Hemoglobin patterns were examined in human fetuses in order to determine the onset of synthesis of adult hemoglobin in fetal red cells. In fetuses younger than 11 gestational wk, hemoglobin A could not be detected with electrophoretic techniques. A small amount of adult hemoglobin was constantly present, however, in hemolysates from fetuses older than 11 gestational wk, and it had the structural properties of adult hemoglobin A.

Heterozygous fetuses had hemoglobins C and A or S and A as early as the 11th and 13th gestational wk. The findings are compatible with onset of beta hemoglobin gene action as early as the 11th gestational wk and synthesis of approximately 5% of adult hemoglobin by the end of the first and throughout the second trimester of gestation.

Knowledge of the time of onset of synthesis of hemoglobin A in fetal red cells, in addition to its biological interest, is of practical importance. Mutations affecting the beta hemoglobin genes, i.e., Hb S, Hb C, Hb E, and the beta thalassemias are frequent in several populations. If the beta hemoglobin genes direct synthesis of adult hemoglobins early in fetal life, fetuses homozygous for beta hemoglobin mutants will contain abnormal hemoglobin fractions in a proportion of their red cells. If techniques for obtaining fetal red cells without a risk to the fetus are developed, antenatal diagnosis of certain hemoglobinopathies could be possible by a demonstration of abnormal, instead of normal adult hemoglobin in the red cells of homozygous fetuses.

However, the time of onset of synthesis of beta chains and consequently of hemoglobin A in fetal red cells is uncertain. It is true that in embryos and young fetuses a hemoglobin with electrophoretic migration similar to that of adult hemoglobin A is observed. Its amount sometimes exceeds 10% of the total hemoglobin. However, the identity of this fraction with Hb A has not been established. On the contrary, evidence has been presented that this A-like hemoglobin of embryos and young fetuses migrates like a fetal hemoglobin on citrate agar gels. It has been indicated that this putative Hb A in fetuses is not Hb A but rather the embryonic hemoglobin Portland-159 and
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that synthesis of Hb A before the 12th or 15th gestational wk is doubtful.4,10

The purpose of this investigation was to examine the hemoglobin patterns in embryos and fetuses of various gestational ages in order to determine the time of appearance of adult Hb A in fetal red cells.

MATERIALS AND METHODS

Embryos and fetuses were collected in the Central Laboratory for Human Embryology, University of Washington, Seattle. The methodology used for collection and classification of conceptual wastage in this laboratory has been described.11,12 For our study 78 intact embryos and fetuses, collected between 1966 and 1971, were used. Blood samples were obtained after a cardiac puncture and, in older fetuses, also by a cannulation of the umbilical vessel. Fetal age was assessed from developmental graphs relating crown-rump (C-R) length to gestational age.11,13

The red cells were washed in isotonic saline at 4°C, lysed with hypotonic shock, and clear hemolysates were prepared after centrifugation at 10,000 rpm for 20 min in 4°C. Hemoglobin electrophoresis was done with horizontal starch gels for 16-18 hr at 4°C using a Tris-EDTA-borate (TEB) buffer system at pH 8.6. Electrophoresis on horizontal agar gels (concentration of agar 1.0 g/100 ml, citrate buffer at pH 6.0) was done in 29 cm x 14 cm x 4 mm agar plates at 4°C for 12-16 hr or in 8 cm x 5 cm x 2 mm agar plates at room temperature for 2 hr. The hemoglobins in starch gel electrophoretic preparations were localized with benzidine and amido black staining solutions. The agar gels were stained with a buffered aqueous benzidine solution for 1-2 mm, were thoroughly washed, and counterstained with amido black solution. The proportion of hemoglobin fractions in these electrophoretic preparations was estimated by visual comparison with artificial mixtures of pure hemoglobins A and F.

Chromatographic separations of hemoglobin fractions were done using Carboxymethyl (CM)-Sephadex (C-50) 0.8 x 10 cm or 1.2 x 40 cm columns with a 0.05 M Tris-maleic buffer (0.001 M in KCN) and a pH gradient from 6.3 to 7.5.14 The chromatographic fractions were concentrated by vacuum dialysis in the cold and were identified by agar gel and starch gel electrophoresis. Globin was prepared with acid acetone precipitation,15 and chains were separated in a CM-cellulose (Whatman CM-23) 1.0 x 10.0 cm column.16 Electrophoretic separation of globin chains using 8 M urea starch gel electrophoresis was done with the method of Chernoff and Pettit.17 After tryptic digestion16 of the isolated chains, peptide maps18 were prepared by subjecting 1.5-mg portions of the tryptic digest to paper electrophoresis using a pyridine-acetic acid-water buffer (10:0.4:90, by volume) at pH 6.4, followed by paper chromatography in a solution of pyridine-isooamyl alcohol-water (35:35:30, by volume). Peptide maps were stained with 0.3% ninhydrin in acetone. Specific amino acid residues were located by spraying the peptide maps with Ehrlich (tryptophan), iodoplatinate (sulfur-containing amino acids), Sukaguchi (arginine), and Pauly (histidine) reagents.19

RESULTS

"Hemoglobin A" between 5th and 11th Gestational Wk

Hemoglobin patterns were examined in 15 embryos and fetuses with C-R lengths 20-75 mm. Nine of these were embryos smaller than 55 mm. In their hemolysates, no hemoglobin fraction was observed on the position occupied by Hb A on agar gel electrophoresis. A hemoglobin A-like fraction was, however, consistently observed in electrophoresis on alkaline starch gels. Its approximate amount was estimated by inspection of stained gels and ranged from 5% (Fig. 1A) to 20% (Fig. 1B) of the total hemoglobin. The electrophoretic behavior of this fraction resembled that of Hb Portland-1 in two
Fig. 1. (A) Starch gel electrophoresis in a Tris-EDTA-borate system (pH 8.6), benzidine stain. Line 1, hemolysate from a 31 mm (54 gestational days) human embryo; line 2, hemolysate of an adult. (B) Starch gel electrophoresis in TEB system (pH 8.6), benzidine stain. Line 1, adult control; line 2, hemolysate from 29 mm (52 gestational days) human embryo.

respects. On starch gel electrophoresis at alkaline pH, its migration rate was slightly faster than that of hemoglobin A (Fig. 1), while on citrate agar gel it displayed a mobility characteristic of a fetal hemoglobin. In these embryos an additional hemoglobin fraction was observed on starch gels. It had a migration rate intermediate between hemoglobins Gower-i and F (fraction X of Fig. 2). Its quantity varied considerably, from traces (Fig. 1) up to quantities almost equal to that of Hb Portland-i (Fig. 2, position 2).

In hemolysates from six fetuses 55–75 mm long, with starch gel electrophoresis “hemoglobin A” was barely visible (Fig. 3, position 2). With agar gel electrophoresis, either a very faint stain or no staining was seen in the position expected to be occupied by Hb A.

Fig. 2. Hemoglobin patterns in human embryos. Line 1, hemolysate from a 37 mm (56 gestational days) embryo; line 2, hemolysate from a 41 mm (58 gestational days) embryo. Starch gel electrophoresis in TEB system (pH 8.6), benzidine stain.
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Fig. 3. Starch gel electrophoresis in TEB system (pH 8.6), benzidine stain. Lines 1 and 4, hemolysate from adult controls; line 2, hemolysate from 69 mm (75 gestational days) fetus; line 3, hemolysate from 81 mm (81 gestational days) fetus; line 5, hemolysate from 126 mm (104 gestational days) fetus.

Hemoglobin A After 11th Gestational Wk

Sixty-three fetuses were older than 11 gestational wk. Their distribution in various age groups and findings concerning presence of Hb A in their hemolysates appear in Table 1.

In all the samples from fetuses aged 80 gestational days and older, hemoglobin A appeared as a distinct electrophoretic zone (Fig. 3, positions 3 and 5). On alkaline starch gels, its electrophoretic migration was identical to that of hemoglobin A. In the hemolysates of these fetuses, a fraction occupying the position expected for adult hemoglobin A was seen with electrophoresis on citrate agar gels (Fig. 4). Identity of this electrophoretic fraction to adult hemoglobin A was indicated from chromatographic and structural findings.

Chromatographic patterns were studied in 18 fetuses ranging in length from 81 to 199 mm. CM-Sephadex chromatography with a pH gradient 6.5–7.5 was used because it permits a wide separation of the hemoglobins that migrate ahead of Hb F on starch gel electrophoresis. Thus, in this chromatographic system, hemoglobins Portland-1 and Bart's are eluted as a single fraction with a pH peak of 6.58, while the Hb A fraction is eluted after Hb F0. A typical chromatographic pattern under the conditions applied in the study of fetuses is depicted in Fig. 5. The fraction of fetal hemolysates that was eluted at the position expected for Hb A had a migration rate identical to that of Hb A on starch gel and on citrate agar gel electrophoresis. Its proportion ranged from 4 to 10% with some age-dependent variation. Electrophoresis of the chromatographically isolated hemoglobin A on urea gels indicated that it consisted

<table>
<thead>
<tr>
<th>Gestational Age (days)</th>
<th>Crown-Rump Length (mm)</th>
<th>No. of Fetuses Examined</th>
<th>Hb A</th>
</tr>
</thead>
<tbody>
<tr>
<td>40–77</td>
<td>20–75</td>
<td>15</td>
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</tr>
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<td>80–90</td>
<td>80–100</td>
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<td>121–140</td>
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<tr>
<td>123–150</td>
<td>161–199</td>
<td>18</td>
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of alpha and beta chains. Peptide maps of the nonaminoethylated beta chains contained all the peptides recognized in a beta chain from adult individuals (Fig. 6).

DISCUSSION

The findings of the present study support the suggestion\textsuperscript{4,10} that in embryos and small fetuses hemoglobin A is absent. Instead, the hemoglobin fraction previously\textsuperscript{1-3} considered to be adult most probably is the embryonic hemoglobin Portland-1. These conclusions are based on the following considerations. First, no hemoglobin fraction occupies the position of Mb A on citrate agar electrophoresis of samples from fetuses younger than 11 gestational wk. Second, an A-like hemoglobin is present in hemolysates from embryos, but it has the electrophoretic characteristics of Hb Portland-1 (hemoglobin $\gamma\zeta$). Third, the quantity of this hemoglobin decreases with growing of the fetuses, and it virtually disappears after the 8th gestational wk. Fourth, in embryos, a hemoglobin X is observed that has the electrophoretic behavior of the tetramer $\alpha_2\xi$.\textsuperscript{7,9,20} Considered together, these findings are compatible with synthesis of zeta rather than beta chains in embryonic erythroid cells.
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Fig. 6. Peptide maps of nonaminoethylated beta chain from (a) normal adult, and (b) isolated from Hb A of fetuses. Identified peptides are numbered. All peptides expected for nonaminoethylated beta chain are present in fingerprint of fetal sample.

In contrast to the findings in embryos and young fetuses, electrophoretic, chromatographic, and structural evidence indicates that hemoglobin A is present in the red cells of fetuses older than 11 gestational wk. Thus, a fraction with an electrophoretic migration identical to that of Hb A is always observed with starch gel electrophoresis of hemolysates of fetuses older than 11 gestational wk. This fraction displays the electrophoretic behavior of Hb A on citrate agar gels. It has the chromatographic behavior expected for an adult hemoglobin. It consists of alpha and nonalpha chains, and, finally, it has nonalpha chains with a fingerprinting pattern identical to that of beta chains isolated from normal adults. Observations in 63 fetuses permit the conclusion that the normal beta hemoglobin genes are active at the end of the first and throughout the second trimester of fetal life.

The observations in normal fetuses mark the probable onset of action of the mutant beta chain genes. There is no priori reason to suggest that the regulatory mechanism that is responsible for the initiation of action of the beta hemoglobin genes does not turn on a $\beta^S$ or a $\beta^C$ hemoglobin gene. One expects that $\beta^S$ and $\beta^C$ genes will express at the same period with $\beta^A$ genes and that, after the 80th gestational day, heterozygotes and homozygotes for these genes will manifest with production of their abnormal hemoglobin. In support are the following electrophoretic and genetic observations.

Fetuses at the 13th, 15th, 16th, and 19th gestational wk were found to have small amounts of hemoglobin A and of a hemoglobin fraction that, on agar gel electrophoresis, displayed the electrophoretic migration of Hb S. Structural studies with this hemoglobin were not done, so the possibility that it was a fetal hemoglobin variant cannot be formally excluded. Genetic evidence, however, indicates that it is hemoglobin S. One of these fetuses with small amounts of hemoglobins A and S was an obligatory heterozygote for the Hb S gene; his
mother was a homozygote for sickle cell anemia. In the sample of another fetus whose mother was a double heterozygote for Hb S and Hb C, hemoglobins S and A were detected on agar gel electrophoresis (Fig. 7). In a third fetus, examined at the 80th gestational day, approximately 2% of the hemoglobin had the electrophoretic behavior of hemoglobin C. Examination of the mother of this fetus disclosed that she was a Hb C heterozygote.

Demonstration of Hb A in a fetal hemolysate does not necessarily indicate synthesis of adult hemoglobin in the fetal erythroid cells. Adult hemoglobins will also be found in the hemolysate of the fetus when maternal red cells contaminate the fetal blood. This possibility can be excluded as an explanation of our findings for several reasons. Intact fetuses were studied, and fetal blood samples were obtained after a cardiac puncture. With such methodology for collection of fetal blood, a contamination by maternal red cells is unlikely. If a contamination by maternal blood had systematically occurred, the presence of Hb A in fetal hemolysates should not correlate with the age of the fetus; Hb A should have been detected randomly in fetuses of older, as well as of younger, age groups. In our study, Hb A was observed only in one group of fetuses, those who were older than 11 gestational wk. Furthermore, contamination will more likely be random regarding quantities of the maternal blood infused into the fetal blood. Since the blood volume of these fetuses is small, with a small variation in the quantity of the maternal red cells entering the

![Fig. 7. Agar gel electrophoresis in citrate buffer pH 6.0. (1a and 1b) Hemolysate of 67 mm (73 days) fetus. (2a and 2b) Hemolysate of 120 mm (100 days) fetus whose mother was doubly heterozygous for hemoglobins S and C. Note that Hbs S and A and not C are present in hemolysate of fetus. (3a) Artificial mixture of Hbs F, S, and C. (3b) Artificial mixture of hemoglobins F, A, and S.](#)
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fetal blood, striking differences in the quantities of Hb A in the hemolysates of the fetuses will be found. On the contrary, all the 63 fetuses aged more than 11 gestational wk had similarly low quantities of adult hemoglobins in their hemolysate. Against contamination by maternal blood argue also the findings in certain fetuses with abnormal hemoglobin. When the mother of the fetus has a distinctive hemoglobin pattern, a contamination of the fetal blood by maternal blood should result in an identical hemoglobin pattern in the hemolysate of the fetus. Thus, when the mother of the fetus is a sickle cell disease homozygote, in the hemolysate of the fetus hemoglobins F and S but no hemoglobin A will be found. When the mother is a Hb S/Hb C double heterozygote, in the fetal hemolysate hemoglobins F, S, and C and no Hb A must be found. As it was already indicated (Fig. 7), the actual findings in such cases were that the offspring of homozygous or doubly heterozygous mothers had almost equal small amounts of hemoglobins A and S, i.e., it had the distinctive hemoglobin pattern of a sickle cell trait heterozygote.

Our findings permit the conclusion that normal and abnormal beta hemoglobin genes do operate at a period during which procedures aiming at a prenatal diagnosis of genetic disorders are performed. If a harmless technique for obtaining fetal red cells were developed, a prenatal diagnosis of certain hemoglobin abnormalities like sickle cell anemia could become possible by an electrophoretic or immunochemical demonstration of presence of Hb S and absence of Hb A, either in single cells or in hemolysates of suspect fetuses.

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Hemoglobins in Human Fetuses: Evidence for Adult Hemoglobin Production After the 11th Gestational Week

Haris A. Pataryas and George Stamatoyannopoulos