Comparative Biochemistry of Hemoglobins VII. On the N-terminal Structures of Two Main Components in Fetal Bovine Hemoglobin

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Citation

Issue Date
1966-10-25

URL
http://hdl.handle.net/10069/17429
Comparative Biochemistry of Hemoglobins

VII. On the N-terminal Structures of Two Main Components in Fetal Bovine Hemoglobin*

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Received for Publication August 4, 1966

The subunits and N-terminal structures of the protein moieties of the two main components in fetal bovine hemoglobin which were named HbF₀ and HbF₁ were studied in the following ways, and then the results were compared with those from adult bovine hemoglobin. First of all, the two main components in the hemoglobin isolated from the blood of seven-day-old newborn bovine were confirmed using cellulose-acetate membrane electrophoresis, and were separated by CM-cellulose column chromatography. From these, F₀ globin and F₁ globin were obtained and A globin were prepared from adult bovine hemoglobin. These three kinds of globins were comparatively examined by urea dissociation cellulose-acetate membrane electrophoresis. Each globin was observed to migrate dissociating into two kinds of subunits. One subunit of every globin, supposed to correspond to α-chain and migrating faster toward the cathode, showed the same migration rate. On the other hand, the other subunit supposed to correspond to β-chain or γ-chain and migrating more slowly, showed the different migration rate respectively. Moreover, each globin was dinitrophenylated and hydrolyzed with 6 N HCl for 6 hrs, 12 hrs, and 24 hrs respectively. DNP-amino acids and DNP-peptides extracted from these hydrolysates with ether were separated from each other, identified by paper chromatography and determined quantitatively. The restults of these experiments prove that both of F₀ globin and F₁ globin consist of four polypeptide chains, that is, two polypeptide chains (α-chains) with Val-Leu N-termini and two polypeptide chains (γ-chains) with methionine N-termini. Concerning N-termini, it is just like adult bovine globin. It is considered that like the case of human hemoglobin, the two main components of fetal bovine hemoglobin are respectively composed of two α-chains equivalent to adult bovine's α-chain and two γ-chains different from adult bovine's β-chain. Moreover, it is considered that γ-chains both components of fetal bovine hemoglobin are different from each other, however, bovine hemoglobin is
different from human hemoglobin in point of N-terminal structure; the N-terminal structure of the two components of fetal bovine hemoglobin are quite similar to that of adult bovine hemoglobin. Therefore, the difference between β-chain of adult and γ-chain of fetal component is presumed to be in their inner amino acid sequences in the polypeptides.

In 1886, Körber discovered, in fetal human blood, the hemoglobin which has extremely stronger resistance to alkali than the hemoglobin in adult human blood. Since then a lot of researches concerning these two hemoglobins have been made and the functional difference between them was recognized. It was also reported that the replacement of fetal human hemoglobin to adult human hemoglobin already begins before birth and ends within about four months after birth. Moreover according to the determination of the primary structure of adult human hemoglobin by Braunizer et al. and Konigsberg et al., and also according to the successive determination of the primary structure of fetal human hemoglobin by Schröder et al., the structural difference between them was confirmed.

It is well-known that many fetal mammals have different type of hemoglobins from their adult hemoglobins as in case of man. This phenomenon arouses our interest not merely because it is observed very often in animal kingdom but because it permits us to recognize distinctly the alternation of biosynthetic ability of protein at the molecular level as one of the differentiation phenomena in the process of ontogeny of living things. The research work on this phenomenon using human hemoglobin, however, seems to have many difficulties and also from the standpoint of comparative biochemistry of hemoglobin and comparative embryology, it is indispensable to study the replacement of other fetal animals' hemoglobin to their adult one, to research into the structural difference between both, and consequently to compare them with human fetal and adult hemoglobin. Nevertheless, very few reports on the primary structure of hemoglobin of other fetal mammals except man have been presented.

Concerning fetal bovine hemoglobin, Brinkman and Jonxis first suggested its existence through their study on alkali resistance of hemoglobin. Later, Wyman reported that there is a distinct difference between fetal and adult bovine hemoglobin, recognizing that the former has higher solubility than the latter. Furthermore, Cabannes and Serain, Barak, and Monnier and Fischer recognized the difference between both by electrophoresis. Fuisman et al. also reported that hemoglobin in blood of fetal bovine is isolated into one major and three minor components by CM-cellulose column chromatography and that the behaviours of these components on chromatogram are all different from those of adult bovine. In our laboratory, we have been making comparative biochemical studies on various kinds of hemoglobins.
Recently, as a part of these studies, Mutai studied on newborn Holstein bovine hemoglobin using the methods of electrophoresis, CM-cellulose column chromatography, and alkali denaturation. He reported the existence of the two kinds of major and five kinds of minor components which are different from those of adult bovine hemoglobin. Furthermore, he investigated the quantitative change of these components and adult bovine components of fetal bovine hemoglobin in the process of time after birth. As a result of this investigation, he reported as follows; directly after birth, blood almost consists of hemoglobin whose group of components are specific to newborn bovine but little adult bovine hemoglobin. As days go by after birth, those components peculiar to newborn bovine decrease, to the contrary, adult bovine components increase. After about a month adult bovine components almost replace the fetal bovine components. The sum of these investigations allows us to consider that these components peculiar to fetal bovine play a leading part of respiration in the fetus.

As the first step to the study on the primary structure of fetal bovine hemoglobin, the present author isolated hemoglobin from the blood of seven-day-old newborn Holstein bovine which was considered to preserve sufficient fetal components; next, the existence of the two main fetal components was confirmed by electrophoresis and separated from each other with CM-cellulose column chromatography. Then, as for the protein moiety of these components, the structures of their subunits were investigated by urea dissociation electrophoresis, and the analysis of N-terminal structure was carried out by the DNP method.

MATERIALS AND METHODS

1) Preparation of Hemoglobin Solution

By the jugular vein puncture of the Holstein adult and seven-day-old newborn bovine, the venous bloods were obtained. The hemoglobin solution of each blood was prepared by Drabkin's method; that is, from each blood which was added sodium citrate as anticoagulant, red blood cells were isolated by centrifugation at 2,000 r.p.m. for 2 min at 0° to 5°C, washed with 0.9 % NaCl solution three times, and centrifuged again in order to remove plasma. Then this red blood cells were hemolyzed with 2 volumes of deionized water and 0.4 volume of toluene. The hemolysate was separated into three layers by centrifugation at 1,500 r.p.m. for 60 min at 0° to 5°C. From this middle layer the desired hemoglobin solution was prepared.

2) Cellulose-acetate Membrane Electrophoresis of Hemoglobin

Electrophoresis of each hemoglobin solution was performed by Kohn's method modified by Mutai; that is, it was carried out at
the constant current of 0.4 mA/cm for 60 min at room temperature on cellulose-acetate membrane, Oxoid (manuf. at Oxo Co., Eng.) which was cut into 1 x 5 cm strips. The buffer used was Tris-EDTA-boric acid solution, pH 8.0 [6.05 g of Tris-(hydroxymethyl)-aminomethane + 6.00 g of ethylenediamino tetraacetate 2 Na + 4.60 g of boric acid + deionized water to be 1 L solution]. Then it was stained with Ponceau 3 R (0.4 % Ponceau 3 R 3 % TCA solution) for 30 sec and excessive dye was removed with 5 % acetic acid.

3) Separation of Components of Fetal Bovine Hemoglobin (F₀ and F₈)

The method of CM-cellulose column chromatography used by MUTA was modified its scale so as to treat larger amount of material at a time. The procedure was as follows: CM-cellulose (0.7 meq/g, manuf. at Serva Co.) was thoroughly washed, in order, with acetone, deionized water, 1 N aqueous ammonia, deionized water, 1 N hydrochloric acid, and deionized water. Moreover it was washed with 0.01 M sodium phosphate buffer pH 6.4 (containing 0.01 % KCN), and then, packed into 2 x 65 cm chromatographic column up to the height of 50 cm and equilibrated thoroughly with the buffer. Against 2 L of the buffer used previously, 10 ml of hemoglobin solution (containing 490 mg of hemoglobin) which had been dialyzed for a whole day, was added to the column. Into the mixing chamber, 1 L of the buffer used previously was put, and also into the supplying bottle 1 L of 0.01 M sodium phosphate buffer pH 7.0 (containing 0.01 % KCN) was poured. Gradient elution was carried out at the flow rate of 1 ml/min. After the buffer in the supplying bottle had flowed over, 2 L of 0.01 M sodium phosphate buffer pH 8.5 (containing 0.01 % KCN) was added and gradient elution was continued. Chromatography described above was carried out at 3°C to 4°C in the low temperature room. The effluent from the column was collected in 10 ml fractions and its extinction at 415 μm was measured by Spectronic 20 (manuf. at Bausch & Lamb Co.). The portions of the peaks of the main components of fetal bovine hemoglobin, HbF₀ and HbF₈, which were named by MUTA, were collected respectively, dialyzed for a whole day with deionized water, and finally lyophilized.

4) Preparation of Globins

According to ANSON and MIRSKY'S method, 15 ml of adult bovine hemoglobin solution prepared previously, HbF₀ solution, and HbF₈ solution, were subjected to dehemeing; HbF₀ and HbF₈ solution were made by dissolving 150 mg of HbF₀ and HbF₈ respectively into 15 ml of deionized water. Into the mixed solutions of 200 ml of acetone and 6 ml of concentrated HCl, which were being stirred and cooled at -20°C, hemoglobin solutions mentioned above were respectively added drop by drop. After each mixture was stirred for 20 min more, the globin was isolated by centrifugation, washed several times with cold
acetone. The isolated globin was dissolved in deionized water, and finally lyophilized to prepare A, F\textsubscript{A} and F\textsubscript{B} globin.

5) *Urea Dissociation Cellulose-acetate Membrane Electrophoresis of Globins*

Each globin prepared previously was subjected to urea dissociation electrophoresis by Kobayashi's method\textsuperscript{13} using cellulose-acetate membrane. After small portion of each globin dissolved into small portion of 8 M urea solution was allowed to stand for 30 to 60 min at room temperature, it was subjected to electrophoresis on 1 x 5 cm strip of cellulose-acetate membrane at the constant current of 0.4 mA/cm for 40 min, using veronal buffers of various pH (ionic strength: 0.045) which contained 6 M urea. The buffers were prepared by the method of Holt and Gaede\textsuperscript{10}; that is, 2.21 g of sodium 5,5-diethylbarbiturate and 1.17 g of sodium acetate were both dissolved into deionized water; pH of this solution was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 with acetic acid respectively. Deionized water was again added to each buffer to be 500 cc solution. To these buffers, urea was added so that their concentration were 6 M. After finishing electrophoresis, like the case of electrophoresis of hemoglobins, the protein moiety on the membrane was stained with Ponceau 3R for 30 sec and washed with 5% acetic acid in order to remove excessive dye.

6) *Analysis of N-terminal Amino acids and Peptides*

The following experiments were performed under fluorescent lamps in the dark room.

a) Preparation of DNP globins

According to the method of Rhinesmith et al\textsuperscript{30}, F\textsubscript{A} globin and F\textsubscript{B} globin were dinitrophenylated; that is, 100 mg of each globin was dissolved into 20 ml of deionized water; this solution was adjusted to pH 9.0 with 0.1 N NaOH and was added 25 mg of Na\textsubscript{2}CO\textsubscript{3} and 0.25 ml of DNFB. Then it was stirred for 3 hrs and adjusted to pH 2.0 with 0.1 N HCl. Furthermore, it was subjected to centrifugation at 12,000 r. p.m. for 20 min at 1° to 5°C and removed its supernatant. After the residue of DNP globin was washed by centrifugation with 0.1 N HCl three times, and once with acetone, it was put on the glass filter, washed again with acetone and ether respectively, and finally dried in vacuum desiccator.

b) Hydrolysis of DNP globins

In case of the two components of fetal bovine hemoglobin, the molecular weights of DNP globins were both assumed to be almost equal to that of fetal or adult human hemoglobin. So, the experiments and calculations were carried out regarding 8.8 mg of DNP globin as 0.1 \( \mu \)M. 2.0 ml of constant boiling HCl was added to 8.8 mg (0.1 \( \mu \)M) of each DNP globin. Hydrolysis was performed in a sealed tube at 110°C for 6 hrs and 12 hrs respectively.
c) Extraction of DNP amino acids and DNP peptides

After hydrolysis described previously, each hydrolysate was diluted to 8.0 ml of deionized water and transferred quantitatively to separating funnel. DNP derivatives in each hydrolysate were extracted four times with 10.0 ml of ether. The combined extracts were washed four times with 4.0 ml of 0.5 N HCl and the washings were also extracted with 10 ml of ether. This ether layer combined with the ether extract solution prepared previously was transferred into the flask of Mills' apparatus and evaporated to dryness under reduced pressure.

d) Removal of Dinitrophenol

The ether extract prepared previously was dissolved in small portions of acetone, and was evaporated in a current of warm air from a hair dryer in the flask of Mills' apparatus that was revolved in order to spread DNP-derivatives on its inner surface as a thin layer. The cold finger condenser of Mills' apparatus loaded with crushed solid carbon dioxide was fitted to the flask, which was warmed in a water bath at 55°C and evacuated by oil pump. After 20 min the pump was stopped; cold finger condenser was unfixed from the flask; dinitrophenol which adhered by sublimation to the cold finger condenser was wiped with absorbent cotton damped with acetone. The procedure mentioned above was repeated four times.

e) Paper Chromatography of DNP amino acids and DNP peptides

All the residue in Mills' flask diluted to small portion of acetone was quantitatively transferred on Toyo filter paper (No. 51, 40 x 40cm), drying between addition in a current of warm air from a hair dryer. The spot was placed 5 cm from both sides of the paper and this part of paper where the residue was placed was exposed to ammonium vapor. According to the method of Koh and Wiedel, chromatography was carried out; the first dimension was developed with the upper layer of the mixture of n-Butanol and 0.1 % aqueous ammonia (1:1); the second dimension was developed with 1.5 M phosphate buffer, pH 6.0 (1 M of NaH₂PO₄ + 0.5 M of Na₂HPO₄ + deionized water to make up 1 L solution). After the filter paper spotted the sample was allowed to stand at 25°C for 4 hrs in the vapor of the solvent for the development of the first dimension, the first dimension was developed by ascending procedure at 25°C for about 20 hrs. Afterward the filter paper was dried in a current warm air at 40°C for 3 hrs. The second dimension was developed by ascending procedure using 1.5 M phosphate buffer at 25°C for about 15 hrs, and the filter paper was dried at 40°C for 2 hrs.

f) Identification of DNP amino acids and DNP peptides

After the ether extract free from dinitrophenol was added the authentic sample of DNP valine, DNP methionine, and DNP valylleucine
respectively, each mixture was subjected to paper chromatography. As the spots were completely accorded with each other, the identification of DNP valine, DNP methionine, and DNP valylleucine was performed. The authentic samples of DNP valine and DNP methionine manufactured by Wako Pure Chemical Industries were used here. The authentic sample of DNP valylleucine, however, was obtained from adult human hemoglobin; that is, dinitrophenylated adult human hemoglobin was hydrolyzed with 6 N HCl for 1 hr, and the ether extracts of this hydrolysate were subjected to chromatography using silica gel-celite column which was devised by Matsuda et al22). Consequently DNP valylleucine was isolated and refined.

\[ \text{g) Quantitative Determination of DNP amino acids and DNP peptides} \]

Each yellow spot of DNP valine, DNP methionine, and DNP valylleucine identified on the paper chromatogram was cut 2 to 3 mm beyond its visible periphery; each blank paper of similar size was also cut from the adjacent part of the filter paper for the contrast. Each paper strip was put into 5.0 ml of deionized water in a test tube, which was placed in a water bath at 55°C for 15 min. In order to allow complete elution of yellow color, the test tube was shaken every five min. Furthermore, after it was allowed to stand at 25°C for 15 min, its extinction at 360 mp was measured by spectrophotometer (HITACHI EP-2). By the difference between the extinction of each DNP derivative and that of each blank paper, the quantity of each DNP derivative was determined by Levy's method18) in which millimol extinction coefficient was assumed to be 15.6/F. In this case, according to Koh and Weidell14), F value of DNP valine was determined to be 1.00; that of DNP methionine, 1.21. F value of DNP valylleucine was also determined to be 1.00 like DNP valine.

\[ \text{h) Correction for Losses of DNP amino acids during Experimental Treatment} \]

The losses of DNP amino acids during the procedures such as hydrolysis, extraction, paper chromatography, and so forth were corrected by the method of Frankel-Conrat et al8), and the method of Iwai and Fujikaka12); here, the quantity of DNP amino acids which was determined quantitatively during the procedures of only DNP globin (0.1 \( \mu \text{M} \)) is termed \( a \mu \text{M} \); \( b \mu \text{M} \) is the quantity of DNP amino acid which was determined quantitatively during the procedures of the mixtures of DNP globin (0.1 \( \mu \text{M} \)) and 0.1 \( \mu \text{M} \) of pure DNP amino acid (DNP valine or DNP methionine) under the same condition as the above-mentioned experiment of DNP globin only. So, the number of N-terminal amino acid residues per 1 mol of DNP globin, \( N \), (mol) was calculated according to the following formula,
\[ N = \frac{a}{b - a} \]

The correction for losses of DNP valylleucine during the procedures was not especially performed. In addition, experiments of quantitative determination described previously were repeated several times and the mean quantity of several experiments conformable within 10% was used in the calculation.

RESULTS AND DISCUSSION

Fig. 1 shows the results of cellulose-acetate membrane electrophoresis of adult and seven-day-old newborn bovine hemoglobin solution. As shown in the figure, only one component (HbA) can be recognized in adult bovine hemoglobin. Consequently, A globin was prepared not by more isolation and refinement, but only by deheming, and was used in the experiment hereafter.

In newborn bovine hemoglobin solution, as the figure shows, three components are found: of these, one component which exists less than others has the similar migration rate to that of adult bovine hemoglobin (HbA), therefore, it was decided to be HbA. Moreover, judging from the fact that the others which exist more than the one cannot be found in adult bovine hemoglobin solution at all, and referring to MUTA'S report\(^{27}\), they were also considered to be the components peculiar to fetal bovine hemoglobin.

The two components were separated by CM-cellulose column chromatography; MUTA'S chromatography column was too small to treat...
larger portion of the materials, so the present author modified its
diameter larger. As Fig. 2 shows, the results of the chromatography
was almost same as MUTA’s. However, the separation of $F_1$-group was performed better than in
MUTA’s experiment. It may depend on the larger diameter of the
column. $F_n$ and $F_3$ overlaps each other. Each component is named
according to MUTA’s report.

Table 1. Percentage of the different hemoglobin components
in seven-day-old newborn bovine blood

<table>
<thead>
<tr>
<th>Hb components</th>
<th>V</th>
<th>$F_1^A$</th>
<th>$F_1^B$</th>
<th>$F_1^C$</th>
<th>$F_1^D$</th>
<th>$F_2$</th>
<th>$F_3^B$</th>
<th>$A_1^A$</th>
<th>$A_1^B$</th>
<th>$A_0$</th>
<th>$F_{III}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>0.06</td>
<td>0.25</td>
<td>2.49</td>
<td>6.78</td>
<td>27.17</td>
<td>2.46</td>
<td>44.0</td>
<td>0.79</td>
<td>1.22</td>
<td>12.81</td>
<td>1.10</td>
</tr>
</tbody>
</table>

In the Table 1, the relative quantities of the separated components
are given. These quantities almost agree with the results of MUTA’s
report, however, the value of the minor component of $F$-group is
generally smaller and those of the major components are generally
larger than the correspondents of Mutai's report. Especially $F_{a}^{A}$ has nearly 10% larger quantity. This fact suggests that there are differences among individual bovines as for the relative quantity of major and minor components of fetal hemoglobin. From the two main components ($Hb F_{a}^{A}$ and $Hb F_{a}^{B}$) which were separated by chromatography, $F_{a}^{A}$ globin and $F_{a}^{B}$ globin were prepared by deheming and used in the experiment hereafter.

![Fig. 3](image)

Fig. 3 Urea dissociation cellulose-acetate membrane electrophoresis of adult and fetal bovine globins (6M urea veronal buffers pH 8.5, $\mu=0.045$, 0.4 mA/cm., 40 min.)

Fig. 3 shows the result of urea dissociation cellulose-acetate membrane electrophoresis of the protein moieties of adult and fetal bovine hemoglobin, $A$, $F_{a}^{A}$ and $F_{a}^{B}$ globin. As shown in the figure, each globin migrates dissociating into two subunits. Take39), by urea dissociation filter paper electrophoresis, recognized that adult bovine globin migrates dissociating into two subunits. Furthermore, Sasakawa31) reported that one subunit which migrates faster toward the cathode is $\alpha$-chain with Val-Leu N-terminus and the other which migrates slower toward the cathode is $\beta$-chain with Met N-terminus. The relative electrophoretic behaviours both on the filter paper and on cellulose-acetate membrane are considered to be similar, therefore, the present author decides that one subunit which moves faster to the cathode corresponds to $\alpha$-chain and the other which moves slower corresponds to $\beta$-chain likewise on cellulose-acetate membrane electrophoresis.

The two main components of fetal bovine globin are expected to have two kinds of subunits respectively like $A$ globin, because they migrate dissociating into two subunits. Of these subunits, one which moves faster toward the cathode has the same migration rate as $\alpha$-chain of $A$ globin, on the other hand, the other has the different rate from that of $\beta$-chain of $A$ globin. Likewise, slight difference can be found
even between the migration rates of slower subunits of both fetal components. Further, by urea dissociation electrophoresis using buffers whose pH varied from pH 6.0 to pH 9.0, the migration distance of the subunits of Fα globin and Fβ globin was compared with that of the subunits of A globin. As Fig. 4 shows, the faster subunits of fetal components have similar migration distance to that of α-chain of adult component at every kind of pH. On the other hand, the slower subunits have migration distance close to, but a little different from that of β-chain of A globin. And also slight difference can be found between the migration distances of slower subunits of both fetal components.

Above-mentioned facts suggest that both components of fetal bovine hemoglobin have two kinds of subunits. One is similar to α-chain of adult bovine hemoglobin, the other different from β-chain. In case of man, adult hemoglobin consists of α-chain and β-chain. Fetal one has same α-chain with adult one, but γ-chain instead of β-chain. In the same way, fetal bovine hemoglobin may be assumed to have α-chain
similar to that of adult hemoglobin, the other subunit different from β-chain of adult bovine hemoglobin, which can be called γ-chain like the correspondent of human hemoglobin. It is more interesting that the following can be presumed. α-chains of the two main components of fetal bovine hemoglobin are similar, but γ-chains are different from each other. γ-chain of human fetal hemoglobin and β-chain of human adult hemoglobin are different from each other originally on the stage of N-terminal amino acid; namely, β-chain has valine as N-terminal amino acid, but γ-chain has glycine. The number of amino acid residues which composes the peptide chain, β-chain or γ-chain, is 146. Of these, the exchange of amino acids can be recognized in 39 parts\(^{34}\). Furthermore, it is known that in fetal human hemoglobin, the minor component (Hb F\(_0\)) exists besides Hb F (or called Hb F\(_\alpha\))\(^{13}\). In this minor component, N-terminus of one of the two γ-chains is acetylated\(^{35}\).

In case of bovine hemoglobin, the same differences as in case of human hemoglobin can be expected between β-chain of adult hemoglobin and γ-chain of fetal one, and also between γ-chains of the two main components of fetal hemoglobin, judging from the result of urea dissociation electrophoresis. The analysis of N-terminal structure of adult bovine hemoglobin was already made by Porter and Sanger\(^ {29}\), and Osaawa and Satake\(^ {28}\). They confirmed that per 1 mol of hemoglobin, 2 mols of valylleucine (α) and 2 mols of methionine (β) exist. Here, the analysis of N-terminal structure of the two main components of fetal bovine hemoglobin (Hb F\(_\alpha\) & Hb F\(_\beta\)) was performed by the method of dinitrophenylation. Fig. 5 is the paper chromatogram of ether extracts from DNP-F\(_\alpha\) globin and DNP-F\(_\beta\) globin which were hydrolyzed for 12

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![Paper chromatograms of ether extracts from hydrolysates of DNP-F\(_\alpha\) and DNP-F\(_\beta\) globin (6 N HCl, 110°C, 12 hrs.)](image)
hrs. Both Fδ and Fβ globin on paper chromatogram always showed almost same pattern. Both main components which were hydrolyzed for 6 hrs and 24 hrs respectively were also subjected to paper chromatography, in which DNP valine, DNP valylleucine, DNP methionine, DNP methionine sulfon, and DNA are always found in the same way shown in Fig. 5. DNP valylleucine was produced by incomplete hydrolysis. DNP methionine sulfon was a by-product which yielded from DNP methionine during HCl hydrolysis. It is known that DNA was almost always produced as a by-product when analysis of N-terminal structure of the protein is performed by DNP method, and that it is not to relate N-terminal structure. Therefore, from above-mentioned facts, it is expected that both components of fetal bovine hemoglobin have at least two kinds of N-termini; one is of valylleucine and the other, of methionine.

Table 2. Numbers of N-terminal residues in the two main components of bovine fetal hemoglobin

<table>
<thead>
<tr>
<th>Hydrolysis time (hr)</th>
<th>DNPcomp.</th>
<th>DNP-Val</th>
<th>DNP-Val-Leu</th>
<th>DNP-Met</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Fδ</td>
<td>0.81</td>
<td>1.29</td>
<td>1.05</td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>Fβ</td>
<td>0.87</td>
<td>1.27</td>
<td>1.12</td>
<td>3.26</td>
</tr>
<tr>
<td>12</td>
<td>Fδ</td>
<td>1.31</td>
<td>0.65</td>
<td>1.35</td>
<td>3.31</td>
</tr>
<tr>
<td></td>
<td>Fβ</td>
<td>1.24</td>
<td>0.63</td>
<td>1.51</td>
<td>3.38</td>
</tr>
<tr>
<td>24</td>
<td>Fδ</td>
<td>1.63</td>
<td>0.14</td>
<td>1.62</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td>Fβ</td>
<td>1.74</td>
<td>0.14</td>
<td>1.78</td>
<td>3.65</td>
</tr>
</tbody>
</table>

for a detailed explanation, see text

DNP valine, DNP valylleucine, and DNP methionine were determined quantitatively. DNP valine and DNP methionine were corrected their losses during the procedures. They were converted into mol number per 1 mol of the globin (66,000 g) and the result can be seen in Table 2. It indicates that N-terminal structures of the two main components of fetal bovine hemoglobin respectively consist of 2 mols of valylleucine and 2 mols of methionine per 1 mol of hemoglobin exactly like adult bovine hemoglobin; that is, it is considered that both components of fetal bovine hemoglobin are composed of two polypeptide chains with Val-Leu N-termini which assumed to be a-chain, and two polypeptide chains with methionine N-termini which assumed to be γ-chain.

On the primary structure of fetal bovine hemoglobin, the results of
the experiments described previously suggest as follows: First, from
the result of urea dissociation electrophoresis, it is presumed that like
human hemoglobin, α-chain of fetal bovine hemoglobin is similar to
the correspondent of adult hemoglobin, and that ρ-chain is dissimilar
to β-chain of adult one. It is also presumed that even between two
main components of fetal bovine hemoglobin, α-chains are same but
γ-chains are different from each other. It is considered that this fact
corresponds with the relation between the major component HbF and
the minor HbFt of fetal human hemoglobin. In case of human hemog-
lobin, however, as described previously, the difference is recognized
not only in the inner amino acid sequences but also between N-terminus
of β-chain of adult hemoglobin and the correspondent of γ-chain of fetal
hemoglobin.

In case of bovine hemoglobin, the difference between fetal and adult
hemoglobin is not recognized in N-terminal amino acids. Therefore,
it is surmised that the difference between them may be found in the
inner amino acid sequences. Furthermore, the relation between the
two components of fetal bovine hemoglobin resembles the relation
between the major component (Hb F) and the minor component (Hb Ft)
in point of the similarity of α-chains, however, they are quite different in
point of γ-chains. The difference between γ-chains of fetal human
hemoglobin can be found only in their N-termini, however, γ-chain of
fetal bovine hemoglobin cannot be recognized to be different in their
N-termini. Therefore, it is presumed that the difference between them
can be found in the inner amino acid sequences in their polypeptide
chains.

Satake and Sasakawa already reported concerning the primary
partial structure of adult bovine hemoglobin. Further studies on fetal
bovine hemoglobin compared with their results on adult bovine
hemoglobin will much contribute to the explanation of the relation
between the structure and function of these two hemoglobins.

CONCLUSION

Hemoglobin was isolated from newborn Holstein bovine blood. By
the method of cellulose-acetate membrane electrophoresis, the existence
of the two main components (Hb Fα and Hb Fγ) was recognized in fetal
bovine hemoglobin, and were separated by CM-cellulose column chroma-
tography.

The protein moieties of Hb Fα and Hb Fγ were respectively subjected
to urea dissociation cellulose-acetate membrane electrophoresis in order
to investigate their subunits. It was confirmed that both components
had two kinds of subunits respectively. By comparing these subunits
with those of adult bovine hemoglobin (α-chain and β-chain), it was
recognized that both hemoglobins were common in one subunit (α-chain) but different in the other (β-chain and γ-chain). Moreover, it was recognized the similarity of α-chain of HbF₀ and HbF₉, and the difference of γ-chains of these two components.

Next, analysis of N-terminal structures of HbF₀ and HbF₉ was performed by DNP method to prove that 1 mol of fetal bovine hemoglobin has 2 mols of valylleucine and 2 mols of methionine as N-terminal amino acids just like adult bovine hemoglobin. This fact demonstrates that like adult bovine hemoglobin, the two main components of fetal bovine hemoglobin are respectively composed of four polypeptide chains, that is, two polypeptide chains with Val-Leu N-termini (α-chains) and two polypeptide chains with Met N-termini (non α-chains, γ-chains). Of these chains, α-chains are the same with the correspondent of adult bovine hemoglobin and N-termini of γ-chains of both components are methionine like the correspondent of β-chain of adult bovine hemoglobin. But there will be difference between β-chain and γ-chain in the inner amino acid sequences of their peptide chains. Likewise, even between γ-chains of the two main components of fetal bovine hemoglobin, there will be difference in the inner amino acid sequences in their peptide chains.

ACKNOWLEDGEMENT

The author wishes to express his cordial gratitude to Prof. Dr. G. Matsuda and Assist. Prof. Dr. T. Maekawa who gave him constant and useful advice and encouragement during this work and also to Miss S. Arakawa who helped him preparing this manuscript.

* This report was presented at the 1st Chugoku, Shikoku Kyushu Branch Meeting of the Japanese Biochemical Society on May 29th, 1966 in Yonago.

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