HEMOGLOBINS OF EMBRYONIC SHEEP

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
BRUCE HAMMERBERG
1974

ABSTRACT

HEMOGLOBINS OF EMBRYONIC SHEEP

By

Bruce Hammerberg

The ontogeny of hemoglobin in sheep was further delineated to the early embryonic stages of gestation. Cell morphology and tissue sites of hematopoiesis were included in this study of erythrocytes and hemoglobin.

Immune sera developed in the horse specific for the different hemoglobin types of sheep were used to study the degree of molecular similarity among sheep hemoglobins and the hemoglobins of other mammals.

New techniques for peptide mapping on a microscale using fluorescent compounds and thin layer plate chromatography were adapted for use with the small amounts of embryonic hemoglobin available. These techniques, in conjunction with isoelectric focusing on polyacrylamide gel, were employed to produce peptide maps of embryonic hemoglobin.

The formation of a novel hemoglobin type, not reported in mammals outside of man, was discovered in tissue lysate suspensions. This hemoglobin, which is analogous to hemoglobin Koelliker in man,

was found to result from the enzymatic action of a protease which removes only the carboxy terminal arginine from the α chain polypeptide.

HEMOGLOBINS OF EMBRYONIC SHEEP

By

Bruce Hammerberg

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1974

ACKNOWLEDGEMENTS

I wish to acknowledge the inspiring guidance of Hyram Kitchen, the expert technical advise of Irene Brett, and the moral support of JoAnn Harian.

TABLE OF CONTENTS

LIST OF TABLES

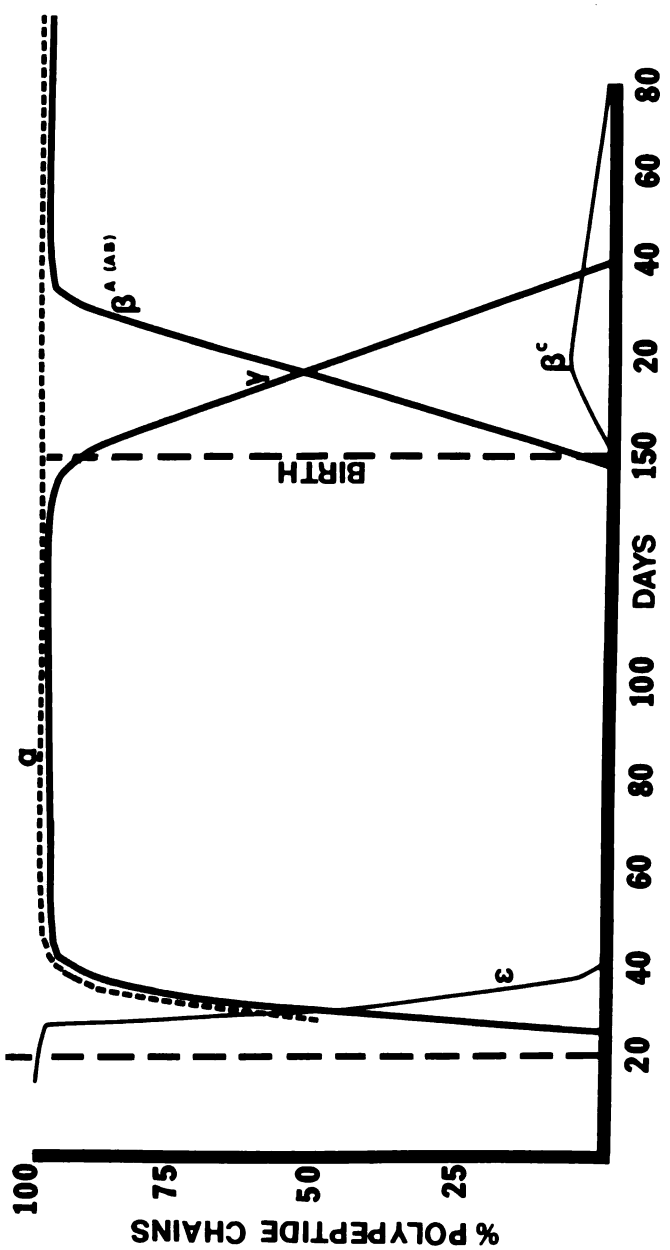
LIST OF FIGURES

INTRODUCTION

In the domestic sheep three distinct stages of hemoglobin production have been identified in the time span from the 20 day old embryo up to the adult animal (Figure 1). This ontogeny, including the two transition events, is almost completely analogous to the ontogeny of hemoglobin occurring in man.

In sheep, the transition of hemoglobin occurring at birth involves the disappearance of fetal hemoglobin ($\alpha_2\gamma_2$) and appearance of the adult hemoglobins over a time space of about two months (4). In sheep of hemoglobin B, HbB ($\alpha_2\beta_2^B$) type, the transition involves only fetal hemoglobin, Hb F, and HbB; i.e., a disappearance of γ chains and appearance of β^B chains. In sheep of HbA ($\alpha_2\beta_2^A$) type, the transition at birth involves the disappearance of γ chains as in all sheep; however, the appearance of β^A chains at birth is accompanied by the onset of HbC ($\alpha_2\beta_2^C$) production which peaks at 14-20 days making up only about 5-10% of circulating adult type hemoglobin at this peak (4). Hemoglobin C, termed an inducible hemoglobin due to its appearance during anemic states (5) and when erythropoietin is administered (6), has no analogy in man and its allelic site seems to be associated exclusively with the loci for HbA. It is apparent that the HbF to HbA transition occurring in sheep occurs in a much shorter time space than that same transition in man, and that it is

Figure 1. The relative composition of hemoglobin subunits during the time span from 20 days' gestation to the 80-day-old lamb (age at which blood is entirely of adult type and HbC has disappeared in the non-anemic lamb). The relative amounts and exact time of onset of α chains is not known; however, at 20 days' gestation ε chains comprise almost 100% of the subunits. Globin composition of circulating hemoglobin in embryos less than 80 days old is graphed from data described in this thesis. Globin composition after 80 days' gestation is graphed from data obtained from literature as referenced in the body of the thesis.



ONTOGENY of SHEEP HEMOGLOBIN - PHENOTYPE A or AB

Figure 1

more closely associated with birth. In man the transition is begun several months prior to birth and is completed by 6 months after birth (3).

The comparatively short period of time required for the HbF to HbA transition in sheep implies an event occurring concurrently in all hematopoietic tissue, an event which is initiated very close to birth.

The other transition event occurring in sheep involves the switch from embryonic hemoglobin to fetal hemoglobin. Just as in man the transition involves the termination of embryonic hemoglobins and the onset of fetal hemoglobin. This transition of hemoglobin production is accompanied by a change in red blood cell morphology (from large nucleated cells to small anucleated cells), which is quantitatively parallel to the decrease in circulating embryonic hemoglobins. Also involved in this transition is the termination of hematopoiesis in one tissue (yolk sac blood island) and the initiation of hematopoiesis at a new tissue site (embryonic liver). It may also be that this transition period in sheep involves the onset of a chain production not seen in concurrence with e chains; i.e., a Gower I type hemoglobin, ε_4, in sheep may precede all other hemoglobin production including Gower II ($\alpha_2\varepsilon_2$), although this is yet to be proven.

The question of the subunit composition of the embryonic hemoglobins in sheep remains in question and even the work done with human embryonic hemoglobin by Huehns (26, 38) is not of an unambiguous nature. Huehns' early conclusions about embryonic hemoglobin

structure based on hybridization and electrophoretic data remain unchallenged. The structure of slow migrating (pH 8.6) embryonic hemoglobin, termed Gower II, is fairly certain to be $\alpha_2\varepsilon_2$ by peptide mapping (26). Less work has been done with Gower I and all conclusions about polypeptide components are based on hybridization of Gower I with Hb-β_4 and with human fetal hemoglobin, Hb-$\alpha_2\gamma_2$. Gower I plus Hb-β_4 did not result in any Hb-A, $\alpha_2\beta_2$ as would be expected if Gower I contained a dimer of chains. Gower I plus fetal hemoglobin, $\alpha_2\gamma_2$, produced Gower II-like hemoglobin.

Thus, no definitive structural evidence (such as peptide mapping) exists to substantiate the subunit structure proposed before Gower I. Whether an analogous arrangement of subunits occurs in embryonic sheep hemoglobin remains to be proven; yet dansyl-peptide mapping as reported in this thesis may suggest the Gower I analogue in sheep is not merely an tetramer.

In order to pursue these questions further and develop knowledge about the onset of hemoglobin production when it first appears in embryos, techniques commonly in use which are designed for larger sample amounts must be replaced by methods and techniques sensitive to nanamole amounts of hemoglobin. Also when using tissue lysates as a source of embryonic hemoglobin, as is the common practice for most laboratory animals, special considerations are warranted with regard to artifacts being produced by the action of protease of tissue origin on the hemoglobin.

After describing the findings on hemoglobin and hematopoietic tissue from early sheep embryos, techniques adapted to further

investigation of embryonic hemoglobin on a micro-scale will be described and the early results of their use detailed. Finally a hemoglobin degradation product is characterized and its occurrence in experiments dealing with tissue lysates described so that workers in this area do not describe it as an inherited hemoglobin variant.

Each of these topics will be given a chapter in this thesis so that the continuity of the individual topic is maintained and related material not scattered throughout the text.

CHAPTER I

ONTOGENY OF HEMOGLOBINS IN SHEEP EMBRYOS

Introduction

Sheep have been used extensively by many workers in almost all aspects of research involving the use of hemoglobin; that is, structure-function studies (39, 41), genetic studies (40), and studies on the differential induction of hemoglobin (42, 42a). From this popularity the knowledge concerning sheep hemoglobin, including its ontogeny, is quite extensive; however, due to technical and other difficulties, including the logistics of obtaining sufficient sample and the costs in maintaining pregnant animals incurred when dealing with early embryos, very little has been reported on the embryonic hemoglobin (1) of sheep. For that matter, very little has been reported on embryonic hemoglobin in any species (3) and only in man have structural studies been carried out (2). This study was undertaken in order to make the picture of the transitions in hemoglobin from early embryonic stages to the mature animal more complete.

The hemoglobin type, erythrocyte morphology, and tissue sites of hematopoiesis were investigated in individual sheep embryos from 20 days to 35 days of gestation. The small amount of hemoglobin available at these stages of development allows individual

characterization such that heterogeneities due to genotype may be related to known hemoglobin types of the sire and dam. This precaution against missing possible heterogeneities in embryo hemoglobin was deemed necessary since embryonic hemoglobin as it is obtained from the mouse and hamster, with which most of the work on this type of hemoglobin has been carried out, is a composite of many tens of embryos.

Methods and Materials

Crossbred and Dorset purebred sheep were watched carefully for exact breeding dates. In this procedure the ram was marked on the brisket with chalk such that when a ewe was bred it would be marked over the rump. Morning and evening the flock was checked for marked ewes, thus giving breeding times which would be within 24 hours of the actual event. Embryos of the desired age were obtained by cesarian section under sterile conditions so that the postoperative ewe could be returned for breeding. Twenty ewes were bred, of which 16 had embryos (8 ewes had twins). All twenty postoperative ewes were rebred within two months of surgery (most were rebred within one month). Eleven ewes conceived successfully after the first surgery.

Contamination of placental membranes by maternal bleeding from the uterine incision was minimized by injecting 1/1000 epinephrine along the incision site (about 3 cm long) and by repeatedly washing the intact embryo and membranes in 1.2% saline and 0.1% EDTA. The membranes of the washed embryo were torn and the embryo decapitated while in the saline and EDTA solution in order to collect peripheral blood. The liver was excised under a dissecting microscope.

Peripheral blood cells were washed 4 times in 1.2% NaCl, then treated with CO for 15 min. before a volume of distilled water equal to the packed cell volume was added. Lysis was completed by freeze-thawing.

Identification of hemoglobin components was determined by electrophoretic mobility at pH 8.6 on starch gel in a discontinuous Tris-buffer system according to the methods of Smithies (8).

Identification of individual polypeptide chains upon precipitation of globin in acid acetone was obtained by comparing mobilities at pH 9.0 on cellulose acetate in 8 M urea and .028 M mercaptoethanol after 1 hour and 40 minutes of electrophoresis at 300 volts.

Microscopic examination of peripheral cell population, liver sections and yolk sac was accomplished by using a combination of Benzidine and Wright's stains. All embryos were measured in order to relate crown-rump length to the embryo age. This measurement corresponded well to gestation time and appears to be an accurate measurement of embryonic age in sheep. Hematopoietic activity of the liver and yolk sac when present was checked on all embryos by impression smears of cut surfaces and of entire tissue in the case of the yolk sac.

Results

When the peripheral blood collected from five 25-day-old embryos was electrophoresed on starch gel at pH 8.6, no bands were seen which migrated like any of the adult hemoglobins or like fetal hemoglobin (Figure 2). However, a very slowly migrating major band with a slightly slower and a slightly faster minor band was observed. None

Figure 2. Hemoglobin electrophoresed on starch gel shows the distinct mobility of embryonic hemoglobin from 25- to 28-day-old embryos when compared to all adult types and fetal hemoglobin. The appearance of fetal hemoglobin is at 26 days' gestation. The origin is marked "0".

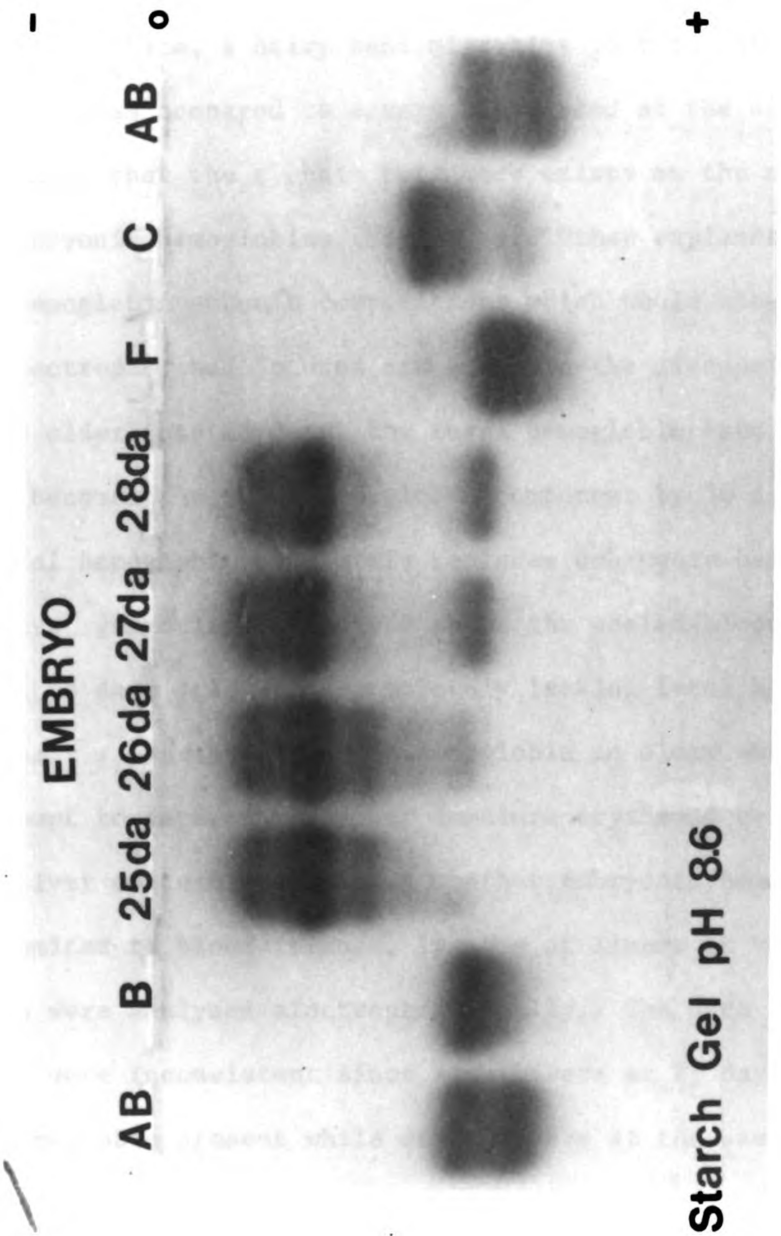


Figure 2

of these bands approached the migration rates of any other sheep hemoglobins. This observation is contrary to that reported by Kleihauer and Stoffler (1), who found that sheep embryos have less than 50% of this slow migrating hemoglobin at 25 days' gestation. On urea cellulose acetate, a heavy band migrating just to the anodic side of the origin, as compared to a very light band at the α chain position, indicates that the ε chain tetramere exists as the major component of embryonic hemoglobins (Figure 3). Other explanations for embryonic hemoglobin subunit compositions which would show this pattern when electrophoresed in urea are given in the discussion.

In embryos older than 25 days, the fetal hemoglobin band appears and eventually becomes the major hemoglobin component by 30 days' gestation. Fetal hemoglobin completely replaces embryonic hemoglobin by 37 days' gestation. Figure 2 shows the pooled blood of five embryos at 25 days' gestation completely lacking fetal hemoglobin and the subsequent appearance of fetal hemoglobin in older embryos.

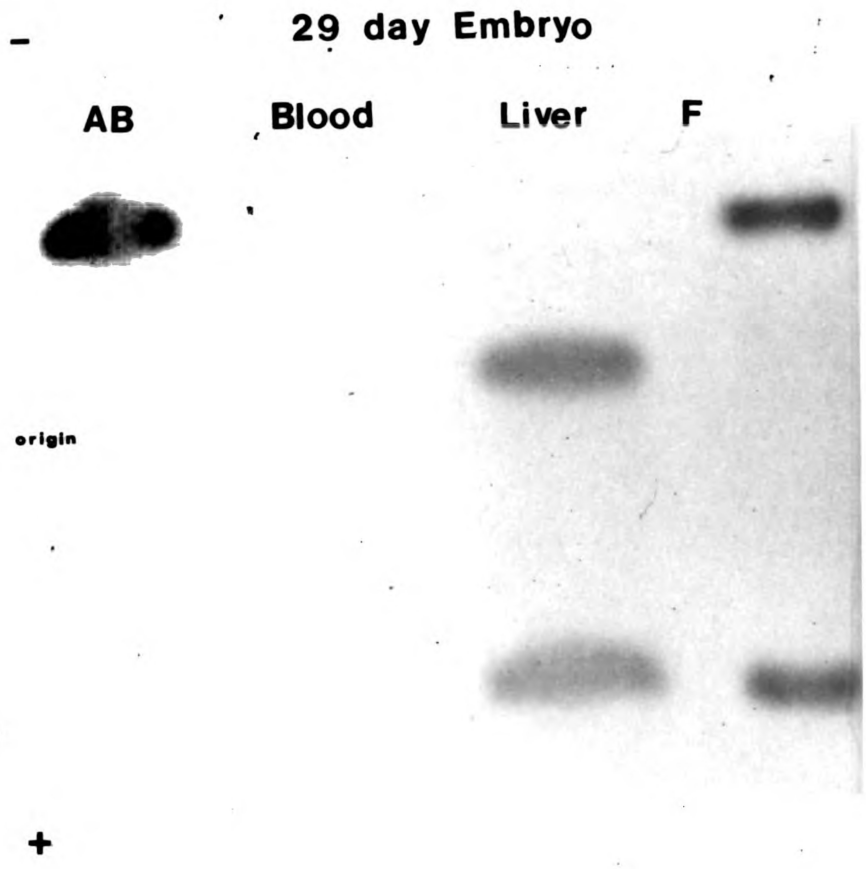
In an attempt to determine whether immature erythroid cells of the embryonic liver contain ε chains or whether embryonic hemoglobin synthesis is limited to blood islands, lysates of livers at various days' gestation were analyzed electrophoretically. The data from this experiment were inconsistent since some livers at 27 days showed no embryonic hemoglobin present while other livers at the same stage showed 20-30% embryonic hemoglobin present. This inconsistency may be due to variable success in completely washing away peripheral blood cells, in exact breeding times, or biological variation in development.

The definition of embryonic hemoglobin in sheep is based upon three criteria: 1) it occurs early in embryo development prior to and independent of fetal hemoglobin as seen in starch gel in Figure 2, 2) it exists only during embryonic and early fetal development and cannot be detected in later fetal development or in the newborn and adult animal, 3) its source is the nucleated, nondefinitive (48) erythrocyte (Figure 4), as evidenced by the correlation demonstrated in Table 1.

Establishing the parameters of an embryonic to fetal hemoglobin switch cannot be limited to merely following the change in production of the hemoglobin molecule type, for at this dynamic transition stage there are also involved changes in location of hemopoietic centers and even changes in the morphology of the red blood cell.

Blood smears from embryos of 25 to 29 days' gestation were examined by staining with Benzidine and counter staining with Giemsa in order to identify and compare populations of the large, nucleated embryonic red blood cell and non-nucleated adult type red blood cell (Figure 4). These two types of cells were easily distinguishable. Even nucleated red blood cells of the adult cell line, i.e., those produced in the liver which are in the stage prior to nucleus extrusion, could be recognized as distinct from embryonic red blood cells on the basis of size and appearance of the nucleus. The embryonic cells are larger and show a less pyknotic nucleus. No significant numbers (counts ranged from zero to 2%) of non-nucleated red blood cells of the adult cell line were seen in peripheral blood prior to 26 days' gestation.

Figure 3. Globin subunits dissociated in 8 M urea separated electrophoretically. Left to right the sources are: adult hemoglobin type AB (α chains migrate to the negative pole; β^A and β^B migrate to the positive pole); peripheral blood from a 29-day-old embryo (ε chains migrate close to the origin on the negative side); liver lysate from a 29-day-old embryo (modified α chains migrate less rapidly than normal α chains while γ chains are not modified); and fetal hemoglobin, F.



Sheep Globin on

Urea Cellulose Acetate pH 8.9

Figure 3

Figure 4. Peripheral red blood cells from a 25-day-old embryo showing the large mottled nucleus and generally larger cell size of the embryonic or non-definitive red blood cell line.

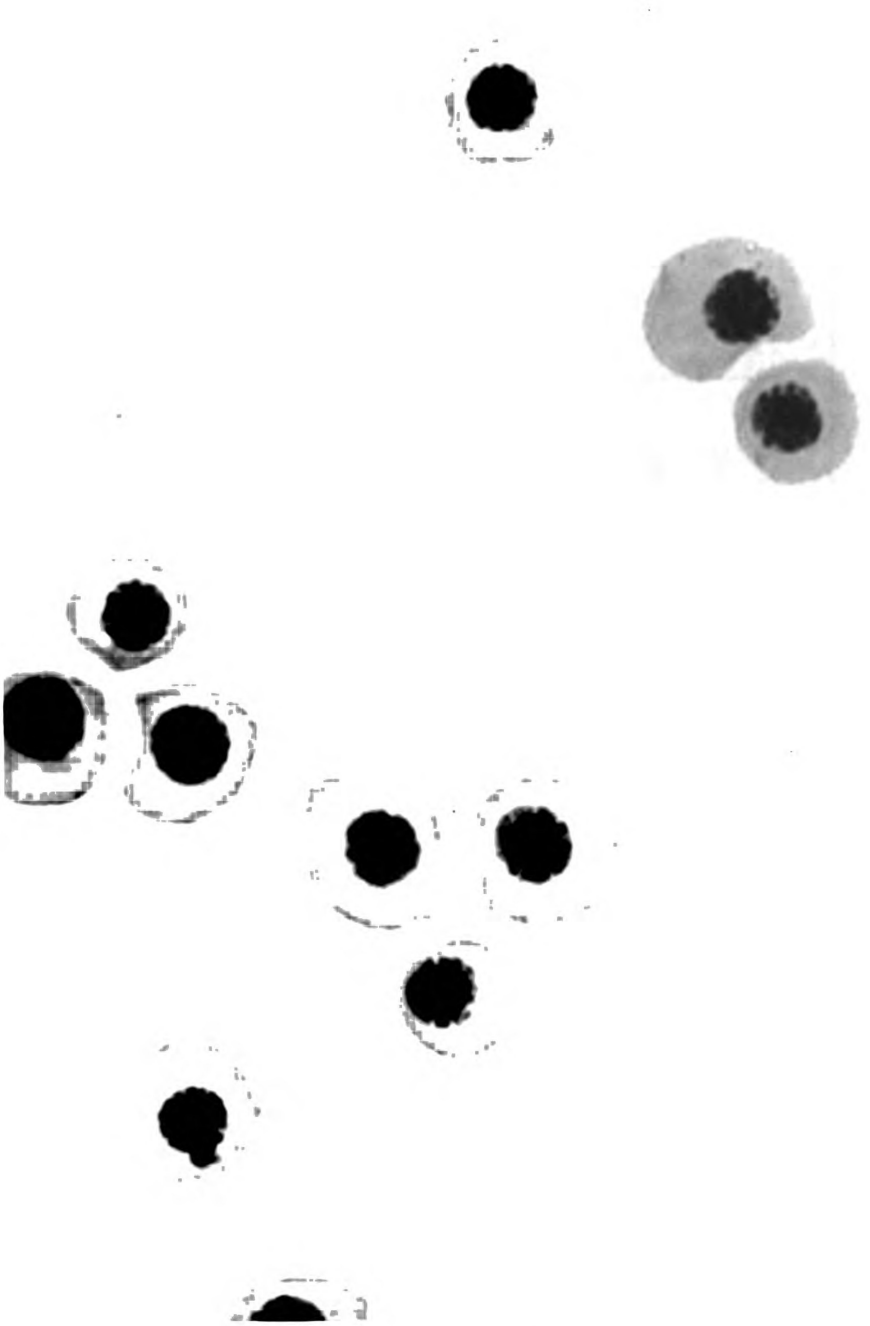


Figure 4

Table 1. The appearance of fetal hemoglobin in embryonic circulating blood

Age of embryo in days	25	26	27	28	29
Number of embryos	5	4	4	4	6
Crown-rump length in mm	9-10	10-12	13-15	15-16	16-18
Relative density of Hb bands on starch gel electrophoresis	fetal 0% embryonic 100%	fetal <10% embryonic 90%	fetal <15% embryonic >80%	fetal 25% embryonic 75%	fetal 50% embryonic 50%
Relative density of subunit chains on cellulose acetate in 8M urea	α chain 5% ε chain 95% γ chain 0%	α chain 5-10% ε chain 90% γ chain 5%	α chain 10% ε chain 80% γ chain 10%	α chain 15% ε chain 60% γ chain 15%	α chain 30% ε chain 40% γ chain 30%

Table 1 shows the parallel relationship between decreasing embryonic red blood cells and decreasing relative density of the embryonic hemoglobin band on starch gel electrophoresis.

At 20 days' gestation embryonic liver imprints show few nucleated embryonic erythrocytes and large, light-staining stem cells, none of which stains with Benzidene. At 25 days' gestation the liver contains a large population of orthochromatic cells plus the stem cells noted earlier; however, no non-nucleated erythrocytes appear. By 26 days there are appreciably fewer stem cells and more of the immature erythrocyte cell types at all stages of maturation. Also at this time large numbers of non-nucleated erythrocytes are present for the first time.

Discussion

The electrophoresis of 25- to 28-day-old embryonic hemoglobin on starch gel (Figure 2) shows a progressive loss of the fast, minor, embryonic hemoglobin band. At later times, 29 days (Figure 5), a single embryonic hemoglobin component remains or only a slight trace of the slow, minor, embryonic hemoglobin band is detected. This pattern could be explained if a novel combination of epsilon and alpha polypeptides were to be accepted as the major embryonic hemoglobin component. If the major component consisted of $\varepsilon_3\alpha_1$, the slow, minor component $\varepsilon_2\alpha_2$ and the fast, minor component ε_4, then based on the relative net molecular charges of the polypeptides α and ε as determined from urea cellulose acetate electrophoresis, the migration pattern would fit over all molecular charges of the hemoglobins as indicated by starch gel electrophoresis. The possibility of three

Figure 5. Hemoglobins from various sources comparing electrophoretic mobility. Placental membranes of embryos from two different ewes show varying amounts of contaminating maternal hemoglobin. The placental membranes of the sheep with B type hemoglobin were grossly contaminated (so that embryonic hemoglobin was completely diluted out) while placental membranes of the sheep with AB type hemoglobin show only slight maternal blood contamination. Liver lysates from both embryos show the rapidly migrating degradation product of fetal hemoglobin.

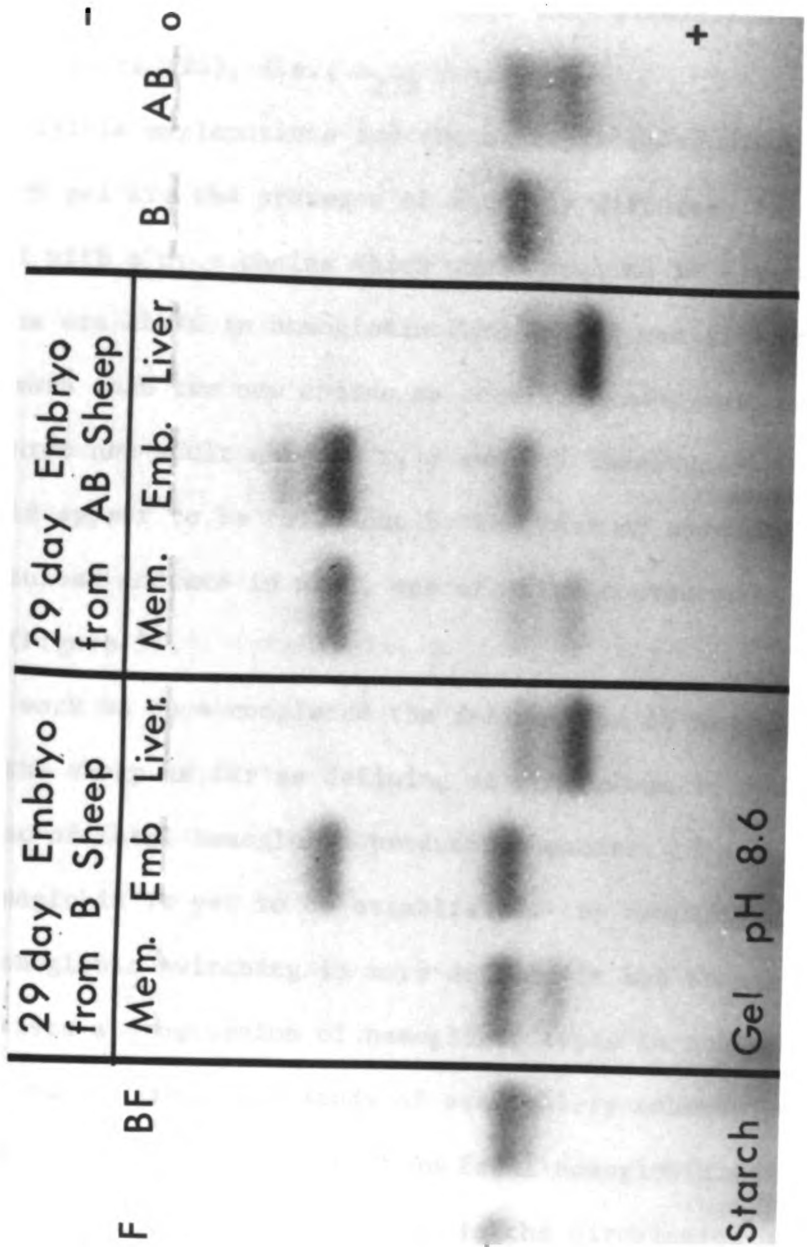

29 day Embryo from B Sheep
29 day Embryo from AB Sheep
F
BF
Mem.
Emb
Liver.
Mem.
Emb.
Liver
B
AB
O
-
+
Starch Gel pH 8.6

Figure 5

epsilon chains associated with one alpha chain would be a novel arrangement for naturally occurring hemoglobins studied to date. However, this possibility is supported by studies showing that when polypeptide chains of hemoglobin dissociate they dissociate into heterogeneous dimers (24); i.e., $\alpha_2\varepsilon_2 \rightleftarrows \alpha\varepsilon + \alpha\varepsilon$.

Other possible explanations for the multiple embryonic bands seen on starch gel are the presence of entirely different polypeptides not identical with α or ε chains which would combine in a manner similar to the eta chain in hemoglobin Portland of man (50) or the presence of more than two new chains as occurs in embryonic mice (11) which have three non-adult chains, x, y and z. These possibilities, however, would appear to be ruled out by the lack of more than two bands on cellulose acetate in urea, one of which corresponds to the alpha chain (Figure 3).

In this work we have completed the delineation of hemoglobin ontogeny in the sheep as far as defining at what stage in development the onset of fetal hemoglobin production occurs. The onset of embryonic hemoglobin is yet to be established. By completing this picture of hemoglobin switching in more detail, it has become obvious that there exists a progression of hemoglobin types in sheep almost identical to that in man. Our study of over thirty embryos between 20 and 40 days' gestation showed that no fetal hemoglobin was detectable by starch gel electrophoresis in the circulation of embryos younger than 26 days.

Other researchers have reported the presence of fetal hemoglobin as 50% of circulating hemoglobin at 25 days' gestation (1). I could

detect no fetal hemoglobin at this stage in development. The reason for this discrepancy may lie in the fact that contamination by maternal blood of placental membranes is inevitable without special precautions as described. Without special care in washing all maternal blood off the placental membranes that would be incurred from uterine incision bleeding, it is easy to confuse the common adult hemoglobin, HbB, for HbF on starch gel electrophoresis. Confusing HbB for HbF would lead to the impression that HbF is being produced at early embryo ages when it may actually be contamination by maternal HbB. A period in embryo development during which no fetal hemoglobin is produced is essential for studies on the induction of fetal hemoglobin.

The fact that sheep have only one new type of subunit, ε, associated with embryonic hemoglobin, instead of three new subunits as in the mouse (11) or an undetermined number as in the chicken (12), provides reason to consider the sheep as a superior animal model for studying parameters controlling hemoglobin transitions. No laboratory animal model currently being used has the degree of similarity that sheep hemoglobin ontogeny has to human hemoglobin ontogeny.

The study of protein induction and the study of coordination of alpha chain production with non-alpha chain production are at the core of understanding the blood diseases known as Thalessiemias (49). In these disease syndromes the relative production of the alpha and non-alpha subunit proteins is not proportional or the presence of one of the subunits may be completely lacking. The lack of a one-to-one ratio between α and ε chains in the nondefinitive cell line of

embryonal sheep and man is a normal and not a disease state. How this disproportionality arises is unknown, for no level of protein production (gene number, transcription translation, or product stability) has been studied in the nondefinitive cell line erythrocyte (also known as the primitive cell line or embryonal erythrocyte line). The obvious reason for this is the limited number of germinal cells of the primitive cell line. Studies on the induction of a clone of cells to begin producing fetal erythrocytes as occurs after 20 days' gestation in the sheep embryo or studies on hemoglobin production in the nondefinitive cell line with its unbalanced α and ε production are feasible using cells of sheep embryo origin. At this same time of dynamic hematopoietic changes the embryonic placental membranes are not tightly adhering to the uterine wall, so that the embryo, up to 27 days' gestation, may be removed with its placental membranes which may make it feasible for extrauterine embryo cultures to be carried out modeled after the work of New *et al.* (13).

CHAPTER II

ANALYSIS BY ANTIBODY

Introduction

Up to this point I have given only electrophoretic migration rates as evidence for distinct hemoglobins. Other means of differentiating these closely related proteins must reflect primary structure more closely and yet require small amounts of hemoglobin in order to show comparison with limited embryonic samples. In the following two sections, two methods of comparing proteins on a structural basis will be described as they relate to the study of hemoglobin ontogeny in sheep. This section deals with specific antibodies to hemoglobin molecules.

Employing specific antibodies made against individual proteins allows comparison of protein structure by the number of homologous antigenic sites (27, 51). Homology may not be strict but more closely a measure of side group functional homology; that is, substitutions of glycine for alanine or aspartic acid for glutamic acid may still allow a site on the protein molecule to be antigenically homologous (27). This is not always the case, however, as seen in the substitution of valine for leucine as the immunological differentiating factor between the immunoglobins Gm and InV of man.

The ideal approach to comparing hemoglobins of an ontogenical series in one species with those of another species is via comparison of primary amino acid sequences among these hemoglobins. Unfortunately this type of data is compiled only after a great deal of time and effort and where the hemoglobin is in ample supply; in the case of sheep we are fortunate in having sequence data on fetal hemoglobin and all adult types (20). The limited amounts of HbE available preclude its immediate sequencing and only a limited number of fetal animal hemoglobins have been sequenced. Thus we must rely on the specificity of antisera in order to measure the degree of similarity in hemoglobin structure when comparing various species at different levels of development.

With the use of specific antibodies to sheep hemoglobins, the degree of homogeneity may be described for hemoglobins which are available in such minute quantities as to make sequencing and classical peptide mapping techniques impossible. Cross reactivity, sites of homology, between two hemoglobins can be determined on as little as one microgram. The ease and rapidity of this method also allow extensive inter-species comparisons in order to determine the degree of relative variability of hemoglobin at different stages of development when compared with other species at similar stages of development.

Using an immunization schedule communicated by Dr. John Robbins of NIH, I was able to induce serum antibodies in the horse to sheep fetal hemoglobin and to the adult sheep hemoglobins A, B and C. Horse serum antibodies which show no reactivity to other sheep hemoglobins were produced against fetal sheep hemoglobin, HbF, and against sheep

hemoglobin A, HbA, by absorbing out cross reacting factors. This absorption was accomplished by a means not to this date reported in the literature.

This thesis describes the means by which the antisera were prepared and made specific and also describes the initial results of a planned comparison of fetal and adult hemoglobins of sheep with comparable hemoglobins in many other mammalian species. Adult hemoglobins have been collected from many species. However, only pig, sheep and cow fetal hemoglobins have been collected and studied.

Materials and Methods

All hemoglobins used for immunization were purified by the following steps: a) carbon monoxide treated hemoglobin from lysed cells was centrifuged at 20,000 gs for twenty minutes, b) dialyzed against 0.01 M Tris-HCl buffer pH 8.6 overnight, c) placed on a column of DEAE Sephadex equilibrated in the same buffer (100 mg was placed on a column 2.5 cm by 50 cm). The column was run with the beginning buffer at a rate of 30 ml/hour for six hours prior to beginning a pH gradient with the same buffer to a pH of 7.3. The peak fraction of 100 ml was concentrated to a volume of 2 ml containing about 50 mg of hemoglobin. This solution was dialyzed against phosphate buffer 0.01 M pH 6.3 overnight before centrifugation as above and being placed on a column of Sephadex G-75 1 cm x 30 cm in the same buffer. Hemoglobin eluted from this column was placed directly on a column of Whatman CM-32 (2.5 cm x 50 cm) equilibrated with the same phosphate buffer. This column was run at various flow rates but always with

200 ml of starting buffer prior to the beginning of a gradient to pH 7.6 in the same buffer.

Two milligrams of this purified hemoglobin were used for each immunization or booster injection of both rabbits and horses. The immunizing solution for rabbits came to a volume of 5 ml, 1 to 1 with Freund's complete adjuvant FCA (2.5 ml distilled water containing 2 mg hemoglobin plus 2.5 ml FCA). The immunizing solution for horses came to a volume of 15 ml with the same amount of hemoglobin and the same amount of FCA.

Rabbits were inoculated with intradermal injections on the abdomen (more than 20) and into the toe pad. They were boosted with the same regimen at ten days and bled at this time and again ten days later in order to test antibody titer.

Horses were inoculated by intradermal injections (more than 60) over the rump and both shoulders. They were bled and boosted at monthly intervals by the same method.

Immune sera were stored at minus 50°C as were the purified hemoglobins. All tests for precipitating antibody against hemoglobin were done by double diffusion in agar gels as described by Ouchterlony (47). The antibody source was in all cases unfractionated serum and the hemoglobin used at a concentration of about 1 gm/100 ml. All hemoglobin was treated with carbon monoxide by bubbling for 15 minutes prior to storage.

Based on the fact that all sheep hemoglobins bind tightly by electrostatic forces to Sephadex A-50 at a pH of 8.1 in 0.01 M Tris-HCl buffer and will remain bound in the presence of immune serum

directed against the hemoglobin, the Sephadex resin was used, when coated with hemoglobin, as an immunoabsorbant of high specificity. The technique developed was as follows: hemoglobin at a concentration of 8-10 gm/100 ml obtained after centrifugation at 20,000 gs to remove particulate material was dialyzed against 0.01 M Tris-HCl buffer then added to Sephadex A-50, equilibrated in the same buffer, in a batch-wise method such that excess hemoglobin was present in the supernatant; this charged batch of resin was then washed 5 times with the Tris-HCl buffer. The buffer was then filtered off and 1 ml of serum previously dialyzed against the same buffer was added to 3 ml of resin. Incubation of the immune serum and Sephadex-bound hemoglobin was done at room temperature with gentle rocking for eight hours, then continued for 16 to 24 hours at 4°C without agitating. The absorbed serum was recovered by filtration of the incubation mixture.

Results

Efforts to produce high titer of antibodies toward hemoglobin in the rabbit were unsuccessful while those antibodies produced in low titer showed no specificity with respect to the different hemoglobins, as would be indicated by spurs on Ouchterlony plates. Thus, this source of antibody was abandoned and the use of the horse begun.

Within three months (initial immunization and two boosts) the horse inoculated with purified hemoglobin F had a high precipitating antibody titer for all sheep hemoglobins but, in addition, showed spurs for fetal hemoglobin compared to all other hemoglobins, indicating specific antibodies for fetal hemoglobin (47). The horse immunized

with hemoglobin A produced a high titer for sheep hemoglobins by the fourth month. The horses immunized with hemoglobin C and hemoglobin B both failed to develop high titers after five months of boosting. In both these cases I was unable to absorb out cross reactivity to other hemoglobins without losing all titer against the specific hemoglobin.

The highest titers obtained from the horses immunized against hemoglobin F and A occurred when they were reboosted six and nine months later, respectively. These were the sera used in all of the following work.

Once high titers had been achieved, the next step was to absorb out cross reactivity such that the serum would recognize only those antigenic sites which are unique to the specific hemoglobin to which it was directed. Attempted adsorption by direct addition of hemoglobin to immune serum was unsuccessful due to incomplete precipitation of horse antibodies. This characteristic of horse sera (failure of all antigen-antibody complexes to precipitate out) is reported by Kabat (46). Thus a means of immobilizing the hemoglobin was needed which would be rapid and simple.

The immobilization of hemoglobin on Sephadex A-50 by electrostatic forces was easily tested in our system. The use of an affinity 9 column was tried but failed to give satisfactory results. The batch technique described in Materials and Methods, however, gave excellent results for antisera directed against hemoglobin F and hemoglobin A but completely depleted all detectable titer of antisera directed against hemoglobin C and hemoglobin B. The most effective

absorbant of anti-hemoglobin F cross reacting components was hemoglobin B. This hemoglobin was also the most effective absorbant for making anti-hemoglobin A specific. Hemoglobins A, B, C and F were used as absorbants in the unsuccessful attempts to produce specific anti-hemoglobin C and anti-hemoglobin B.

The fact that all the sheep hemoglobins used for adsorption of antisera to hemoglobins C and B removed all antibodies to hemoglobin indicates that only those shared antigens of all the hemoglobins have elicited an immune response in the two horses immunized with hemoglobins C and B. It would be expected upon examining the sequence data for the four sheep hemoglobins that the only antigens consistently shared between all the hemoglobins would be those of the alpha chain.

Non-absorbed anti-hemoglobin A shows several bands for non-purified hemoglobin A while demonstrating but one band against purified hemoglobin A. Antiserum against hemoglobin A absorbed with hemoglobin B shows only one band against both non-purified and purified hemoglobin A (Figure 6). This indicates that adsorption with non-purified hemoglobin on DEAE Sephadex has removed reactivity against non-hemoglobin factors.

In Figure 7 non-absorbed anti-hemoglobin F shows single band reactions with all sheep hemoglobins and, after absorption with hemoglobin B, this same serum is specific for hemoglobin F when compared with other sheep hemoglobins, but it has cross reactive antibodies for bovine fetal hemoglobin.

The hemoglobins reacted with the absorbed antisera directed against hemoglobins A and F are listed in Table 2. The absorbed antisera against hemoglobins A and F gave no reactions with any

Figure 6. Ouchterlony plate showing the reactivity of non-absorbed antiserum against hemoglobin A on the left in the center well and absorbed antiserum against hemoglobin A on the right in the center well. Hemoglobins in the small wells starting at the top and going clockwise are: A, B, C, F, bovine fetal, and bovine adult.

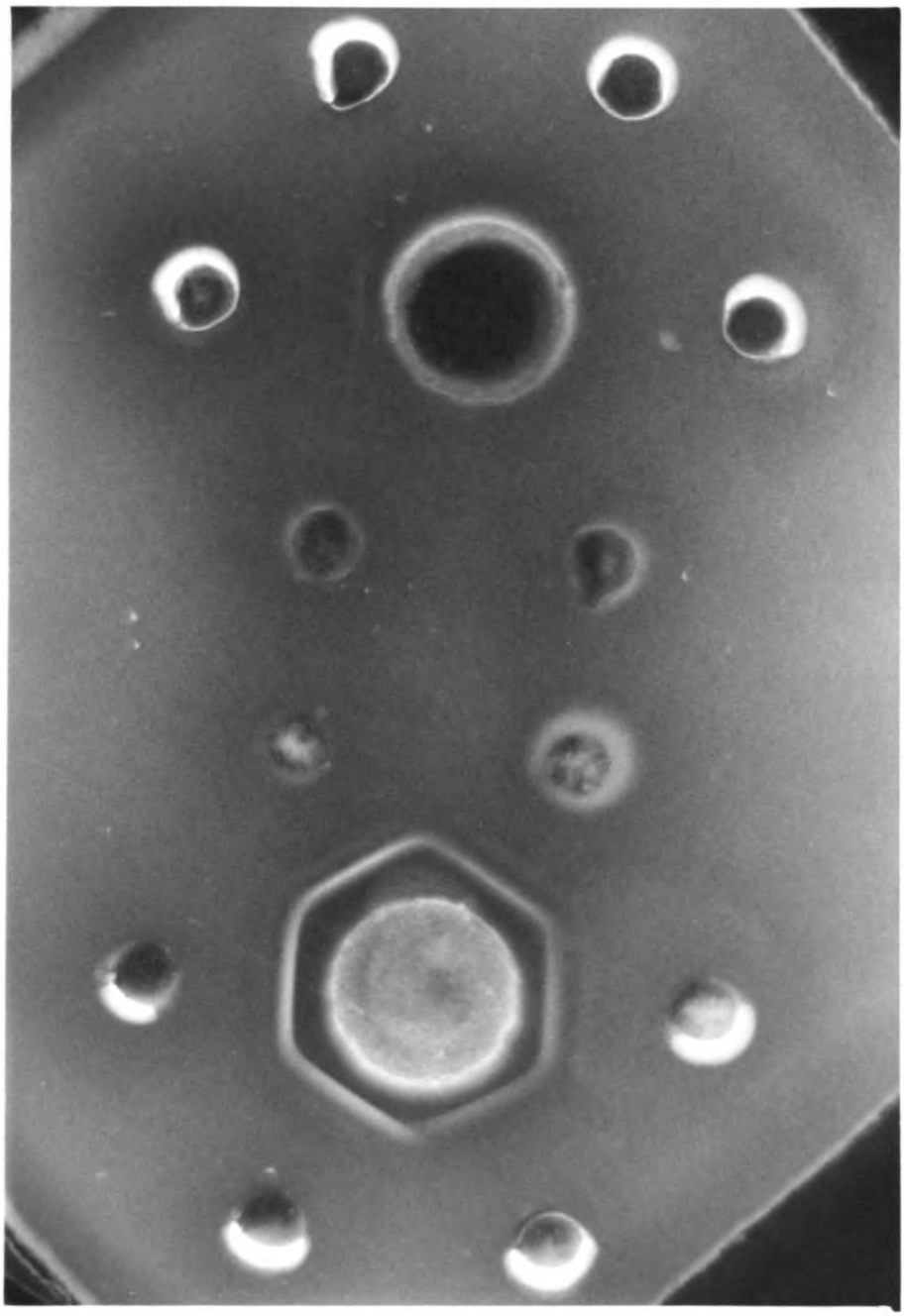


Figure 6

Figure 7. Ouchterlony plate showing the reactivity of non-absorbed antiserum against fetal sheep hemoglobin on the left in the center well and absorbed antiserum against fetal sheep hemoglobin on the right in the center well. Hemoglobins in the small wells starting at the top and going clockwise are: A, B, C, F, bovine fetal, and bovine adult.

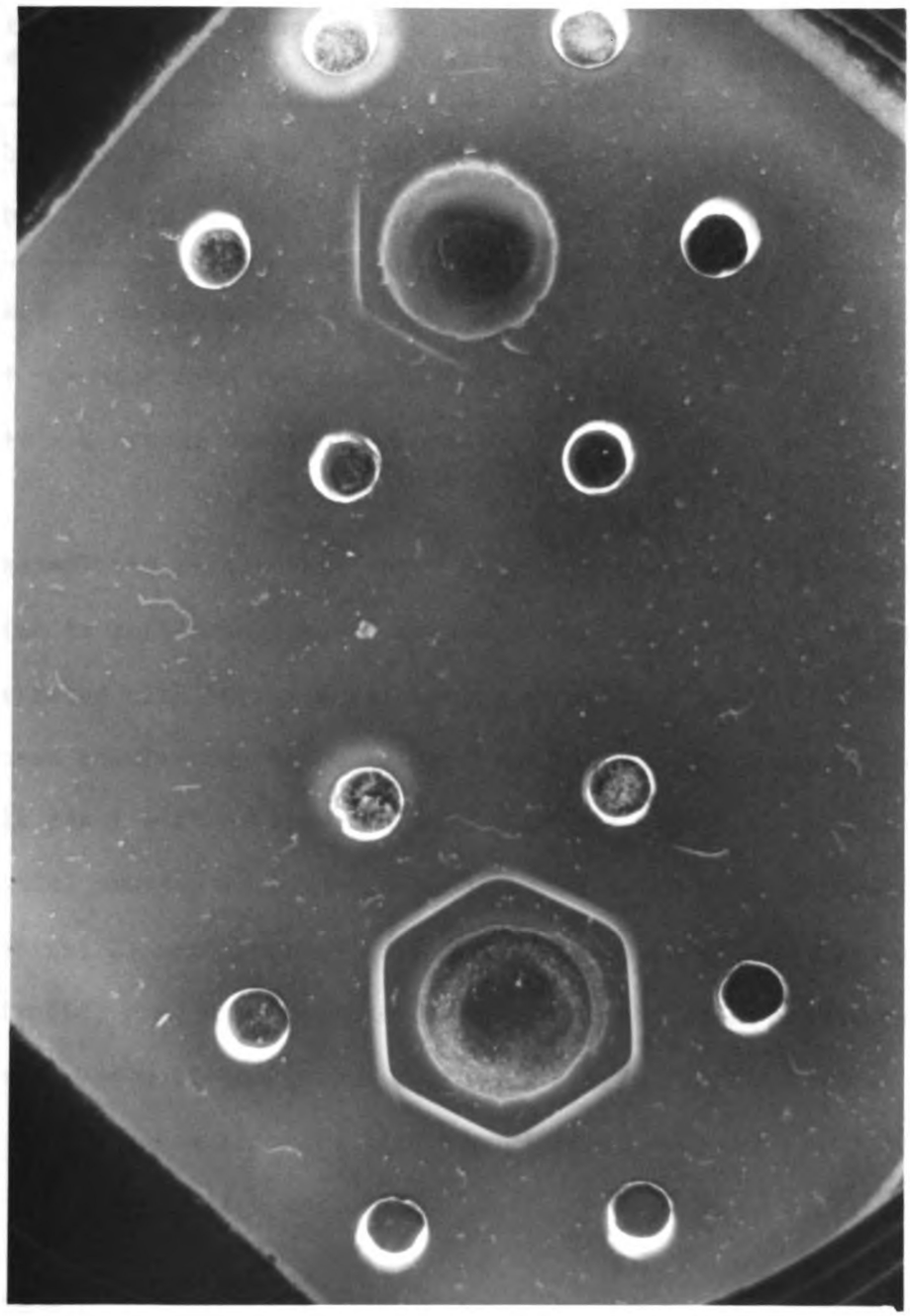


Figure 7

Table 2. Reactivity of specific antisera (absorbed) against sheep and bovine hemoglobins as measured by double diffusion precipitin reaction

Antisera absorbed	Sheep A	Sheep B	Sheep C	Sheep fetal	Sheep embryonic	Bovine	Bovine fetal
anti-Hb A	++[c]	N.B.[a]	N.B.	N.B.	N.B.	N.B.	N.B.
anti-Hb F	N.B.	N.B.	N.B.	++	N.B.	N.B.	+[b]

[a] N.B. - no bands formed

[b] + - a weak band was formed

[c] ++ - a strong band formed early

other hemoglobin except that which they were directed. The notable exception is anti-hemoglobin F absorbed with hemoglobin B, which cross reacted with bovine fetal hemoglobin.

These results can be summarized as follows: 1) ruminant hemoglobins all react with nonabsorbed sera strongly (Table 3), which suggests substantial numbers of homologous antigenic sites between these hemoglobins; 2) non-absorbed anti-hemoglobin F reacts more strongly with giraffe, pig, hippopotomus and elephant than does anti-hemoglobin A; 3) absorbed anti-hemoglobin F cross reacts with fetal bovine hemoglobin, whereas absorbed anti-hemoglobin A does not react with bovine adult or fetal hemoglobin.

Discussion

The degree of homology in primary structure, as measured indirectly by homologous antigenic sites on the hemoglobin molecule,

Table 3. **Reactivity of antisera (non-absorbed) against various mammalian hemoglobins as measured by double diffusion precipitin reaction**

Antisera	Sheep A	Sheep B	Sheep C	Sheep fetal	Sheep embryonic Gower I type	Goat (pooled)	Antelope	Eland	Salile	Buffalo	Giraffe	Elephant	Hippopotamus	Pig
anti-Hb A	++[d]	++	++	++	N.B.[a]	++	++	++	++	++	+[c]	N.B.	+ faint	+ faint
anti-Hb B	++	++	++	++	N.T.[b]	++	++	++	++	++	+	N.B.	+ faint	+ faint
anti-Hb C	++	++	++	++	N.T.	++	++	++	++	++	++	+	N.B.	N.B.
anti-Hb F	++	++	++	++	N.B.	++	++	++	++	++	++	+	+	+

Antisera	Zebra	Dog	Cat	Primate (man and Rhesus)	Seal	Pig fetal	Horse	Horse fetal
anti-Hb A	N.B.	N.B.	N.B.	N.B.	N.B.	+ faint	N.B.	N.B.
anti-Hb B	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.
anti-Hb C	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.
anti-Hb F	N.B.	N.B.	N.B.	N.B.	N.B.	+	N.B.	N.B.

[a]N.B. - no bands formed; [b]N.T. - this reaction was not tested; [c]+ a weak band was formed;

[d]++ - a strong band formed early.

indicates that among ruminant hemoglobins there is a great deal of homology. These homologous sites are not present, as detected by immune sera, in hemoglobin of other species tested. On a molecular level, then, the relative homology of these hemoglobins supports the phylogenetic relationship as determined by anatomical studies.

When the comparisons described above are extended to include the fetal animal, we begin to look at the degree of homology between hemoglobins of the fetus and the adult of the same species and the degree of homology among the fetal hemoglobins of various species. In this manner with more data in the future, the evolution of the fetal animal can be compared with the evolution of the adult on a molecular basis.

Thus it was interesting to find that, when comparing the non-absorbed sera for hemoglobins A, B, C and F in the reaction against non-ruminant species, the anti-hemoglobin F reacts more strongly against giraffe than any of the anti-adult hemoglobin sera. Against pig and hippopotamus, only slight reaction is seen by anti-hemoglobin A and anti-hemoglobin B, while anti-hemoglobin C does not react at all; however, anti-hemoglobin F shows a strong reaction with both pig and hippopotamus. In the case of the elephant, only anti-hemoglobin F shows a precipitation band; all other antisera do not react with elephant hemoglobin. This is illustrated in Figure 8.

This same pattern of anti-hemoglobin F sera reflecting greater homology between fetal hemoglobin of different species than between the adult hemoglobins of the different species is indicated in the results using absorbed, specific antisera. In this case absorbed

Figure 8. Ouchterlony plate showing the reactivity of non-absorbed antiserum against fetal sheep hemoglobin on the left in the center well and non-absorbed antiserum against hemoglobin A on the right in the center well. Hemoglobins in the small wells starting at the top and going clockwise are: pig, giraffe, hippopotamus, elephant, F and A.

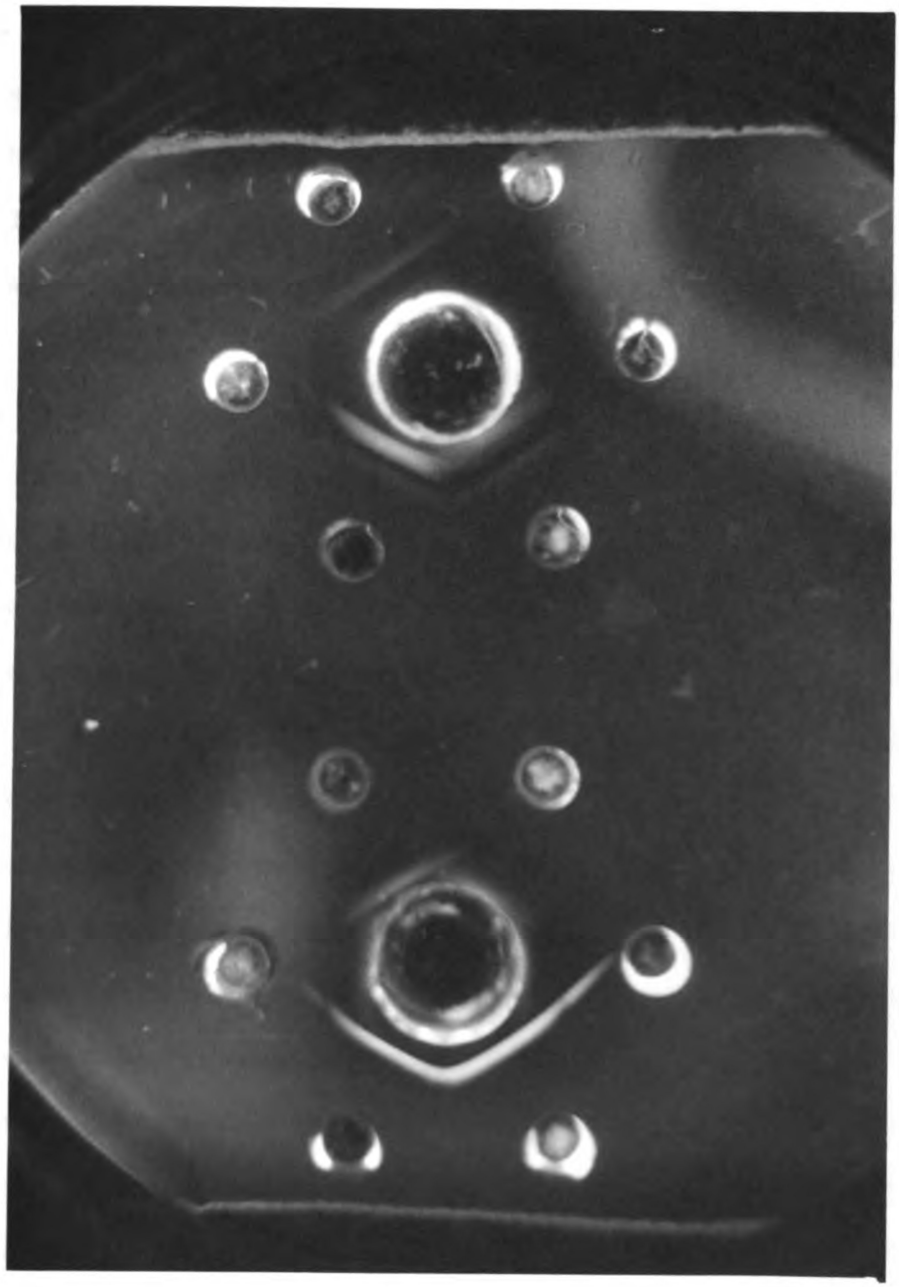


Figure 8

anti-hemoglobin F does not react with any of the adult sheep hemoglobins or the adult bovine hemoglobin; however, it does react with fetal bovine hemoglobin (Figure 7). Specific antiserum to the sheep hemoglobin A does not react with bovine fetal or adult hemoglobin. This implies a greater homology, structural conservativeness, in the fetal hemoglobins than in the adult hemoglobins of these two species. In order to test the general application of this hypothesis for other mammals (in particular for ruminants), more fetal samples of various species are required.

Babin *et al.* (17) realized the need for comparative primary structural data on hemoglobin from different stages of development in different species of animals. This type of information is needed in order to determine which parts of the hemoglobin molecule are responsible for adapting the function of the molecule to its environment. For this reason Babin chose to sequence bovine fetal hemoglobin and compare structural differences with human fetal hemoglobin which had previously been sequenced. He found bovine fetal gamma chains to differ from human gamma chains by 40 amino acids and from human adult β chains by 32 amino acids; also, more amino acid substitutions occurred between human gamma chains and human adult β chains than between bovine gamma chains and human adult β chains. He concluded that the type of amino acid substitution must be considered as well as its location in the molecule as determined by comparison with a structural model of globin chains (17). I have carried this type of comparison further using more recent sequence data (20) and my own results from specific antibody reactivity.

From amino acid sequence data showing differences between sheep Hb A and Hb F, it is possible to assign the cross reacting antigenic site to a probable position at 54 to 58 amino acids from the N terminus and/or 73 to 77. These areas are identical between the sheep fetal gamma chain yet completely different from adult sheep A β chain. From the Perutzian model of β chains (29, 30), the former site is located at the bend between the D helix and E helix and protrudes out from the molecule surface, whereas the latter site is part of the E helix and less prominent on the molecular surface. Interestingly, the 54 to 58 site is much less variable than the 73 to 77 site when comparing γ and β chains of ruminants and primates. The possible functional importance of the 54 to 58 site in ruminant (it is also the same sequence in fetal goat gamma chain) fetal hemoglobin cannot be extended to human fetal hemoglobin since this site is invariant between adult human beta chain and human fetal gamma chain except for one amino acid substitution at 54 where all fetal ruminants and fetal human have isoleucine and all adult species of animals have valine. Exceptions are the mouse and the frog, which have isoleucine in their adult hemoglobins.

As one of many possible explanations for the close homology between the fetal hemoglobins of sheep and cattle may be a similarity in environments which the hemoglobins are functioning in uptake of oxygen from maternal tissue. The environment to which I refer is at the molecular level; for it may be that fetal hemoglobin is required to interact molecularly in a much more specific manner than adult hemoglobin in its uptake of oxygen, thus allowing less molecular

heteroteneity for effective function. Longmuir (43) has proposed that the oxygen carrier between maternal and fetal circulation is cytochrome P-450, found in placental tissue, which Gurtner and Burns (25) could inhibit with drugs such as analine and morphone and observed a marked decline in oxygen flux transplacentally. This type of selection pressure, that is, specific interaction with another protein molecule, has not been proposed for the uptake of oxygen from the lungs and thus would present a limitation on fetal hemoglobin structure not to be found in the adult air-breathing animal. The theory that the highly integrated interaction of molecules as they function in specialized roles in higher animals provides a selective pressure against protein heterogeneity is put forth by Goodman (51).

In further investigation of the non-adult hemoglobins, embryonic hemoglobin of the Gower I type, purified by isoelectric focusing on polyacrylamide gel as described later was reacted with all the non-absorbed antisera and the two absorbed antisera, as shown in Tables 2 and 3. No precipitin bands were produced with any of the antisera. This rather surprising result may be explained by a lack of alpha chains (shared by all other sheep hemoglobins) in the embryonic hemoglobin which would be consistent with previous results indicating epsilon chain tetramers exist in sheep embryonic hemoglobin as in man (3). Also this lack of reaction between embryonic hemoglobin and any of the antisera indicates that the epsilon chain itself is less closely related to fetal sheep hemoglobin than is fetal bovine hemoglobin. In addition to the direct comparisons of hemoglobins,

specific antibodies may be used in a variety of techniques ranging from purification methods by specific precipitation or affinity column to intracellular identification of hemoglobins by fluorescent labeled antibodies. For identifying hemoglobins or any protein, antibodies have certain advantages over electrophoretic methods.

It has been my experience that specific antisera ignore many forms of non-genetic heterogeneity in determining the identity or non-identity between hemoglobins which may be susceptible to conditions which alter their overall charge and thus their migration on electrophoresis. Thus where electrophoresis would indicate more than one hemoglobin from a sample, stored over a long period, due to valence state of iron, polymerization or phosphate binding a specific antibody would not show more than one hemoglobin if the primary structure were still intact and the tertiary structure not altered since these are the factors determining antigenic sites (27). A specific antiserum is not a final proof of amino acid sequence identity between two proteins; however, it can be used on a micro-scale to check for homology or non-identity between proteins.

CHAPTER III

PEPTIDE MAPPING OF DANSYL-PEPTIDES

Introduction

Many workers examining the hemoglobin of embryonic animals have reported numerous electrophoretically distinct hemoglobins existing in the embryos of some species. These reports have been based primarily on electrophoresis data alone, with the exception of the extensive study of chicken hemoglobin ontogeny by Schalekamp *et al.* (12), which employed specific antisera in verifying electrophoretically distinct bands. The recent use of polyacrylamide gel isoelectric focusing has led to the reporting of numerous embryonic hemoglobins in a single species, notably the hamster (18). Other authors have made similar reports of multiple embryonic hemoglobins in the rabbit (28).

There are several explanations for the inordinately large number of embryonic hemoglobin types being found within a single species: firstly, there may be some advantage to the developing organism at this stage in having numerous hemoglobins; secondly, the electrophoretically distinct bands may be reflecting a propensity for polymerization by embryonic hemoglobin subunits [as is the case with adult chicken (12) and mouse (44)]; thirdly, embryonic hemoglobin subunits may readily hybridize with subunits common to the adult or

fetal stages; fourthly, the method of sample collection, preparation and storage may cause changes in heme iron oxidation state or in phosphate binding by hemoglobin that would show up as distinct bands on isoelectric focusing.

It is obvious that the apparent heterogeneity, as revealed by isoelectric focusing and electrophoresis, of these early hemoglobins must be verified by a method which more closely detects changes in primary structure. The method must be especially adaptable to micro- and ultramicroscale analysis since the samples of hemoglobin available from individual embryos are extremely small. This limitation in sample size rules out the use of classical peptide mapping techniques in most instances.

The use of 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dans-Cl) in the analysis of proteins on a microscale involving less than 10 nmoles has been reported by several authors and the detailed description of its use in N-terminal amino acid and amino acid sequencing determinations is given by Gray (23). Though these methods have many advantages over classical sequencing techniques, my concern with a more general and rapidly acquired picture of the primary structure of the hemoglobin types to be differentiated led me to employ peptide mapping of dansylated peptides following tryptic digestion of the hemoglobin. Only in recent years has the solvent combinations necessary to resolve dansylated peptides been developed to a stage where satisfactory maps are achievable (36).

The ability to elute isolated bands of hemoglobin from polyacrylamide gels after isoelectric focusing in amounts of 100 to 200

μg and the sensitivity of the dansyl peptide mapping procedure allows the primary structure of these bands to be examined as a more accurate measure of the degree of identity between bands of hemoglobin separated by isoelectric focusing. In the following section we applied the dansyl peptide mapping technique to comparing sheep embryonic hemoglobin with fetal hemoglobin and adult hemoglobin and to determining which hemoglobin bands separated on isoelectric focusing gels due to primary structure changes.

The purpose of the work described in this section is twofold: first of all, an experiment was conducted to determine whether multiple bands forming on isoelectric focusing gels when blood from a sheep homozygous for hemoglobin A was used show similar or dissimilar dansyl peptide maps, and secondly, the structural analysis of embryonic hemoglobin was begun by comparing dansyl-peptide maps of embryonic hemoglobin with fetal and adult hemoglobin.

Materials and Methods

All hemoglobin used in this study was collected and prepared in a non-purified form as described in the previous section. This method was carefully followed except in those experiments where artifacts of storage were desired.

Rapid preparation of globin from hemoglobin on a microscale was accomplished by adding one or two drops of hemoglobin (concentration of about 5 mg/ml) to 2 ml of acid acetone (3 ml of 2 N HCl/100 ml acetone) in a 15 mm x 75 mm test tube which had been cooled in dry ice acetone bath. After the hemoglobin had been added, the tube was allowed to come to room temperature prior to centrifuging down the

precipitated globin at 15,000 gs for 5 min. The precipitate was washed with acetone and allowed to dry in the tube.

Tryptic digestion was carried out as follows: to the globin in the 75 mm x 15 mm tube, 50 µl of deionized water, 13 µl of 1 M $NaHCO_3$ (to make 0.2 M), and 5 µl of trypsin at 1 mg/ml were added before incubating at 37°C with agitation for 2 hours. Immediately following tryptic digestion the samples were lyophilized.

Dansylation of the tryptic peptides was carried out after the method described by Gros and Labouesse (35) by adding 10 µl of deionized water, 20 µl of $KHCO_3$, and 30 µl of 0.1 M dansyl-Cl in acetone. The dansylation of N-terminal amine is complete within 30 minutes (35). After this time 5 1 of the mixture was spotted directly onto a TLC plate while the rest was stored at -20°C for future use. Dansylated peptides were stored for up to six weeks at -20°C in the dark with no change in the map pattern.

The preparation of TLC plates was as follows: silica gel (Kiesilgel G nach Stahl) from Merch A.G. was applied in a slurry of 30 g gel in 73 ml deionized water to glass plates (20 cm x 20 cm) at a depth of 250 microns; these plates were aired, dried, and stored before use. They were heated at 110°C for 2 hours and cooled to room temperature (15 minutes). The plates were spotted immediately upon cooling with the dansylated peptides 3 cm from the bottom and 3 cm from the edge (bottom in the second dimension chromatography). The spot was never larger than 4 mm in diameter and was dried by a hair dryer set on "cool" between applications.

Chromatography was based on the method used by Atherton and Thomson (16), the first dimension being in ethyl acetate-isopropanol-aqueous ammonia 9:7:4 by volume and the second dimension in chloroform 95% (v/v) ethanol acetic acid 38:4:3 by volume. This is a slight modification of the authors' method in that ethyl acetate was substituted for methyl acetate. The chromatography was run for 15 cm in both directions. Between the first and second developments the plate was air dried 5 min, heated at 110°C for 10 min, then cooled 15 min to room temperature.

For the best visibility of the fluorescent spots, the plate was examined immediately upon removal from the chromatography tank before it had dried or, if this was not possible, the fluorescence could be preserved up to 48 hours exposed to dioxane vapor in the dark. Fading of the spots was very rapid while viewing the plate under ultraviolet light, making it mandatory to work with all possible speed in recording the location of the spots on the plate (this can be done by scratching a ring around the spot and later tracing this map with tracing paper under white light).

The separation of hemoglobin samples for this study was carried out by isoelectric focusing on polyacrylamide gel using techniques from several sources (18, 19, 28, 31). For preparative isoelectric focusing, plastic tubes with an inside diameter of 0.9 cm by 27 cm were used. These tubes were set up on a Buchler polyacrylamide gel electric apparatus.

Stock solutions were as follows: #1 - 20% acrylamide plus 0.8% N-N-methylene bisacrylamide, #2 - 40% ampholyte (commercial

preparation by LKB pH range 6 to 8), #3 - 0.08% NNN'N'-tetramethyl-ethylenediamine, #4 - 0.34% ammonium persulfate, #5 - 0.2 M NaOH (diluted 1/10), #6 - 0.1 M H_3PO_4 (diluted 1/10). Solutions 1, 2 and 3 were stored at 4°C, while solution 4 was made fresh prior to using.

The solutions were mixed in a vacuum flask as follows: 3 ml of solution 1, 3 ml of distilled water, 3 ml of solution 3, 0.6 ml of solution 2, and 2.4 ml of solution 4. This total of 12 ml was enough for one tube. As soon as the #4 solution was added, the solutions were mixed well and degassed rapidly; then they were immediately added to the column which was standing upright with dialysis membrane over the bottom end.

Five minutes after the columns were poured, 100 µl of distilled water was layered very gently onto the top of the gel, with a Hamilton syringe, in order to give a flat surface. After 30 to 60 minutes, the gel had set. The gels were then added to the apparatus set up in a cold room, 4°C, and the bottom tray was filled with 0.1 M H_3PO_4 at a volume of 500 ml and the top tray filled with 1 liter of 0.2 M NaOH. The negative pole of the power source was connected to the top tray and the positive pole to the bottom tray.

The $(NH_4)_2S_2O_8$ was removed from the column by passing one mA per tube for 10 to 20 minutes.

Twenty-five to thirty microliters of 5% sucrose were layered on each column. Under this was layered a solution containing hemoglobin at a concentration of 5 mg/ml or greater in a 2:1 ratio with 15% sucrose. The total volume of this latter solution did not exceed 60 µl. During this procedure the current was maintained at 1 mamp/tube.

Constant current at the above setting was maintained for 4 to 6 hours unless the voltage exceeded 400 V, in which case a constant voltage of 400 was maintained.

The bands to be studied were eluted and concentrated by the following method: bands were cut from the gel and quartered, then placed in 5 ml of distilled water and stirred overnight, or until no hemoglobin could be seen in the gel at 4°C. The eluted hemoglobin now contained peptides from the ampholytes, which were removed by dialysis for 24 hours against three changes of deionized distilled water. The dialyzed solution was then lyophilized and the hemoglobin resuspended in one or two drops of deionized distilled water prior to making of globin.

Pictures of the fluorescent dansylated peptides separated by chromatography on thin layer plates were taken on Tri Pan X film (Kodak) at a distance of about 1 ft, using 6 sec exposures with the lens set at f 5.6. The camera used was a Mirauda Sensorex fitted with a 35mm lens (F 2.8 with an A+1 diopter, auxiliary close-up) and a Y2 yellow filter (Hoya). The fluorescent light was of both long and short wave length ultraviolet light.

Results

The following experiment was conducted to test the effectiveness of dansyl-peptide mapping as a means of determining whether bands which separate on isoelectric focusing in polyacrylamide gel have distinct primary structures or whether net molecular charge differences are due to nonprimary structural differences. While the former are indeed significant and important differences, the latter

are often the result of experimental conditions and outside of having an importance in understanding biological phenomena.

Hemoglobin prepared as described previously and stored for one month at -20°C was repeatedly freeze-thawed (5 times) prior to being placed on polyacrylamide gel for isoelectric focusing. The hemoglobin source was a sheep homogeneous for hemoglobin A; however, the blood was not purified over ion-exchange resin before freezing at a concentration of 10 gm/100 ml. The resulting migration pattern is seen in Figure 9. Globin from each of the major bands was tryptic digested and the dansyl peptides mapped. The maps of each band were identical (Figure 12).

The characteristic migration of sheep embryonic hemoglobin, as seen in starch gel electrophoresis (Figure 2) is analogous to its migration in polyacrylamide gel isoelectric focusing. The major band of embryonic hemoglobin compared to fetal hemoglobin (in the same sample) and adult hemoglobin A is closest to the origin (negative pole). The well separated bands were easily cut out and the hemoglobin eluted as described.

The hemoglobin sample used as a source of embryonic hemoglobin was from a 29-day-old embryo; it shows the presence of substantial amounts of epsilon chains as well as alpha and gamma chains on cellulose acetate in urea (Figure 3). Alpha chains appear to be the major component, as would be expected if they were associated with both gamma and epsilon chains.

Dansylated peptides from hemoglobins A and F separated by isoelectric focusing gave identical maps to homologous hemoglobins not

Figure 9. Unstained bands of hemoglobin separated by isoelectric focusing in polyacrylamide gel. Run for 4 hours at 400 volts and 3 mamps using ampholytic with a pH range of 6 to 8. From left to right: the tube on the left contained less than 100 µg of sample; next is approximately 500 µg of hemoglobin from the peripheral circulation of several 29-day-old embryos; next is hemoglobin from a 100-day-old fetus showing characteristic migration of fetal hemoglobin. On the far right is hemoglobin from an adult sheep homozygous for hemoglobin A, the sample being stored for one month at -20°C and repeatedly thawed.

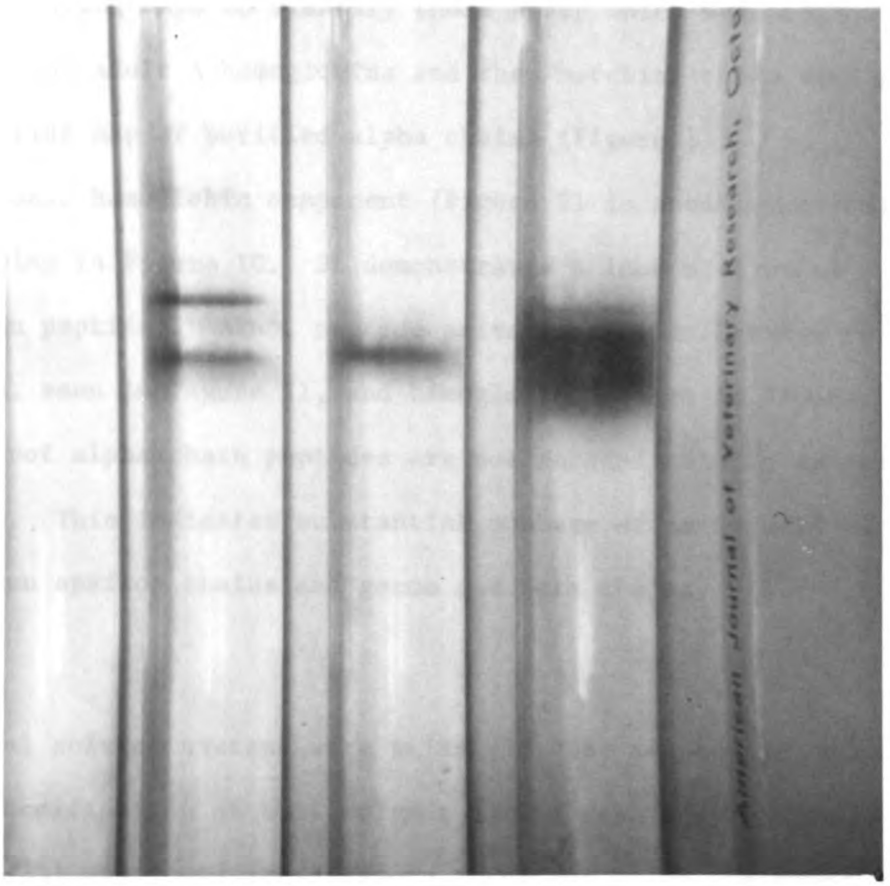


Figure 9

purified by isoelectric focusing. This indicates that the procedures involved in isoelectric focusing and band elution do not alter the peptide composition of the hemoglobin.

The identification of the spots as α chain polypeptides was made by comparing maps to identify those spots which were common to both fetal and adult A hemoglobins and then matching these with a dansyl-peptide map of purified alpha chains (Figure 13).

Embryonic hemoglobin component (Figure 9) is seen immediately after mapping in Figure 10. It demonstrates a lack of typical alpha chain peptides. Also, peptide patterns similar between fetal hemoglobin, seen in Figure 11, and hemoglobin A, seen in Figure 12 which are not alpha chain peptides are not recognizable in embryonic hemoglobin. This indicates substantial numbers of amino acid variation between epsilon chains and gamma and beta chains.

Discussion

Several solvent systems were tried (16, 33, 34) and rejected until the modification of that solvent system reported by Gros and Labouesse (35) was found satisfactory. Further trial and error experimentation would probably yield even greater resolution of peptides since the system reported by Zanetta *et al.* (36) appears to be a promising possibility. As is evident from the figures showing the dansyl peptide maps, further resolution in the second dimension is needed for those peptides carried less than 7 cm by the first dimension solvent. This may be accomplished by Zanetta's method in that he finds by raising the dialectric constant of the second dimension, solvent peptides remaining close to the origin

Figure 10. Dansyl-peptide map of embryonic hemoglobin eluted from polyacrylamide gels. Actual size of the map as viewed under ultraviolet light. The maps were spotted in the lower right-hand corner and the first dimension chromatography run in the upward direction, as pictured here, to the horizontal solvent line, while the second dimension chromatography was to the left to the vertical solvent line. Spots representing dansyl ammonia and hydroxylated dansyl compound are worked. These spots were consistent landmarks on all maps. The lack of most typical alpha chain spots and presence of unique spots indicate the distinct structural difference between this embryonic hemoglobin component and fetal hemoglobin.

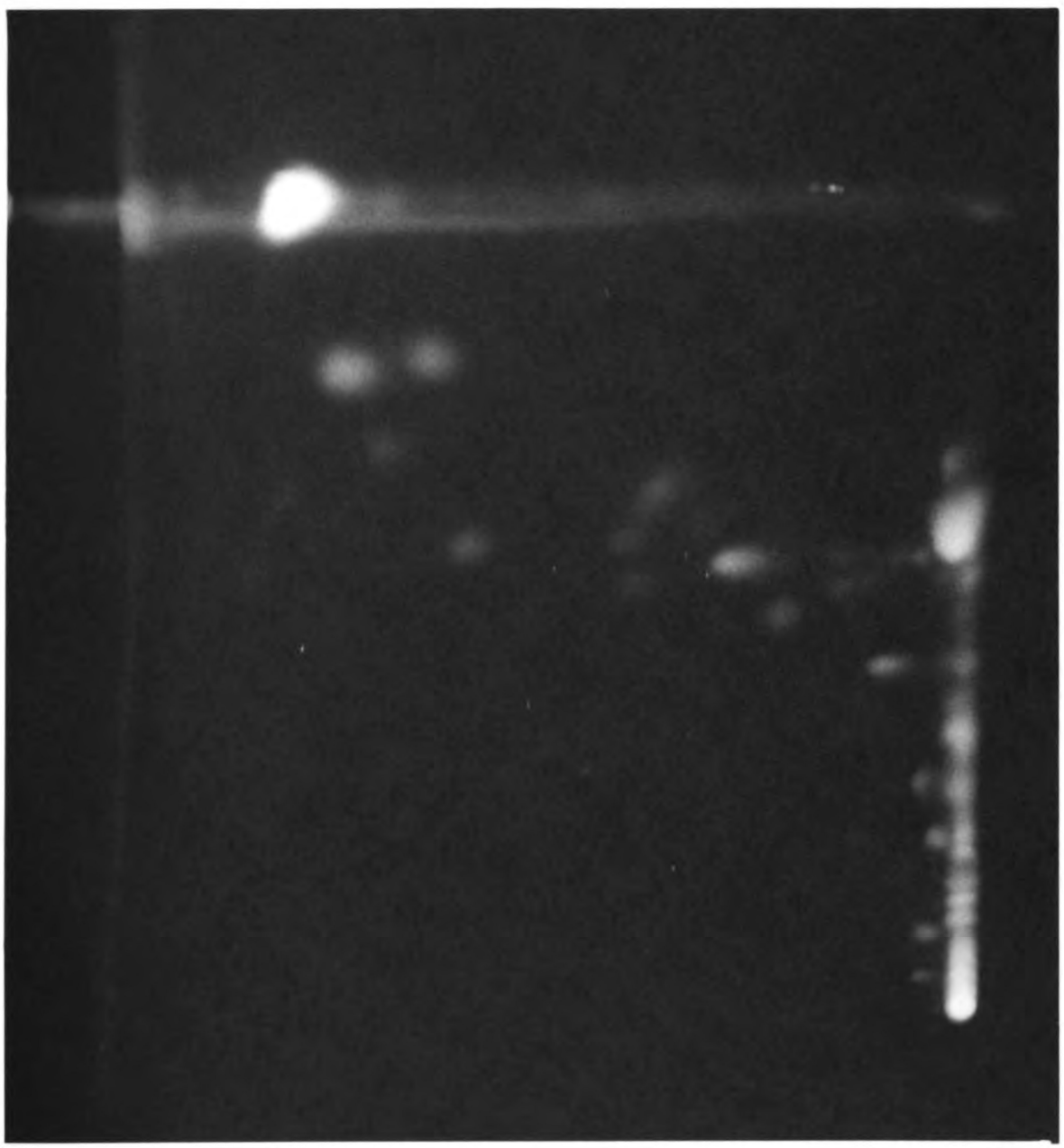


Figure 10

Figure 11. Fetal hemoglobin eluted from the same gel as the embryonic hemoglobin mapped in Figure 10 shows characteristic alpha chain spots and the other differences from embryonic hemoglobin not due to alpha chain spots.

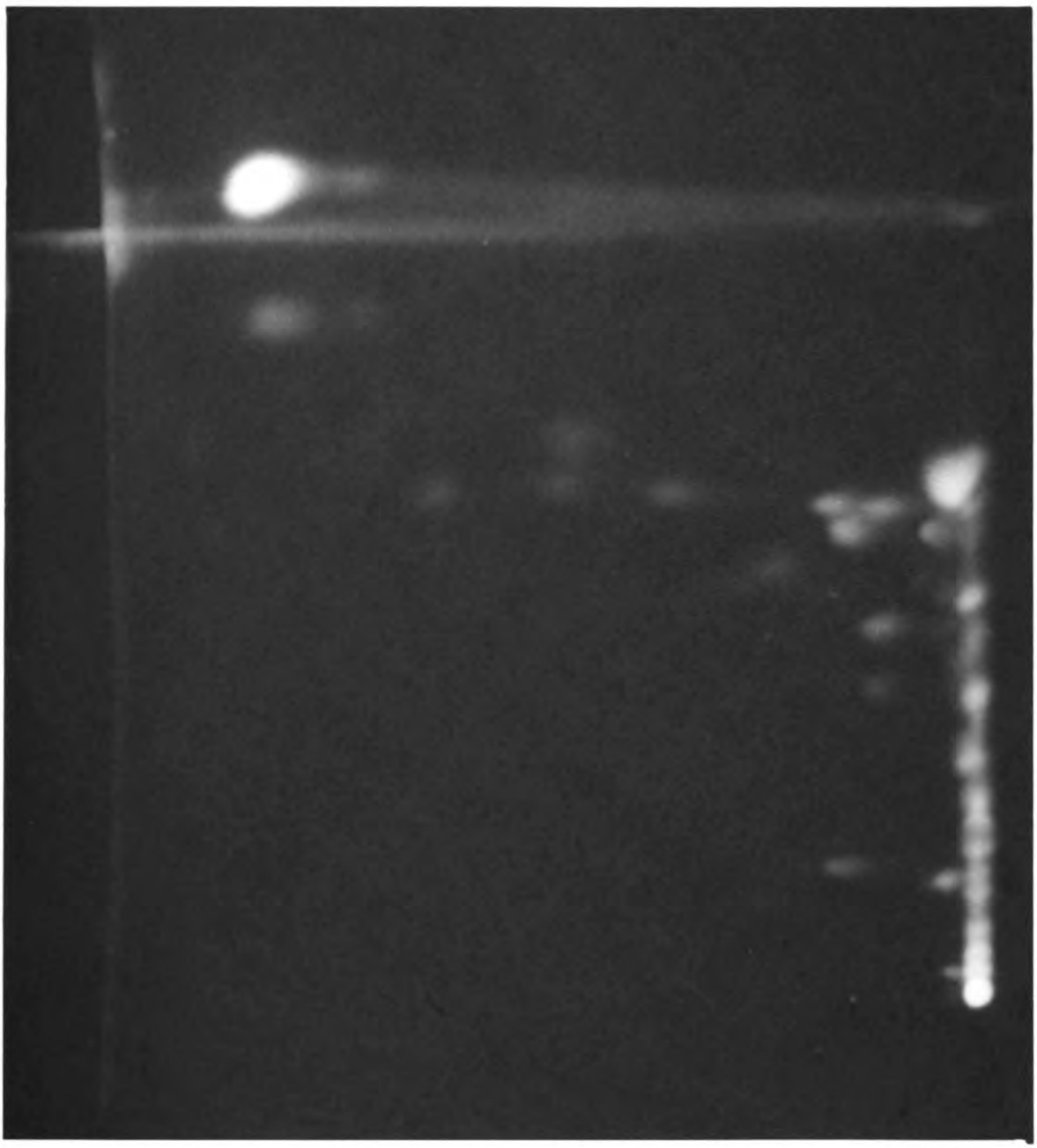


Figure 11

Figure 12. Adult hemoglobin A, not eluted from gel, in this map is in deliberately large amounts to demonstrate the alpha chain spots. The non-alpha chain differences between it and fetal hemoglobin are apparent; however, many of the α peptides are not carried to the left by the second dimension chromatography and, in this sample, are indistinct along the line of the first dimension chromatography. The large, bright spot below dansyl ammonia is often resolved into two spots as in both fetal and embryonic globin.

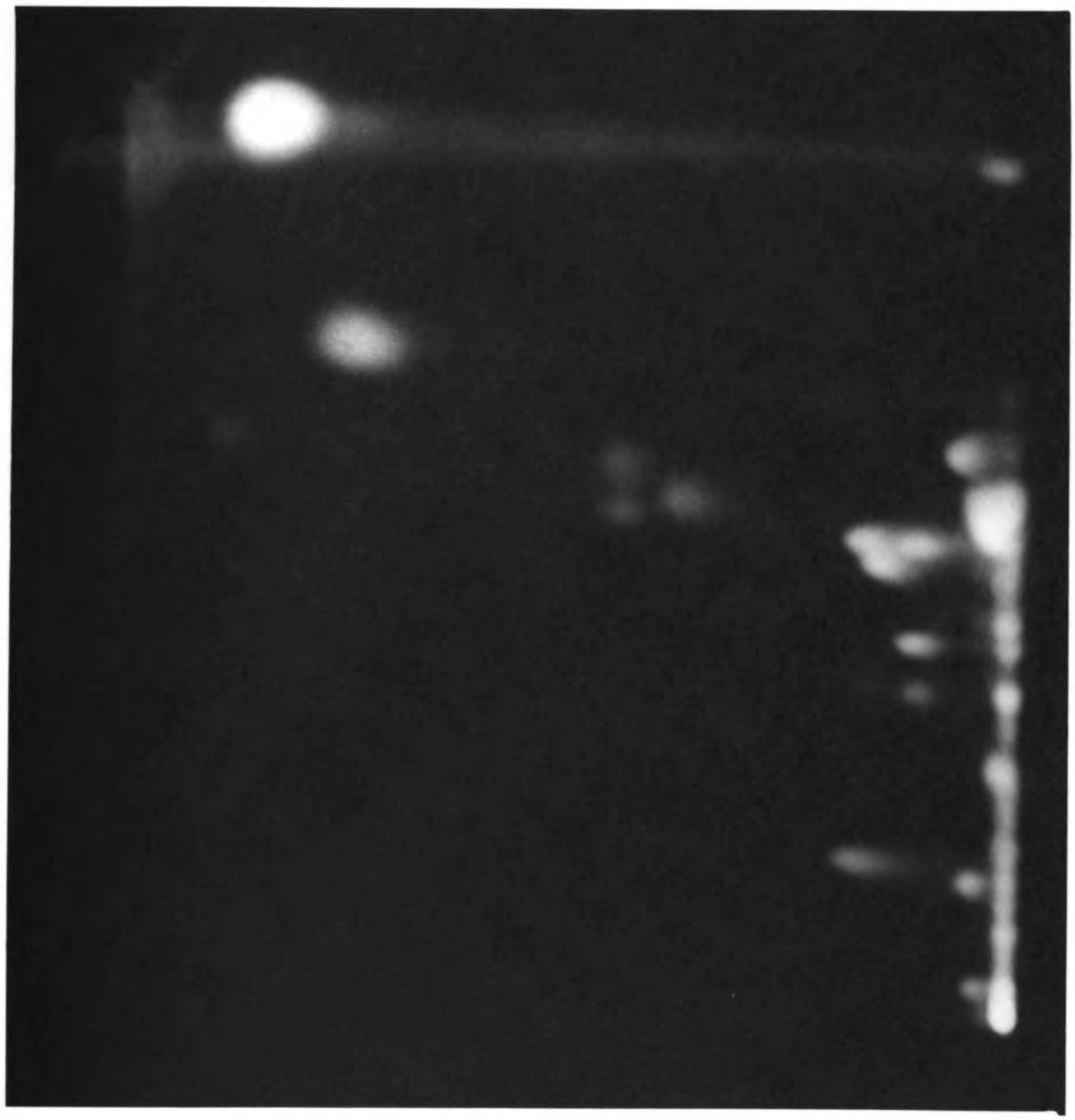


Figure 12

after the first dimension are carried out by the second dimension solvent. Specifically, this means substituting isobutanol for chloroform and reducing its proportion to acetic acid and water.

It must be emphasized that, in addition to the correct solvent system, the preparation of the TLC plates is critical to reproducibility and resolution. The plates must be heated at 110°C for more than 2 hours and spotted and placed in the chromatography tank within one hour after removal from the oven.

In examining all the adult sheep hemoglobins and fetal hemoglobin, it was found that certain peptides were consistently present upon mapping of alpha chains (Figure 13) isolated after the methods of Clegg (54).

The ability of this method to rapidly distinguish whether bands separating on isoelectric focusing are closely similar or contain distinctly different peptides provides a means of confirming suspected amino acid heterogeneities or indicating caution in accepting different electrophoretic migration rates as the sole evidence for a difference in primary structure.

The differences in peptides between embryonic and fetal hemoglobin cannot be related to specific amino acids until analogous peptides can be established between hemoglobin E and the already sequenced sheep hemoglobins. This step is accomplished in classical peptide mapping based on much previous work and is aided by specific staining methods which identify amino acid side groups. In the use of an entirely new technique for separation of peptides, the classical

Figure 13. Composite drawing of the location of alpha chain peptides and the by-products of the dansylation process. This map is representative of alpha chains separated from both fetal sheep hemoglobin and adult hemoglobin A.

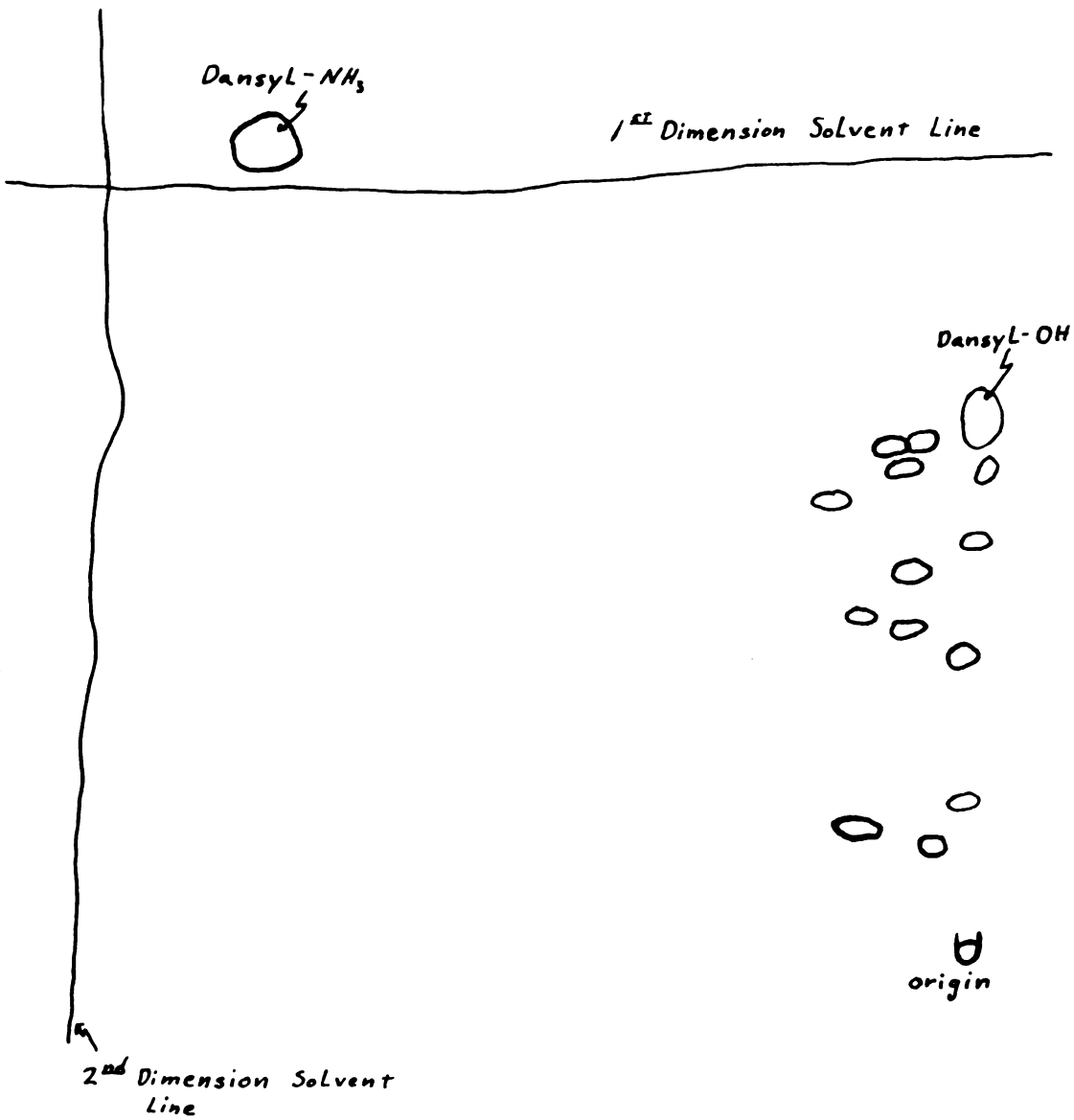

Dansyl-NH3
1st Dimension Solvent Line
Dansyl-OH
origin
2nd Dimension Solvent Line

Figure 13

patterns of peptides on paper chromatography are of little use; however, identification of peptides derived from dansyl peptide maps may be carried out using techniques of spot elution and end group determination of peptides as reported by many authors (34, 25, 36).

These techniques employ the unique migration patterns of dansylated amino acids to identify N terminal amino acids of the peptide after acid hydrolysis. The N-terminal amino group is the primary reactive group which dansyl chloride attacks under the conditions described, so that those peptides being mapped are primarily labeled at the N terminal only. Though this technique was not employed, the potential for establishing the analogous peptides between embryonic hemoglobin and any of the already sequenced hemoglobins of sheep is very great.

CHAPTER IV

KOELLIKER-LIKE HEMOGLOBIN IN SHEEP AND OTHER MAMMALS

Introduction

During studies of hemoglobin isolated from embryonic liver lysates it was found that electrophoretically there existed a band running faster than fetal hemoglobin in starch gel electrophoresis at pH 8.6 and that the alpha subunits showed less positive charge (slower cathodal migration) on cellulose acetate in 8 M urea. The characterization of the origin of this hemoglobin type from a number of tissue sources and in several species is described.

The importance of reporting on the occurrence of this artifact of hemoglobin isolation in this thesis on embryonic hemoglobin and sheep hemoglobin ontogeny lies in the fact that the source of embryonic hemoglobin from laboratory animals is a whole embryo lysate. If valid comparative studies are to be made on hemoglobin ontogeny among mammalian species, then accurate data must be available regarding the hemoglobin components at all stages of development.

The ubiquity of the occurrence of this type of hemoglobin modification is illustrated by the fact that I found it in horse, dog, guinea pig, hamster, and sheep while the original report of its occurrence was in man. Marti and Lehman described the occurrence of hemoglobin Koelliker in serum and urine of patients with hemolytic

anemia. There was isolated a hemoglobin which was found to be lacking the carboxy terminal arginine. The amount of modified hemoglobin was proportional to the length of time hemoglobin was in solution with serum or urine (10).

Materials and Methods

All tissue sources of hemoglobin were treated the same. Homogenates of liver, spleen or bone marrow were made and the cells washed three times in 1.2% saline and bubbled for 15 min with carbon monoxide. Circulating erythrocytes were also washed in saline three times and treated with CO for 15 min. Lysis of cells was carried out by adding an equal volume of distilled water to the packed cells, which were then freeze-thawed three times.

Electrophoresis was done on cellulose acetate in Tris-Borate EDTA buffer pH 9.0 and 8 M urea and on starch gel in barbital buffer, pH 8.6.

Timed incubation of the cell lysate was done at room temperature following freeze-thaw lysis and high speed centrifugation.

Inhibition studies of alpha chain modification were carried out with 2,2' dipyridyl and acetoacetamide by adding varying concentrations prior to cell lysis.

All tissue samples and peripheral blood were collected in 1.2% saline and 0.1% EDTA except in those experiments designed to determine the effect of EDTA on modification of hemoglobin in tissue lysates.

Peptide mapping followed the technique described by Smithies.

Purification of hemoglobin from tissue lysate was done on Sephadex A-50 over pH gradient from 8.6 to 7.3 in 0.01 M Tris-HCl buffer.

Results

In Figure 5 is the comparison of peripheral blood and liver lysate from a 29-day-old embryo: perhipheral blood contains 50% or more embryonic hemoglobin, while the liver lysate shows a minor component corresponding to fetal hemoglobin and a major band running faster than any of the known sheep hemoglobins. The identification of this fast migrating band is the subject of this chapter, since it corresponds to no other sheep hemoglobin previously identified. The migration patterns of the hemoglobin isolated from placental membranes are variable and of limited significance due to difficulties in washing contaminating maternal hemoglobin from them. In the membranes from the B sheep most of the hemoglobin is maternal and the minor band is likely to be a Koelliker type derived from hemoglobin B by the action of the placental tissue proteases. The placental membranes from the AB sheep show much less maternal contamination such that the major component of embryonic circulation is represented. In order to determine which of the globin subunits is involved in the formation of this new hemoglobin, electrophoresis on cellulose acetate was carried out at pH 9.0 in 8 M urea. The results of the electrophoresis of the same sample as used above are seen in Figure 3. Peripheral blood shows a preponderance of epsilon chains which migrate just off the origin with a slight amount of gamma chains (positive pole) and alpha chains (negative pole). The

liver lysate shows no alpha chains, while the migration of the gamma chains appears unchanged; however, there is a new band which corresponds to no other sheep globin. This new band is distinct from epsilon and has less net positive charge than alpha chains.

After sampling various aged embryos and fetuses, it was discovered that varying degrees of the new hemoglobin were present in tissue lysated and that the variations could not be correlated to age or tissue type (only hematopoietic tissue was sampled). Further investigation of newborn lambs and even other species indicated that a fast migrating band relative to the normal hemoglobin component from circulation could be the result of a modification of the normal hemoglobin, after lysis of mature erythrocytes, by some agent common to erythropoietic tissue.

To test the time dependence of this agent, incubation at room temperature was done with liver lysate from a 2-month-old lamb homozygous for hemoglobin A. Figure 14 shows the results of a sample freeze-thaw lysed without incubation and an aliquot of the same sample incubated for 20 minutes. The greater concentration of modified alpha chains appearing in the incubated sample prove time dependence. The question now arose as to the nature of the modification. Was it an alteration in the primary structure of the globin chain or a noncovalent binding of some charged molecule such as an organic phosphate? To answer this question the modified hemoglobin was purified over DEAE Sephadex and then peptide mapped after tryptic digestion. The peptide mapping results seen in Figure 15 show that the peptide corresponding to $\alpha 14$ is missing and that no new peptides

are present. This peptide is the carboxyl terminus composed of tyrosine-arginine (arginine being the carboxy terminal amino acid). At the pH at which the electrophoresis is run, arginine has a positively charged ε amine group which, if removed, would give the globin molecule a net loss of one positive charge. This corresponds to the migration seen for the alpha chain on cellulose acetate and for the entire hemoglobin on starch gel electrophoresis.

The removal of a carboxyl terminal amino acid having an ε amine group is characteristic of the enzymatic action of carboxypeptidase B as described in the pig (14). These workers also reported that 2,2' dipyridyl at a concentration of 6.6×10^{-4} M produces complete inhibition of this enzyme. The inhibition of the formation of the Koelliker-like hemoglobin in sheep liver lysates was complete at concentrations above 6.0×10^{-4} M of 2,2' dipyridyl.

Other factors affecting this enzyme's action are metal ions and chelating agents of these ions (15). In order to determine if the method of collecting samples might be modified so as to minimize the action of carboxypeptidase B, EDTA was replaced by heparin as the anticoagulant. It is reported that EDTA enhances the enzyme activity of carboxypeptidase B. There was no difference in the amount of Koelliker-like hemoglobin formed under identical conditions of sample collection and preparation.

Discussion

The importance of showing the occurrence of the Koelliker in the lysates of various tissues (liver, spleen and bone marrow) in sheep at all stages of development and in several different species

Figure 14. Cellulose acetate in urea electrophoresis of spleen lysate samples taken at various times of incubation at room temperature: left, the sample stored for four hours at 4°C; middle, the sample incubated for four hours at room temperature; right, the sample incubated for 20 minutes at room temperature.

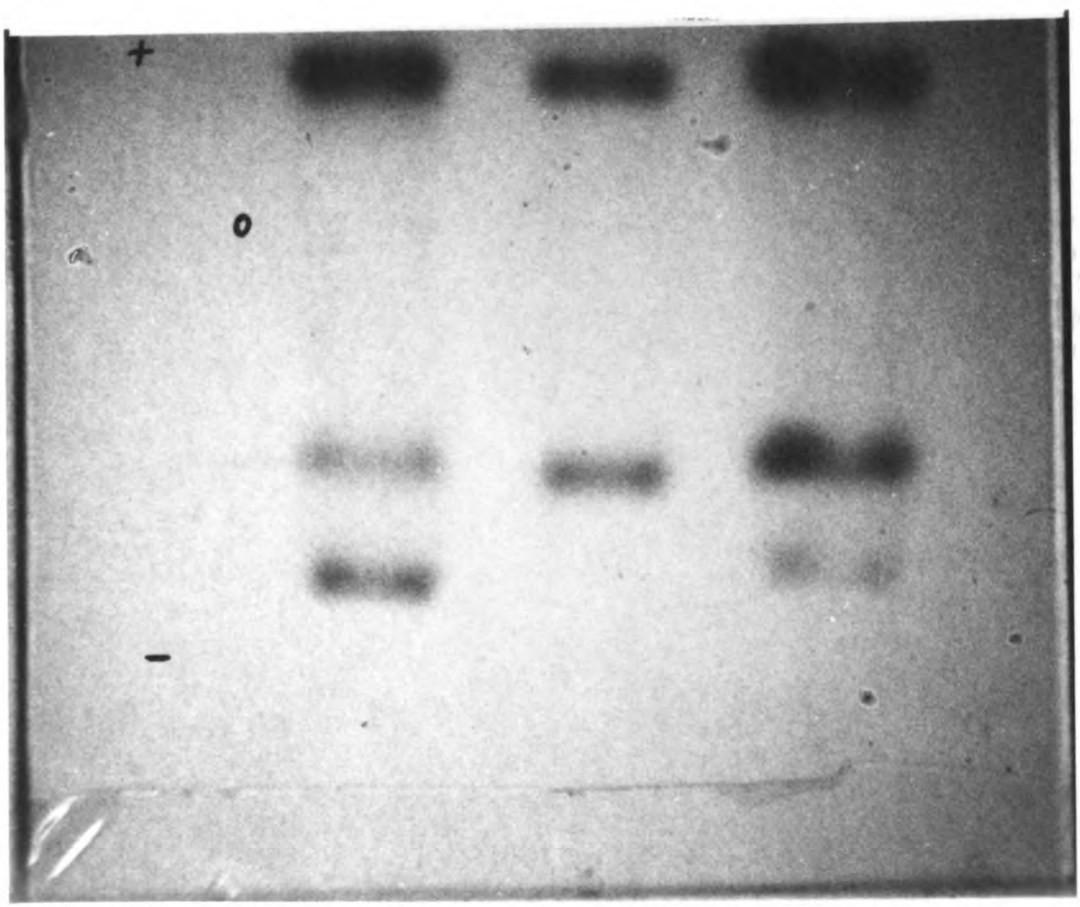


Figure 14

Figure 15. Peptide maps of spleen lysate, purified on DEAE-Sephadex, and peripheral blood of the same lamb are compared. The missing peptide, $\alpha 14$ (tryptic digest product, tyrosine-arginine), is marked by a star while the corresponding peptide present in normal HbA is marked by an arrow.

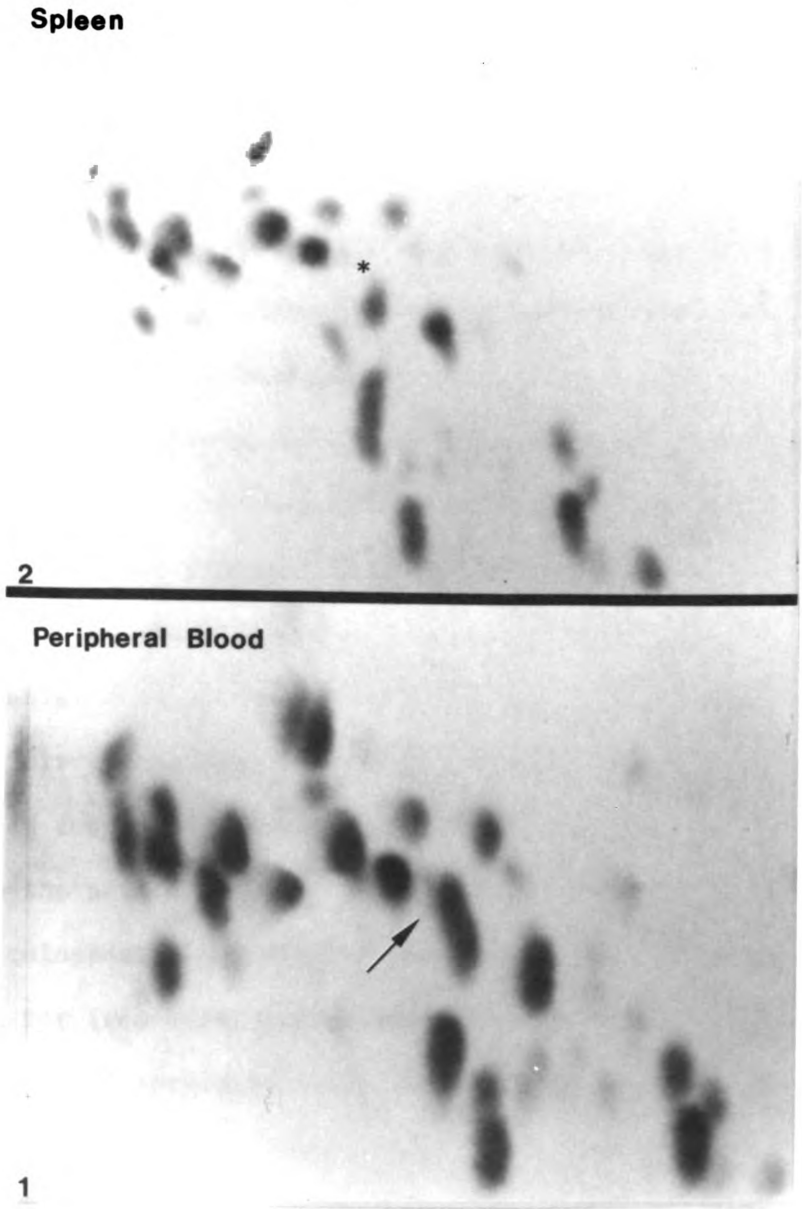


Figure 15

of mammals is not that it has as yet proven to have physiological function but that it presents a potential source of error in those experiments using other than circulating erythrocytes as a source of hemoglobin. Thus, those experiments using lysates of erythropoietic tissue or whole embryo lysates to study the onset of hemoglobin production must be extensively controlled in order to avoid attributing hemoglobin heterogeneity to differential gene induction when indeed the source of hemoglobin heterogeneity may be merely the specific action of a protease.

The unique specificity of carboxypeptidase allowing it to selectively remove an arginine group from the α chain should serve as a warning that the products of proteases need not necessarily be random peptides which do not appear as a single band on electrophoresis.

It is interesting that almost all mammalian species hemoglobin that has been sequenced has an α chain ending in tyrosine-arginine (20), while the action of carboxypeptidase B is prevalent in organs of the reticuloendothelial system in a wide variety of mammals. It may be that far from being just a pitfall to workers in hemoglobin, carboxypeptidase B serves as a key initial step in the turnover of hemoglobin.

CONCLUSION

The study of the ontogeny of a protein during the development of an animal species from conception to death is far from merely an academic exercise. Accumulated knowledge concerning transition events in protein synthesis and comparative protein structure should eventually lead to answers to questions about control of gene induction and structure-function relationships related to changing environments in which the protein functions.

My studies relating circulating hemoglobin type and red blood cell type (definitive versus non-definitive) and hematopoietic centers at very early stages of embryo development indicate that no detectable levels of fetal hemoglobin are present in non-definitive (embryonic) red blood cells which are primarily produced in the yolk sac blood islands. Impression smears suggest that none or very few non-definitive cells were of liver origin. Starch gel electrophoresis and urea cellulose acetate electrophoresis showed no fetal hemoglobin in circulation prior to 26 days' gestation, which can be correlated with non-nucleated (definitive) cells' appearance in the embryonic liver at 26 days' gestation. These observations suggest that fetal hemoglobin production is limited to the definitive cell line and that this line of cells is first induced in the liver at about 24 days' gestation. Further studies on fetal hemoglobin

induction and the induction of the definitive red blood cell line must center around this stage in embryo development in the sheep.

Thepossible differences between the transition of embryonic to fetal hemoglobin and from fetal hemoglobin to adult hemoglobin must not be overlooked since recent evidence indicates that these two events may involve very different mechanisms.

Recently Kazazian (53) and Boyer (52) have detected the presence of minute amounts of adult hemoglobin in human embryos as early as weeks' gestation and in sheep (personal communication, Kazazian) as early as 35 days' gestation. This implies that genes for adult hemoglobin present in erythroid cells of early embryos are not completely turned off and that induction is a process of turning these genes on more fully while turning fetal hemoglobin genes off. Contrasted with this process is the process of switching from embryonic hemoglobin to fetal hemoglobin, which seems to involve a switch from one clone of cells producing embryonic hemoglobin (non-definitive) to a second clone of cells producing fetal and adult type hemoglobins.

I have presented data on the time period involved in the dynamic change from embryonic to fetal hemoglobin; however, more thorough knowledge of the structural relationships of embryonic hemoglobin to the other hemoglobins is necessary in order to understand its function as a respiratory protein.

Electrophoretic data indicate the presence of three components of embryonic hemoglobin at very early stages which resolve into two components (one much more prominent than the other). The major

component at 29 days' gestation was isolated by isoelectric focusing on polyacrylamide gel and found to contain no cross reacting antigenic sites with fetal hemoglobin, whereas adult sheep hemoglobin and many other adult ruminant hemoglobines did cross react with fetal hemoglobin when tested with an antiserum directed against fetal hemoglobin. This lack of similarity between embryonic hemoglobin and fetal and adult sheep hemoglobin was borne out in dansyl-peptide maps.

Embryonic hemoglobin did not display the typical alpha chain peptide spots; although some spots were analogous, most alpha chain spots were lacking. Also the non-alpha chain spots showed substantial variation from both fetal and adult A hemoglobin. The spots analogous to neither alpha chain nor non-alpha chain of fetal or adult hemoglobins may be either peptides from epsilon chains or peptides from an entirely new alpha type chain which would be electrophoretically indistinguishable from fetal and adult type alpha chain. Further studies will need to determine more definitively the peptide composition of separated embryonic hemoglobin subunits, since electrophoretic patterns of whole hemoglobin following hybridization may not detect some structural heterogeneities. Though samples are severely limited in amount, this problem may be alleviated if it can be shown that bovine embryonic hemoglobin has a close homology with sheep by dansyl-peptide mapping. Bovine embryos at comparable stages in development are substantially larger. The homology between fetal hemoglobins of cattle and sheep detected by specific antisera to fetal sheep hemoglobin leads me to suspect that a great degree

of homology exists between embryonic hemoglobin of cattle and sheep.

It appears that the degree of homology between fetal hemoglobin of cattle and sheep determined by comparison of amino acid sequences corresponds well with the degree of homology determined by specific antisera. Reactivity of specific antisera seems to be a valid means of detecting homology in hemoglobin structure and is a highly valuable technique in the absence of definitive sequence data.

REFERENCES

REFERENCES

1. Kleihauer, Enno and Stoffler, G. 1968. Embryonic Hemoglobin of Different Animal Species. Molecular and General Genetics. 101:59.

2. Szelenyi, Judit, G. and Hollan, S. R. 1969. Studies on the Structure of Human Embryonic Haemoglobin. Acta Biochimica et Biophysica Academia. Scientiarum Hungaricae. 4:47.

3. Huehns, E. R. and Shooter, E. M. 1965. Human Hemoglobins. Journal of Medical Genetics. 2:48.

4. Huisman, T. H. J., Lewis, J. P., Blunt, M. H., Adams, H. R., Miller, A., Dozy, A. M., and Boyd, E. M. 1969. Hemoglobin C in Newborn Sheep and Goats: A Possible Explanation of Its Function and Biosynthesis. Pediatrics Research. 3:189.

5. Kitchen, H., Eaton, J. W., and Taylor, W. J. 1968. Rapid Production of a Hemoglobin by Induced Hemolysis in Sheep: Hemoglobin C. American Journal of Veterinary Research. 29:2.

6. Boyer, Samuel H., Crosby, E. F., and Noyes, A. N. 1968. Hemoglobin Switching in Non-Anemic Sheep. I. Mediation by Plasma from Anemic Animals. Johns Hopkins Medical Journal. 123:85.

7. Leyko, W., and Gondko, R. 1963. Separation of Hemoglobin and Cytochrome C by Electrophoresis and by Chromatography on DEAE-Sephadex. Biochemica et Biophysica Acta. 77:500.

8. Smithies, O. 1959. An Improved Procedure for Starch-Gel Electrophoresis: Further Variations in the Serum Proteins of Normal Individuals. Biochemical Journal. 71:585.

9. Kitchen, Hyram, Easley, C. W., Putnam, F. W., and Taylor, W. J. 1968. Structural Comparison of Polymorphic Hemoglobins of Deer with Those of Sheep and Other Species. The Journal of Biological Chemistry. 243:6:1204.

10. Marti, H. R., Beale, D., and Lehmann. Haemoglobin Koelliker: A New Acquired Haemoglobin Appearing after Severe Haemolysis: $\alpha_2^{\text{minus 141 arg}}\beta_2$. Acta Haematologica. 37:174.

11. Fantoni, Antonio, Bank, A., and Marks, P. A. 1967. Globin Composition and Synthesis of Hemoglobins in Developing Fetal Mice Erythroid Cells. Science. 157:1327.

12. Schalekamp, M., Schalekamp, M., Van Goor, D., and Slingerland, R. 1972. Re-evaluation of the Presnce of Multiple Haemoglobins During the Ontogenesis of the Chicken. Journal of Embryology and Experimental Morphology. 28:3:681.

13. New, D. A. T., and Brett, R. L. 1972. Effect of Yolk-Sac Antibody on Rat Embryos Grown in Culture. Journal of Embryology and Experimental Morphology. 27:3:543.

14. Folk, J. E. 1970. Carboxypeptidase B (Porcine Pancreas) Chapter 32, *Methods in Enzymology*. Academic Press, New York and London.

15. Folk, J. E., Pietz, K. A., Carroll, W. R., and Gladner, J. A. 1960. Carboxypeptidase B: Purification and Characterization of the Porchie Enzyme. Journal of Biological Chemistry. 235:2272.

16. Atherton, R. S., and Thomson, A. R. 1969. "Fingerprinting" of 1-Dimethylaminonaphthalene-5-sulphonyl-Labelled Protein Digests. Biochemistry Journal. 111:797-98.

17. Babin, D. R., Schroeder, W. A., Shelton, J. R., Shelton, J. B. and Robberson, B. 1966. The Amino Acid Sequence of the Chain of Bovine Fetal Hemoglobin. Biochemistry. 5:1297-1310.

18. Boussios, T., and Bertles, J. F. Ontogeny of Hamster Hemoglobins Determined by Isoelectric Focusing in Polyacrylamide Gel. In Press.

19. Drysdale, J. W., Righetti, P., and Bunn, H. F. 1971. The Separation of Human and Animal Hemoglobin by Isoelectric Focusing in Polyacrylamide Gel. Biochimica et Biophysica Acta. 229:42-50.

20. Dayhoff, M. 1972. *Atlas of Protein Sequence and Structure*, Vol. 5. National Biomedical Research Foundation.

21. Easley, C. W., Godwin, B. P., and Kitchen, H. 196 . Characterization of Peptide Subgroups Obtained by Gel Filtration. Automation in Analytical Chem., Technicon Symposia I, p. 427, Mediad, Inc., White Plains, NY.

22. Fantoni, A., De La Chapelle, A., and Marks, P. A. 1969. Synthesis of Embryonic Hemoglobins During Erythroid Cell Development in Fetal Mice. Journal of Biological Chemistry. 244:675-81.

23. Gray, W. R. 1967. Dansyl Chloride Procedure. *Methods in Enzymology*, Vol. XI, page 139-151. C. H. W. Hirs (ed.). Academic Press, New York and London.

24. Guidotti, G., Konigsberg, W., and Craig, L. C. 1963. Proceedings of the National Academy of Science. 50:774.

25. Gurtner, G. H., and Burns, B. 1972. Possible Facilitated Transport of Oxygen Across the Placenta. Nature. 240:473-75.

26. Huehns, E. R., Dance, N., Beaver, G. H., Hecht, F., and Motulsky, A. G. 1964a. Human Embryonic Hemoglobins. Cold Spring Harbor Symposium on Quantitative Biology. 29:327.

27. Kabat, E. A. 1968. *Structural Concepts in Immunology and Immunochemistry*. Holt, Rinehart and Winston, Inc.

28. McIlwaine, I., Rodbard, D., and Chranbach, A. 1973. Characterization by Polyacrylamide Gel Electrophoresis of Hemoglobins in Rabbit Embryo, Fetus, and Adult. Analytical Biochemistry. 55:521-38.

29. Perutz, M. F. 1970. Stereochemistry of Cooperative Effects in Hemoglobin. Nature. 228:726-39.

30. Perutz, M. F., and Lehmann, H. 1968. Molecular Pathology of Human Hemoglobin. Nature. 219:902-909.

31. Righetti, Piergiorgia and Drysdale, J. W. 1971. Isoelectric Focusing in Polyacrylamide Gels. Biochimica et Biophysica Acta. 236:17-28.

32. Schmer, G., and Kreil, G. 1967. Fingerprints of DNS-Labeled Protein Digests on a Millimicromole Scale. Journal of Chromatography. 28:458-61.

33. Schmer, G., and Kreil, G. 1967. Micro Method for Detection of Formyl and Acetyl Groups in Proteins. Analytical Biochemistry. 28:458-61.

34. Spivak, V. A., Levjant, M. I., Katrukha, S. P., and Varshavsky, J. M. 1971. Quantitative Ultramicroanalysis of Amino Acids in the Form of Their DNS-Derivatives. Analytical Biochemistry. 44:503-18.

35. Gros, C., and Labouesse, B. 1969. Study of the Dansylation Reaction of Amino Acids, Peptides, and Proteins. European Journal of Biochemistry. 7:463-70.

36. Zanetta, J. P., Vincendon, G., Mandel, P., and Gombos, G. 1970. The Utilization of 1-Dimethylaminonaphthalene-5-Sulphonyl Chloride for Quantitative Determination of Free Amino Acids and Partial Analysis of Primary Structure of Proteins. Journal of Chromatography. 51:441-458.

37. Kazazian, H. H. Jr., and Woodhead, A. P. 1973. Hemoglobin A Synthesis in the Developing Fetus. New England Journal of Medicine. 289:58-62.

38. Huehns, E. R., Dance, N., Beaven, G. H., Hecht, F., and Motulsky, A. G. 1964. Human Embryonic Hemoglobins. Symposium on Quantitative Biology. 29:327.

39. Chamley, J. H., and Holland, R. A. B. 1969. Some Respiratory Properties of Sheep Hemoglobins A, B, and C. Respiration Physiology. 7:287-94.

40. Balani, A. S., Ranjekar, P. K., and Barnabas, J. 1968. Structural Basis for Genetic Heterogeneity in Hemoglobins of Adult and New Born Ruminants. Comparative Biochemical Physiology. 24:809-815.

41. Huisman, T. H. J., and Kitchens, J. 1968. Oxygen Equilibria Studies of the Hemoglobins from Normal and Anemic Sheep and Goats. American Journal of Physiology. 215:140-146.

42. Baldy, M., Gaskill, P., and Kabat, D. 1972. Expression of the Silent Hemoglobin Gene in Sheep. Studies of the Globin Messenger Ribonucleic Acids. Journal of Biological Chemistry. 247:6665-6670.

43. Longmuir, I. S., in Microcirculatory Approaches to Current Therapeutic Problems. Symposia, Sixth European Conference on Microcirculation, Aalborg, 1970, 3 Karger, Basel, 1971.

44. Riggs, A. 1965. Hemoglobin Polymerization in Mice. Science. 147:631.

42a. Barker, J. E., Last, J. A., Adams, S. L., Nienhuis, A. W., and Anderson, W. F. 1973. Hemoglobin Switching in Sheep and Goats: Erythropoietin-Dependent Synthesis of Hemoglobin C in Goat Bone-Marrow Cultures. Proceedings of the National Academy of Science U.S.A. 70:1739-43.

45. Huisman, T. H. J., Schillhorn Van Veen, J. M., Dozy, A. M., and Nechtman, C. M. 1964. Studies on Animal Hemoglobins. II. The Influence of Inorganic Phosphate on the Physico-Chemical and Physiological Properties of the Hemoglobin of the Adult Chicken. Biochimica et Biophysica Acta. 88:352-366.

46. Kabat, E. A., and Mayer, M. M. 1967. In *Experimental Immunochemistry*, page 343-345. Charles C. Thomas, Springfield, Illinois.

47. Ouchterlony, O. 1968. *Handbook of Immunodiffusion and Immunoelectrophoresis*. Ann Arbor Science Publishers, Inc.

48. Metcalf, D., and Moore, M. A. S. 1971. *Haemopoietic Cells*. North-Holland Publishing Co., Amsterdam.

49. Fink, H. (consulting ed.), Whipple, H. E. (ed.). 1964. *Problems of Cooley's Anemia*. Annals of the New York Academy of Sciences, Vol. 119, art. 2, pp. 369-850.

50. Capp, G. O., Rigas, D. A., and Jones, R. T. 1967. Hemoglobin Portland 1: A New Human Hemoglobin Unique in Structure. Science. 157:65.

51. Goodman, M., and Peters, H. (ed.). 1964. The Specificity of Proteins and the Process of Primate Evolution. *Protides of the Biological Fluids*. Elsevier Publishing Co., Amsterdam.

52. Boyer, S. H., Boyer, M. L., Noyes, A. N., and Belding, T. K. Immunological Basis for Detection of Sickle Cell Hemoglobin Phenotypes in Amniotic Fluid Erythrocytes. To be published by the New York Academy of Sciences in the Proceedings of the Conference on Hemoglobins: Comparative Molecular Biology - Models for the Study of Disease (November, 1973). To be published 1974.

53. Kazazian, H. Adult Hemoglobin Synthesis in the Developing Human Fetus. To be published by the New York Academy of Sciences in The Proceedings of the Conference on Hemoglobins: Comparative Molecular Biology - Models for the Study of Disease (November, 1973). To be published 1974.

54. Clegg, J. B. 1968. Separation of the Alpha and Beta Chains of Human Haemoglobin. Nature. 219:69-70.