation. Differentiation is defined here as the appearance of a specialized structure, function or biochemical pattern, particular for a given cell type. Such a stage of development evolves only when the corresponding organ is already

recognizable as a primordium, consisting of a definite mass of cells - clusters - (Grobstein, 1959). Therefore it was assumed that the formation of such cell clusters plays a role in cellular differentiation by a mechanism of group action. Here we touch upon an embryological problem which has dominated embryological thinking in the period when the preformation-epigenesis debate had found an answer in which neither of the opposed camps proved to be fully right or wrong. The notion that the formation of cell masses plays a role during differentiation, stimulated in the next decades studies on histo- and organogenesis. Studies were carried out on the movement of individual cells to special parts of the embryo- migration-, on the interaction of cells in the formation of sheets, on the spreading and folding of the formed sheets and on the contact between such sheets (see review Trinkaus, 1969). Such studies taught that in very early embryos, the first movements of cells leads to the formation of polarity and gradient fields, i.e. the embryo becomes orientated. Special systems of order, involving progressively increasing or decreasing concentrations of substances between the two poles of the embryo, are held responsible for the formation of such fields (Needham, 1950; Raven, 1954). At a later stage of development a circumscript organiser, a term indicating a special part of the living embryo, seemed to exert influences, leading to the formation of the neural tube,thereby causing the determination of the embryonic axis. This idea was based on classical experiments of Spemann (1918) with amphibians. However later experiments with amniotes taught that the formation of neural tube could be stimulated with several arbitrary parts of the embryo and even with very unspecific stimuli. In such cases an embryonic axis was not always formed, this proved to be a more complex process than neural tube formation.

Cell contacts at still later stages of development were found to cause so-called induction, a process whereby a short contact between tissues from different embryonic origin evokes further differentiation. A typical example is the induction of the lens vesicle, when the eye-cup touches the ectoderm (described in each embryology textbook). Such studies although very important to learn about the pathways of development, do not fully unveil the

signals that initiate them. Therefore more research on the processes in the cells themselves are necessary to obtain ultimate understanding of the factors leading to embryonic differentiation. On this, even Trinkaus - a front rank cell movement man - agrees. When reviewing cell movement studies (1969) he wrote: "The investigation of various aspects of cytodifferentiation is of course basic, and it is only by such research that we will eventually achieve understanding of how the embryo gives rise to so many different types of cells". Such considerations are the reason that the present-day embryologist shifted his interests from the field of histo- and organogenesis largely to that of cytodifferentiation.

Modern approaches in embryology, current views.

Descriptions of the modern developments in embryology (Davidson, 1968; Teas, 1969; Loomis et al., 1970; Markert and Ursprung, 1971; Brachet, 1974; Gurdon, 1974; Lewin, 1974; Davidson, 1976; Graham and Wareing, 1976) teach that advances have been made especially in the field of cytodifferentiation. These advances have been aided greatly by progress in adjacent research fields as biochemistry and genetics. Use of such disciplines by embryologists, led to a new line of investigation generally known as chemical embryology, a term used first by Needham (1931). In the Netherlands this line of research was picked up by ten Cate (review 1966) and his school.

Advances based on chemistry were mainly due to the use of chemical techniques, more specifically those of histochemistry and of biochemistry. Biochemical analysis of embryonic material was always difficult, as only minute amounts of material are available from the small embryos. The development of new micro-modifications, especially of electrophoresis and chromatography however, allows presently the demonstration of more components in complex mixtures of proteins. Immunochemical methods became another extremely useful tool, especially for the tracing during development of organ-specific proteins in small amounts of biological material. With these methods mixtures of proteins can

be analysed without prior treatment. Organ-specific proteins - sometimes called luxury proteins, in contrast to the more ubiquitous household proteins - are hoped to have morphogenic properties or at least to be closely related to the acquisition during embryonic development of differentiated characteristics. Therefore information on these proteins opens a whole new line in embryology, sometimes referred to as immuno-embryology.

Genetics is another field that greatly contributed to embryology. It accounted for the notion that the genome is identical in all cells of an organism- genome equivalence-. Nevertheless each cell develops along a certain pathway and the organism ends up as a mass of totally different specialized cells. Therefore it was felt, that the genome must encode a developmental program for sequential gene activity. This theory of variable gene activity was highly appealing to embryologists, as it revived on a molecular base the debate between the old preformation and epigenesis theories. In a similar way, other pre-existing theories were modernized to fit newly acquired genetic knowledge. A mosaic theory in molecular terms arose, explaining cell specialization by unequal cleavage of cells, causing yet unknown instructor substances, to become unequally distributed among daughter cells (Graham, 1976). Such instructor substances are thought to be localized in circumscript parts of the cytoplasm and membranes of the mother cell. The particular organiser of Spemann found its counterpart in a reference point, a circumscript instruction centre within the cell exerting a gradient field. Epigenesis at its extreme found its translation in a theory, called - in view of the theories just outlined - the non-instructive theory. This theory accounts for differentiation by growing complexity of intra-cellular environment, without previously existing instruction centres (Graham, 1976). Such theories clearly show how traditional embryological thinking lives on.

Apart from a contribution to the formulation of new theories, genetics also prompted a new line of investigation in embryology. This most modern view on gene activity in embryonic development has just developed and looks very attractive and promising. It will be discussed now. The idea that the onset of gene activity directly

causes the appearance of new structural or functional proteins via a relatively short biochemical pathway is one of the keys that opened the door to this type of research. It implies that now the question how gene expression is controlled can be approached so-to-say going backwards by studying protein patterns. The proteins - being the end products of active genes betray undeniable that the corresponding genes are operative. Information on the protein pattern is thought to lead then back to the understanding of the major levels at which gene expression during development may be controlled. Such control levels are already known from genetic studies and are briefly mentioned here, with their expected relevance to embryological problems.

1. Translational control. Message translation may be verified by establishing the presence of protein-specific messenger RNA. This messenger RNA may be present at a stage where the organ-specific protein is not yet synthetised. In this case only the translation is blocked and this may be due to purely epigenic factors. Effects on translation of messenger RNA can be studied in cell-free systems or by the technique of micro-injection, an experimental method in which protein-specific messenger RNA(mRNA)is injected in e.g. amphibian eggs or other embryonic cells. By such studies Heywood and Kennedy (1976) found myosin-specific mRNA stored in an inactive form in non-differentiated chick muscle.

2. Transcriptional control. The synthesis of protein-specific messenger RNA, its processing, its transport and its stability may be studied in succession. Workers in this field are concerned with the influence of agents, which are called - on the basis of their mode of action- inhibitors, pro-inhibitors, repressors, co-repressors and inducers. In this way it is attempted to elucidate the complex system and to show the individual contribution of these specific agents. An example of such systems is shown by Wareing and Graham (1976) and reproduced in fig. 2 as a modern counterpart of fig. 1. Such studies may contribute in the future to the understanding of the effects on gene translation of the growing environmental complexity in embryos and vice versa. Interesting in this respect are e.g. the studies on the development of the chromatin

proteins - histones and non-histones - which seem to impose directly on the genome its differentiated pattern of gene repression; template restriction being celltype specific.

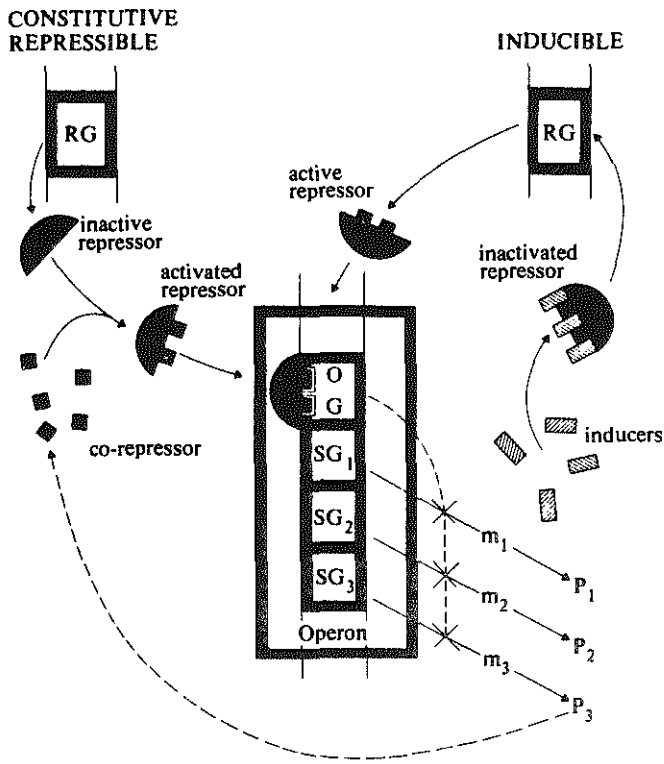

Fig. 2. A representation of the action of the constitutive repressible and inducible systems of gene regulation. The active and activated repressors produced by the regulator genes (RG) are represented as fitting into the operator gene (OG) of the operon. They then block the transcription of the structural genes (SG) of the operon in producing the mRNAs (m_1, m_2, m_3) which would specify the synthesis of the protein enzymes (P_1, P_2,P_3). One of the enzymes is concerned in activating a repressor in the repressible system. In the inducible system, the inducers are represented as inactivating the repressors by preventing them from fitting the operator genes. Reproduced from Wareing and Graham (1976).

The level of transcription is thought to be the fundamental process underlying differentiation and development (Davidson, 1976).

3. Chromosomal control. Protein specific DNA's may be detected and studied using new techniques in which single stranded DNA-copies are used that code specifically for the protein under study. Such DNA- copies are prepared by the use of reversed transcriptase and a protein specific messenger RNA. They are highly labeled with radioactivity and used in hybridization studies, where they hybridize to complementary DNA- or RNA- molecules, which can than be recognized. These techniques can give clues on the number of specific genes involved in the genome—testing of the specific gene amplification theory - and possibly also on the mechanisms acting in eventual gene segregation during development - testing of the directed somatic mutation theory - (Ford, 1976).

The above shows that many techniques are available for the research fields pointed out. These techniques have been developed and are extensively used in studies on monokaryotic systems. Eukaryotes have only lately been introduced to this type of study. A limited numer of models were in use some years ago, when we started our project, but presently more systems become available. Usually they comprise the translation of "adult" gene products, as the experimentators were interested only in questions related to protein synthesis per se. Sometimes the coding messenger RNA's are offered to embryonic translation systems. Examples of models in which "adult" protein-specific messenger RNA's are successfully translated are: adult globin messenger RNA from rabbits (Baglioni, 1963; London et al., 1967; Hunt et al., 1968, 1969; Lane et al., 1971; Lodish, 1971; Lodish and Jacobson, 1972; Giglioni et al., 1973; Lane et al., 1974; Hendrick and Dekloet, 1975; Hendrick and Buch, 1975) and mice (Gilmore and Paul, 1972; Williamson et al., 1973, 1974), translation of globin gene sequences in cytoplasmic DNA fragments (Williamson et al., 1976), translation of myosin-specific messenger RNA's from chickens (Heywood, 1969; Rourke and Heywood, 1972; Thompson et al., 1973), immuno-globulin specific messenger RNA from mouse myeloma cells (Stavnezer and Huang, 1971),

alpha-crystalline-specific messenger RNA from calf lens (Berns et al., 1972 a.b) and delta-crystalline-specific messenger RNA from chick lens (Millstone and Piatigorsky, 1977). It would be of interest to extend such studies and to compare the translation of "adult" and "embryonic" gene products in such systems.

Much work with eukaryotic embryonic cells has already been done on the level of more complex genetic material, as whole DNA or transplanted whole cell nuclei. Briggs and King (1952) were the first to transplant animal cell nuclei. Work has also been done on whole RNA inventarisation and translation (reviewed by Lewin, 1974). From such studies however no information on the level of regulation of protein-specific embryonic genes during differentiation can be expected.

It is the major goal of the present-day embryologist to obtain information on this subject and to correlate such information with pre-existing knowledge on the morphological events during embryonic development.

The present study, orientation.

Which model?

A first approach of embryologists in the field of genetics in relation to development as deliniated above, is to determine whether the appearance of structural and functional properties of developing tissues is correlated with changes in protein composition of the constituent cells. A strict correlation of chemical and histological data is only possible when both the tissue and its chemical make-up are well defined and specific: i.e. the tissue must be easily recognizable, also in immature form and the chemical substances involved must be specific for the tissue and detectable in small quantities without interference from unspecific substances.

Most organs are composed of many cell types, including unspecific ones, such as those of connective tissue, blood-vessels and nerves. Also, most organs are, when not carefully washed, " contaminated" with blood and lymph and in the case of embryos, also with yolk and albumen. The choice of experimental models is therefore

limited. Embryonal tissues which have been screened for protein composition because of a suitable morphological appearance are e.g. eye-lens, heart- and skeletal muscle, cartilage, neural tissue and the melanocytes of retina and neural crest (see reviews Ebert, 1959; Schalekamp, 1963; ten Cate, 1966; Croisille, 1966; Markert and Ursprung, 1971). Such analyses revealed the presence of tissue-specific proteins in these tissues, when differentiated. Crystallins, myosin and actin, chondroprotein, melanin and brain specific proteins were demonstrated respectively. The first appearance and quantitatively changing pattern of such tissue-specific proteins during ontogeny has been studied. One of the interesting results was, that tissue-specific proteins were almost always already present at a stage preceeding cellular differentiation. This is in agreement with the idea that tissue-specific proteins play a role in cytodifferentiation.

Another approach in this line of research has been - because of the relative ease in which chemical constituents of a very specific nature can be detected - the study of the development of specific enzymes or enzyme systems in more complex tissues. Enzyme systems investigated are: dehydrogenases, esterases, kinases, aldolases, transferases, synthetases, phosphatases and polymerases (reviewed by Markert and Ursprung, 1971 and compiled by Markert as editor, 1975). Here also specific shifts in enzyme patterns were observed during ontogeny. Some of these shifts will be discussed later on in relation to our own work.

Our own previous work on proteins specific for the eye-lens (Langman et al., 1957; van Dam et al., 1963) and brain (Schalekamp, 1960 a,b; Schalekamp and Kuyken, 1961; Schalekamp, 1963) during development indicated that a very simple model was preferable to demonstrate a link between morphology and genetics. The blood tissue seemed appropriate.

Composition of blood tissue.

In blood over 90% of the cells are of one cell type. The contaminating leuco- and thrombocytes are easily removed and the

remaining erythropoietic cells can be accuratedly washed,eliminating most of the above mentioned contaminants. Also, there are obvious advantages on the chemical side, in that the erythrocytes contain one predominant highly specific type of protein: haemoglobin, a protein type described in detail in biochemistry, where it is used often as a model for protein synthesis.

Adult haemoglobin.

Haemoglobins are composed of two pairs of identical polypeptide (globin) chains, each linked to a haem group and arranged in a tetrahydral shape with a cavity in the middle to accomodate the haem molecule, which carries oxygen (fig. 3a). This shape, which is essential for haemoglobin function, is maintained by bonds between certain amino acid stretches on the globin chains (fig. 3b), which stretches are therefore little subject to variation. This relative conservatism of the molecule has made it also interesting to geneticists, and normal adult haemoglobin variability is intensively studied genetically today. For a review of early work on haemoglobins in genetics and evolution see Ingram (1963). More recent work has been collected by Kitchen and Boyer (1974). It is now known that each haemoglobin polypeptide chain is coded for by a specific gene, which is present in only a single or a few copies in birds (Bishop and Rosbach, 1973).

The study of pathological haemoglobins, in which mutations have occurred which impair normal functional properties, have played an important role in the understanding of the interaction of genetic regulation and physiological functioning of haemoglobins. Such studies raised the interest of pathologists in normal haemoglobin synthesis.

Recently, oncologists have become also interested in haemoglobin synthesis, this because some RNA-virusses appeared to be able to induce tumors, from which cells can be grown in tissue culture, that are capable to synthesize haemoglobin after stimulation with various inductive compounds. Such cells can be used to study artificially activated haemoglobin genes.

The interest of all these disciplines in haemoglobin

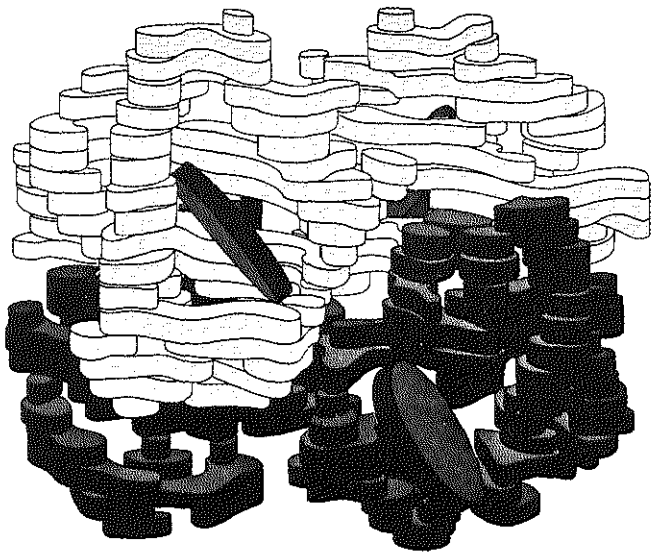

Fig. 3[a]. Haemoglobin molecule as deduced from X-ray diffraction studies. The molecule is built up from four subunits; two identical alpha- and two identical beta-chains. Each chain enfolds a haemgroup (disk), that binds oxygen to the molecule.

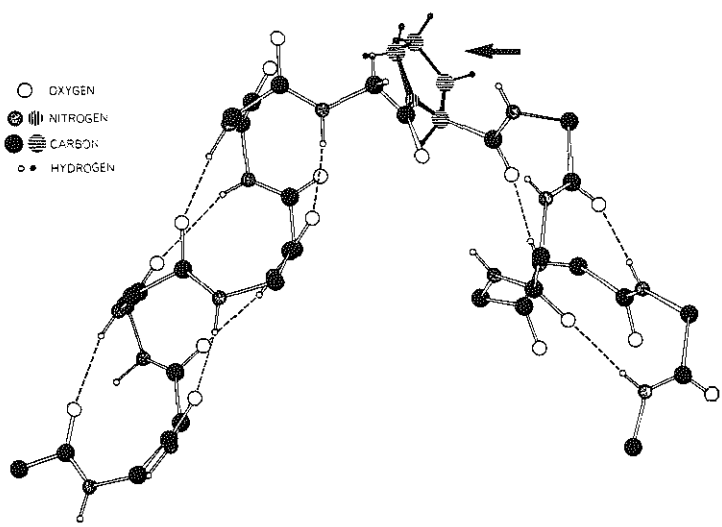

Fig. 3[b]. Corner in haemoglobin molecule occurs where a subunit of the amino acid proline (arrow) falls between two helical regions in the beta chain. Reproduced from Perutz (1973).

synthesis adds to the value of studying this process during embryonic development, as activation of globin genes can be expected to occur naturally here.

Embryonic and foetal haemoglobins.

Embryonic, foetal and adult haemoglobins appear successively in man and various animals (Kitchen and Brett, 1974). Whether the diversity of haemoglobins during embryonic development is functional is as yet not clear. It has been suggested that the oxygen affinity of immature haemoglobin types is higher than that of adult types. This would be functional, as embryos are enabled in this way to pick up enough oxygen in the placenta from the circulation, where pO_2 is lower than in the lungs. Purified human foetal haemoglobin however, shows a lower oxygen affinity than the adult human haemoglobin A; the higher affinity in vivo being due to less binding of haemoglobin in the cells to diphosphoglycerate, which reduces oxygen affinity (Tiyuma and Schimizu, 1969). Foetal blood is anyhow in a priviliged position for oxygen uptake, as at reaching the placenta it undergoes a rise in pH, increasing its Bohr effect (Walker, 1959). Maternal blood in the placenta on the contrary gets a lower pH and therefore the Bohr effect is diminished. With such explanations of facilitation of oxygen exchange in the placenta, there is no need for a special type of haemoglobin in foetuses. Therefore haemoglobin diversity during ontogenic development may just as well reflect phylogenic diversity.

Another interesting aspect of the occurence of foetal and embryonic haemoglobins is the fact that sometimes foetal haemoglobins persist or reappear in blood cells under pathological conditions (Lorkin, 1973). Especially the observation of delayed postnatal disappearance of foetal haemoglobin in infants with D_1 trisomy (Huehns et al, 1964) may be important for the localization of a gene, regulating switches in haemoglobin synthesis.

Much work has been done on the description of embryonic and foetal haemoglobins in various species. However, due to the technical difficulties in handling minute amounts of proteins from small

numbers of small embryos, no definitive conclusions on the number and time of appearance of haemoglobin types have been reached as yet (see table of Kitchen and Brett, 1974).

Which test animal?

Blood cells from the peripheral blood of chicken embryos can be grown economically and synchronized, in very large numbers. Additional advantages of the use of chicken blood cells, especially with regard to the genetic approach are 1. chicken blood cells remain nucleated throughout their lifetime. This will facilitate future work, where protein patterns will be traced back to the relevant RNA- and DNA- fragments of these cells. This may be done in vitro, using for instance copies of DNA- or RNA- molecules in hybridization experiments, or in vivo by re-activation of the dormant nuclei in fusion experiments, as has been done by Alter and Ingram (1975), who were able to fuse erythroblasts to fibroblasts. 2. In chicken blood, primitive and definitive cell strains are morphologically different (Lucas and Jamroz, 1961), which opens the possibility to determine in which cell type the various haemoglobins occur. One example of such a possibility is shown in fig. 4, where in a mixture cell types can be recognized. Another possibility taking advantage of this feature is to separate the cells in differential centrifugation and to compare in that way their genetic make-up.

In one study analyses were performed of haemolysates from mouse erythropoietic cells derived from the peripheral blood or the foetal liver and of haemolysates from leucaemic mouse cells, which were stimulated to initiate haemoglobin synthesis. Liver originating and leucaemic cells reveal more immature cell types, which is attractive as here the initiation of haemoglobin synthesis can be more readily studied.

The present study; scope of the papers.

The body of this thesis consists of three studies. The first two studies (Schalekamp et al., 1972; Schalekamp et al, 1976)

describe the various haemoglobins appearing during embryonic development of chickens and the time at which these haemoglobins first appear. The third study (Schalekamp et al, 1975) deals with mouse and Friend cell haemoglobins and with observations on the synthesis of haem and the handling of iron by immature blood cells.

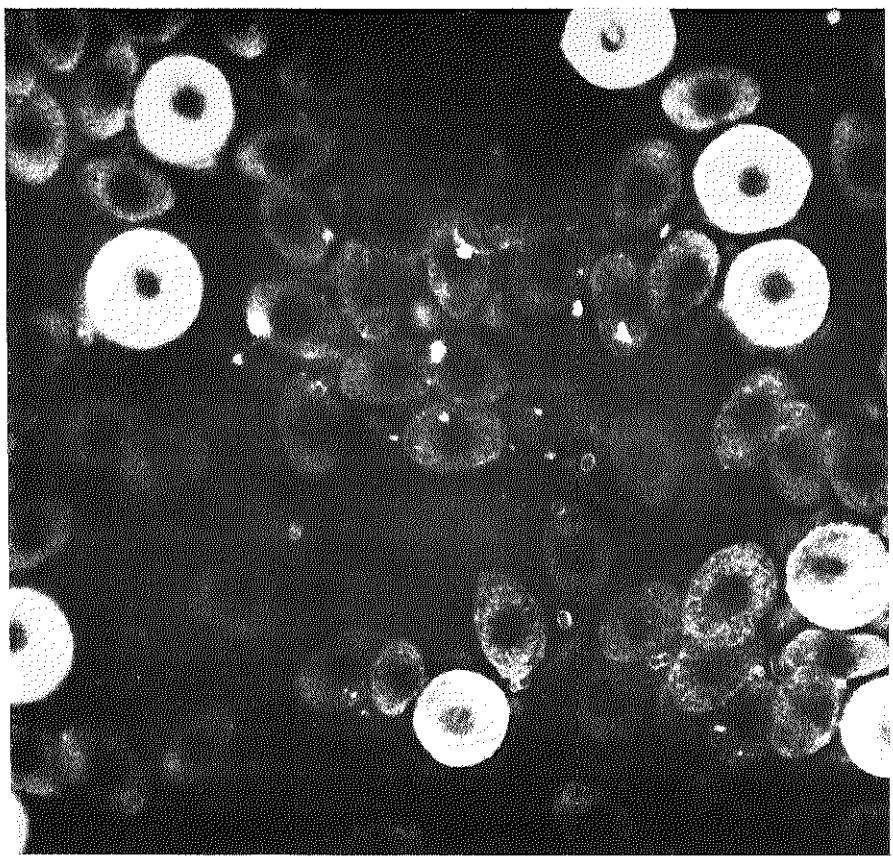

Fig.4.Blood smear of the peripheral blood of a 12 day incubated chick embryo, stained with specific anti-embryonic haemoglobin serum. Large round cells of the primitive blood cell strain show fluorescence. Smaller oval cells from the definitive strain only show background

fluorescence. This indicates that embryonic haemoglobins ($E_3$and E_4) are synthesized exclusively in cells of the primitive strain.

Results of first two studies (chickens).

Three periods of haemoglobin synthesis during chicken embryogenesis could clearly be deliniated. The first period is characterized by the presence of four haemoglobins, two quantitatively major and two minor types. At day six of incubation a sudden switch in haemoglobin synthesis occurs. The two major embryonic haemoglobins disappear and are replaced at once by two other more mature haemoglobin types. A second switch is found at day twelve of incubation, when again two haemoglobins are replaced. After hatching in the post-embryonic period, two more haemoglobins disappear, leaving in adult animals two haemoglobins. These findings suggest activation of globin genes on day six and twelve of incubation. However, in work, described in this paper and dealing with the globin chain composition of the various embryonic and adult haemoglobin types, no new globin chains were found at the time of the first switch. The disappearance of embryonic globin chains on the other hand was obvious enough. The new haemoglobins arising at day six of incubation appeared to be composed of pre-existing globin chains. Only the combination of the globin chains and their quantitative manufacturing were specifically altered. This would mean that inactivation, rather than activation of genes leads to the formation of new, more mature haemoglobin types during this first switch.

This idea received support in the second paper, which describes how the appropriate haemoglobins were isolated and more thoroughly analysed. On the basis of these data, a scheme was designed to explain the formation of the new haemoglobins at day six of incubation by recombination of remaining globin chains after inactivation of embryo-specific globin genes. The inactivation of genes is thought to be responsible for the change in production rate of the remaining globin chains and this in turn to cause recombination.

Similar findings in the literature.

Our findings do not stand alone. Exploration of literature on developmental biochemistry shows parallel findings. This holds for many structural proteins as well as for enzymes, especially the multilocus isozymes. Enzymes mostly represent so-called household proteins, i.e. proteins which are necessary for the basic metablolism of cells. Therefore it would not be surprising if they did not change their nature during differentiation. However, when multiple genes govern such enzymes, giving rise to a number of isozymes, shifts in isozyme pattern during embryonic development occur. Recent literature on isozymology during embryonic development is collected in a voluminous book edited by Markert (1975). Some parallels to our work are listed below.

1. The notion that parts of adult proteins are already present at early embryonic stages in so-called embryo-specific proteins. Adult polypeptide chains have been described in the human embryonic haemoglobins Gower 2 (alpha chain) and Portland (gamma-chain) and in several animal embryonic haemoglobins (summarized by Kitchen and Brett, 1974). Also lens proteins, such as foetal bovine alpha-crystalline have been found to contain adult as well as embryo-specific polypeptide chains (Kamp et al., 1973; 1974). Several embryo- and stage-specific isozymes in embryonic tissues have also been reported to contain adult subunits. Especially during the transition from an embryonic to an adult population of subunits heteromeric enzymes, containing the adult subunit, may exist temporarely as e.g. in the case of aldolase and creatine kinase isozymes during the development of chick skeletal muscle (Eppenberger, 1964; Lebherz, 1975). Such transitory isozymes also arise in cultures of embryonic cells and are then used as a marker of differentiation (Turner, 1975; Kozak, 1977; Lough and Bischoff, 1977).

2. The loss of embryo-specific proteins is well known. Examples are the loss of embryonic globin chains from foetal and embryonic haemoglobins (Kitchen and Brett, 1974) and the loss of crystalline subunits from embryonic alpha - crystalline (Kamp et al., 1973; 1974). Also, the disappearance of embryo-specific enzyme types or subunits has been observed. This has been described

e.g. for L-glycerol 3 phosphate dehydrogenase isozymes from mouse brain (Kozak and Jensen, 1974), for creatine kinase and fructose dephosphate aldolase isozymes from avian and mammalian skeletal muscle (Eppenberger, 1964; Lebherz and Rutter, 1969) and for malate dehydrogenase-1 in teleost fishes (Champion et al., 1975).

3. Specific shifts in the relative proportion of proteins during development. This is known in the case of each newly appearing structural protein. Such a protein gradually or suddenly increases its absolute amount, causing the relative amounts of other proteins to change simuntaneously. The classical lactodehydrogenase profile of developing mouse heart muscle (Markert and Ursprung, 1971) and the more recently described profiles of the five isozymic aminopeptidases during the development of the sea snail Nassarius (Nelson and Scandalios, 1977) and the lactodehydrogenases of very easly mouse embryos (Monk and Ansell, 1976) are only a few examples of such shifting patterns in the case of isozymes.

4. Combination blocks between subunits. An example can be found in the study of Lebherz (1975) on fructose diphosphate aldolase in chicken skeletal muscle. He describes two subunits A and C, able to form five types of isozyme (A_4, A_3C, A_2C_2, AC_3, C_4); the complete molecule being a tetramer. In the muscle of young animals all five isozymes are present, but during development the amount of some of them decreases and eventually becomes zero. This he explains by a gradual loss of activity of the gene coding for subunit C. The relative quantity of the produced polypeptides thus governs the type of isozyme formed; C_4- isozyme type for instance is not formed even when the C unit is made, as demonstrated in the A_3C type enzyme, which is present at the same stage where C_4 is missing. He, and many others explain subunit assembly by a dosage effect in a non-randomized way. However, also discriminating selection of isozyme types has sometimes been claimed. In the Green Sunfish, Champion and Whitt (1976) note only three lactodehydrogenase isozymes: A_4, A_2B_2 and B_4. The other possible intermediates, A_1B_3 an A_3B_1, lack which leads them to the statement, that this is "most likely the result of restricted

subunit assambly or instability of the asymmetrical heteropolymers".

5. A last and very striking parallel to our findings are the studies of Thompson (1975) on the haemoglobin profile of Chironimus Tentans during the last instar. These insects possess monomeric haemoglobin types. There is a striking similarity between Thompson's representation of haemoglobin changes during the development of larvae (fig. 5) and ours of globin chains during chicken development (paper 2, fig. 1b).

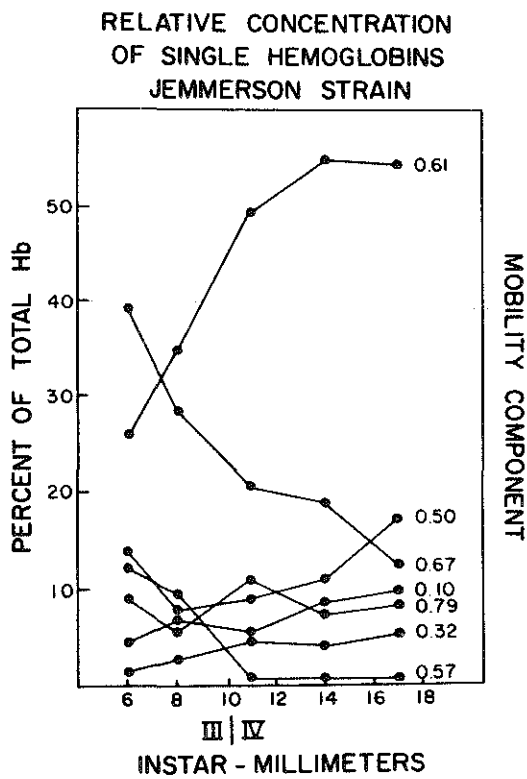

Fig. 5. Shifts in relative quantities of haemoglobins in Chironimus larvae. Reproduced from Thompson (1975).

Here also, a sudden increase in globin production, rather then activation of a gene for a new globin chain seems to accompany a developmental critical switch.

The data cited add to the credibility of our own findings and also point to the possible validity-as a more common principle of the mechanisms proposed to play a role during ontogeny. Our hypothesis adds a new notion in that it proposes that some new, more mature- multiple gene governed- proteins may arise during embryonic development without the need to accept derepression of an extra structural gene. It also provides a means to explain the coordinate nature of changes, when an embryonic subunit is replaced by its adult counterpart. This is more complex when direct activation of two separate genes is considered to be the main regulative power.

Results of third study (mouse).

In this study haemolysates of mouse peripheral blood, of liver originating blood cells and of Friend cells were analysed. This study was prompted by the view, that findings could be obtained, which when integrated, would contribute to the whole project. Especially the observations on the more immature cells would give us a hint for experimental set-up and better understanding when studying in the future the translational control of haemoglobin synthesis during ontogeny. The following information was obtained:

First we could test the value of our methods using mouse haemoglobins. We were able to find with these methods a hitherto undescribed foetal haemoglobin in mice. Several investigators had searched for this haemoglobin, because it seemed unlikely that mice would be exceptional in not having such foetal haemoglobins. A foetal haemoglobin in mice was simultaneously found by Krauss et al. as reported in a conference on haemoglobins (1974).

Secondly, the value of the development of accurate and sensitive methods for haemoglobin determinations became very clear from these studies, where errors were demonstrated, when haemoglobin calculations were based on haem estimation, as for instance in using Drabkin's method. The possibility to determine specific globin chain production separately, will be an even more powerful tool in sutdies on gene activity during development or during

neoplastic deraillement. Globin chain specific antibodies will be very promising in this respect. In studies performed at the moment such anti-globin chain sera have been prepared.

Thirdly, in this study we worked on foetal liver as an additional source for foetal blood cells. The analyses of blood cell fractions, enriched in this way by immature cells, suggested an important role for some haem-containing, non-haemoglobin proteins in these cells. A similar protein fraction was described in immature chicken blood cells (stromal protein, paper 1). However, in this third study we were able to evaluate this protein fraction more thoroughly. Here we established its non-haemoglobin nature more convincingly and also estimated its molecular weight to be very high. This makes it likely that this protein fraction contains the so-called haemin controlled translational repressor substance, which acts as protein kinase, phosphorylating met-$tRNA_f$ binding factors (Gross and Mendelewsky, 1977). This substance is thought to initiate and regulate globin chain translation and further work on it is hoped to unveil some of the mysteries of translational control in haemoglobin synthesis.

Concluding remarks.

This thesis contains three papers, which carry information on haemoglobin synthesis during embryonic development. Haemoglobin synthesis is a model for protein synthesis and as such for gene expression. The use of an embryonic model adds the possibility to study naturally occurring gene switches. The study of such switches is important for the fields of embryology, biology, biochemistry, genetics, neonatology, haematology, pathology and oncology. Although this study does not pretend to be a stride forward in all these fields, it may represent a wobbly step.

SUMMARY

Haemoglobin synthesis during embryonic development is studied since it is a model of protein synthesis in a system in which switches in genetic activity naturally occur. The results are reported in three papers, which are included in this thesis.

In the first paper (Schalekamp et al., 1972) an analysis of the haemoglobin types of chickens in various developmental stages is described. Various techniques such as starch-gel electrophoresis, immuno-electrophoresis and ion-exchange chromatography were used in this study. In the youngest stage investigated (2 days incubated) four haemoglobin types are present (E_2, E_3 E_4, E_5). Two of them (E_3, E_4) are quantitatively the most important (85% of the total amount). At day 6 of incubation the two major embryonic haemoglobins abruptly disappear and two new haemoglobins - an adult type (A_1) and a foetal type (E_6) - appear, replacing haemoglobins E_2 and E_5. After hatching haemoglobins E_1 and E_6 disappear, leaving only haemoglobins A_1 and A_2 in adults.

The appearance of new haemoglobins is mostly interpreted in terms of activation of genes coding for new globin chains. Therefore in the present study the globin chain composition of the various haemoglobins was analysed. This was done on urea-starch gels. However no new globin chains could be detected at the time of appearance of the new foetal and adult haemoglobin types; in contrast, the most striking change at that time was the disappearance of embryo-specific globin chains.

The second paper (Schalekamp et al., 1976) describes a more detailed characterization of the globin chains before and after the haemoglobin switch at day 6 of development of chickens. Using urea-amberlite chromatography the globin chains of various purified adult and embryonic haemoglobins were separated. Each purified globin chain was fingerprinted on thin layer silica gels, also the amino acid composition was determined. The results were in agreement with our first study, in that indeed all the globin chains involved in the formation of haemoglobins A_2 and E_1 were already found to be present before these haemoglobins could be demonstrated i.e. before day 6.

Again the only event at the time of the switch, which could be firmly established, was the disappearance of two embryonic delta chains, indicating repression of embryonic globin genes.

On the basis of these findings we propose a recombination hypothesis, suggesting that new, more mature - multiple gene governed - proteins may arise during ontogeny, not by derepression of a structural gene coding for a new foetal or adult globin chain, but by repression of a gene coding for embryonic globin chains, followed by stimulation of the production of remaining globin chains. This may be illustrated by the following simplified example: Haemoglobin O consists of chains x and y, haemoglobin P consists of chains x and z. Chain x disappears. A new haemoglobin Q with chain composition y and z arises. It is necessary in this concept to assume discriminating combining properties of the globin chinas, since haemoglobin Q is not formed at an earlier stage, despite the presence of its constituing chains. This phenomenon may be caused either by a dosage effect i.e. the quantitative proportions of chains before and after the switch are different, as proposed for isozymes, or by selective discrimination i.e. a greater affinity of chain x for y and z than of chain y for z.

Some aspects of haemoglobin synthesis have been studied with mouse haemolysates as a model. The results of these experiments have been reported in the third paper (Schalekamp et al., 1975). One part describes the analysis of the haemoglobins of adult and foetal peripheral blood cells and of blood cells from foetal liver, which are at a more immature stage. We were able to establish in these blood cells a hitherto not described foetal haemoglobin. It showed a specific globin chain composition. The second part of the paper describes a study on other iron-containing proteins in haemolysates, especially from immature foetal liver blood cells and from virus infected leucaemic cells (Friend cells) in which haemoglobin synthesis was induced. In earlier work we noticed that apart from haemoglobins, other haem-containing proteins were present in the stromal proteins. In the experiments with mice a similar protein peak from immature mouse blood cells from foetal livers and Friend virus infected leucaemic cells was subjected to more elaborate analyses. It was found

that in this protein peak - X-fraction - relatively more iron-containing protein was present in the more immature blood cells. Moreover relatively more iron was present in the X-fraction as compared to the haemoglobin fraction in the very young cells. Chromatography over sephadex G200 showed that iron was bound to high molecular weight proteins. Treatment with acidified Drabkin's solution established that 30-50% of the iron was present in the form of haemin. Single haem-containing globin chains or haemoglobin aggregates did not seem to contribute to the peak. Part of the peak represents catalase, which is present in foetal haemolysates in greater amounts than in adult haemolysates. The remainder is formed most probably of proteins acting as carrier for haemin, transporting it from the mitochondria where haemin is formed, to the polysomes where the globin chain is growing. This group of proteins is also likely to contain the haemin controlled translational repressor substance, which is a protein with a high molecular weight, that acts as a protein kinase, phosphorylating transfer RNA_f binding factors, thus initiating and regulating globin chain synthesis.

SAMENVATTING

Haemoglobine synthese tijdens de embryonale ontwikkeling staat model voor een onderzoek naar de eiwitsynthese in een systeem waarin op natuurlijke wijze veranderingen in gen-expressie optreden. De resultaten zijn weergegeven in drie opeenvolgende publikaties, die in dit proefschrift zijn bijgesloten.

De eerste publikatie (Schalekamp c.s., 1972) beschrijft een onderzoek naar de veranderingen in haemoglobine samenstelling tijdens de embryonale ontwikkeling van kippen. Deze waarnemingen werden gedaan met behulp van electroforese, immuno-elektroforese en chromatografie. In het jongste stadium dat werd bestudeerd (2 dagen bebroed) werden vier haemoglobines (E_2,E_3,E_4,E_5) aangetoond, waarvan twee (E_3, E_4) in een grote hoeveelheid (85%). Op dag 6 verdwijnen plotseling deze twee quantitatief belangrijkste haemoglobines, twee andere haemoglobines komen ervoor in de plaats, een volwassen (A_2) en een foetaal (E_1) type. Op dag 12 treedt er weer een verandering in de haemoglobine samenstelling op, er verschijnen weer een volwassen (A_1) en een foetaal(E_6) haemoglobine in plaats van de verdwijnende haemoglobines E_2 en E_5. Na het uitkomen verdwijnen ook de laatste foetale haemoglobines E_1 en E_6. In haemolysaten van volwassen dieren komen alleen de volwassen haemoglobines A_1 en A_2 voor.

Het verschijnen van nieuwe haemoglobines wordt meestal geassocieerd met het aktief worden van nieuwe genen. Als gevolg daarvan zouden dan nieuwe globine ketens verschijnen. Wij onderzochten daarom ook de globine samenstelling van de geïsoleerde volwassen en embryonale haemoglobines. Dit werd gedaan met behulp van ureum-zetmeel gel elektroforese. Wij vonden echter geen enkele nieuwe globine keten op het tijdstip waarop de nieuwe haemoglobines verschenen. Opvallend was wel het verdwijnen van twee globine ketens tijdens die periode.

De tweede publikatie (Schalekamp c.s., 1976) beschrijft een meer uitgebreid onderzoek van de globine ketens voor en na de haemoglobine switch op dag 6 van de embryonale ontwikkeling. Hier werden de globines van verschillende gezuiverde volwassen en embryonale haemoglobines gescheiden met behulp van chromatografie over ureum-amberlite kolommen. Van de geïsoleerde globines werden peptide kaarten op

dunnelaag silica gels gemaakt. Ook werd de aminozuur samenstelling van elke gezuiverde globine keten bepaald. Op grond van deze gegevens kwamen wij weer tot dezelfde conclusies als in onze eerste publikatie, namelijk: alle globines, die nodig zijn voor de vorming van haemoglobine A_2 en E_1 worden reeds aangemaakt in embryonen voordat de haemoglobine switch op dag 6 optreedt, dat is de dag waarop deze haemoglobines voor het eerst kunnen worden aangetoond. De enige duidelijk vastgestelde verandering op dat tijdstip was weer het verdwijnen van de embryonale delta ketens, wijzend op inactivatie van embryonale genen.

Op basis van deze gegevens construeerden wij een recombinatie hypothese. Hierbij wordt verondersteld dat nieuwe, rijpere vormen, van bepaalde eiwitten, die gevormd worden onder invloed van meerdere genen (doordat zij zijn samengesteld uit meerdere eiwitstukken, die elk door een eigen gen worden gecodeerd) tijdens de ontwikkeling kunnen ontstaan, niet door het actief worden van een gen voor een nieuw eiwitstuk, maar juist door het inactief worden van een gen voor een bestaand eiwitstuk en een daarop volgende stimulatie van de produktie van de overgebleven globines. Het ontstaan van een nieuw haemoglobine wordt verklaard door recombinatie van de overgebleven ketens te veronderstellen op bijvoorbeeld de volgende wijze: haemoglobine O is samengesteld uit de ketens x en y, haemoglobine P bestaat uit de ketens x en z. Globine keten x wordt niet meer gesynthetiseerd en een nieuw haemoglobine Q, dat is samengesteld uit de ketens y en z verschijnt. Hierbij moet worden aangenomen dat haemoglobine Q - waarvoor immers alle ketens aanwezig waren - tevoren niet werd gevormd. Deze discriminative combinatie van ketens kan het gevolg zijn van het feit dat de ratio waarin deze ketens worden aangemaakt - eerst komen zij in quantitatief onbelangrijke, later in de quantitatief belangrijkste haemoglobines voor - een factor is, die de combinatie van de ketens beïnvloedt (doseringseffect), zoals dat ook voor bepaalde isozymen wordt aangenomen. Een andere mogelijkheid is dat de keten x een grotere affiniteit voor y en z toont dan y en z voor elkaar (selective discriminatie).

Een aantal aspecten van de haemoglobine synthese hebben wij bestudeerd bij muizen.De resultaten hiervan vindt u in de derde

publikatie (Schalekamp, 1975). Een deel van het onderzoek betreft de analyse van de haemoglobines in volwasssen en foetaal perifeer muizebloed en in bloedcellen afkomstig uit foetale muizelevers. Wij vonden hierbij een tot dan toe niet beschreven foetaal muizehaemoglobine met een eigen specifieke globine samenstelling. Het tweede deel beschrijft een onderzoek naar andere ijzer- en haem-houdende eiwitten, speciaal uit onrijpe bloedcellen (foetale levers) en tumorcellen (door Friend virus geïnfecteerde leucemische cellen). Uit eerdere onderzoekingen was ons gebleken, dat behalve haemoglobines ook andere soms ijzerhoudende eiwitten, aanwezig waren in hemolysaten, in een fractie die wij hebben aangeduid met stromaeiwitten. Bij het in publikatie drie beschreven onderzoek onderwierpen wij deze fractie aan een nadere analyse. Daarbij zagen wij, dat in jongere bloedcellen, deze fractie - nu X-fractie genoemd - hoger was naarmate de cellen onrijper waren en dat er ook meer ijzer in terecht kwam ten opzichte van de haemoglobine fracties. Chromatografie over sephadex G200 kolommen toonde aan, dat het ijzer gebonden was aan hoogmoleculaire eiwitten en behandeling met een aangezuurde Drapkin oplossing, dat het voor 30 - 50% in de vorm van haem aanwezig was. Verder werd uitgemaakt, dat het haemoproteïne waarschijnlijk geen haemoglobine aggregaat of haem-houdende losse globines bevatte. Een gedeelte van de haem-houdende eiwitten representeert catalase, de rest betreft waarschijnlijk eiwitten, die betrokken zijn bij het haem transport van de mitochondria, de plaats waar haem gevormd wordt naar de polysomen met de juist gevormde globine keten. In deze fractie komen waarschijnlijk ook hoogmoleculaire eiwitten voor die voor de initiatie en regulatie van de globine synthese van belang zijn, zoals bijvoorbeeld de zogeheten haemin-controlled translational repressor substance, een eiwit dat werkt als eiwitkinase en de fosforylering van transfer RNA_f - bindende factoren bewerkstelligt, waardoor deze vrij komen voor de globine synthese.

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PAPERS

J. Embryol. exp. Morph. Vol. 28, 3, pp. 681–713, 1972
Printed in Great Britain

Re-evaluation of the presence of multiple haemoglobins during the ontogenesis of the chicken

Electrophoretic and chromatographic characterization, polypeptide composition and immunochemical properties

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SUMMARY

Haemolysates of red blood cells from embryos of several developmental stages ranging from 2 to 21 incubation days and from post-hatching chickens of various age groups were analysed by ion-exchange chromatography, agar- and starch-gel electrophoresis, immuno-electrophoresis with specific antisera and polypeptide chain electrophoresis.

With these methods two adult (A_1 and A_2) and six embryonic (E_1–E_6) haemoglobin types were identified. Antisera specific for the major adult haemoglobins (A_1 and A_2) as well as antisera specific for the major embryonic haemoglobins (E_3, E_4) could be prepared. Throughout embryogenesis the haemoglobin types contribute in varying amounts to the total haemoglobin pattern. Three periods of haemoglobin synthesis could be recognized, the transition between these periods occurred at the 6th and 12th incubation day. The first period is characterized by the presence of two major embryonic haemoglobins (E_3 and E_4) and two minor embryonic haemoglobins (E_2 and E_5). During the second period E_3 and E_4 are largely replaced by a major adult haemoglobin (A_2) and a new embryonic haemoglobin (E_1). The third period is characterized by the appearance of a second adult haemoglobin (A_1) and a new minor embryonic haemoglobin (E_6) with a concomitant decrease of E_2 and E_5. At the time of hatching two embryonic haemoglobins (E_1 and E_6) are still present.

Besides A_1 and A_2, several minor haemoglobin fractions were inconsistently found in adult chickens. Evidence has been obtained that these additional fractions are reflecting a so called minor heterogeneity or separation artifacts.

The haemoglobins A_1, A_2 and E_1–E_6 show different polypeptide chain compositions. Three embryo-specific chains could be demonstrated ($\beta E_2 E_5$, γE_4 and δE_3). The production of the polypeptide chains appears to be correlated with the aforementioned periods of haemoglobin synthesis.

The genetic and morphological implications of the findings are discussed.

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INTRODUCTION

During ontogenesis activation and repression of genes take place continuously, causing the process of differentiation. The factors involved are still difficult to study due to complexity of the developmental events. Emergence of new proteins is thought to be a rather direct expression of gene activity. A system in which changes in protein composition are readily demonstrable would therefore be useful as a model.

Haemoglobin maturation heterogeneity may be such a system. Embryonic and foetal haemoglobins, different from adult haemoglobins, occur in representatives of several vertebrate classes (Manwell, 1960; Ingram, 1963; Manwell, Baker, Rolansky & Foght, 1963). In a number of avian species, embryonic haemoglobins have been demonstrated: turkey and partridge (Manwell, Baker & Betz, 1966), white Peking duck (Borgese & Bertles, 1965), and house sparrow (Bush & Townsend, 1971). The presence of embryonic haemoglobins in chickens is still controversial. Some authors (Fraser, 1961, 1964, 1966; Wilt, 1962; Simons, 1966) state that the changes in haemoglobin composition as observed during ontogenesis are essentially quantitative, others (D'Amelio & Salvo, 1959, 1961; D'Amelio, 1966; Manwell *et al.* 1963; Manwell *et al.* 1966; Hashimoto & Wilt, 1966; Schürch, Godet, Nigon & Blanchet, 1968; Denmark & Washburn, 1969) report the presence of distinct embryonic haemoglobins, but do not agree about the number of haemoglobin types and the time of their appearance. There is no agreement even about the number of haemoglobins present in adult chickens (Godet, Schürch & Nigon, 1970). These discrepancies are possibly due to the fact that most authors have used only one or two different techniques. Therefore, a thorough re-analysis of the haemoglobin types present in adult and embryonic chickens seems to be necessary.

In the present study the different haemoglobin types have been characterized by agar- and starch-gel electrophoresis, by chromatography on cation and anion exchangers, by immunochemical techniques and by the analysis of the polypeptide chain composition. Furthermore, the developmental stages at which the haemoglobin types appear and disappear in the circulation have been determined.

MATERIALS AND METHODS

Preparation of the haemolysate. Blood samples were obtained from the wing vein of White Leghorn chickens of various age-groups, ranging from 1 day to 3 years post-hatching and from the vitelline vein or the heart of White Leghorn embryos at various developmental stages, ranging from 2 to 21 incubation days. Care was taken to avoid contamination with yolk, as washing of the blood cell suspension with saline does not sufficiently remove the yolk particles. For the 3- to 5-day embryonic stages the haemolysates from 300 animals were used. For the later embryonic stages haemolysates from 3–30 animals were pooled. Post-

hatching samples were obtained from individual chickens. The blood cells were suspended and washed three times in a tenfold volume of ice-cold saline. They were allowed to lyse for 12 h in 0·025 M-NaCl at 4 °C. After centrifugation the haemoglobins in the supernatant were converted into the CO-form. Samples were stored at 4 °C until analysed. Analyses were started within 48 h after preparation of the haemolysate, as haemoglobins are known to alter by ageing as well as by freezing and thawing (Manwell *et al.* 1966).

Electrophoresis. Agar-gel electrophoresis was carried out using 0·05 M barbiturate buffer, pH 8·6, following the method of Wieme (1959). Vertical starch-gel electrophoresis was performed according the Smithies (1959) using a discontinuous buffer system containing 0·017 M Tris, 0·07 M EDTA and 0·025 M boric acid, pH 8·9 (gel) and 0·3 M boric acid and 0·06 M-NaOH, pH 8·2 (electrode vessels), or as described by Scopes (1963), who used 0·25 M sucrose, 0·0015 M citric acid and 0·020 M Tris, pH 9·1 (central part of the gel), 0·010 M boric acid and 0·06 M Tris, pH 8·6 (ends of the gel) and 0·3 M boric acid and 0·06 M-NaOH, pH 8·2 (electrode vessels). All analyses were carried out in the cold room at 4 °C. In order to trace the haemoglobins, the agar- and starch-gel strips were stained by a standard peroxidative procedure, in which benzidine in acetic acid and hydrogen peroxide were used (Dessauer, 1966). Amido black or Ponceau S were used to visualize other protein components. Relative electrophoretic mobilities in agar were calculated according to Wieme (1959), using as standards 1 % human serum albumin (Behring), mobility 100, and 1 % dextran (M.W. ± 135000), mobility 0.

Column chromatography. Carboxmethyl (CM)-cellulose was used as cation exchanger in combination with a discontinuous buffer system. In the first elution step 0·01 M sodium phosphate buffer, pH 6·9, was used. The second step was performed with 0·02 M sodium phosphate buffer, pH 7·5, and the third with 0·02 M sodium phosphate buffer, pH 8·5, to which 0·4 M-NaCl was added.

The anion exchanger diethylaminoethyl (DEAE)-Sephadex was developed with 0·05 M trihydroxymethylaminomethane (Tris) HCl buffer in a linear gradient from pH 8·6 to 7·4. All buffers were saturated with CO. The optical density (O.D.) of the effluent fractions was measured at 280, 419 and 540 nm, using a Zeiss spectrophotometer. Approximate haemoglobin concentrations were calculated from 419 nm readings. The ratio O.D. 419/280 was determined for each peak in order to locate non-haemoglobin contamination. Absorption spectra of all haemoglobin fractions were recorded.

Polypeptide chain analysis. Isolated lyophilized haemoglobins were submitted to vertical starch-gel electrophoresis according to (1) the method of Bucci & Fronticelli (1965) using *p*-chloromercuribenzoic acid (PCMB), (2) the method of Muller (1961) using pH 1·9, and (3) the method of Gilman & Smithies (1968), who used 8 M urea at pH 3·2. The latter method proved to be preferable. The haemoglobins were used as such or after conversion into haemless globins by the acetone precipitation method of Rossi-Fanelli, Antonini & Caputo (1958) or

by the method of Teale (1959) using ethyl-methyl ketone. In our hands globin preparation always caused a considerable loss of material which became insoluble. As, apart from a distinct haem zone, no difference was seen in the starch patterns obtained when either total haemoglobins or haemless globins were used, total haemoglobins were used in later experiments. Mercaptoethanol was added in all experiments in order to prevent minor fractions to arise from oxidation of SH groups (Chernoff & Petitt, 1964*a*).

Immunochemical techniques. Micromodifications of the double-diffusion technique of Ouchterlony (1958) and immuno-electrophoresis following the method of Scheidegger (1955) were used (Schalekamp, 1963). Antisera were prepared in rabbits according to the technique of Freund *et al.* (1948) with total haemolysates or purified haemoglobins and with total adult chicken serum as antigens. Absorption of antisera was performed as recommended by Boyd (1956). An adequate amount of a purified haemoglobin as calculated from microtitration readings (precipitin reaction), together with an equal amount of stromal non-haem proteins was added to the antiserum; the mixture was allowed to stand for 1 h at 37 °C and then overnight at 4 °C. After centrifugation, the supernatant was absorbed once more in the same way. The antisera were concentrated by ultrafiltration before use. In order to guarantee that the antibodies were directed against haemoglobins and not against accompanying impurities, titration experiments were performed in which increasing amounts of anti-haemolysate serum were added to fixed amounts of haemolysate. In a control series anti-haemolysate was substituted by anti-chicken ovalbumin serum. The O.D. of the supernatant of the tubes, as measured at 419 nm, showed that haemoglobin was precipitated only by anti-haemolysate serum. The precipitation lines in the immuno-electrophoresis and Ouchterlony plates were further identified as haemoglobins by a positive benzidine reaction.

RESULTS

The presence of eight qualitatively different haemoglobins (A_1, A_2, E_1, E_2, E_3, E_4, E_5, E_6) could be established by combining several methods, including electrophoresis and polypeptide chain analysis (Fig. 1). The embryonic stages at which haemoglobins appear and disappear could be ascertained accurately with immunochemical methods (Fig. 2). The relative quantities of the haemoglobins at different developmental stages were estimated from chromatographic graphs and are presented in Fig. 3 (embryonic haemolysates) and Table 1 (post-hatching haemolysates).

Electrophoretic and chromatographic characterization of adult and embryonic haemoglobins and stromal proteins

Post-hatching haemolysates (Figs. 4, 5). Two major condensations of benzidine-positive material could be easily distinguished after agar- and starch-gel

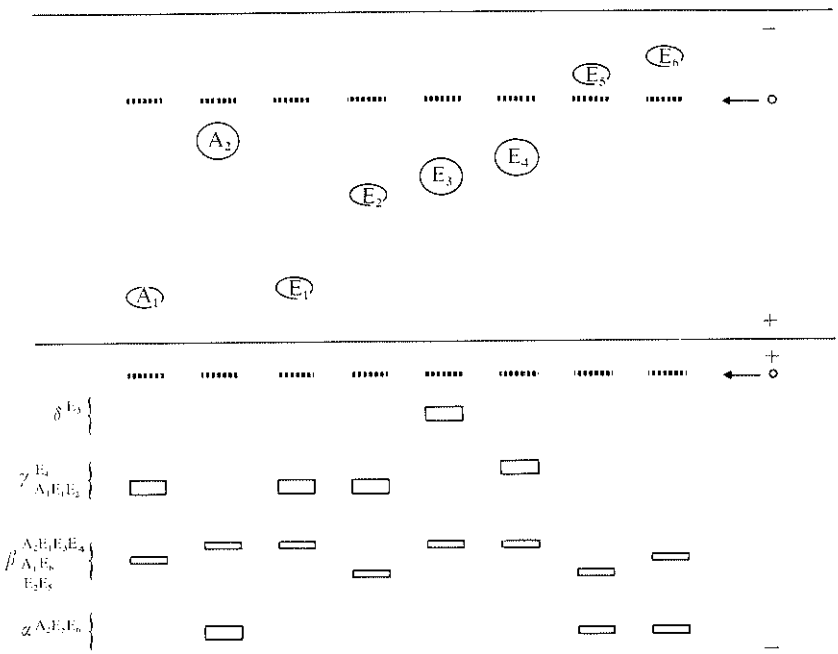


Fig. 1. Upper part: diagrammatic representation of the electrophoretic mobilities of the haemoglobins present in haemolysates from post-hatching and embryonic chickens in pH 9·1 starch gel. Lower part: polypeptide chains of each of the haemoglobins in pH 3·2 urea starch gel. ○ indicates the slot.

Abbreviations: + = anodic side; capital letters = haemoglobins; greek letters = polypeptide chains; *ad* = haemolysate from an adult chicken; *day post-h.* = day post-hatching at which the haemolysate was taken; *day embryo* = day of incubation at which the haemolysate was taken; *anti-*Hb_{ad} = antiserum prepared to total haemolysate of an adult chicken; *anti-*A_1 *spec.* = antiserum reacting exclusively with A_1; *anti-*A_2 *spec.* = antiserum reacting exclusively with A_2, E_5 and E_6; *anti-*E_3, E_4 *spec.* = antiserum reacting exclusively with E_3 and E_4.

electrophoresis in the haemolysates from chickens aged 1 month and older. In starch gels an additional minor benzidine-positive condensation was found, when haemolysates from chickens aged between 1 day and 1 month were investigated. On the basis of further investigations these condensations were thought to contain two *major adult haemoglobins* and one persistent *minor embryonic haemoglobin*. They were called A_1, A_2 and E_6 in the order of their relative electrophoretic mobilities in agar which were 25, 10 and 0 respectively. It may be noted here that all chicken haemoglobins behave more alkaline than human haemoglobin A (mobility 45).

CM-cellulose and DEAE-Sephadex chromatography also revealed two major peaks in haemolysates from chickens older than 1 month. An additional minor peak was found in haemolysates from the younger chickens when using DEAE–Sephadex chromatography. CM–cellulose chromatography in our hands

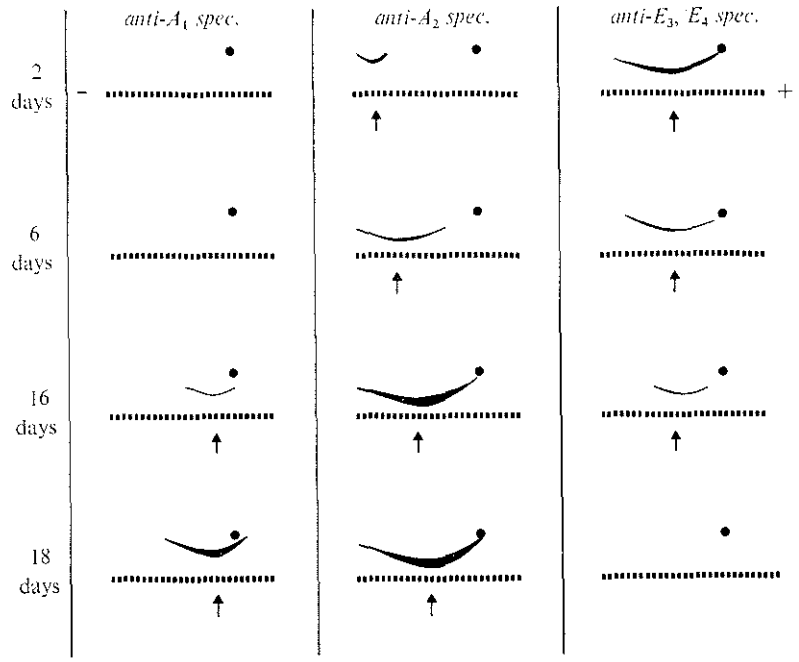


Fig. 2. The time of appearance or disappearance of some major haemoglobins as demonstrated by immuno-electrophoresis with specific antisera. Abbreviations: see Fig. 1.

--- = Antiserum reservoir; ● = antigen well; ← = nadir of precipitation line.

was not suitable to separate this peak. When isolated the three chromatographic peaks showed a typical CO-haemoglobin absorption spectrum and an absorbance ratio (O.D. 419/280) of ±4 which means that the main constituent was haemoglobin. All haemoglobins showed a tryptophan notch at 290 nm in their absorption spectrum. Electrophoresis identified A_1 and A_2+E_6 in the first and second CM-cellulose peak and E_6, A_2 and E_1 in the first, second and third DEAE-peak respectively. The relative amounts of the three haemoglobins as calculated from chromatographic readings at 419 nm are given in Table 1.

Besides these three haemoglobins starch-gel electropherograms occasionally displayed two more zones in which proteins were detectable. This material could be further characterized as follows.

Additional *group I* proteins were situated electrophoretically more anodic than A_1 and contained about eight distinct lines which were benzidine-negative and were therefore considered to be non-haem *stromal proteins*. The most cathodic portion of this protein group was inconsistently benzidine-positive; it is suspected that ageing of the haemolysate (although maximally 48 h) might somehow have been responsible for the presence of an extra haemoglobin component in these cases. In one out of the 25 adult chickens used for this

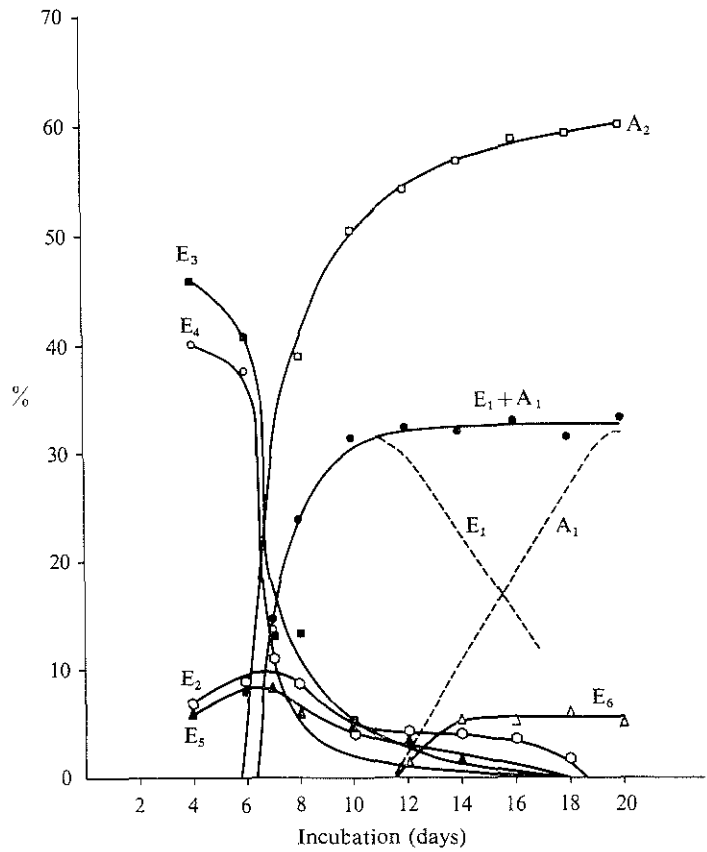


Fig. 3. Percentages of the haemoglobins found at different embryonic stages, as calculated from planimetric analyses of chromatographic curves measured at 419 nm. Each dot represents the mean of at least three chromatographic samples. The lines are extrapolated to a certain day of appearance or disappearance on the base of semi-quantitative immunochemical studies. The dotted lines indicating the appearance of A_1 and the disappearance of E_1 are also based on immunochemical studies. Abbreviations: see Fig. 1.

investigation a 'rare haemoglobin' peak was consistently found to be present in amounts of about 2 % in this electrophoretic zone of stromal proteins, even in freshly prepared haemolysates.

CM-cellulose chromatography revealed the group I stromal proteins in the first elution step. The non-haemoglobin nature of the larger part of this peak was demonstrated by the ratio O.D. 419/280, which was less than 1. The peak was often slightly reddish, due to withdrawal of haemoglobins with the break-through volume. Degraded haemoglobin pigments may appear in this peak. The 'rare haemoglobin', when present, was also eluted in the first elution step, causing a raise in the ratio O.D. 419/280 to about 2.

After chromatography on DEAE-Sephadex the group I stromal proteins

Table 1. *Relative amount of the haemoglobins in blood of post-hatching chickens*

Age of chickens	Haemoglobins (%)*		
	A_1	A_2	E_6
1 day	33·2	61·7	5·1
2 weeks	31·7	67·0	1·3
1 month	28·9	71·1	±
4 months	21·1	78·9	—
6 months	22·0	78·0	—
1 year	21·7	78·3	—
3 years	19·4	80·6	—

Each number represents the mean of at least five chromatographic samples.
* Calculated from planimetric analyses of chromatographic curves measured at 419 nm.

remained on the column, while the 'rare haemoglobin' appears as a shoulder behind the A_1 peak containing maximally 2 % of the total amount of haemoglobin.

Additional *group II* proteins were situated electrophoretically between A_1 and A_2, and also contained mainly non-haem stromal proteins. Catalase activity was often found in this region. A benzidine-positive component in this group was occasionally present, but only in haemolysates which also showed benzidine positivity in the region of the group I stromal proteins; i.e. in cases of suspected ageing of the haemolysate or in the case of the one out of 25 chickens with the 'rare haemoglobin' in group I. This indicates the presence of more 'rare haemoglobins' in this chicken.

In CM-cellulose chromatography the group II stromal proteins were eluted in the third step together with A_2.

DEAE-Sephadex chromatography revealed the haem-containing component of group II, when present, as a shoulder in the A_2 peak containing maximally 18 % of the total amount of haemoglobin.

Embryonic haemolysates (Figs. 6, 7). Two or three major condensations of benzidine-positive material were present in these haemolysates also. Here again the mean electrophoretic mobilities were 25, 10 and 0. However, observation of the pH 9·1 starch-gel electrophoresis patterns now permitted further subdivision. With this method seven haemoglobin fractions could be distinguished. Two of these fractions had exactly the same mobilities as A_1 and A_2 and thus seemed to represent the two *major adult haemoglobins*. Further analysis indicated that the A_1 fraction may also contain a *major embryonic haemoglobin*, E_1. This E_1 haemoglobin behaved electrophoretically and chromatographically identical to A_1, but could be distinguished with the other methods used.

The A_1–E_1 fraction and the A_2 fraction were present in increasing amounts in embryonic haemolysates from the 6th incubation day onward. The other five

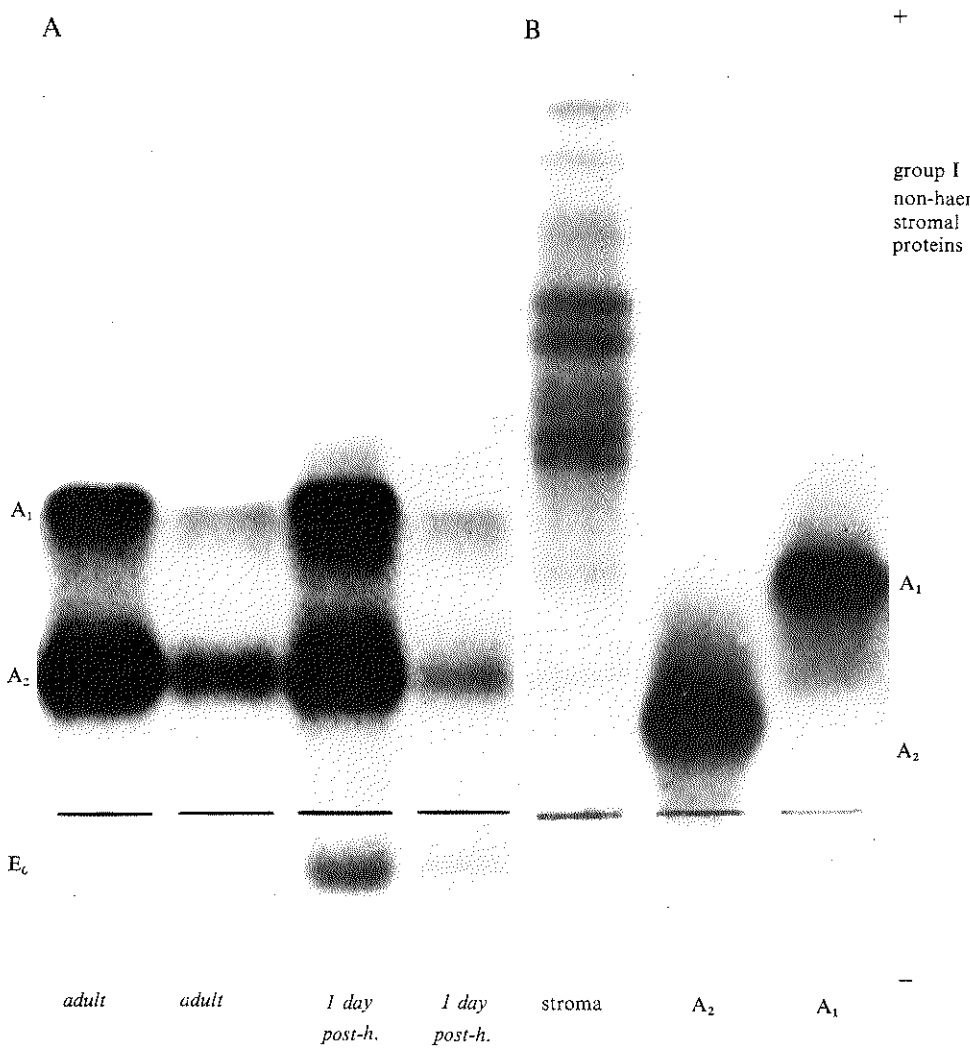


Fig. 4. Electrophoretic patterns of haemolysates from post-hatching chickens in pH 8·9 starch gels.

(A) Normal adult haemolysate and haemolysate from a 1 day old chicken, both inserted at two different concentrations. Benzidine staining.

(B) Fractions isolated from total haemolysate by CM-chromatography. The non-haem stromal proteins present in the first eluting peak and the A_1 and A_2 fractions from the same column are shown. Amido black staining. The duration of electrophoresis was not the same for plate A and plate B. Abbreviations: see Fig. 1.

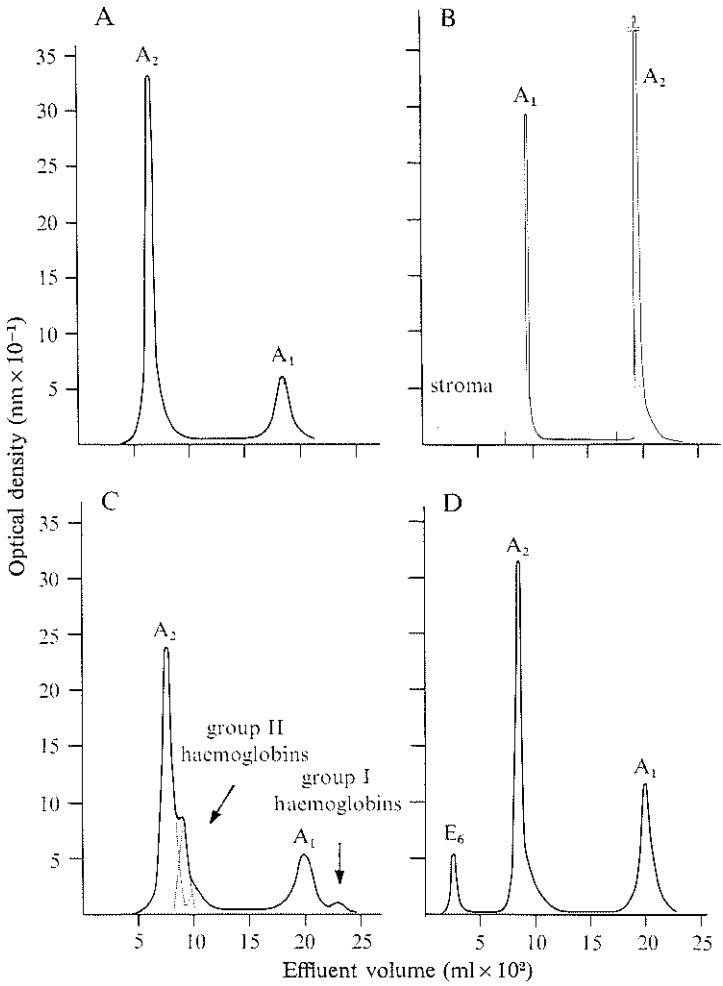


Fig. 5. Chromatographic patterns of haemolysates from post-hatching chickens.

(A) DEAE-Sephadex chromatogram of a haemolysate from a normal adult chicken. Optical density (O.D.) at 419 nm.

(B) CM-cellulose chromatogram of a haemolysate from a normal adult chicken. Solid line: O.D. at 419 nm.; dotted line: O.D. at 280 nm.

(C) DEAE-Sephadex chromatogram of a haemolysate containing the group I and group II haemoglobins. O.D. at 419 nm.

(D) DEAE-Sephadex chromatogram of a haemolysate from a 1-day-old chicken. O.D. at 419 nm.

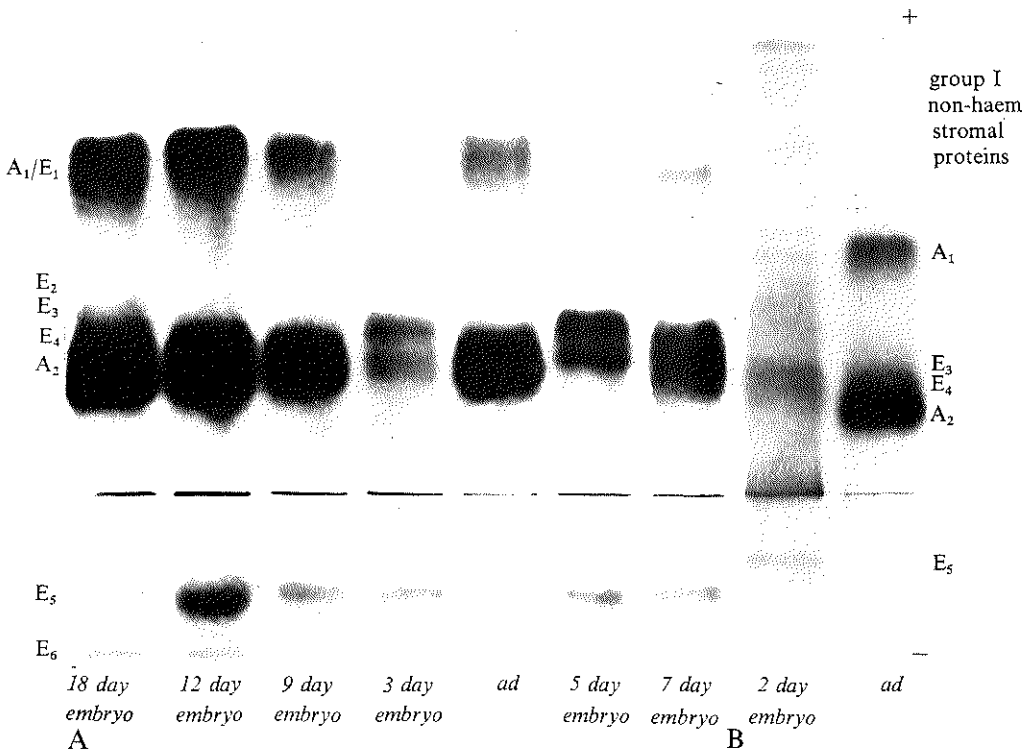


Fig. 6. Electrophoretic patterns in pH 9·1 starch gels of haemolysates from embryos at various developmental stages. (A) Benzidine staining; (B) amido black staining. The duration of electrophoresis was not the same for plate A and B. Abbreviations: see Fig. 1.

haemoglobin fractions were all regarded as embryo-specific. The most anodic of these fractions, a *minor embryonic haemoglobin*, E_2, was visible as a faint smear on the anodic side of the middle benzidine positive group up to the 18th incubation day. Furthermore two *major embryonic haemoglobins*, E_3 and E_4, were found in this group which were situated between E_2 and A_2. There was considerable overlap between E_3, E_4 and A_2; these haemoglobins were only observed as separate bands after long electrophoretic runs. They were most easily recognized when haemolysates with a low haemoglobin concentration from the youngest embryos (2–5 incubation days) were analysed, since no A_2 is present at these stages. Both major embryonic haemoglobins were visible in decreasing amounts until the 18th incubation day with a steep fall in concentration at the 6th incubation day. In the most cathodic benzidine-positive group, well separated from A_2, two more *minor embryonic haemoglobins*, E_5 and E_6, were found. E_5 was present in young embryos up to the 18th incubation day. E_6 in contrast was only detected in older embryos from the 12th incubation day onward. This haemoglobin persisted until one month post-hatching.

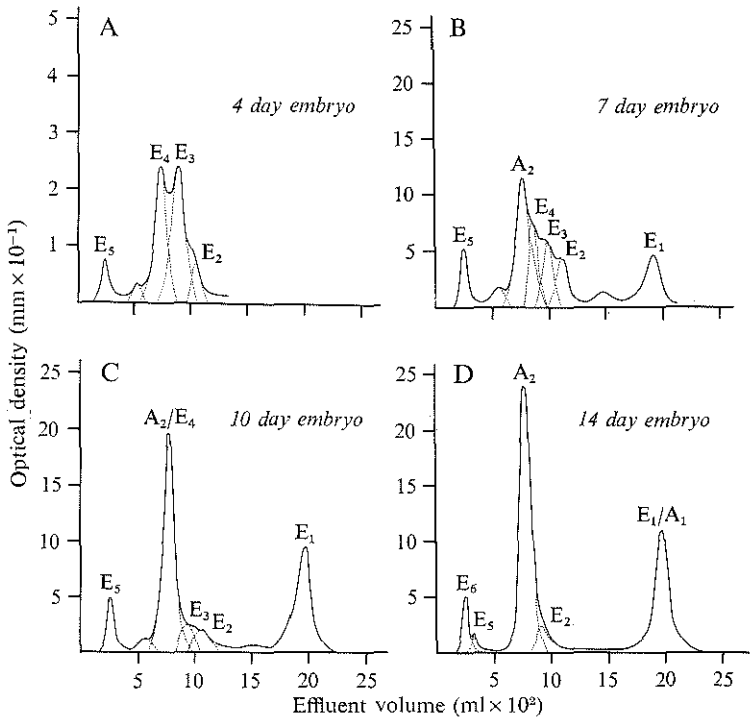


Fig. 7. DEAE-Sephadex chromatography, patterns of haemolysates at four developmental stages. O.D. was measured at 419 nm. Dotted lines represent the haemoglobin fractions, which are measured by planimetry to get the percentages given in Fig. 3. Abbreviations: see Fig. 1.

It was not possible to obtain a satisfactory separation of the embryonic haemoglobins using CM-cellulose chromatography. In the first elution step the larger part of the stromal proteins was eluted. In the second step the haemoglobins A_1, E_1, E_2, E_3 and E_4 were eluted together, while in the third elution step the haemoglobins A_2, E_5 and E_6, sometimes contaminated with some more E_3 and E_4, appeared.

DEAE-Sephadex chromatography provided good separations. Seven different haemoglobin peaks were detected in which starch-gel electrophoresis identified the haemoglobins E_6, E_5, A_2, E_4, E_3, E_2 and A_1 and (or) E_1. The relative quantities of these haemoglobins, as estimated from optical density readings at 419 nm, are changing during development (Fig. 3). Obviously the major embryonic haemoglobins are E_1, E_3 and E_4. The minor embryonic haemoglobins E_2, E_5 and E_6 never exceed the relative amount of 10 %.

In embryos incubated for 6–10 days there is another small peak between E_1 and E_2; moreover, in embryos incubated 4–10 days a shoulder in the foot of the E_4 peak was consistently found. These haemoglobin peaks could not be evaluated as their concentration was too low.

When using DEAES-ephadex chromatography for preparative purposes, several difficulties were encountered. The peaks A_2, E_4, E_3 and E_2 showed a great overlap. Therefore, rechromatography was considered. However, a sample applicated for the second chromatographic cycle always remained on the column for more than 80 %. Apparently the preparation decomposed by ageing during the dialysing period or by the lyophilization which was performed for sampling. The denaturation of the samples could be verified with the absorption spectra of these preparations in which all peaks shifted to a longer wavelength. Similar difficulties were encountered with the first eluting peaks E_5 and E_6, which occasionally showed separation artifacts, and with the haemoglobin peaks A_1 and E_1 which showed a total overlap. As rechromatography thus seems to be impossible we had to purify the haemoglobins by selecting a haemolysate of an appropriate embryonic stage, i.e. from embryos younger than 5 incubation days (E_2, E_3 and E_4), from embryos younger than 7 incubation days (E_1), from embryos younger than 10 incubation days (E_5), from young post-hatching chickens (E_6) and from adult animals (A_1 and A_2). The absorption spectra of all haemoglobin fractions, isolated in such a way, showed a tryptophan notch at 290 nm, besides the normal haemoglobin absorption peaks.

Stromal proteins were detected in the haemolysates from all embryonic stages. After starch-gel electrophoresis seven to eight fractions were observed in the electrophoretic region of the group I proteins in post-hatching haemolysates. Proteins corresponding to the group II proteins of post-hatching haemolysates, when present, were masked by the E_2, E_3, and E_4 haemoglobins, as these haemoglobins have the same electrophoretic properties.

Polypeptide chains

Adult and embryonic haemoglobins (Figs. 8, 9). Three clearly distinguishable groups of chains became visible when total haemolysates from adult chickens were analysed with the urea pH 3·2 starch-gel method. A fourth group was found in total haemolysates from 8-day embryos, a haemolysate containing 6 out of the 8 haemoglobins found in embryonic haemolysates. The groups of chains were named α, β, γ and δ chains, according to their decreasing electrophoretic mobility from the anode. Furthermore, they received in their index the name of the haemoglobins in which they were found. The nomenclature used in this study does not imply any similarity with human globin chains. In pH 1·9 starch gels only three groups of chains were visible, corresponding to α, $\beta+\delta$ and γ respectively. With both methods the groups showed a subdivision. When isolated, each haemoglobin separated into two zones which was best demonstrated in the urea pH 3·2 gels. The colour intensity of the two zones, as stained with amido black, was not identical. This may have been due to a difference in affinity for the dye or to a difference in solubility of these chains in acid media rather than to a difference in molecular weight. Benzidine negativity indicated haem release in both chains even when total haemoglobin was applied.

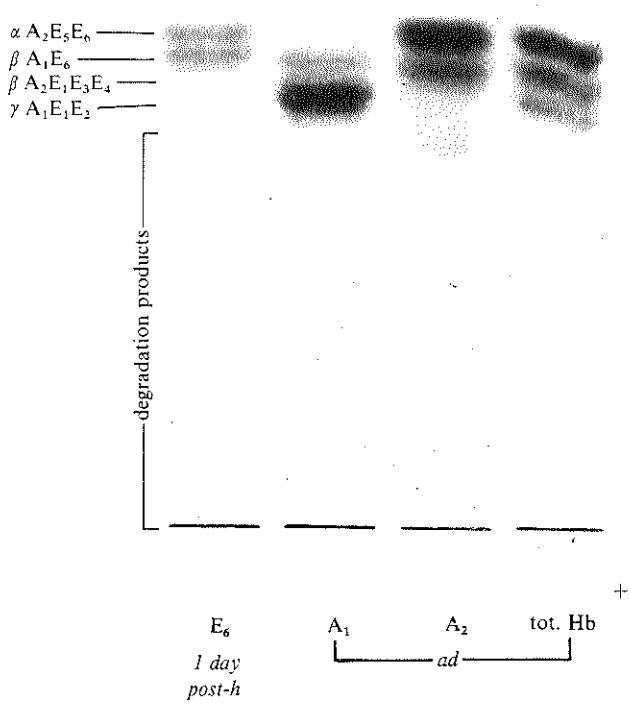


Fig. 8. Electrophoretic patterns in urea pH 3·2 starch gel of an isolated E_6, A_1, A_2 fraction and of a total haemolysate from a normal adult chicken. The two β chains show a minor difference in electrophoretic mobility and are therefore not separately visible when total haemolysate is analysed. Amido black positive zones, anodic of the α, β and γ chains are sometimes visible. As these are variable in electrophoretic position and colour intensity, they were thought to be due to degradation products. Abbreviations: see Fig. 1.

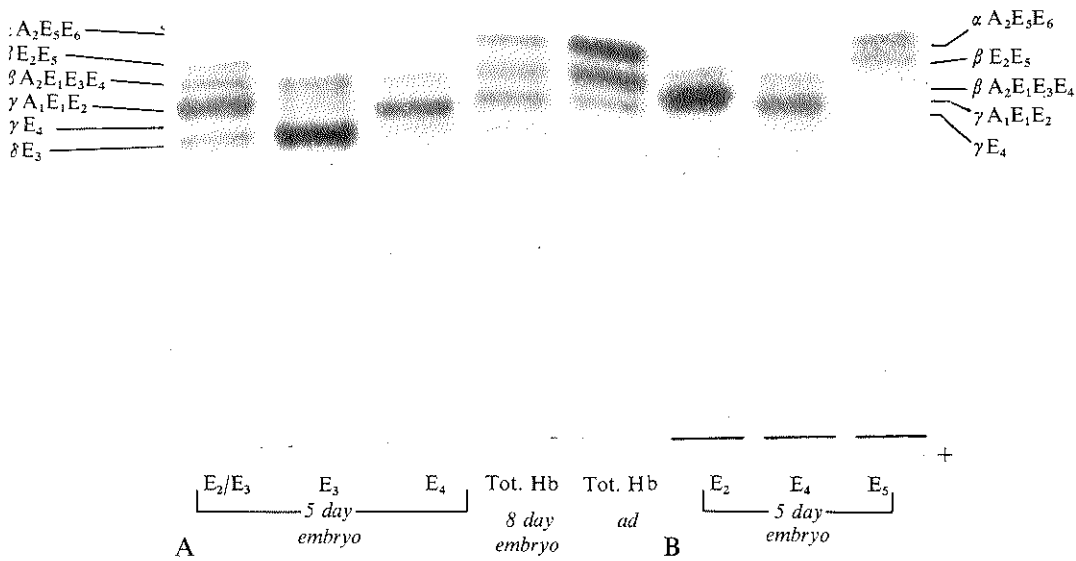


Fig. 9. (A) Electrophoretic patterns in urea pH 3·2 starch gels of the polypeptide chains of total haemolysates from embryos incubated for 8 days and from adult chickens, compared with haemoglobin fractions obtained by DEAE-Sephadex chromatography of haemolysate from embryos incubated for 5 days. These fractions contain the haemoglobins E_2–E_3, E_3 and E_4 respectively.

(B) Haemoglobin fractions of another haemolysate from embryos incubated for 5 days. These fractions contain the haemoglobins E_2, E_4 and E_5. The duration of electrophoresis was not the same for plates (A) and (B). Abbreviations: see Fig. 1.

On the base of comparison of the two chains of each isolated haemoglobin with the 'total' chain pattern the presence of at least seven distinct chains was assumed. This conclusion was based mainly on the pattern in urea pH 3·2 starch gels. In pH 1·9 starch gels, seven zones could be found also. However, although all the chains showed up as circumscript lines in urea pH 3·2 gels, one of the chains formed a smear, interfering with other chains in pH 1·9 gels. Moreover, in pH 1·9 gels the distance between some chains was small in comparison to the distances obtained in urea pH 3·2 gels. The chains belonging to one of the above-mentioned three groups showed only small differences in electrophoretic mobility. Nevertheless such small differences may be caused by a difference in primary structure as has been shown by fingerprint analyses of βA_1E_6 and $\beta A_2E_1E_3E_4$ (Muller, 1961; Saha, 1964; Moss & Thompson, 1969). Additional evidence for this view was obtained with immunochemical techniques: preliminary studies with an antiserum to a purified $\beta A_2E_1E_3E_4$ chain revealed that this antiserum showed specificity when the reactions with $\beta A_2E_1E_3E_4$ and βA_1E_6 preparations were compared in immuno-electrophoretic analyses.

Fig. 9A illustrates that it was possible to decide about the chain pattern of each haemoglobin, even when the haemoglobin fractions were not quite pure. For example, comparison of the E_3 and E_4 fraction indicates that E_4 contains a γ chain, whilst E_3 contains a δ chain. The corresponding faint line of a γ chain in the E_3 preparation and of a δ chain in the E_4 preparation is most likely due to contamination of the E_3 preparation with E_4 and vice versa (see also Fig. 7A), in which case the most intensely stained chain of E_3 or E_4 will show up in the preparations of E_4 and E_3 respectively. The only remaining chain for E_4 as well as for E_3 may be identified as a β chain. In the same way the pattern of chains in E_2 may be deduced by subtraction of the E_3 chains from the pattern found for the E_2 fraction. That the γ chain present in E_2 is not identical to the γ chain found in E_4 could be demonstrated in gels in which the E_4 and the E_2 samples were run next to each other (Fig. 9B). The sequence of the globin chains as found in urea pH 3·2 gels was from cathode to anode: $\alpha A_2E_5E_6$–βE_2E_5; βA_1E_6; $\beta A_2E_1E_3E_4$–$\gamma A_1E_1E_2$; γE_4–δE_3. In pH 1·9 gels all the β chains and the δE_3 and the γE_4 chain were grouped together in the fast migrating fraction. The $\gamma A_1E_1E_2$ and the $\alpha A_2E_5E_6$ chain were situated more anodically as clearly separate spots. It may be concluded that the following chain combinations are present in the respective haemoglobins (see also Fig. 1):

Adult haemoglobins:	$A_1 = \beta A_1E_6$, $\gamma A_1E_1E_2$	(major Hb),
	$A_2 = \alpha A_2E_5E_6$, $\beta A_2E_1E_3E_4$	(major Hb).
Embryonic haemoglobins:	$E_1 = \beta A_2E_1E_3E_4$, $\gamma A_1E_1E_2$	(major Hb),
	$E_2 = \beta E_2E_5$, $\gamma A_1E_1E_2$	(minor Hb),
	$E_3 = \beta A_2E_1E_3E_4$, δE_3	(major Hb),
	$E_4 = \beta A_2E_1E_3E_4$, γE_4	(major Hb),
	$E_5 = \alpha A_2E_5E_6$, βE_2E_5	(minor Hb),
	$E_6 = \alpha A_2E_5E_6$, βA_1E_6	(minor Hb).

The chains βE_2E_5, δE_3 and γE_4 are exclusively embryonic, and the haemoglobins E_2, E_3, E_4 and E_5 may therefore be considered as embryo-specific. The haemoglobins E_1 and E_6 on the other hand appear to be composed of chains also available in the adult haemoglobins A_1 and A_2. These haemoglobins may be considered as hybrid forms.

The haem-containing components of *group I* and *group II*, and the slightly asymmetric foot of A_1 and A_2, when isolated by chromatography, showed minor variations in chain composition when compared to A_1 and A_2 (top) respectively. The most cathodic chain of A_1 (βA_1E_6) shifted slightly more to the anode, while the most anodic chain of A_2 ($\beta A_2E_1E_3E_4$) was slightly more cathodic in these samples. This means that these 'haemoglobins' are of a quite other nature than the embryonic haemoglobins which were found in the same electrophoretic zone (E_2, E_3 and E_4).

Surprisingly it was not possible to separate monomeric chains with the

PCMB method of Bucci & Fronticelli (1965). Both haemoglobins A_1 and A_2 formed one dimeric zone, different in electrophoretic mobility from each other and from the untreated haemoglobins. Embryonic haemoglobins were not submitted to this method. The inability to form monomeric chains with PCMB was also reported by Rosemeyer & Huehns (1967) for human foetal haemoglobin and for the haemoglobins of rabbit, horse and pig, and may be due to a different location of the sulphydryl groups in these haemoglobins when compared to human haemoglobin A.

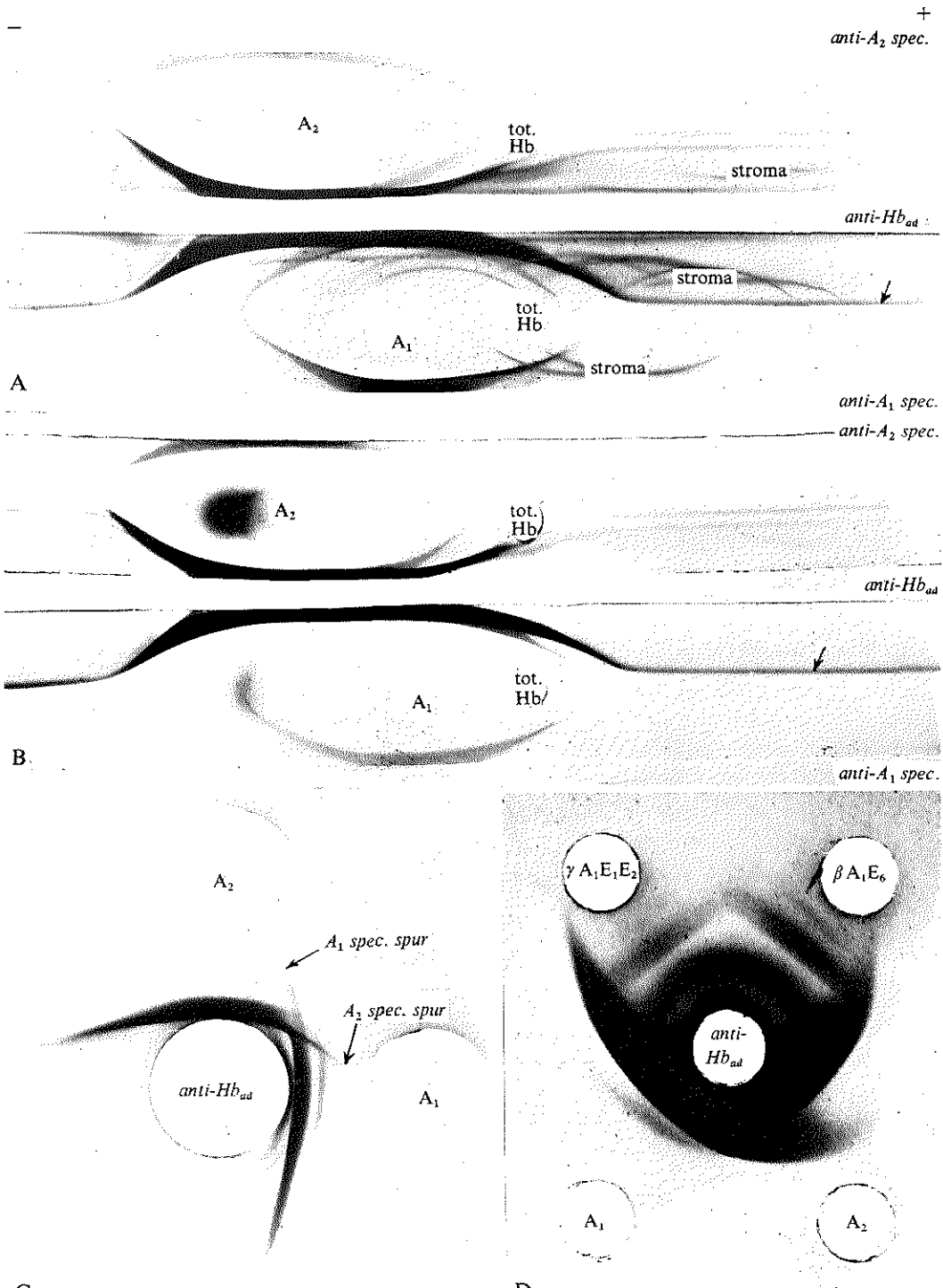
Immunochemical characterization of adult and embryonic haemoglobins and their polypeptide chains

Post-hatching haemolysates (Fig. 10). A total haemolysate of adult chicken erythrocytes formed one main precipitation line when it was allowed to react with anti-total-adult haemolysate (anti-Hb_{ad}) serum in immuno-electrophoresis. When a suitable haemoglobin concentration was used, this line showed two nadirs. The electrophoretic position of these nadirs corresponded with the electrophoretic position of the sites of the highest concentration of the *major adult haemoglobins* A_1 and A_2. Proof that the line actually belonged to both haemoglobins and was not just the extending precipitate of one of them could be obtained by the reaction of anti-Hb_{ad} with chromatographically purified A_1 and A_2.

Using the Ouchterlony technique a fusion of the main part of the precipitation lines of isolated A_1 and A_2 also occurred. With this method, however, it was clear that a tiny part of the precipitates did not fuse (Fig. 10C). The formation of spurs indicated a reaction of 'partial identity' between both haemoglobins, each spur representing specificity for one or more antigenic determinants. Closer observation of the immuno-electrophoretic patterns revealed similar spurs in the zone between A_1 and A_2, but with this technique the spurs are parallel to the main precipitation line and tend to confluate with it. Especially the A_2 specific spur is only visible within a narrow range of antigen–antibody concentration ratios.

To support the immunochemical specificity of A_1 and A_2, absorption experiments were performed. Anti-Hb_{ad} serum was mixed with an adequate amount of A_2 and of stromal proteins, in order to remove the antibodies directed against A_2 specifically, against the common antigenic determinating groups of A_1 and A_2, and against the stromal proteins of haemolysate. The so absorbed (anti-A_1 spec.) serum reacted only with A_1 (Fig. 10A, B), thus proving the individual immunochemical specificity of A_1.

In a similar way antisera specific for A_2 could be prepared (Fig. 10A, B). This (anti-A_2 spec.) serum did not react with A_1. However, it reacted with the *embryonic haemoglobin* (E_6), which was found during the early post-hatching period. Absorption of the anti-A_2 spec. serum with E_6 removed all antibodies against A_2 and E_6, indicating a closer immunochemical relationship between


–
+
anti-A2 spec.
A2
tot. Hb
stroma
anti-Hbad
stroma
tot. Hb
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A2
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B
anti-A1 spec.
A2
A1 spec. spur
A2 spec. spur
anti-Hbad
A1
C
γ A1E1E2
β A1E6
anti-Hbad
A1
A2
D

these two haemoglobins than between A_2 and A_1. This was also suggested by the chain composition of these haemoglobins.

The stromal proteins of *group I* and *group II* showed about seven precipitation lines when reacted with anti-Hb_{ad} (Fig. 10A). Some of these lines had catalase activity. None was benzidine-positive, or could be developed with either anti-A_1 spec. or anti-A_2 spec. serum (Fig. 10B). Anti-chicken serum did not develop these precipitation lines, excluding the possibility of contaminating serum proteins.

In the 'rare haemoglobins' of group I and group II, only haemoglobin determinants were demonstrable which were common to A_1 and A_2.

Embryonic haemolysates (Fig. 11). All embryonic haemolysates (2–21 incubation days) showed precipitation lines when they were allowed to react with anti-Hb_{ad} serum. Haemolysates from the youngest stages (2 incubation days) showed three precipitation lines corresponding to E_3, E_4 and E_5 (Fig. 11A). With haemolysates from older embryos these lines became confluent in one common precipitate in which E_1, E_2, A_1, A_2 and E_6 participated (Fig. 11B, C). These results indicate an immunochemical relationship between the adult and embryonic haemoglobins on the base of one or more common antigenic determinant groups. The presence of more than one line in the haemolysates from the youngest embryonic stages may be due to the presence of more than one determinant group, to which different antibodies are directed.

FIGURE 10

Immunochemical studies.

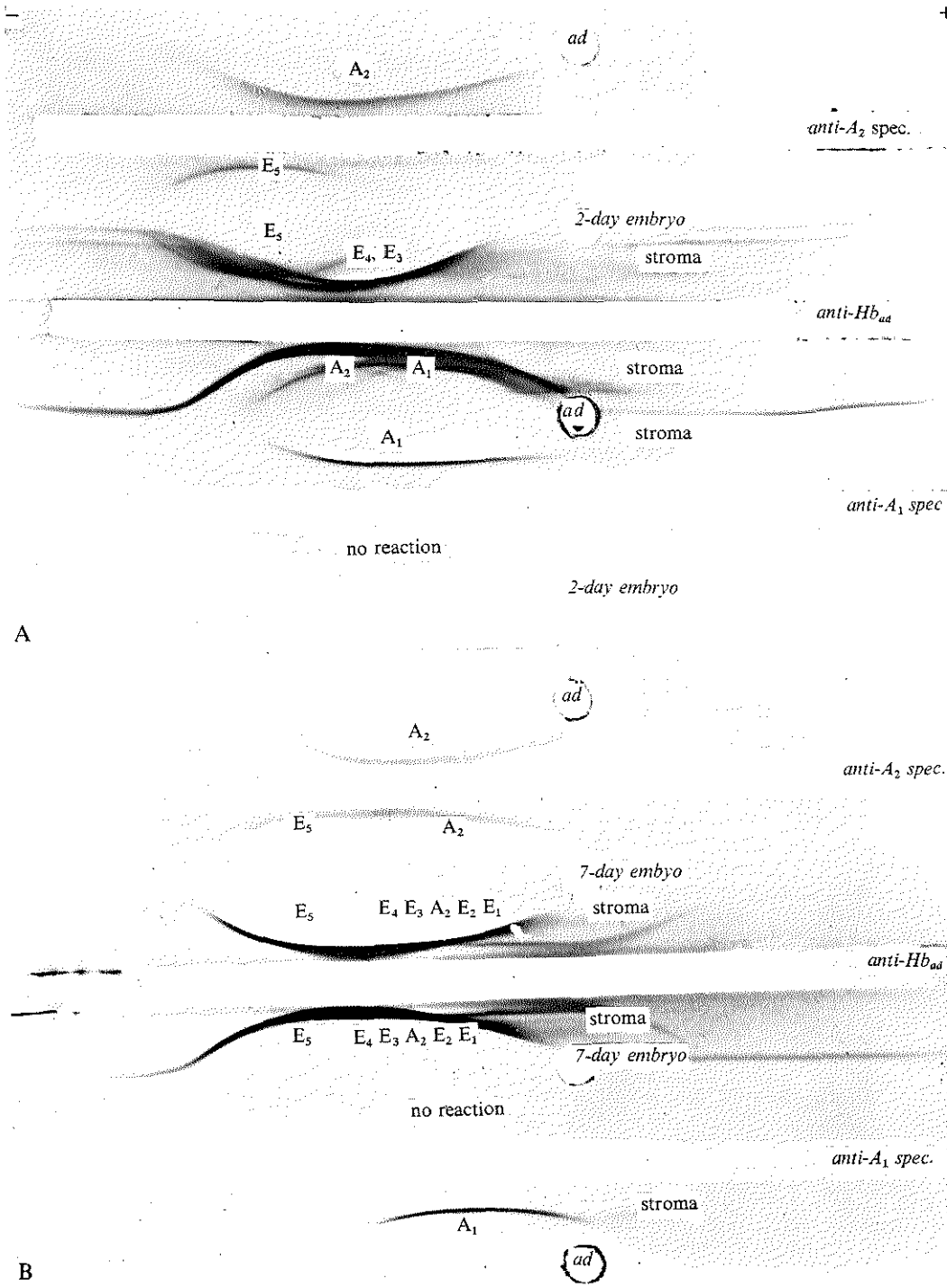
(A) Immuno-electrophoresis. Total haemolysate at two different concentrations from a normal adult chicken (circular wells) reacting with (1) antiserum to total adult haemolysate (central longitudinal reservoir), (2) the same antiserum adequately absorbed with an isolated A_1 fraction and stromal proteins (upper longitudinal reservoir), (3) the same antiserum, here adequately absorbed with an isolated A_2 fraction, but insufficiently absorbed with stromal proteins (lower longitudinal reservoir). Photographic representation of an unstained strip.

(B) The same immuno-strip stained with benzidine reagent, no stromal proteins are visible now. The horizontal precipitation line ($\downarrow$) which extends the common precipitation line represents a reaction of double diffusion between the excess A_2, added during the absorption of the anti-A_1 spec. serum (lower longitudinal reservoir) and anti-Hb_{ad} (central longitudinal reservoir). Similar horizontal precipitation lines are formed with anti-A_2 spec. serum.

(C) Ouchterlony plate. Isolated A_1 (lower right well) and A_2 (upper well) reacting with anti-Hb_{ad} (lower left well). The spurs indicate individual immunochemical specificity of A_1 and A_2. The confluent precipitates indicate antigenic determinants common to A_1 and A_2.

(D) Ouchterlony plate. The polypeptide chains $\gamma A_1E_1E_2$ (upper left well) and βA_1E_6 (upper right well), cut out of urea pH 3·2 starch gel and inserted without further treatment, an isolated A_1 (lower left well) and A_2 (lower right well) reacting with anti-Hb_{ad} (central well). The precipitation lines in this plate are more diffuse than in (C), which is most probably due to the interference of urea.

Abbreviations: see Fig. 1.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+
ad
A2
anti-A2 spec.
E5
2-day embryo
E5
E4, E3
stroma
anti-Hbad
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anti-A1 spec
no reaction
2-day embryo
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E5
A2
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FIGURE 11

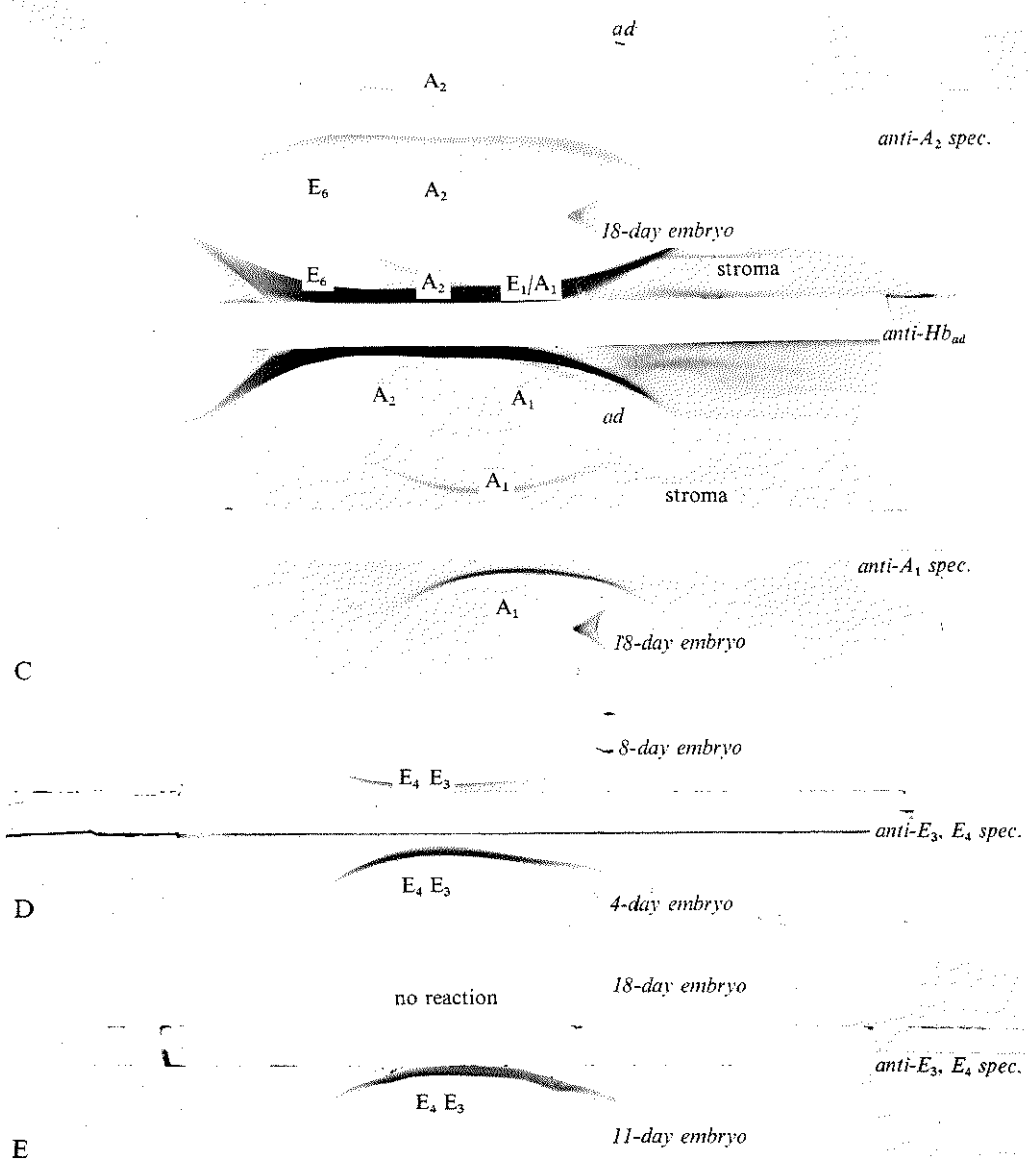

Fig. 11. Immuno-electrophoretic patterns of haemolysates from various embryonic stages and of a haemolysate from an adult chicken (round wells), reacting with several antisera (longitudinal reservoirs). Photographs of uncoloured strips. The same strips were stained with benzidine afterwards; the precipitation lines not reacting with benzidine are stromal proteins. Abbreviations: see Fig. 1.

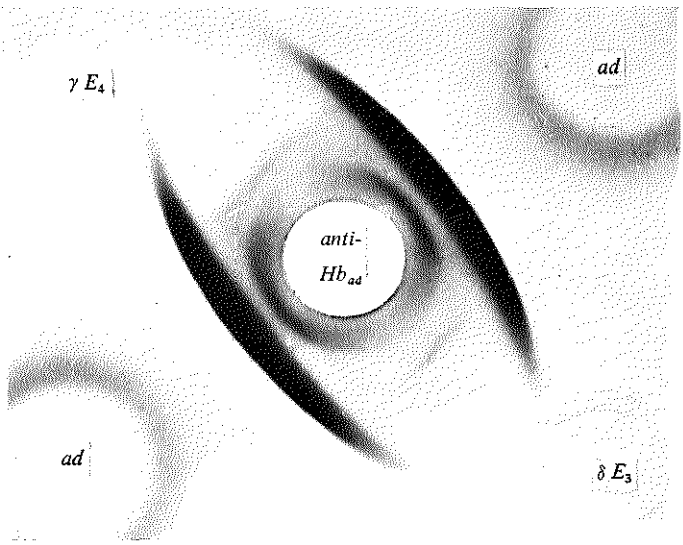

Fig. 12. Ouchterlony plate. The polypeptide chains γ E_4 and δ E_3, cut out of urea pH 3·2 starch gel and inserted without further treatment, show a faint reaction with anti-Hb_{ad}, indicating antigenic determinants common to these chains and the chains of adult haemoglobins. Abbreviations: see Fig. 1.

Antisera which are specific for embryonic haemoglobins were difficult to obtain. This may be due to the presence of antigenic determinants which are common to both adult and embryonic haemoglobins and which have a relative high potency to induce antibody production. We were only successful in the preparation of some antisera containing antibodies specific for E_3 and E_4. Absorption of these antisera with total adult haemolysate left into solution only these specific antibodies (anti-E_3, E_4 spec. serum). With these antisera it could be determined that E_3 and E_4 have disappeared on the 18th incubation day (Fig. 11 D, E).

Anti-A_1 spec. serum only reacted with A_1 as could be found in tests in which isolated embryonic haemoglobins were used. With this antiserum it was possible to demonstrate A_1 to appear on the 12th incubation day (Fig. 11 A, B, C) in spite of a haemoglobin fraction with virtually the same electrophoretic and chromatographic properties which is present from the 6th incubation day onward (Fig. 3). This fraction which does not react with anti-A_1 spec. is considered to be an additional embryonic haemoglobin and has been designated E_1 in view of its electrophoretic position. Anti-A_2 spec. serum appeared to react with A_2 but also with E_5 and E_6. Due to the difference in electrophoretic mobility of A_2 as compared to E_5 and E_6 (mobilities 10 and 0 respectively) it was possible to determine with this antiserum the time of appearance of A_2 as early as the 6th incubation day. The shift of the nadir of the precipitation line was used as a criterion in these studies (Fig. 11 A, B). The results obtained with specific anti-sera are summarized in Fig. 2.

Polypeptide chains (Figs. 10D, 12). Polypeptide chains cut from the urea pH 3·2 gels were analysed in Ouchterlony plates using anti-Hb_{ad}. The results are of a preliminary nature, as only a very limited number of analyses could be carried out. A difficulty encountered in these studies was the interference of urea with the precipitation reaction. The polypeptide chains α $A_2E_5E_6$, β $A_2E_1E_3E_4$, β A_1E_6 and γ $A_1E_1E_2$ from adult haemoglobins as well as the chains β E_2E_5, γ E_4 and δ E_3 from embryonic haemoglobins produced a precipitation line with anti-Hb_{ad}, indicating that all chains possess a considerable part of the common antigenic haemoglobin determinants (Fig. 10D). It must be emphasized that the reaction of the embryonic chains (Fig. 12) was poorer than the reaction of the adult chains. No specific determinants could be demonstrated with anti-A_1 spec., anti-A_2 spec. or anti-E_3, E_4 spec. serum. This may be due to the fact that the specific antigenic determinants which produce the weakest lines have disappeared from the patterns because urea is present, which is known to inhibit precipitation reactions. On the other hand, it is also possible that some determining sites do not belong to a single chain, but are only present in stereometric complete haemoglobin molecules as has been reported by Ovary (1964), who prepared polypeptide chains from human haemoglobin A by counter-current distribution and found poor reactions of these chains with anti-total haemoglobin in comparison with the native haemoglobin molecule.

DISCUSSION

Technical and general considerations

Inventarization and characterization of the haemoglobins during ontogenesis

Our studies indicate a sequential synthesis of haemoglobins during the ontogenesis of the chick (Fig. 3). Three periods of haemoglobin synthesis with transitions at the 6th and 12th day of incubation could be recognized. In the earliest embryos investigated (2–5 incubation days) four haemoglobins were found which are all different from the adult haemoglobins. Two of these, E_3 and E_4, are present in relatively large quantities and are considered to be major embryonic haemoglobins. Their electrophoretic and chromatographic properties are much like those of A_2, but their globin chain composition is markedly different (E_3: β $A_2E_1E_3E_4$, δ E_3; E_4: β $A_2E_1E_3E_4$, γ E_4; A_2: α $A_2E_5E_6$, β $A_2E_1E_3E_4$). Their immunochemical properties are also different from A_2, as could be demonstrated by two specific antisera, anti-A_2 spec. and anti-E_3, E_4 spec. The globin chains δ E_3 and γ E_4 are embryo-specific. Two minor embryonic haemoglobins, E_2 and E_5, could be distinguished from E_3 and E_4 and from A_1 and A_2 by electrophoresis and chromatography as well as by their globin-chain composition (E_2: β E_2E_5, γ $A_1E_1E_2$; E_5: α $A_2E_5E_6$, β E_2E_5). β E_2E_5 represents an embryo-specific chain. Immunochemical specificity of these embryonic haemoglobins has not been demonstrated.

The second period of haemoglobin synthesis appears to start at the 6th

incubation day. At this time the haemoglobins A_2 and E_1 appear and increase rapidly (Fig. 3) to become the predominant haemoglobins of this period. Haemoglobin A_2 is the normal major adult haemoglobin, consisting of the chains $\alpha A_2E_5E_6$ and $\beta A_2E_1E_3E_4$. Haemoglobin E_1 is considered to be a major embryonic haemoglobin which could not be distinguished from A_1 in electrophoretic and chromatographic studies. Its immunochemical properties, however, are different from those of A_1, as could be demonstrated with specific anti-A_1 serum. Furthermore, its β-chain is electrophoretically somewhat different from the β-chain of A_1 (E_1: $\beta A_2E_1E_3E_4$, $\gamma A_1E_1E_2$; A_1: βA_1E_6, $\gamma A_1E_1,E_2$). In the same period a rapid decrease of the major embryonic haemoglobins of the first period, E_3 and E_4, occurs. The haemoglobins E_2 and E_5 remain present in about equal relative amounts.

The third developmental period appears to start around the 12th incubation day, when replacement of E_1 by A_1 and of E_5 by E_6 occurs. E_6 is a minor embryonic haemoglobin, slightly different from E_5 in electrophoretic and chromatographic properties. Furthermore, its β-chain is different from that of E_5 (E_6: $\alpha A_2E_5E_6$, βA_1E_6; E_5: $\alpha A_2E_5E_6$, βE_2E_5). Immunochemical specificity of this embryonic haemoglobin has not been detected. During this period haemoglobin E_2 disappears.

After hatching the embryonic haemoglobin E_6 disappears during the first month. The time of disappearance of E_1 could not be determined exactly since the electrophoretic and chromatographic properties of E_1 are very similar to those of A_1. On the other hand, the use of anti-A_1 spec. serum enabled us to pinpoint the time of appearance of A_1 rather precisely. A further shift in the amount of A_1 and A_2 takes place in the post-hatching period.

The post-hatching haemoglobins

The presence of two *major adult haemoglobins* in the haemolysate of post-hatching chickens has been reported by almost all authors working on the subject (Godet, 1970, review). However, some of them emphasize the presence of additional minor haemoglobins (D'Amelio & Salvo, 1959, 1961; Buschmann, 1963; Matsuda & Takei, 1963; Schall & Turba, 1963; Alekseenko & Orekhovich, 1964; Marchis-Mouren & Lipman, 1965; D'Amelio, 1966; Hashimoto & Wilt, 1966; Simons, 1966; Godet, 1967; Schürch *et al.* 1968; Godet *et al.* 1970).

At least four fundamentally different explanations for the finding of these extra haemoglobin fractions may be considered.

(1) *Separation artifacts* resulting from the procedure by which the haemolysate was prepared. Freezing and thawing of the blood cells, ageing of the haemolysate, oxidation of the haemoglobins to methaemoglobin with or without the use of a ligand like cyanide have been described to induce hybrids, aggregation products and conformationally changed or degraded haemoglobin products (Manwell *et al.* 1963, 1966; Matsuda & Takei, 1963; Chernoff &

Pettit, 1964*b*; Hammel & Bessman, 1965; Riggs, Sullivan & Agee, 1964). Moreover, the technique which is used for separating the haemoglobins may cause artificial components. Column chromatography especially is notorious in this respect, because initial pH anomalies (Huisman, Martis & Dozy, 1958; Felland & Snyder, 1968) as well as overloading of the column and withdrawal of haemoglobin with the break-through volume (Bargellesi, Callegarini & Conconi, 1969) have been described to cause artificial peaks.

(2) *Separations resulting from minor heterogeneity*. As discussed by Huisman (1969), the extra haemoglobins are thought to be derivates of the major haemoglobins or of their precursors. Since the primary structure is not different from those of the major haemoglobins, these derived haemoglobins may be determined by the same genetic mechanism. In humans, derived haemoglobins are shown to be already present in the circulating blood cells, especially in case of severe anaemia (Horton & Huisman, 1965). Ageing of the haemolysate, however, is known to cause an increase in the number and concentration of these derived haemoglobins which suggests that metabolic activity plays a role. For human haemoglobins, blockade of the *N*-terminals of one or more of the polypeptide chains is described as a cause (Holmquist & Schroeder, 1964, 1966*a*, *b*). Especially in case of an acetyl loaded *N*-terminal, as found in human haemoglobin F_1 (Schroeder, Cua, Matsuda & Fenninger, 1962) and also in chicken haemoglobins (Satake, Sasakawa & Maruyama, 1963; Matsuda, Maita & Nakajima, 1964; Marchis-Mouren & Lipman, 1965), loading with more complex structures seems possible (Huehns & Shooter, 1966). Complex formation of β-chain SH groups with glutathione residues, as has been found in the case of human haemoglobin A_{1d} (Huisman & Dozy, 1962; Huisman, Dozy, Horton & Nechtman, 1965), provides another possibility for chain loading.

(3) *Separations resulting from genetic heterogeneity* of the animals from which the blood was taken. The incidence of genetic polymorphism in avian haemoglobins seems to be rare. The avian haemoglobins are regarded as relatively conservative on the species level (Brush & Power, 1970; Saha, 1964). Only Washburn (1968*a*) reported two allelic co-dominant chicken types: type I possessed 2 haemoglobins comparable to our A_1 and A_2, type II showed 3 haemoglobins, one comparable to our A_2, a different 'A_1' and an additional haemoglobin in the region of our group II stromal proteins. This author was able to breed the heterozygote which had all these haemoglobins.

(4) *Separations resulting from maturation heterogeneity*. The use of chickens of different ages may introduce also extra haemoglobin fractions. Young chickens are shown to contain a minor haemoglobin, more cathodic than the adult haemoglobin A_2 (Huisman & Schillhorn van Veen, 1964; Washburn, 1968*b*). Following severe anaemia by repeated bleeding or by treatment with phenylhydrazine, embryonic haemoglobins may reappear in the haemolysate of older chickens (Stino & Washburn, 1970). Similarly, haemoglobin F is found in humans under several pathological conditions (Huisman, 1969)

Taking into account the above-mentioned arguments, we conclude that the minor haemoglobins which have occasionally been found by several authors and by ourselves in the region of our group I and group II stromal proteins are due to artificial separations or minor heterogeneity. The varying number and amount of these extra haemoglobins also point strongly in this direction. The haemoglobins found on the base of artificial and minor heterogeneity are believed to possess an identical primary structure as the haemoglobins A_1 and A_2, from which they are derived. They seem therefore to be irrelevant from genetic point of view. Our chicken with 'rare' haemoglobins might have been genetic polymorph, but we have not carried out breeding experiments.

Three groups of authors characterized the extra haemoglobins in chicken haemolysates more extensively. Alekseenko & Orekhovich (1964) found after freezing and thawing of their haemolysates an extra haemoglobin in the region of our group II stromal proteins. They performed amino acid analyses and found small differences between all chains. However, they mention on the base of the presence of six disulphide bonds, the possibility of glutathione binding in this haemoglobin. Unfortunately, up to now they were unable to identify glutathione in their preparations. Nevertheless we consider their haemoglobin to be most probably a derivate of the major haemoglobin A_2. Hashimoto & Wilt (1966) reported three haemoglobins in the electrophoretic zone of our component I. They performed polypeptide chain analyses and found one polypeptide chain of these haemoglobins to have a comparable electrophoretic mobility as our γ $A_1E_1E_2$ chain, whereas the other polypeptide chains are only slightly different from our β A_1E_6 chain. Therefore we consider these haemoglobins to be derived from haemoglobin A_1. D'Amelio (1966) found an extra haemoglobin in the region of our group II stromal proteins. This haemoglobin was reported to contain the chains of A_1 as well as of A_2. This may point to an aggregation product in their preparations.

In conclusion it may be said that until now no absolute evidence has been adduced in the literature for the existence of extra haemoglobins which are different in primary structure from the two major haemoglobins, and which are situated in the zone of our group I and group II stromal proteins. On the other hand, the major haemoglobins (A_1 and A_2) which have been found in previous studies and by us are in our opinion different in primary structure. This view is supported by the following observations: (1) the haemoglobins A_1 and A_2 are consistently present in freshly prepared haemolysates; (2) the electrophoretic and chromatographic properties of these haemoglobins are markedly different; (3) these haemoglobins have different antigenic properties; (4) the electrophoretic properties of the polypeptide chains γ $A_1E_1E_2$ and α $A_2E_5E_6$ are also markedly different.

The minor haemoglobin (E_6) which is found in the haemolysates of young chickens is considered to be due to maturation heterogeneity and is also thought to be different in primary structure from A_1 and A_2 on the base of their electro-

phoretic and chromatographic properties and their polypeptide composition. This haemoglobin, however, might be a natural hybrid of A_1 and A_2.

The embryonic haemoglobins

Our results on the molecular forms of haemoglobins in the chick at different stages of development confirm and extend the results of the authors who agree about the existence of embryonic haemoglobins and are in contrast with those authors who report only quantitative changes in haemoglobin composition during the ontogenesis. (It seems of interest to compare our data in greater detail with these pertinent previous studies.)

D'Amelio & Salvo (1959, 1961) investigated the haemoglobins of chicken embryos with the Ouchterlony technique and immuno-electrophoresis, but did not report the presence of embryo-specific antigenic determinants. After agar-electrophoresis they observed two embryonic haemoglobins or groups of haemoglobins, in the electrophoretic region of our E_2–E_3–E_4 and E_5–E_6 respectively. In a later study D'Amelio (1966) reported on the presence of one embryo-specific polypeptide chain on the base of pH 1·9 starch-gel electrophoresis of haemolysates from 3 to 7 days incubated embryos. Using starch-gel electrophoresis at pH 8·6 Hashimoto & Wilt (1966) compared the fractions of haemolysates from 5 days incubated embryos with those from adult animals and described the presence of three embryonic haemoglobins which are comparable to our E_3, E_4 and E_5. A more extensive study has been carried out by Manwell *et al.* (1966) by means of starch-gel electrophoresis at pH 8·5. Their electrophoretic results are in agreement with those of Hashimoto & Wilt (1966). Both groups of authors isolated their embryonic haemoglobins from the pH 8·5 starch gels and separated the polypeptide chains in pH 1·9 starch gels. As the resolving power of pH 8·5 starch-gel electrophoresis for chicken haemoglobins is low, in comparison with DEAE-Sephadex chromatography, the peptide patterns which were obtained by both groups of authors are somewhat difficult to interpret, the more so because the patterns in pH 1·9 starch gels are less distinct than in the urea pH 3·2 starch gels used in our study. These difficulties may explain the conflicting conclusions of Hashimoto & Wilt (1966) and of Manwell *et al.* (1966) concerning the polypeptide chain patterns of embryonic haemoglobins. Yet these authors also conclude to the presence of three embryo-specific chains. They further report a switch-over in the synthesis of haemoglobins at the end of the 5th incubation day, when both adult haemoglobins are thought to appear. However, the fraction which was considered by these workers to be A_1 appeared to be different from A_1 in our study both in polypeptide-chain composition and immunochemically.

Qualitative changes in the haemoglobin patterns during ontogensis have also been claimed by Schürch *et al.* (1968). Their poly-acrylamide-gel electrophoresis patterns indicate the presence of at least one embryonic haemoglobin. Denmark & Washburn (1969) analysed haemolysates from 4 to 21 days incubated

embryos by means of cellulose-acetate electrophoresis. Their patterns are very similar to our starch-gel electrophoresis patterns. The interpretation by these authors, however, is quite different. They report the presence of only two embryonic haemoglobins which correspond to our E_2 and (or) E_3, and E_5 and (or) E_6, respectively. Furthermore, they observed in embryonic haemolysates two fractions called M and m which they thought to represent the adult haemoglobins A_2 and A_1, but which in the present study turned out to be E_4 and (or) A_2, and E_1 and (or) A_1 respectively. Therefore, we do not agree with the conclusions of Denmark & Washburn that A_2 and A_1 are present from the 3rd and 6th day of incubation respectively.

No equivocal evidence for the presence of embryonic haemoglobins was obtained by Fraser (1961, 1964, 1966) who used electrophoresis on paper and cellulose-acetate, chromatography on CM-cellulose and polypeptide chain analyses and by Wilt (1962) who carried out Ouchterlony's method and immuno-electrophoresis with an anti-total adult haemolysate serum. More recently, Simons (1966) was also unable to detect qualitative changes in CM-cellulose chromatographic patterns of embryonic haemolysates. Applying the elution scheme of this author (potassium phosphate, pH 6·8, in a linear gradient from 0·004 to 0·080 M) we found no clear separation between the major embryonic haemoglobins E_1, E_3 and E_4 and the adult haemoglobin A_1. The haemolysates of embryos incubated for 7 days as studied by Simons (1966) showed two chromatographic peaks, I and II; peak I contained our E_1, E_3 and E_4, peak II contained A_2. Haemolysates from older embryos also showed two peaks which appeared at about the same elution volume as peak I and peak II of the haemolysates from embryos incubated for 7 days. However, from the 14th incubation day onward, A_1 was the main constituent of peak I. Thus, the lack of evidence for the occurrence of qualitative changes in the analyses of Simons (1966) may be explained by the fact that the fractions which he obtained with CM-cellulose chromatography are far from pure.

Genetic and morphological considerations

Evidence from studies on human haemoglobins indicates that each polypeptide chain is coded by at least one gene (Ingram, 1963). On the base of this view the following tentative conclusions on genetic control of haemoglobin synthesis in the chicken embryo may be put forward.

During the first period of haemopoiesis (3–5 incubation days), most of the structural haemoglobin genes involved in haemoglobin synthesis are already active. This conclusion is based on the observation that six of the seven chains found in this study are present at these stages. Quantitative data indicate that the structural genes for $\beta\ A_2E_1E_3E_4$, $\delta\ E_3$ and $\gamma\ E_4$ transcribe at a high rate, and that the genes coding for $\beta\ E_2E_5$, $\alpha\ A_2E_5E_6$ and $\gamma\ A_1E_1E_2$ are only permitted to transcribe at a very low rate.

The second period (6–12 incubation days) starts with two events: a repression

of the activity of the genes coding for the major embryo-specific chains δE_3 and γE_4, and an activation (or derepression) of the genes for the adult chains $\alpha A_2E_5E_6$ and $\gamma A_1E_1E_2$. The high activity of the $\beta A_2E_1E_3E_4$ gene seems to be unaffected. When the chains produced at the highest rate combine, they form the new haemoglobins A_2 ($\alpha A_2E_5E_6$, $\beta A_2E_1E_3E_4$) and E_1 ($\beta A_2E_1E_3E_4$, $\gamma A_1E_1E_2$) which appear during this period. The major embryonic haemoglobins disappear, because the δE_3 and γE_4 genes become inactivated. The βE_2E_5 gene seems unaffected, resulting in the persistence of the haemoglobins E_2 (βE_2E_5, $\gamma A_1E_1E_2$) and E_5 ($\alpha A_2E_5E_6$, βE_2E_5).

The third period (12–21 incubation days) starts with the activation of a new gene producing the chain βA_1E_6. This chain was not observed before, but may have been already present in one or both of the haemoglobins which were observed in the foot of the A_2 peak and between the peaks of E_1 and E_2 after DEAE-Sephadex chromatography. These haemoglobins were not studied in detail. The βA_1E_6 chain apparently combines with the chains $\alpha A_2E_5E_6$ and $\gamma A_1E_1E_2$ in unequal amounts to form the newly appearing haemoglobins of this period, E_6 and A_1 respectively. At the same time the gene coding for the embryonic chain βE_2E_5 is switched off, resulting in the disappearance of the haemoglobins E_2 and E_5. At the time of hatching four haemoglobins are present again in the circulating blood cells of the chick: the two major adult haemoglobins A_1 (βA_1E_6, $\gamma A_1E_1E_2$) and A_2 ($\alpha A_2E_5E_6$, $\beta A_2E_1E_3E_4$) and the two minor embryonic haemoglobins E_1 ($\beta A_2E_1E_3E_4$, $\gamma A_1E_1E_2$) and E_6 ($\beta A_2E_5E_6$, βA_1E_6). These haemoglobins are combinations composed of only four different chains and may be considered as hybrid forms.

The demonstration of three clearly marked periods of haemoglobin synthesis makes the hypothesis of Hall (1934) that the different molecular forms of haemoglobin might be synthesized in different haemopoietic organs and the suggestion of Craig & Russell (1964) that these forms might be packaged in morphologically distinguishable erythrocytes most interesting. In Fig. 13 the cell strains and haemopoietic organs involved at different stages of development are represented. This figure is based upon data summarized by Romanoff (1960). The correlations between these data and our chemical findings are striking. The sudden decrease in the relative amount of the major embryonic haemoglobins E_3 and E_4 (Fig. 3) occurs when the relative number of primitive erythrocytes shows a steep fall.

The occurrence of the first erythrocytes of the definitive lines seems to correlate with the sudden appearance in the haemolysates of E_1 and A_2. Lucas & Jamroz (1961) point out the existence of several generations of definitive erythrocytes. The last definitive erythrocyte generation is thought not to appear before the end of the second incubation week, the time at which we observed the beginning of the third period in which E_1 and E_5 are replaced by A_1 and E_6 respectively. Similar correlations exist with the periods in which different haemopoietic organs are active. During the first period the yolk sac is the only

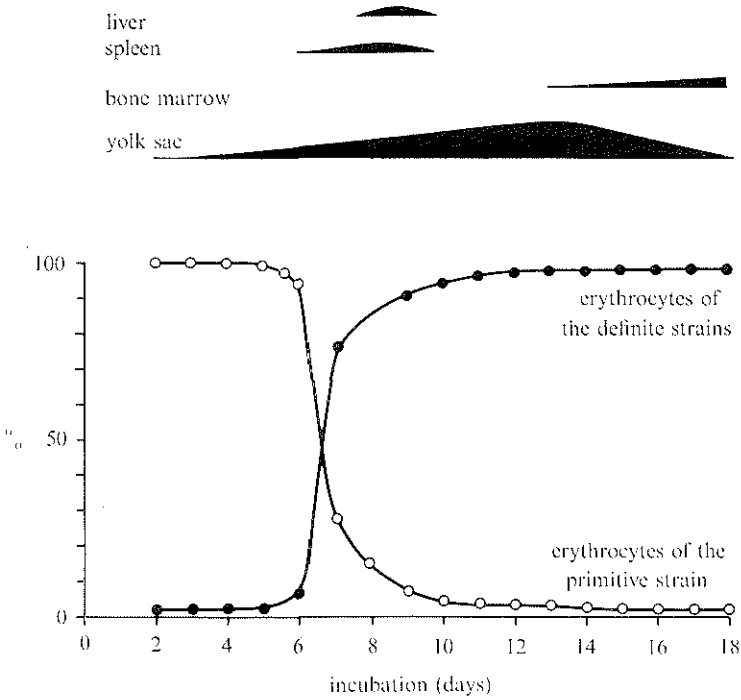

Fig. 13. Morphological data on haemopoiesis in the chicken embryo.

(extra-embryonic) haemopoietic organ. The second period is characterized by the onset of transient erythropoiesis in several intra-embryonic organs, e.g. liver and spleen. The third period starts when the bone marrow initiates definitive haemopoiesis. These correlations suggest a relation between the morphological and chemical differentiation of the haemopoietic system of the chicken, but additional data are needed. Especially the question whether specific haemoglobins are present in special erythrocytes and made in special haemopoietic organs should be answered. Immuno-fluorescence studies, aimed at detecting specific haemoglobins in erythrocytes from different tissue sources, will contribute to the solution of the problem under consideration.

After this discussion on the differences between the haemoglobins found, it seems justified to emphasize again our genetically interesting finding of a striking relationship between these haemoglobins. The possibility that the adult haemoglobins A_1 and A_2 and the embryonic haemoglobin E_1–E_6 might genetically be derived from one monomeric polypeptide-chain ancestor is supported by our observation that these haemoglobins have many antigenic determinants in common. This similarity in immunochemical behaviour was not due to the presence of common α, β, γ, or δ polypeptide chains, but rather to smaller common polypeptide formations which are present in all these chains.

The authors are greatly indebted to the late Professor G. J. ten Cate for guidance in the first phase of this work. Research facilities provided by Professors Moll and Van Doorenmalen are gratefully acknowledged. For critical reading of the manuscript we thank Professors Moll, Raven, Van der Molen and Drs Dullaart and Sanyal. Photos and graphs have been prepared by Mr Van den Oudenalder, Miss Delfos and the Audovisual section of the Medical Faculty.

This work was in part supported by a grant of the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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(Manuscript received 7 March 1972, revised 1 June 1972)

Cell Differentiation, 5 (1976) 263—273

RECOMBINATION OF EMBRYONIC HAEMOGLOBIN CHAINS DURING THE DEVELOPMENT OF THE CHICK

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Accepted 26 July, 1976

Embryonic differentiation is at present interpreted as the expression of variable gene activity. It is commonly thought that derepression of operator gene groups is the main cause of progress during development. However it is equally possible that gene repression plays a role in the appearance of new phenotypic characteristics. This paper illustrates such a possibility. It is known that in chickens embryonic haemoglobins exist which are replaced by other haemoglobins at about the sixth day of incubation. Analyses of globin chain composition of these haemoglobins by chromatography and urea/starch gel electrophoresis as well as TLC-fingerprinting and amino acid analyses of the individual globin chains showed that the haemoglobin switch was not associated with appearance of new globin chains but rather with disappearance of a number of embryonic chains. Moreover the relative proportion of the various chains changed at that time. From these findings we conclude that new haemoglobins arise from a recombination ('hybridization in vivo') of those globin chains which remain after the repression of a gene coding for embryonic chains.

In a previous study on haemoglobins of 2—21 days incubated embryos and adult chickens we reported two adult (A_1 and A_2) and six embryonic (E_1—E_6) haemoglobins (Schalekamp et al., 1972). In young embryos up to 5 days incubation four haemoglobins (E_2, E_3, E_4 and E_5) are present. At day six a marked change in haemoglobin composition occurs, when there is a sharp fall in the quantity of the two major embryonic haemoglobins (E_3 and E_4), and two new haemoglobins (E_1 and A_2) appear (Fig. 1a). Analysis of the globin chains of whole haemoglobins on urea/starch gels in that study did not indicate the appearance of any new type of globin chain at the time of the switch. In contrast, the disappearance of two embryonic globin chains (δE_3, $\delta(\gamma)$ E_4) was very striking (Fig. 1b). Since it seemed unlikely from these studies that the activation of a new globin gene is responsible for onset of production of new haemoglobins, it was postulated that hybridization of pre-existing globin chains explained their appearance (Fig. 2). Repression of embryo-specific genes may be responsible for a quantitative change in the production rate of remaining chains resulting in a shift in chain recombination and thus in the formation of new — hybrid — haemoglobins.

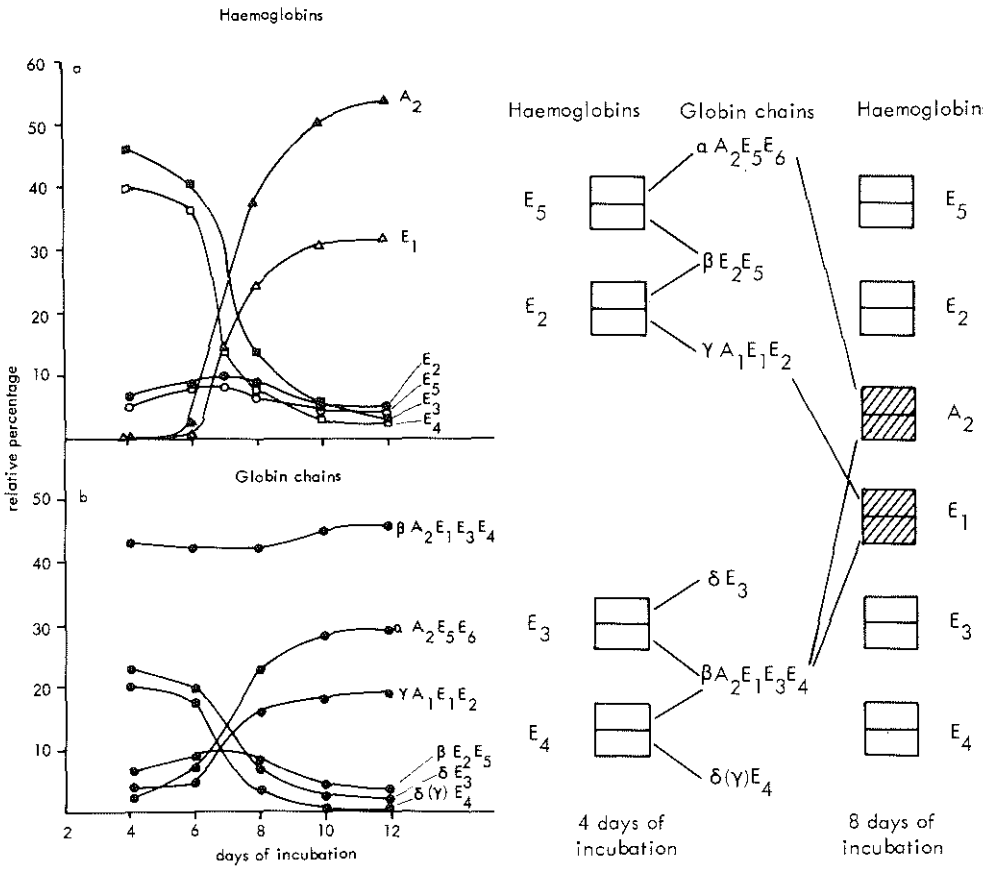


Fig. 1. a) Percentages of the haemoglobins found at different embryonic stages, as calculated from planimetric analyses of chromatographic curves measured at 419 nm as published by Schalekamp et al. (1972). Some new data points have been incorporated since then. b) Percentages of the globin chains found at different embryonic stages, as calculated from the percentages in the above figure on the basis of the known globin composition and a 1/1 ratio of the chains in each haemoglobin. δE_4 was called γE_4 in our previous study (Schalekamp et al., 1972), but will be called δE_4 from this study onward on the basis of the amino acid and peptide composition as reported here.

Fig. 2. Diagrammatic representation of the haemoglobins and their globin composition before and after six days of incubation. Hybridization proposal.

The present study was undertaken to prove this interpretation by more rigorous analysis. We report here the isolation by urea/Amberlite columns of globin chains of several purified embryonic and adult haemoglobins and the characterization of the isolated globin chains by urea/starch gel electrophoresis, TLC-fingerprints and aminoacid analyses.

MATERIAL AND METHODS

Embryonic haemoglobins E_2, E_3, E_4 and E_5 were prepared from haemolysates of 4—7 days incubated embryos. Haemoglobin E_1 was prepared from 8 days incubated embryos. Adult haemoglobins A_1 and A_2 were prepared from one year old chickens.

Isolation of the adult haemoglobins was performed as described previously (Schalekamp et al., 1972). The method by which the embryonic haemoglobins were isolated was modified involving continuous chromatography on different exchangers at 4°C without intermediate freezing or lyophilization stages. Four columns on four consecutive days were used, dialysis against the CO-saturated starting buffers being done overnight in each case. Column 1: CM-cellulose (Whatman CM 52) length 12 cm, diameter 2.5 cm, equilibrated with 0.01 M phosphate buffer pH 6.3. Application of the haemolysate and first elution with same buffer elutes the non-haemoglobin proteins. A second elution with 0.02 M phosphate buffer pH 8.2 containing 0.4 M NaCl elutes the total haemoglobin fraction. Column 2: DEAE-Sephadex (A50, Pharmacia) length 30 cm, diameter 2.5 cm, equilibrated with 0.05 M Tris—HCl buffer pH 8.8. The haemoglobin fraction of column 1 was applied in this Tris buffer and eluted with a 3 l linear pH-gradient of the same buffer (pH 8.8 to pH 7.4). From this column haemoglobins E_1, E_2, E_5 and a mixture of E_3, E_4 and A_2 were collected. Column 3: CM cellulose (Whatman CM 52) length 12 cm, diameter 2.5 cm, equilibrated with 0.01 M phosphate buffer pH 6.3. The mixture of haemoglobins E_3, E_4 and A_2 from column 2 was applied and washed in with the same buffer, a second step, with 0.02 M phosphate buffer pH 7.4 elutes a mixture of haemoglobins E_3 and E_4. Third elution with 0.02 M phosphate buffer pH 8.2 plus 0.4 M NaCl elutes haemoglobin A_2. Column 4: DEAE-Sephadex (A50, Pharmacia) length 30 cm, diameter 2.5 cm. The E_3, E_4 fraction of column 3 is resolved using the same elution scheme as for column 2.

The globin chains of each haemoglobin were separated on Amberlite colums (Amberlite CG 50, type II, The Rohm and Haas Company) length 30 cm, diameter 0.9 cm, equilibrated with 10% formic acid/50 mM mercaptoethanol. The haemoglobins were applied and washed in with the same solution. After additional washing with 80 ml 2 M urea/50 mM mercaptoethanol (brought on pH 1.9 with HCl) the first globin peak (α, γ or δ-chains) was eluted with a gradient of 80 ml of the 2 M urea solution and 1000 ml 6 M urea/50 mM mercaptoethanol (pH 1.9). The second globin peak (β-chains) was eluted with 8 M urea/50 mM mercaptoethanol (pH 1.9). Sometimes the haemoglobins were applied without previous dehaeming; the patterns were not different when dehaemed haemoglobins were analysed.

The isolated chains were dialysed throughout against distilled water/0.005 M HCl, lyophilized and identified by electrophoresis on pH 3.2 urea/starch gels (Schalekamp et al., 1972).

Fingerprints of 250—500 μg lyophilized, trypsinized globin chains were

TABLE I

Amino acid composition of adult and embryonic globin chains.

Amino acid	αA_2	αE_5	βA_1	βA_2	βE_1	$\beta E_3 E_4$ *	γA_1	γE_1	γE_2	$\delta(\gamma)E_4$	δE_3
Aspartic acid	12	12	13	13	13	12	13	13	13	10	10
Threonine	9	8	7	7	7	7	7	7	7	7	7
Serine	6	7	7	7	7	12	8	8	8	12	12
Glutamic acid	9	10	13	12	13	13	16	16	15	14	14
Proline	7	7	6	6	6	6	6	6	6	5	5
Glycine	9	8	8	8	8	7	7	7	7	6	6
Alanine	17	18	16	16	16	16	17	16	16	16	16
Half-cysteine	2	2	3	3	3	2	1	1	2	2	2
Valine	12	11	13	12	12	11	12	12	11	13	13
Methionine	1	1	1	1	1	0	4	3	2	0	0
Isoleucine	6	6	6	6	6	6	3	3	4	9	9
Leucine	16	16	17	18	17	17	15	15	16	15	16
Tyrosine	4	4	2	2	2	4	5	5	5	5	5
Phenylalanine	7	7	8	8	8	7	7	7	7	6	6
Lysine	12	11	10	10	10	11	11	12	11	10	10
Histidine	9	9	7	7	7	6	6	7	6	6	6
Arginine	4	5	6	6	6	5	4	4	5	5	5

Values are given in residues per mole of globin, as calculated by extrapolation to zero time after hydrolysis in 6 M HCl at 110° C. The number of residues and the molecular weight were assumed to be around 142 and 15500 respectively.

* Values for $\beta E_3 E_4$ are calculated from analyses on whole haemoglobins E_3 and E_4 by subtracting δE_3 and $\gamma(\delta)E_4$. A more elongated δ- and β-globin chain in these haemoglobins could account for the larger fluctuation between these values and those obtained with the other isolated β-chains. The deviating values for serine and tyrosine moreover are most probably due to difficulties known to exist in this method, where these values have to be extrapolated from extremely steep lines and are therefore largely dependent on the assumed residue ratio in mixtures of polypeptide chains. In general, however, we consider the $\beta E_3 E_4$-chain to be most comparable to the β-chain group.

made on 200 × 200 × 0.25 mm precoated TLC plates (Silica gel, without fluorescent indicator, Merck). Tryptic digestion was carried out as follows: 2--20 mg purified lyophilized globin chain was denatured in 8 M urea at 65°C for 45 min, dialyzed against distilled water and treated at pH 8.5 with 0.5% trypsin (recrystallized 3 times, Worthington), the trypsin/protein ratio

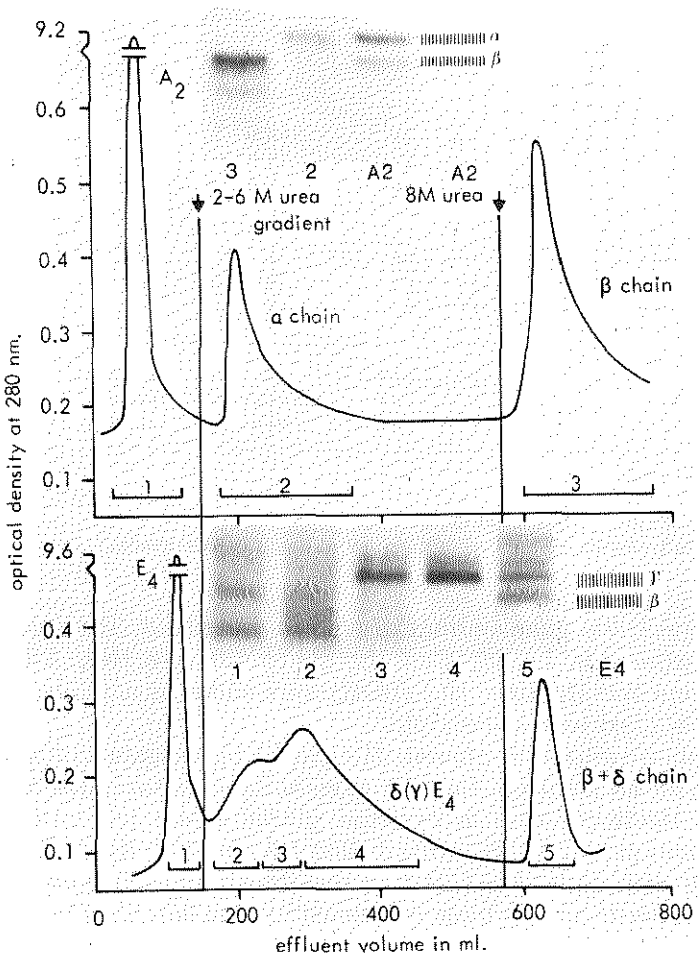


Fig. 3. Separation of the polypeptide chains of isolated A_2 (above) and E_4 (below) haemoglobin on Amberlite columns. The isolated fractions were analysed on urea/starch gels, pH 3.2 (insets) and compared to the appropriate whole haemoglobins. Zones which were seen outside the pattern of the fresh whole haemoglobins (arched zones) were considered artifacts, due to the second exposure to urea at a low pH. On the basis of the pattern of E_4 (see also Fig. 4) we considered the zones of fraction 1 and 2 to contain debris, fraction 3 is a mixture of 2 and 4, fraction 4 contains $\delta(\gamma)E_4$ and fraction 5 contains a mixture of β- and $\delta(\gamma)E_4$-globins.

TABLE II

Peptide composition of the polypeptide chains of adult and embryonic chicken haemoglobins.

Electroph. zone	I										II			
Chromatogr. distance	A	B	D	D/E	E	E/F	F	F/G	G	H	L	B	C	D	E
α															
A_2 prep I					++		++		++		++	+++ [a]	++		
A_2 prep II					+++		++		+++		++	++ [a]	+++		
E_5					++		++		++		++	++ [a]	++		
β															
A_1		++	+++			+++	++	++		++ [b]			+++		
A_2		++	++			++	++	+++		++ [b]			+++		
E_1		++	+++			+++	++	+++		++ [b]			+++		
γ															
A_1	+ [a]			+++			++	++		++		++ [a]		+	+++
E_1	+ [a]			+++			++	+		+++		++ [a]		+++	+
E_2	+ [a]			+++			++	+		++		++ [a]		+++	+
δ															
E_3 prep I						+	+	++ [a]		++		+++ [a]	++		
E_3 prep II						++	+	++ [a]		+++		+++ [a]	+		
E_4							+	++ [a]		+++		+++ [a]	+		

[a] Yellow (proline containing) peptide spots.
[b] Brownish peptide spots.
+++ Indication of colour intensity of the ninhydrin stained spots.
5 peptides appear in common to all polypeptide chains. One of them (IV A) could be identified as lysine.

being 1 : 50. After 30 min the same amount of trypsin was added. The digestion was terminated after 2 h, by adjusting the pH to 6.0 with HCl. After tryptic digestion leucine and arginine—HCl (BDH) were added as markers, so that 5 μg of each was applied with the sample.

Ascending TL chromatography in phenol—H_2O (3 : 1 by weight) containing 20 mg NaCl per 100 ml solution, for 17 h at room temperature, was followed by horizontal electrophoresis in pyridine—glacial acetic acid—water (10 : 5 : 485 by volume) pH 5.2 at 500 volts during 1.5 h at 4°C. Spots were visualized by spraying with ninhydrin (pH 5.0) and drawn on tracing paper. The patterns were compared on the basis of calculations as described under Fig. 4.

Amino acid analyses of isolated globin chains were performed in an automatic amino acid analyser (BC 200, Biocal) as described in Table I.

RESULTS

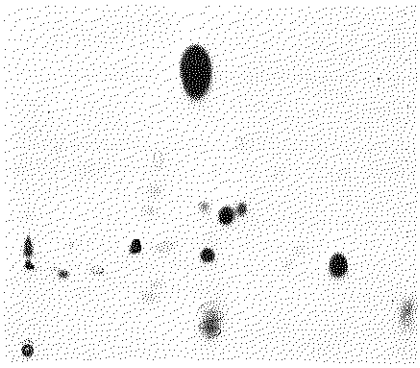
Fig. 1 shows some new data points for haemoglobin A_2 and E_1 from 4 and 6 day-incubated embryos in addition to those previously published (Schale-

							III									IV
F/G	G	I	K	L	N	O	E	F/G	G	G/H	H	J	J/K	K	M	A
	+++	++	++				+	+				+++ [a]	++	++		++
	+++	++	++				++	+				+++ [a]	+++	++		+
	+++	++	++				+	+				+++ [a]	++	++		+
+++	+++			+++				+	+				+	+	++	++
+++	+++			+++				+	+		+			+	++	++
+++	+++			+++				+	+		+			+	++	++
++	++	+++	++ [b]		+++			+						++		++
++	+	+++	++ [b]		++			+						+		++
++	+	+++	++ [b]		++			+						+		++
+	++			+++		+++		+	+	+++				+++		++
++	+++			+++		+++		+	+	+++				+++		++
+	++			+++		+++		+	+	+++				+++		++

kamp et al., 1972). This new evidence, obtained with the improved haemoglobin fractionation procedure, confirms the sudden appearance of haemoglobins A_2 and E_1 at about the sixth day of incubation.

Fig. 3 shows typical chromatographs of the globin chains of the A_2 and E_4 as analysed on Amberlite columns and subsequently identified by urea/starch gel electrophoresis. In all cases, globin chains were isolated for fingerprinting after tryptic digestion, taking care to exclude artefactual degradation products which were not present in fresh haemoglobins subjected only to electrophoresis in urea/starch gels (see legend to Fig. 3 for details). Amino acid changes of these isolated globin chains are summarized in Table I. Fingerprints of tryptic digests of α-, β-, γ- and δ-type chains from different haemoglobins are compared in Fig. 4 and summarized in Table II. These results strongly suggest the identity of the following chains: α in A_2 and E_5; β in A_1, A_2 and E_1, γ in A_1, E_1 and E_2; and δ in E_3 and E_4. Unfortunately, since pure β-chains could not be obtained from E_3 and E_4, it was not possible in this study to establish by fingerprinting the identity of the β-chains in haemoglobins A_2, E_3 or E_4.

However, the second chain of E_3 and E_4 is in chromatographs on Amber-



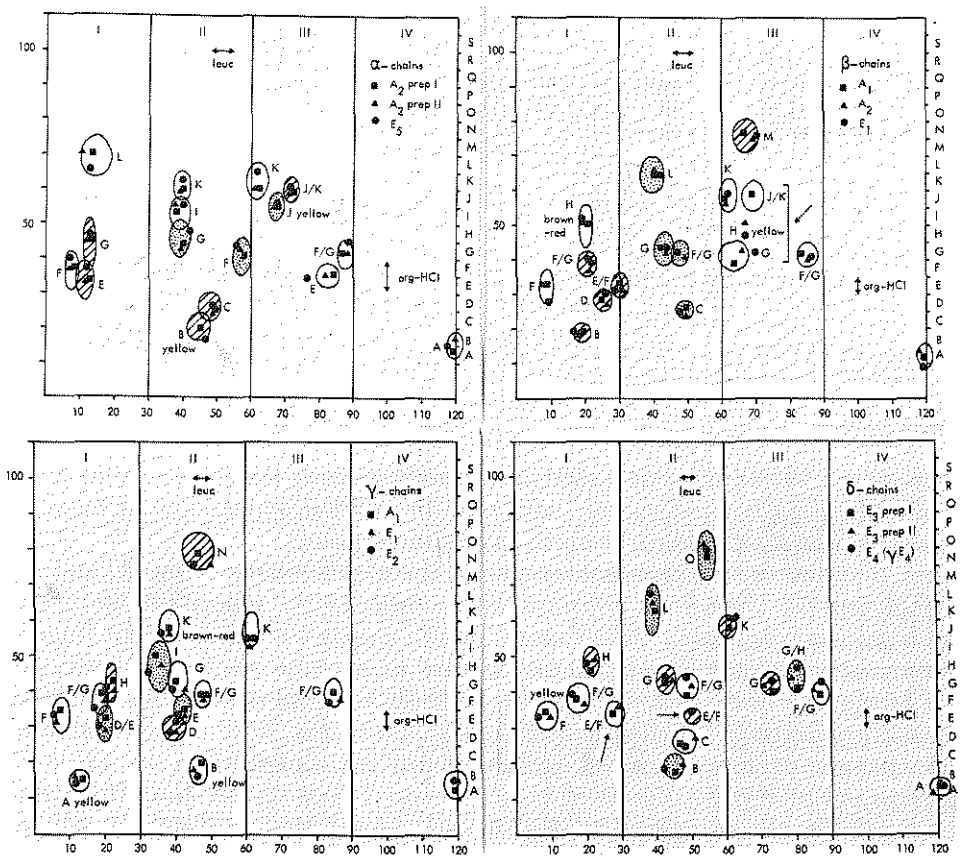


Fig. 4. (Above) Photograph of a fingerprint of an isolated αA_2 polypeptide chain. In this case 600 μg sample and 10 μg leucine and arginine—HCl are applied. This overloading (necessary for photography) caused some retardation in chromatography. Also contaminating dominant β-chain peptides (encircled) show up by this procedure. (Below) Diagrammatic representation of the fingerprints of isolated polypeptide chains. The relative chromatographic and electrophoretic mobilities of the centres of the spots were calcu-

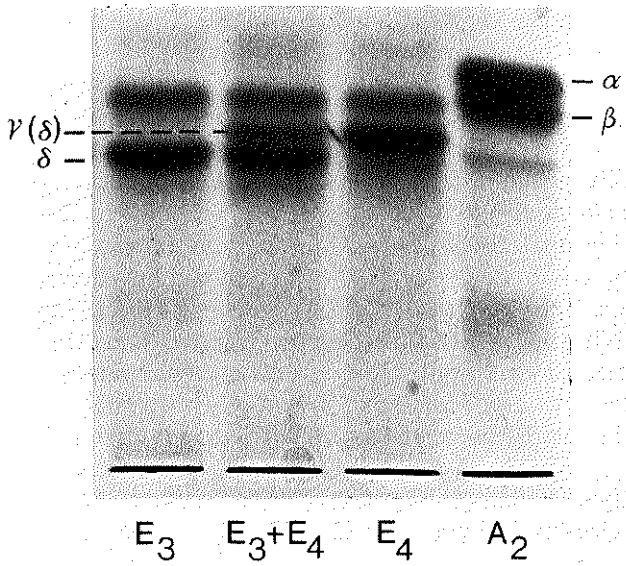


Fig. 5. Globin chain composition of haemoglobins E_3 and E_4 as compared to A_2 in urea starch gels pH 3.2. On the basis of such analyses we considered the β-chains in A_2, E_3 and E_4 to be identical.

lite (Fig. 3) and electrophoresis in urea/starch gels (Fig. 5) identically to the β-chain of A_2. Moreover, the amino acid analyses of the three chains are very similar (Table I). Thus we feel no reason to doubt our previous statement that the β-chains in A_2, E_3 and E_4 seem to be identical.

DISCUSSION

The results presented in this paper are in agreement with our previous findings and with the findings of other authors (Brown et al., 1974; Cirotto et al., 1975; Matsuda et al., 1972, 1973; Moss et al., 1969, 1974; Saha, 1964). A discrepancy in interpretation is only present when the β-chains of the haemoglobins A_1, A_2, E_3 and E_4 are concerned. This is probably due to

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lated using the markers leucine and arginine-HCl (rel. mobs. 100) respectively. The spots were characterized by their electrophoretic (regions I—IV) and chromatographic (regions A—S) mobilities, and compared on that basis. The marks represent the mean of the centres of at least 5 fingerprints of one tryptic digest. The ovals represent the most extreme centre values found for respectively αA_2- prep. I, βA_1, γA_1 and δE_3 prep. I. Dashed ovals are the most intensive spots, hatched ovals are intermediate and open ovals are the weakest spots. Colour differences are also indicated, yellow spots representing proline-containing peptides. The mobilities of the αA_2- and δE_3-chain spots were calculated from two preparations, derived from different animals, to establish the reproducibility of the methods used.

the fact that the β-type chains are very difficult to isolate. However, these considerations do not affect the basic conclusion of our work.

Summarizing our results we would state that four classes of globin chains (α, β, γ, δ) are present in embryonic haemolysates. Only three of them (α, β, δ) persist in adult haemolysates. There are marked chemical differences between the classes of globin chains, as demonstrated by electrophoresis, peptide maps and amino acid analyses. Within one class a family of chains may be present. The chemical differences between chains of the same class are sometimes suggested by electrophoretic methods; but such differences are doubtful in peptide maps. They are probably beyond the resolving capacity of amino acid analyses. Whether or not these minor differences are due to primary amino acid substitutions or to epigenetic modifications remains unclear. Only sequence studies will give definitive information on this point. This, however, is not crucial, as there seems to be little doubt that all the genes associated with the synthesis of the new haemoglobins are already active at a stage in which these haemoglobins are not yet formed. At day 5 of incubation, four haemoglobins are present: E_2 (βE_2, E_5, γA_1 E_1 E_2), E_3 (βA_2 E_1 E_3 E_4, δE_3), E_4 (βA_2 E_1 E_3 E_4, δE_4) and E_5 (αA_2 E_5 E_6, βE_2 E_5). At day six the gene (or class of genes) for the δ-chains (δE_3, δE_4) is switched off. Concomitant recombination of remaining chains results in the appearance of two new haemoglobins: A_2 (αA_2 E_5 E_6, βA_2 E_1 E_3 E_4) and E_1 (βA_2 E_1 E_3 E_4, γA_1 E_1 E_2). That these haemoglobins are not present at an earlier stage, despite the presence of their globin chains, may be due to the fact that the relative production rate of the globin chains in question changes at this time.

Haemoglobins E_2 and E_5 are only minor haemoglobins, whereas haemoglobins A_2 and E_1 are major haemoglobins. Therefore in terms of globin chains there is an increase in the relative production rate of two of the globin chains (αA_2 E_5 E_6 and γA_1 E_1 E_2). The relative amounts of globin chains may be critical for the type of globin chain combination. Whether the production rate of globins is regulated at the translational or at the transcriptional level remains to be clarified.

During the development of chickens a second haemoglobin switch takes place at the 14th day of incubation (Schalekamp et al., 1972), when the haemoglobins E_1 (βA_2 E_1 E_3 E_4, γA_1 E_1 E_2) and E_5 (αA_2 E_5 E_6, βE_2 E_5) are replaced by A_1 (βA_1 E_6, γA_1 E_1 E_2) and E_6 (αA_2 E_5 E_6, βA_1 E_6). Here in addition to the disappearance of a polypeptide chain (βE_2 E_5) a new polypeptide chain (βA_1 E_6) may appear. It is equally possible however that this chain is also already present at an early stage, in the 'minor' haemoglobin which was described in our previous study. Because of the low quantity, analysis of this minor fraction is still not possible.

This postulated role of gene repression in producing new haemoglobins by recombination of remaining chains is supported by other work in the literature. Garrick et al. (1974) demonstrated an increased synthesis of one type of globin chain in rabbit reticulocytes, resulting from inhibition of the synthesis of chains of the opposite type. These authors postulate the same

mechanism to act in the case of thalassaemias, where the inactivity of for instance the β-gene locus would stimulate the production of γ-chains in the case of F-thalassaemia ($\alpha_2\ \gamma_2$) or δ-chains in the case of A_2-thalassaemia ($\alpha_2\ \delta_2$). The possibility of the appearance of a new hybrid haemoglobin under such circumstances may be illustrated by the presence of the pathological haemoglobin H (β_4) in humans with an inactive α-gene locus in the case of H-thalassaemia (Jones et al., 1963).

The presence of fetal or adult globin chains together with an embryo-specific globin chain in embryo-specific proteins has been noticed previously in embryonic human and animal haemoglobins (see the reviews of Kazazian et al., 1974 and Kitchen et al., 1974) and in embryonic bovine α-crystalline (Van der Kamp et al., 1974). Especially the example of haemoglobin F ($\alpha_2\ \gamma_2$) in humans is relevant in this respect, as this fetal haemoglobin is known to be made up of globins that are also present in the embryo-specific haemoglobins Gower II ($\alpha_2\ \epsilon_2$) and Portland ($\zeta_2\ \gamma_2$). Therefore this haemoglobin may be likewise regarded as a hybrid haemoglobin. In spite of these facts, it has not been emphasized sufficiently in previous work that during early embryonic life, qualitatively sufficient adult or fetal globin chains may be present to allow the production of new, more mature, proteins, without the necessity of a new structural gene becoming active.

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J. Embryol. exp. Morph. Vol. 34, 2, pp. 355–371, 1975
Printed in Great Britain

Iron incorporation and haemoglobin synthesis in erythropoietic cells during the ontogenesis of the mouse

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SUMMARY

Iron incorporation (^{59}Fe) into erythropoietic cells from adult and foetal (11- to 15-day) peripheral blood and from foetal (12- to 15-day) livers has been investigated. Ion-exchange chromatography of haemolysates from such cells revealed two groups of ^{59}Fe-containing proteins. The first group (X-fraction) was eluted from CMC-columns in the void volume and was highest in lysates of immature erythropoietic cells. This fraction contained a radiolabelled haemprotein of high molecular weight as well as other ^{59}Fe-containing proteins. The haemprotein does not appear to be related to haemoglobin.

The second group consisted of haemoglobins. One major (A_1) and two minor (A_2 and A_3) haemoglobins were found in adult peripheral blood. In foetal liver lysates two major (F_1 and A_1) and two minor (F_2 and A_3) haemoglobins were present. The relative proportion of the major haemoglobins changed during development. Haemoglobin F_1 was highest in the more mature livers. F_1 proved to be different from A_1 by chromatographic behaviour, in polypeptide chain composition and in fingerprints. A unique foetal polypeptide chain, intermediate in electrophoretic behaviour between the adult α- and β-chain, was identified. In young foetal peripheral blood (11-day), in which 95 % of the cells are of yolk-sac origin, one major (E_1), two intermediate (E_2 and E_3) and one minor (F_1) haemoglobin were demonstrable. Haemolysates of the peripheral blood of older embryos contain haemoglobins from erythroid cells of both yolk-sac and foetal liver origin. The haemoglobin pattern of such lysates is explicable in terms of the decreasing amount of embryonic haemoglobins (E_1, E_2 and E_3) and the increasing amount of foetal haemoglobins (F_1 and A_1). Since A_1 and E_1 are the most prominent haemoglobins of livers from young embryos and yolk-sac erythrocytes respectively, and since they are very similar in chromatographic behaviour, foetal peripheral blood at all stages contains one dominant haemoglobin peak in the A_1–E_1 region. Most authors have neglected the relatively slight elevation of the foetal haemoglobin peak (F_1) in front of A_1–E_1, the more because the F_1–A_1 region has been suspected sometimes to contain artificial haemoglobin components (Riggs, 1965). This probably explains why no foetal haemoglobin (F_1) has been reported previously in the peripheral blood of foetal mice.

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INTRODUCTION

Erythroid cell differentiation represents the process whereby a multipotential precursor cell becomes committed to devoting most of its synthetic machinery to the production of a specific protein, haemoglobin. This process involves the co-ordinated control of many biochemical and morphological events, some of which have been elucidated using *in vitro* cultures of erythropoietic cells stimulated to differentiate by the hormone erythropoietin (for review, see Harrison, Conkie & Paul, 1973). A relatively early event in such cultures is the stimulation of iron incorporation and the synthesis of haem (Krantz & Fried, 1968; Hrinda & Goldwasser, 1969; Gross & Goldwasser, 1970; Shepp, Toff, Yamada & Gabuzda, 1972). This is then followed at a later stage by the synthesis of haemoglobin. However, the mechanism whereby iron is transported from the cell surface, where it is delivered by serum transferrin (Katz & Jandl, 1964), to the mitochondrion, where it is utilized for haem synthesis, has not yet been elucidated. Furthermore, very little is known about the factors involved in transport of haem to the globin chains. Thus more information would be valuable concerning the role of iron and haem-iron-containing proteins, including the haemoglobins, during erythroid cell development. This paper describes experiments concerning the relative proportions and characteristics of iron- and haemproteins in erythroid cells of yolk-sac and foetal liver origin in the mouse. It is likely that the relative proportions of the iron- and haemproteins differ greatly in mature and immature cells. Information about this may be useful, not only as information about embryonic development, but also to explain discrepancies which may arise when calculations of radioactive iron or haem-iron incorporation into haemopoietic cells are used to quantitate haemoglobin synthesis (Harrison *et al.* 1973).

MATERIALS AND METHODS

Collection and lysis of cells. Adult Porton white Swiss mice were bled by decapitation. Foetuses of the same strain aged 11–15 days (the day on which vaginal plugs were observed being taken as day zero) were washed thoroughly and bled from the umbilical vessels for peripheral blood cells. The livers were excised, washed thoroughly and disaggregated by pipetting. Isotonic ice-cold saline was used for washings and cell collection. The blood cell suspensions were washed three times in at least a tenfold volume of saline. Following the final washing the cells were resuspended in a buffer containing 0·81 % NaCl, 0·12 % Tris and 0·03 % Mg acetate, pH 7·0. When all clumps were dissociated, NP_{40} (Nonidet P_{40}, Shell Chemicals) was added to 0·5 %. Lysis was allowed for about 30 min. In some experiments the cells were lysed by adding glass-distilled water, but, especially with foetal liver cells, the NP_{40} method gave better results. The haemoglobins were converted to the CO-form, centrifuged and dialysed overnight against the starting buffer. All manipulations were done at 4 °C. No

freezing-and-thawing, ammonium sulphate precipitation, conversion to cyanmethaemoglobin or ageing of the haemolysate (more than 24 h) was allowed, as all this is known to promote extra haemoglobin peaks (Riggs, 1965; Manwell, Baker & Betz, 1966).

An occasional experiment was done with Friend cells (clone M2) grown in bulk cultures as described by Conkie, Affara, Harrison & Paul (1974). Erythroid differentiation in Friend cells was induced by culturing for 5 days in the presence of 1·5 % dimethyl sulphoxide.

Preparation and estimation of radiolabelled proteins. Ten μCi [^{59}Fe]chloride or citrate (spec. act. 2–10 μCi/μg Fe) was injected intraperitoneally into adult and pregnant animals, 24 h before death. In one experiment [^{3}H]leucine (250 μCi) was added to a cell culture of mouse erythropoietic cells, 5 days before cell harvest. The radioactivity of the ^{59}Fe-labelled fractions was measured by adding aliquots of the separated fractions to Triton X-100/toluene-based scintillator (1:2, v/v) and counting them in a liquid scintillation counter (Beckman LS-100). [^{3}H]leucine-labelled fractions were acid-precipitated on to glass-fibre discs and counted in a toluene-based scintillator.

Chromatography of proteins. Anion-exchange chromatography was performed over carboxymethyl cellulose (Whatman CM 52) columns using 0·01 M sodium phosphate buffer in a linear gradient from pH 6·7 to 8·2 for elution. Cation-exchange chromatography was performed over diethylaminoethyl-Sephadex (A 50, Pharmacia) columns, using 0·05 M TrisHCl buffer in a linear gradient from pH 8·2 to 7·2. Gel-filtration columns were made of Sephadex G-50, G-100 and G-200 (Pharmacia) and developed with 0·01 M sodium phosphate buffer pH 6·7.

Chromatography of polypeptide chains. Acid-acetone precipitated globins of isolated lyophilized haemoglobin fractions were analysed on CM 52 columns made up in 8 M urea and 50 mM mercaptoethanol. The columns were developed with sodium phosphate buffer pH 6·9 in a linear gradient from 0·01 to 0·1 M, these buffers also containing the same amounts of urea and mercaptoethanol (Lingrel, 1972).

Starch-gel electrophoresis of polypeptide chains. Isolated haemoglobins A_1, A_2, A_3 and F_1 were submitted to vertical starch-gel electrophoresis according to Gilman & Smithies (1968), using 8 M urea in barium-lactate buffer (pH 3·2) containing mercaptoethanol.

Digestion of globins. Isolated haemoglobins A_1 (from adult animals) and F_1 (from foetal livers) were denatured in 8 M urea at 65 °C for 45 min, dialysed against water and treated at pH 8·5 with 0·5 % trypsin (recrystallized three times, Worthington), the trypsin/protein ratio being 1:50. After 30 min the same amount of trypsin was added. The digestion was terminated after 2 h, by adjusting the pH to 6·0 with HCl and lyophilization.

Peptide mapping. About 300 μg lyophilized trypsinized A_1 or F_1 was applied together with 5 μg leucine and 5 μg argHCl to activated 200 × 200 × 0·25 mm

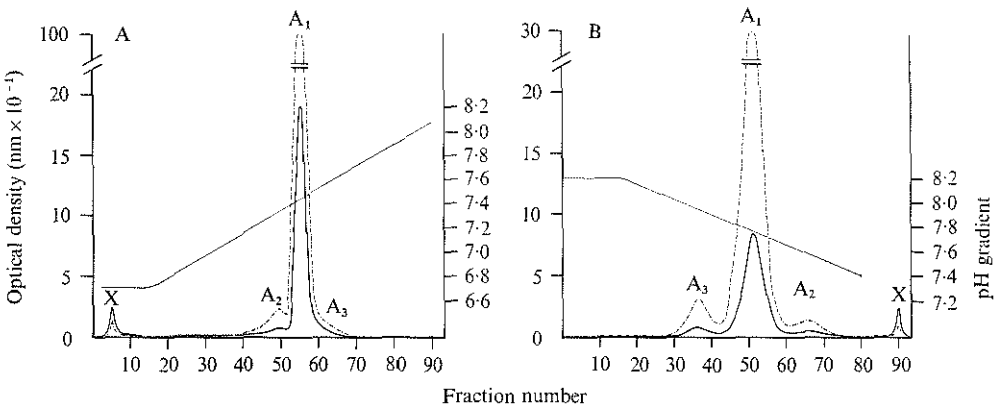

Fig. 1. Haemolysates prepared from mouse adult peripheral blood cells. (A) CM-cellulose chromatogram. (B) DEAE-Sephadex chromatogram. The elution pattern of optical density at 280 nm (——) for protein and at 419 nm (—·—) for haem-protein are shown. The pH-gradient is indicated (—).

precoated TLC plates (Silicagel 60, without fluorescent indicator, Merck). Ascending chromatography in phenol–H_2O (3 + 1 (w/w) containing 20 mg NaCN per 100 ml solution) for 16 h at room temperature, was followed by horizontal electrophoresis in pyridine–glacial acetic acid–water (10 + 5 + 485 (v/v), pH 5·2) at 500 V for 1·5 h at 0 °C. Spots were visualized by spraying with ninhydrin (pH 5·0).

Spectrophotometry. The fractions of each column were measured in a Sp 400 spectrophotometer (Unicam) at 280 nm for protein and at 419 and/or 540 nm for haemprotein content. Approximate relative concentrations of (haem) protein were calculated from these readings. Absorption spectra of some of the fractions were recorded in a Sp 800 spectrophotometer (Unicam).

Haem extraction. ^{59}Fe-labelled haem was extracted in butanone from an acidified aliquot of the fractions in Drabkins solution (Cole & Paul, 1966) and counted on glass-fibre discs in a toluene-based scintillator.

Catalase activity was determined by the rate of breakdown of hydrogen peroxide (Chance & Herbert, 1950) as recorded by the decrease in optical density at 240 nm in an automatically-recording Gilford spectrophotometer. A catalase standard was used to calculate the absolute amounts of catalase.

Ammonium sulphate precipitation was performed according to the method of Scher, Holland & Friend (1971), in which haemoglobin precipitates between 60 % and 85 % saturated ammonium sulphate.

Treatment with iodoacetamide was performed as described by Riggs (1965).

RESULTS

Adult haemolysates (Fig. 1 A, B). Three haemoglobins, one major (A_1) and two minor (A_2 and A_3), were found by chromatography on both DEAE-Sephadex

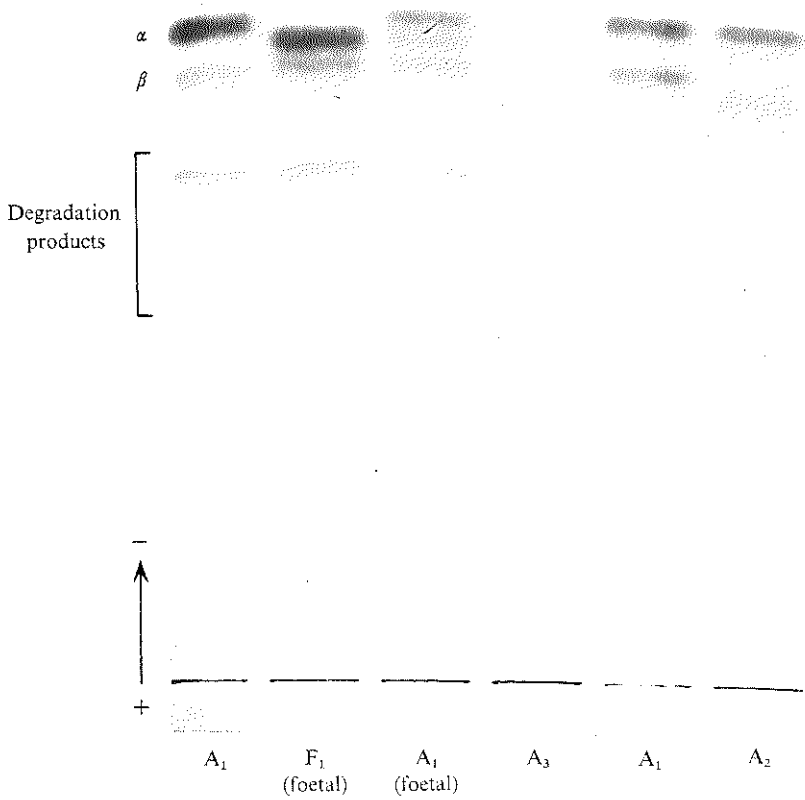

Fig. 2. Polypeptide chain composition, as revealed by urea starch-gel electrophoresis, of adult and foetal haemoglobins isolated by CM-cellulose chromatography. A_1, A_2 and A_3: haemoglobin peaks derived from adult peripheral blood. A_1 and F_1 (foetal): haemoglobin peaks derived from 14-day foetal livers.

and CM-cellulose. Apart from their chromatographic behaviour these haemoglobins were different in polypeptide chain composition as judged by urea starch-gel electrophoresis (Fig. 2). All adult haemoglobins seemed to contain the adult α-chain; however, a normal adult β-chain seemed not to be present in haemoglobins A_2 and A_3. Whether or not the other zones visible represent a different chain, or are due to degradation products, is not clear, owing to the atypical aspect of these zones. A small proportion of the total protein was eluted from CM-cellulose in the void volume (X-fraction). The relative amounts

Table 1. *Percentages of the proteins found in haemolysates of adult peripheral blood cells, foetal liver cells and foetal peripheral blood cells*

Origin of haemolysate	No. of anal.	Protein fractions: X 280	X 419	X cpm	Y 280	Y 419	Y cpm	F_2 280	F_2 419	F_2 cpm	A_2/F_1 280	A_2/F_1 419	A_2/F_1 cpm	A_1 280	A_1 419	A_1 cpm	E_1 280	E_1 419	E_1 cpm	A_3/E_2 280	A_3/E_2 419	A_3/E_2 cpm	E_3 280	E_3 419	E_3 cpm
Ad. periph. bl. CM-cell.	5	5	< 1	—	—	—	—	—	—	—	5	7	—	86	87	—	—	—	—	2*	4*	—	—	—	—
DEAE-Seph.	5	6	< 1	—	—	—	—	—	—	—	4	6	—	84	86	—	—	—	—	7	8	—	—	—	—
Foetal liver CM-cell.																									
13 days	1	88	30	58	< 1	< 1	< 1	1	4	4	4	26	14	6	35	19	—	—	—	1	5	4	—	—	—
14 days	3	87	38	62	—	—	—	1	7	4	8	31	18	5	19	12	—	—	—	1	5	3	—	—	—
15 days	3	87	30	66	—	—	—	1	7	4	6	39	16	4	19	10	—	—	—	2	6	4	—	—	—
Foetal periph. blood CM-cell.														A_1+E_1 280	A_1+E_1 419	A_1+E_1 cpm									
11 days	1	—	—	50	—	—	22	—	—	—	—	—	1	—	—	12	—	—	7	—	—	8				
12 days	1	78	26	39	2	16	11	1	6	4	3	12	10	11	33	30	5	7	6	?	?	?			
13 days	2	58	5	28	6	13	11	—	—	—	3	8	6	21	51	40	12	24	15	?	?	?			
14 days	2	56	7	30	7	10	4	2	3	2	8	17	15	15	40	30	7	15	12	5	8	6			

Components as % of the total amount of protein (280), haemprotein (419) and ^{59}Fe incorporation (cpm).
? means that the values could not be calculated, because at the time of their analysis the chromatograms were not extended to a high enough pH, and this fraction therefore did not elute.
* Resolution of A_3 on CM-cellulose not as good as on DEAE-Sephadex.

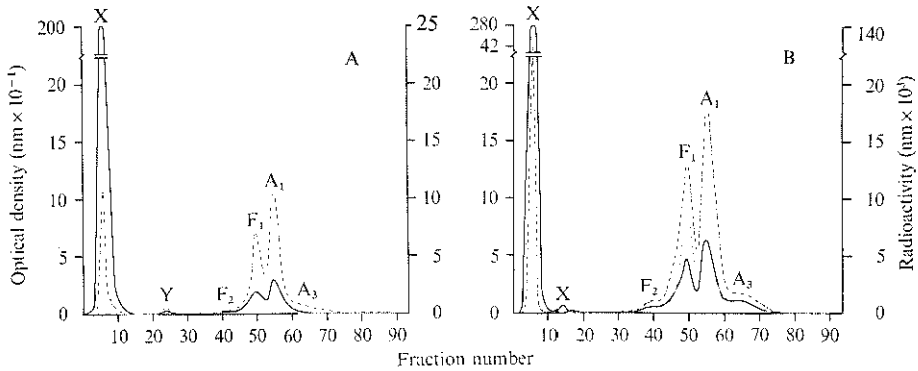

Fig. 3. CM-cellulose chromatography of foetal livers. The patterns of (A) lysate of 13-day radiolabelled livers and (B) lysate of 14-day radiolabelled livers with added unlabelled haemolysate prepared from adult peripheral blood cells. The elution patterns of optical density at 280 nm (——) for protein, at 419 nm (—·—) for haemprotein and of radioactivity (.....) are shown. In (B) only the pattern of radioactivity is representative for the foetal material, the optical density being recorded also from the added adult lysate. The discrepancy in the curves for optical density and radioactivity indicates the position of the unlabelled major adult haemoglobin (A_1).

of these fractions are summarized in Table 1. The three isolated haemoglobin fractions showed a typical CO-haemoglobin absorption spectrum and an absorbance ratio (O.D. 419/280 nm) of approximately three, which is well in accordance with their haemoglobin nature. The first eluted fraction (X) showed a somewhat different absorbance spectrum in that the 260–280 nm region was elevated, which accounted for the inverse absorbance ratio of less than one. However, the peaks in the visible region and the Sorret peak were also present, which means that some haem-containing substance must be present in this fraction.

Foetal livers (Fig. 3A, B). Livers from embryos aged 12–15 days were analysed by CM-cellulose chromatography. The presence of two major and two minor haemoglobins was noted. In order to determine which of the haemoglobins was chromatographically comparable to the major adult haemoglobin, CMC-columns were loaded with a radiolabelled foetal liver lysate plus a non-labelled lysate from adult peripheral blood cells. The position of the major adult haemoglobin can therefore be deduced from the ratio of the radioactivity and the O.D. tracings. In this way the later-eluted major foetal peak was found to co-chromatograph with the major adult haemoglobin and was therefore assumed to be A_1 (Fig. 3B). The proportion of the first major foetal haemoglobin eluted from CMC-columns was found to increase during foetal development (Table 1). As this peak was high in mature foetal livers it was called F_1, although it eluted in the same chromatographic region as adult A_2.

Urea starch gels (Fig. 2) revealed that the polypeptide chain pattern of the

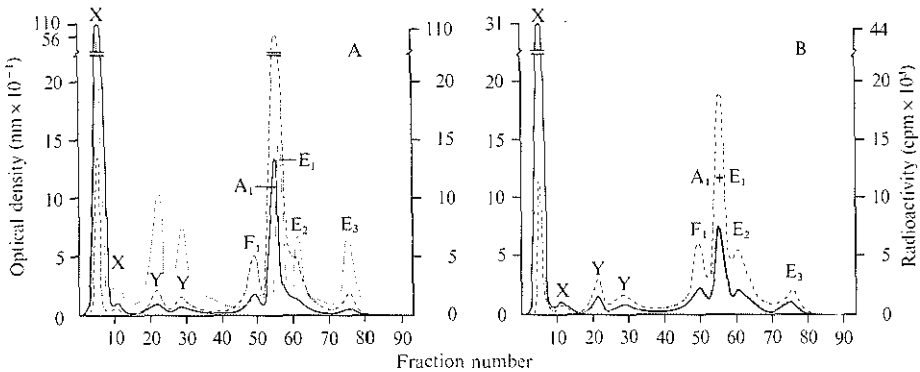

Fig. 4. CM-cellulose chromatography of foetal peripheral blood. The patterns of (A) lysate of 11-day radiolabelled peripheral blood cells with added unlabelled haemolysate prepared from adult peripheral blood cells. (B) Lysate of 14-day radiolabelled peripheral blood cells. See for further legends Fig. 3.

haemoglobin F_1 was different from the adult haemoglobins A_1, A_2 or A_3. Apart from the adult β-chain, a non-adult polypeptide chain intermediate in electrophoretic behaviour between the adult α- and β-chain was present in foetal haemoglobin F_1. Preliminary peptide maps of haemoglobins A_1 (adult) and F_1, although not ideal because whole haem-containing haemoglobins were mapped, also revealed differences between these haemoglobins. Surprisingly the A_1 fraction from foetal livers was not identical to that from the adult A_1 haemoglobin, in that it also contained the extra non-adult polypeptide chain. This could be due to contamination of this fraction with F_1; however, if this were the case, the intensity of the β-chain (which is common to both A_1 and F_1) should be relatively higher. An alternative explanation is that the non-adult chain in haemoglobin A_1 from foetal livers originates from traces of the embryonic haemoglobin E_1 (see below) from small numbers of yolk-sac cells in young foetal livers.

The minor foetal haemoglobins which were recognized as small shoulders on F_1 and A_1 are designated F_2 and A_3 respectively. All the haemoglobins described showed normal CO-haemoglobin absorption spectra. The absorption spectrum of the X-fraction from foetal livers was the same as described for the X-fraction of adult haemolysates.

A very high proportion of the total iron- and haem-containing proteins eluted in the X-fraction, which was sometimes subdivided into more peaks (Fig. 3; cf. the situation in adult blood, Fig. 1, Table 1). The proportion eluting in the X-fraction was greater the younger the embryos from which the livers were derived.

Foetal peripheral blood (Fig. 4A, B). Samples from 11- to 15-day-old embryos were analysed using CMC-chromatography. During this period a change takes place in the erythroid cells of which the blood is composed (Russell & Bernstein,

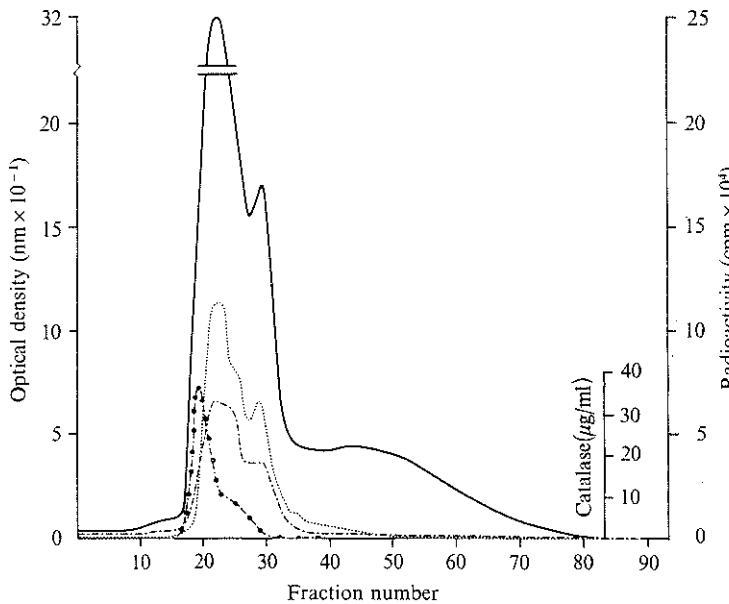

Fig. 5. Sephadex G-200 chromatogram of an X-fraction prepared from radio-labelled foetal livers. The elution patterns of the optical density of 280 nm (——) for protein and 419 nm (—·—) for haemprotein; the radioactivity (. . . . .) and the catalase activity (—·•—·•) are shown.

1966). In 11-day-old foetuses the peripheral blood cells originate entirely (over 95 % in our samples) from the yolk-sac, whereas in later stages an increasing number of erythropoietic cells from the foetal liver is present. The chromatographic pattern of the haemolysates of 11-day peripheral blood is complicated (Fig. 4). Six peaks with a haemoglobin-like absorption spectrum were found. The chromatographic behaviour of the major embryonic peak was very much like that of A_1: the peaks of the A_1 and of the major embryonic peak (E_1) are separated by only one fraction. However, as the A_1/E_1 peak is high in embryonic peripheral blood, lower in foetal livers and higher again in adult blood cells it is likely that E_1 is in fact different from the major adult haemoglobin A_1. A minor haemoglobin fraction eluted immediately prior to E_1 and may be the major foetal haemoglobin F_1. Two further substantial haemoglobin peaks (E_2 and E_3) elute after E_1: E_2 elutes between A_1 and A_3; E_3 eluted after A_3. Between the X-fraction and the region where the haemoglobins usually elute, two more haemoglobin fractions (Y_1 and Y_2) were consistently found. They were visible in all embryonic peripheral blood samples, the amount decreasing as the age increased.

The peripheral blood of the older embryos (14- and 15-day-old) investigated showed a major peak in the A_1/E_1 haemoglobin region. This peak is thought to be composed of a continuously decreasing amount of E_1, which is gradually replaced by an increasing amount of A_1. The haemoglobin eluting in front of this

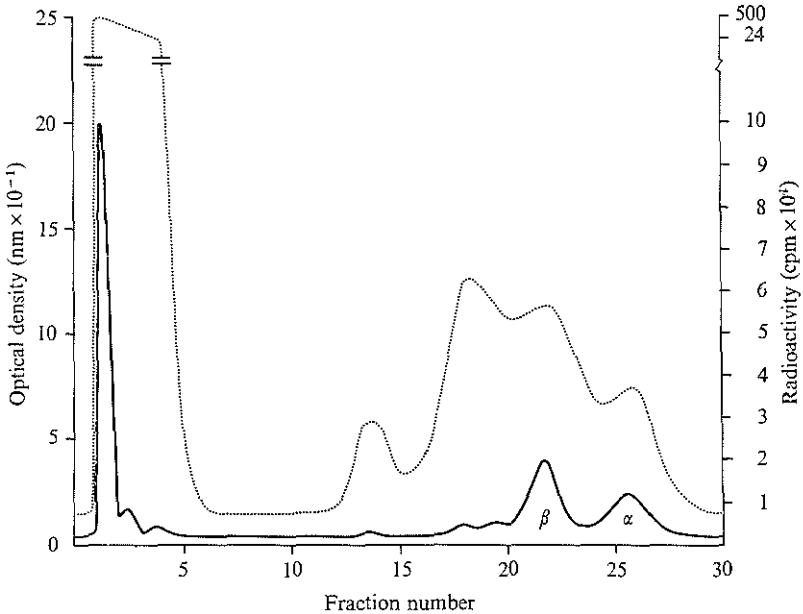

Fig. 6. Elution pattern of the 'globin chains' of an X-fraction. Separated over a column of CM-cellulose prepared in urea. The optical density at 280 nm (——) for protein and the radioactivity (.....) of the fractions are recorded.

peak is relatively greater in amount than in 11-day haemolysates and is thought to contain mainly F_1 derived from foetal liver cells. The peaks eluting after A_1/E_1 are most probably E_2 and E_3 derived from the embryonic yolk-sac cells. The X-fraction in haemolysates of older embryos is considerably smaller than at the younger stage. Absorption spectra of the peaks were similar to the corresponding foetal ones. Quantitative data of the fractions in these haemolysates are shown in Table 1.

The X-fraction. In an attempt to assess the proportion of iron and haem-containing proteins, some X-fractions from immature liver erythropoietic cells were taken up in acidified Drabkins solution and extracted with butanone. It was found that 30–50 % of the ^{59}Fe counts were haem-bound. Since all haemolysates were dialysed, the non-haem ^{59}Fe counts were not due to the presence of free iron. The haem component was not free haem since no ^{59}Fe counts were extracted by butanone from a non-acidified Drabkins solution of the X-fraction. The molecular weight of the protein in the X-fraction was estimated by chromatography on Sephadex G-200 columns, using myoglobin as a molecular weight marker (Fig. 5). In only one of the analyses – a 12-day liver lysate – were ^{59}Fe counts found in the myoglobin region. The bulk of the ^{59}Fe-labelled proteins are of high molecular weight. The possibility that haemoglobin aggregates might be formed was eliminated since treatment of haemolysates with iodoacetamide (Riggs, 1965) did not affect the chromatographic behaviour of the

^{59}Fe proteins in the X-fraction on G-200. Single chains thus may represent a small amount of the iron counts in the X-fraction but the majority of the iron counts in this peak must consist of another haemprotein. To exclude the possibility that the haemprotein under investigation was simply haemoglobin absorbed to a very large non-haemprotein, the X-fraction was subjected to ammonium sulphate precipitation. Less than 0·5 % of the ^{59}Fe counts in this fraction precipitated with 60–85 % ammonium sulphate, i.e. the conditions under which haemoglobin is precipitated. Finally, to determine whether any globin chains could be prepared from this peak, the X-fraction was isolated from erythroid cells (Friend cells) cultivated in the presence of [^{3}H]leucine. This labelled X-fraction was mixed with non-radiolabelled A_1 haemoglobin, precipitated with acid–acetone and the polypeptide chains were separated as described (Fig. 6). The radioactivity pattern was clearly different from the O.D. 280 nm tracing of globin chains from isolated adult haemoglobin A_1. Calculations based on the total radioactivity in the regions where α- and β-globins elute showed that less than 5 % of the X-fraction represented such chains. Since radioactivity eluting in the region of α- and β-globins represents shoulders on the major, non-globin peak, this value for the proportion of globin chains in the X-fraction is clearly over-estimated.

A further possibility is that the haemprotein in the X-fraction is catalase, which is a large protein (M.W. 230000) and contains four haem groups. Moreover, catalase is known to be present in larger amounts in immature than in mature cells. We therefore determined the amount of catalase in the X-fraction after chromatography on Sephadex G-200 (Fig. 5). Assuming that the haem in catalase has the same specific activity as the haem in the haemoglobin from the same haemolysate, we calculated that all of the ^{59}Fe radioactivity in the high M.W. component of the X-fraction eluted from G-200 could be due to catalase. However, the amount of catalase may represent some but not all the ^{59}Fe radioactivity in the lower M.W. components of the X-fraction eluted from G-200. Since catalase activity is not restricted to the catalase protein, but is a property of all iron porphyrin complexes (Jones, Robson & Brown, 1973), the amount of catalase will be over-estimated in these calculations.

DISCUSSION

Chromatographic analysis of the ^{59}Fe-containing proteins in adult and embryonic mouse red blood cells revealed the existence of two separate groups of proteins. The first group eluting in the initial phase of CMC-chromatography apparently consists of a variety of proteins, gathered in this study under the name X-fraction. The second group eluting later consists of haemoglobins. Both groups of proteins show changes during foetal development.

Adult haemoglobins. The haemoglobins from various strains of mice have been termed either 'single' or 'diffuse' according to their electrophoretic pattern in

starch gels. However, using other techniques such as polyacrylamide gels, the so-called 'single' strain haemoglobin sometimes splits into two components (Barker, 1968). The Porton white Swiss mice have the 'diffuse' haemoglobin type, with three haemoglobins. The difference between the haemoglobins may be due to the presence of more α-chain types (Popp & Cosgrove, 1959; Popp, 1965, 1967; Hilse & Popp, 1968) or more β-chain types (Hutton, Bishop, Schweet & Russell, 1962*a*, *b*). In our experiments the three haemoglobins display a common α-chain.

Embryonic haemoglobins in mice have been described by several authors (Table 2). Although the nomenclature is very confusing it may be concluded that specific embryonic haemoglobins are certainly present in erythroid cells from the yolk sac and peripheral blood of 11- to 14-day-old embryos.

Foetal haemoglobins in the peripheral blood of mice have not been reported previously (Table 2), although fraction 6 reported by Barker (1968) may correspond to our haemoglobin F_1. It is not surprising that the existence of a foetal haemoglobin was never noticed in haemolysates from peripheral blood, since here the haemoglobins are derived from both yolk sac and foetal liver erythroid cell types. This confuses any investigation on foetal haemoglobins, as haemoglobins F_1 and A_2 are very similar in chromatographic and electrophoretic behaviour. Moreover the elevation of the F_1/A_2 peak is relatively low, because the major haemoglobin peaks E_1 and A_1 are superimposed with most of the techniques applied. An extra difficulty is that the F_1/A_2 region is notorious in being the region where artificial components, due to degradation of haemoglobins (Riggs, 1965; Bonaventura & Riggs, 1967), are known to chromatograph. Relatively small alterations in the concentrations of this peak in haemolysates of peripheral blood are usually neglected for these reasons.

More reliable information concerning foetal haemoglobins may be obtained from analyses of haemolysates from erythroid cells derived from foetal livers or other erythropoietic organs. In this study a foetal haemoglobin was resolved in haemolysates of foetal-liver origin using chromatography. With this technique it has proved possible to isolate the haemoglobin and to establish that one of its polypeptides is not of adult type, both by urea starch-gel electrophoresis and preliminary fingerprinting of tryptic digests. It has not yet been possible to determine whether the foetal haemoglobin F_1 consists of the adult β-chain together with one of the embryonic chains common to yolk-sac erythropoiesis. The presence of a foetal haemoglobin in foetal livers has not been reported previously either (Table 2), with the possible exception again of fraction 6 reported by Barker (1968) in liver cell haemolysates. In a study of the haemoglobin pattern of erythroid cells from adult spleen, bone marrow and peripheral blood, Kraus (1970) noticed that the relative amount of the haemoglobins was not the same. In particular, the most anodic haemoglobin component was most pronounced in haemolysates originating from spleen and bone marrow. Significantly, phenylhydrazine stimulation of erythropoiesis changed the haemo-

Table 2. *Haemproteins found in erythroid cells from mouse embryos*

Relative mobilities or elution order of haemproteins						Origin (organ)	Age (days)	Analytical method	Authors
1	2	3	4	5	6				
E_1	—	—	E_{II}	E_{III}	—	Yolk-sac	8–12	Chromatography of Hb's and chains	Fantoni, Bank & Marks, 1967
		6	4	2	1	Yolk-sac	9–10	Polyacrylamide gel	Barker, 1968
X	F_2	F_1	A_1	A_3	—	Foetal liver	13–15	Chromatography of Hb's, urea starch-gel of chains	This paper
		—	A	—	—	Foetal liver	?	Chromatography of Hb's and chains	Fantoni *et al.* 1967
		—	A(B)	(C)	—	Foetal liver	15–16	Polyacrylamide gel	Kovach, Marks, Russell & Epler, 1967
		6	5	3	—	Foetal liver	12	Polyacrylamide gel	Barker, 1968
X	F_2	F_1	A_1/E_1	E_2	E_3	Periph. blood	11–14	Chromatography of Hb's	This paper
		—	1	2	3 4	Periph. blood	11–15	Starch gel	Craig & Russell, 1963, 1964
		—	2	3	4	Periph. blood	13	Starch gel	Morton, 1966
		—	A	B	C —	Periph. blood	12–16	Polyacrylamide gel	Kovach *et al.* 1967
		—	A	E_1	E_2 E_3	Periph. blood	12–14	Starch gel of Hb's and chains	Gilman & Smithies, 1968
		6	5	4	3 2 1	Periph. blood	10–17	Polyacrilamide gel	Barker, 1968
		—	A	E_I	E_{II} E_{III}	Periph. blood	10–14	Polyacrylamide gel, chromatography of chains	Fantoni, De La Chapelle & Marks, 1969
E_1	—	—	E_{II}	Minor fraction	E_{III}	Periph. blood	12–13	Chromatography of Hb's and chains	Steinheider, Melderis & Ostertag, 1972

Positions correlated mainly to major adult haemoglobin peak. ( ) means not present in all samples. ? = age not given in paper.

globin pattern in that the same anodic component in haemolysates from peripheral blood became more pronounced. Since the most anodic component would be expected to elute early from CMC-columns, this component might well correspond to our F_1. It is quite likely that this change in the type of haemoglobin synthesized during anaemia is analogous to the situation in sheep (Blunt & Evans, 1963; Van Vliet & Huisman, 1964; Garrick, Reichlin, Mattioli & Manning, 1973) and in man (Huisman, 1969), in which the foetal haemoglobin is shown to reappear under such circumstances.

The X-fraction. The X-fraction contains several proteins, some of them carrying iron in haem-form or otherwise. In a study on chicken haemoglobins (Schalekamp, Schalekamp, Van Goor & Slingerland, 1972) we found a similar peak and showed it to contain eight proteins. In the same study immunochemical evidence was produced that the proteins originated from cell stroma and not from contaminating serum. Extracellular proteins such as serum transferrin therefore do not seem to contribute to the peak. The iron counts are bound to proteins of high molecular weight, 30–50 % being in the form of haem. One of the non-haem iron carriers may be ferritin (M.W. 450000). Although ferritin is mainly thought to participate at a later stage of erythropoiesis (as a latent store for iron from degraded haemoglobin), it is known (Shepp *et al.* 1972; Theil, 1973) that, especially in erythroid precursor cells, an isotype of ferritin is present which is clearly involved in the early iron uptake. This anabolic ferritin fraction increased when haemoglobin synthesis was stimulated. Hrinda & Goldwasser (1969) found, in addition to ferritin, another not yet identified non-haem iron protein in bone-marrow cell lysates. The production of both iron-containing proteins was enhanced when erythropoiesis was induced by erythropoietin, this process being dependent on continued RNA and protein synthesis.

In addition to non-haem iron-proteins we found ^{59}Fe to be present as haemprotein in the X-fraction. A possible haemprotein which might be expected in lysates is catalase. Considerable catalase activity was shown to be present in the X-fraction. Nevertheless, even if all the activity was due to catalase, this would not account for all the haemprotein counts in this peak.

Our finding of a haemprotein other than haemoglobin in cell lysates differs from that of Gallien-Lartigue & Goldwasser (1964), who claimed that essentially all the haem in lysates of nucleated bone-marrow cells was derived from haemoglobin. This paper is often used to state that the determination of haem in lysates would represent the amount of haemoglobin present. Although Gallien-Lartigue and Goldwasser worked with a system in which cultivated cells were labelled *in vitro*, allowing differential selection of cell types, and our finding was established on *in vivo* labelled whole-liver lysates, we feel that the authors may have over-estimated the fraction of total haem synthesis represented by haemoglobin for various other reasons (Harrison *et al.* 1973). Even in more pure erythroblast preparations derived from peripheral blood of mouse foetuses, we

found a relatively high quantity of non-haemoglobin haemprotein in the X-fraction.

Our non-haemoglobin haemprotein could be somewhat similar to serum haemopexin, which may transport haem from the mitochondrion (where it is synthesized) to the nascent globin chains on the polysomes. Alternatively it may represent the translational repressor substance which prevents the initiation of globin chain production unless haem is present (Balkow, Mizuno, Fisher & Rabinovitz, 1973; Gross, 1974). This haem-controlled repressor and its precursor are proteins with an approximate M.W. of 4×10^5 (Gross & Rabinovitz, 1973) and thus may well be present in our X-fraction. Our finding of a high X-fraction in immature foetal livers, before haemoglobion synthesis is fully established, is suggestive for the presence of haem synthesizing and regulating proteins in this peak.

This work was done during a one year stay of the first author in the Beatson Institute for Cancer Research, Glasgow. The interest and kind help of all the Beatson people is greatly appreciated. Professor J. Moll is thanked for his interest during the preparation of this paper. We are grateful to Mr T. Rutherford for separating one batch of foetal liver haemoglobins on CM-cellulose and to Mrs D. Van Goor for performing the polypeptide analyses. The work was supported by the Medical Research Council and the Cancer Research Campaign.

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(*Received 3 February 1975, revised 10 March 1975*)

DE PROMOTIE VINDT PLAATS IN
COLLEGEZAAL 6 VAN LAAGBOUW 3 OOST
VAN DE FACULTEIT DER GENEESKUNDE
DER ERASMUS UNIVERSITEIT ROTTERDAM

RECEPTIE NA AFLOOP VAN DE PROMOTIE
IN DE FOYER ONDER DE COLLEGEZAAL

De schrijfster van dit proefschrift was qua geboorte een kuyken en van nature een broedster. Het is dan ook geen toeval dat het kippe-embryo haar aansprak. Een duidelijke preformatie ligt hieraan ten grondslag. Toch waren er ook zeker regulerende factoren nodig voor de uiteindelijke differentiatie. Daar waren allereerst mijn ouders, die mij gelukkig niet te vroeg zindelijk maakten, zadat ik zonder complexen problemen kon aanvatten. Ook spraken zij mij steeds moed in bij de verschillende ontwikkelingsstadia op achtereenvolgende scholingsinstituten, die blijkbaar nodig geacht worden om tot zo'n onderzoek te geraken. Vele leermeesters aan de Vrije en Gemeentelijke Universiteit te Amsterdam zullen zich mij zeker niet herinneren, hoewel zij eveneens onbewust een bijdrage tot mijn ontwikkeling leverden. Jan Langman was de eerste die mij een kippe-embryo ter hand stelde, hij wilde iets over de ooglenzen weten. Frits van Faassen stelde zich enige tijd welwillend op naast de kippe-breinen, die werden onderzocht door mijn toekomstige echtgenoot Maarten en mijzelf. Daarna ontfermde ten Cate zich over het onderzoek en onszelf en trokken wij naar Utrecht, waar dit onderzoek resulteerde in een proefschrift van Maarten. Hierop starte ik, eerst nog met behulp van Maarten het haemoglobine onderzoek. Het werk deed ik eerst in Utrecht onder leiding van Pim van Doorenmaalen en later in Rotterdam, waar mijn promotor Han Moll zich jarenlang mateloos heeft ingespannen voor de totstandkoming van dit proefschrift. Niet zozeer dat het werk niet gebeurde, dat wel. Schrijven echter bleek niet mijn grootste ambitie te zijn. Zelfs in de tijd dat geen laboratorium faciliteiten hier aan de faculteit beschikbaar waren ontvluchtte ik schrijfwerk en wist ik hem toestemming te ontfutselen om mee te mogen doen aan werk met Maarten in het Zuiderziekenhuis. In het kielzog van dezelfde Maarten verdween ik ook nog een jaar naar Glasgow, waar het muizen onderzoek werd verricht. Welnu, waarde Han, het werkje is toch ter perse gegaan! Evenwel, het zal duidelijk zijn, dat de belangrijkste determinerende factor, de rode draad door deze ontwikkelingsgang, door jou gevormd wordt Maarten. Je enthousiasme, je vermogen om andermans tegenslagen te bagatelliseren, je deskundigheid op allerlei gebied hebben mij het meest geholpen. Daarnaast speelde natuurlijk ook je uithoudingsvermogen en het feit dat je dagenlang op gehaktballen kunt leven een belangrijke rol bij de totstandkoming van dit proefschrift. Veel anderen deden in de loop van de jaren achtereenvolgens mee aan dit werk. Diety, jij was er het langst, misschien had je wel mee moeten promoveren. Ria, Willem, Lous, Edith, Margreet, van den Oudenalder en wie al niet meer, hartelijk dank. Tenslotte blijven wij zitten met het Schalekamp, vier zijn er uitgebroed. Wij hopen dat zij geen irreversibel letsel overhouden van de ambities van hun moeder. Zij zien er niet naar uit. Hartelijk dank voor jullie - meestal - vrolijke gezichten.