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Comparative Biochemistry of Hemoglobins

III. On the Difference between *Macaca mulatta* Monkey Hemoglobin and Human Hemoglobin

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To compare *Macaca mulatta* monkey hemoglobin with human hemoglobin, these two varieties of hemoglobin were examined by means of agar-gel electrophoresis, cellulose acetate membrane electrophoresis, column chromatography on CM-cellulose, and the alkali denaturation method. In addition, these hemoglobins were compared by the fingerprinting method on their tryptic digests. As the results, a clear, though slight, difference was observed between these two kinds of hemoglobin.

In relation to the problem of evolution of the hemoglobin molecule, comparative studies are being made in our laboratory on the hemoglobins from various species of animals. On the other hand, human hemoglobin has been studied in detail, and the primary structure of the α-polypeptide chain of the globin moiety from human adult hemoglobin has been determined by BRAUNITZER et al. and KONIGSBERG et al., that of the β-polypeptide chain by BRAUNITZER et al., and that of the γ-polypeptide chain from human fetal hemoglobin by SCHROEDER et al. The present author, taking interest in monkey hemoglobin, chose as the experimental material the hemoglobin of *Macaca mulatta* monkeys which are usually employed for medical researches. One of the interests in the structure of monkey hemoglobin may be how closely it resembles human hemoglobin in the standpoint of comparative biochemistry. In regard to this point, SCHAPIRA and KRUIJ149 have first confirmed that monkey hemoglobin bears a resemblance to human hemoglobin immunologically. Later, CABANNES and SERAIN and RODNAN and EBAUGH, by using paper chromatography, and FINE et al., by using agar-gel electrophoresis at high pH, have reported a strong resemblance between these hemoglobins. Then CABANNES and
SERAIN\textsuperscript{33} have reported that there is some difference between monkey and human hemoglobins in the solubility curve in various solvents, but to the contrary, ITANO\textsuperscript{89} and BEAVEN and GRATZER\textsuperscript{13} have obtained a result which throws doubt on the difference. MATSUDA and MAITA\textsuperscript{122}, on the other hand, examined the N-terminal amino acid sequences of \textit{Macaca mulatta} monkey hemoglobin and recognized that it, like human hemoglobin, consists of two polypeptide chains with Val-Leu N-terminal residues and two polypeptide chains with Val-His-Leu N-terminal residues. And ZUCKERKANDL et al.\textsuperscript{17} have recognized some difference between human and \textit{Macaca mulatta} monkey hemoglobins comparing the fingerprints of their tryptic digests. But later MASIAR\textsuperscript{114}, also comparing the fingerprints of these hemoglobins, could not find any difference in the tryptic digests. Therefore, in order to inquire into the existence of the difference between \textit{Macaca mulatta} monkey and human hemoglobins, the author performed experiments by means of agar-gel electrophoresis, cellulose acetate membrane electrophoresis, column chromatography on CM-cellulose, the alkali denaturation method, and fingerprinting method as follows:

**MATERIALS AND METHODS**

1) \textit{Isolation of Hemoglobin}

The blood samples were obtained from human adults and \textit{Macaca mulatta} monkey adults. The blood was drawn out of the elbow vein of the human adults and the abdominal aorta of the \textit{Macaca mulatta} monkeys under ether anesthesia, and hemoglobin was prepared by a modified method of DRABKIN\textsuperscript{43} as follows: After addition of 1/5 volume of anticoagulant 3.8% sodium citrate to the blood sample, the blood sample was centrifuged at 1,500 r. p. m. for 20 min. in a cold room below 4°C. The supernatant plasma was discarded and the red blood cells were washed three times with physiological saline solution. After addition of an equal volume of deionized water and 0.4 volume of toluene to the red cells for hemolysis, the mixture was violently shaken and allowed to stand overnight in the cold room. The hemolysate was subsequently centrifuged at 12,000 r. p. m. for 60 min. at a low temperature, and the middle layer, the hemoglobin solution, was taken out and dialyzed against deionized water overnight in the cold room. The concentration of the hemoglobin solution was measured using the spectrophotometer (HITACHI EPU-2), and the solution was kept at -20°C in the refrigerator.

2) \textit{Agar-gel Electrophoresis}

Agar-gel electrophoresis of the hemoglobin solution was carried out according to Shibata's method.\textsuperscript{167} Tris-EDTA-borate buffers at pH 8.6 ([Tris (hydroxymethyl) aminomethane 15.0 g, ethylenediamine-tetra-
acetic acid 2.3 g, and boric acid 3.0 g were dissolved in deionized water to make 1 L] and at pH 7.2 ([Tris (hydroxymethyl) aminomethane 12.3 g, ethylenediaminetetraacetic acid 4.8 g and boric acid 6.7 g were dissolved in deionized water to make 1 L] was used for the electrophoresis, being diluted to three volumes immediately before use.

Bacto-agar (Difco) was added to the buffer at a concentration of 0.8% and the suspension was heated till it got perfectly gelled. Some agar-gel was poured into a petri dish to make a flat horizontal. After the coagulation of the agar-gel, some slide glasses were placed on it and above them some more agar-gel was poured so that it was about 3 mm thick. After standing overnight in the cold room, the upper agar-gel was taken out together with the slide glass being cut along the brim of the slide glass and was used for the later experiment. A 2x4 mm piece of filter paper was soaked in hemoglobin solution and buried at 1 cm from the edge of the agar-gel plate. With the agar-gel plate down and the slide glass up, the plate was placed bridging over across the central compartment of the apparatus. Setting the anode on the side near the origin, and the cathode on the other side, electrophoresis was performed at 100V for 80 min. at pH 7.2 and at 200V for 40 min. at pH 8.6 by the constant voltage in the cold room.

3) Cellulose Acetate Membrane Electrophoresis

Cellulose acetate membrane electrophoresis of monkey and human hemoglobins was carried out by a modified method of Kohn's. As the buffers for electrophoresis, Tris-EDTA-borate buffers at pH 8.6 and at pH 7.2 (the same as the buffers used for agar-gel electrophoresis) were used.

The cellulose acetate membrane used was OXOID (manufactured at OXO Co., England) and was cut into 3x5 cm strips. A strip with hemoglobin on it were subjected to electrophoresis at a constant electric current of 0.4 mA/cm at a room temperature for 60 min, and stained with Ponceau 3R.

4) Column Chromatography

One hundred grams of carboxymethyl (abbreviated to CM hereafter)-cellulose (manufactured at Brown Co.) were washed sufficiently with water, and suspended in water. Then little particles were removed by decantation as much as possible. The CM-cellulose was suspended in 1 N NH₄OH solution for 30 min, put on Buchner's funnel for filtration and washed with 5-10 L of deionized water. After complete removal of NH₄OH, the CM-cellulose was suspended in 1 N HCl solution and was also washed with water through Buchner's funnel. This treatment was repeated once more and after the last thorough washing, the CM-cellulose was suspended in 200 ml. of M/5 phosphate buffer (pH 6.9) and filtered through Buchner's funnel in vacuum. After washing with
4-5 L of M/100 phosphate buffer (pH 6.9), the CM-cellulose was packed into a 1x38 cm column and equilibrated by passing through about 5 L of M/100 phosphate buffer (pH 6.9) in a cold room. On the other hand, hemoglobin (80-120 mg), which had been dialyzed against M/100 phosphate buffer, was applied on the column and was eluted by the concentration gradient with 150 ml of M/100 phosphate buffer (pH 6.9) in the mixing bottle being supplied with M/5 phosphate buffer (pH 6.9). With a flow rate of 5 ml/hr, 1.0 ml fractions were collected by the automatic fraction collector, and the Extinction was measured at 415 m\(\mu\) and 541 m\(\mu\) by the spectrophotometer (HITACHI EPU-2). The whole operation of this column chromatography was performed in the cold room.

5) Alkali Denaturation Method

Each hemoglobin solution was diluted using the spectronic 20 (manufactured at Shimadzu Co.) so that it might have an extinction of about 0.7 at 415 m\(\mu\). A 3 ml portion of this solution was mixed with 1 ml of deionized water and the extinction of the mixture (Eo) was measured. On the other hand, to each 3 ml portion of this hemoglobin solution, 1 ml of phosphate buffer at pH 12.9 (65.3 g of Na\(_2\)HPO\(_4\)12H\(_2\)O was dissolved in 400 ml of deionized water and the solution was adjusted to pH 12.9 with 1 N NaOH solution) was added, and after that the extinction (E) was measured every minute for 15 min. Then, this solution was allowed to stand in a water bath at 37°C for 15 min, and the extinction of the solution at this time (Ee) was measured. Thus, the percentage (P) of the denatured hemoglobin solution was obtained by the following equation:

\[
P = \frac{Eo - E}{Eo - Ee} \times 100(\%)
\]

6) Tryptic Digestion of Hemoglobin

Tryptic digestion was carried out according to INGRAM’s method. A 20 ml portion of about 2.2% hemoglobin solution was adjusted to pH 8.0 with 2 N NaOH solution, and denatured in a water bath at 90°C for 3 min. Then, the hemoglobin solution became white and muddy and yielded some precipitate. Subsequently, the solution was cooled immediately, moved to a reaction bottle which was kept at 38°C and stirred sufficiently with an electric stirrer. To this was added 1 ml of 0.001 N HCl solution in which trypsin (SIGMA Chemical Co., twice crystallized) was dissolved so as to be 0.5%. Adding 0.1 N NH\(_4\)OH to keep pH 8.0 during the progress of digestion, the digestion was continued for 90 min. After the digestion, the pH of the solution was adjusted to pH 6.4 by the addition of 1 N HCl, and the "cores" which were insoluble at this pH were precipitated. After that the solution was centrifuged at 3,000 r.p.m. for 30 min and the supernatant was lyophilized and preserved.
7) Fingerprinting

The water-soluble parts of the tryptic digests of the globin were subjected to fingerprinting by a modified method of Ingram’s⁷⁷, and examined comparatively. Filter paper (TOYO filter paper, No. 51) was cut into a form as shown in Fig. 1. The origin was set at a point 34 cm distant from the end of the cathode side and 3 cm distant from the end of the base, and the sample was applied to this point. The apparatus used for high-voltage filter-paper electrophoresis was the ISHIDAI FE 105-B type, and the buffer used was pyridine-acetic acid-water (100:4:900), pH 6.4. First, the electrophoretic part of the filter paper was soaked in the buffer, and after the removal of excess of the buffer on it with another large sheet of filter paper, the sampled filter paper was attached to the frame of the apparatus. Subsequently, the solution of the tryptic digest was applied to the origin which was previously marked. Then, the filter paper with the frame was placed in the electrophoretic box full of hexane previously cooled below 10°C with ice, and was electrified at 5 KV for 30 min. After the electrification, the paper was dried with hot air and subjected to paper chromatography with pyridine-n-butanol-acetic acid-water (30:45:9:36) as the developer in the vertical direction to the electrophoresis. The paper was taken out after 18 hr and dried with hot air, and various color reactions were tested on the filter paper.

8) Color Reactions on the Filter Paper

a) Ninhydrin reaction
An acetone solution containing 2% ninhydrin was prepared, and in this solution the filter paper which had been fingerprinted was soaked and heated with an iron to produce colors.

b) Pauli’s reaction

A solution composed of 9 g of sulfanilic acid and 1 L of water containing 90 ml of concentrated hydrochloric acid was mixed with 5% sodium nitrite solution at the rate of 2 to 1, and to the mixture, 20% NaOH solution was added at the rate of 1 to 3. This agent was sprayed upon the chromatographed paper to reveal reddish orange spots.

c) α-Nitroso-β-naphthol reaction

The chromatographed paper was sprayed with 90% ethanol solution containing 0.1% α-nitroso-β-naphthol, and after drying with hot air, was sprayed again with 10% nitric acid solution. The chromatographed paper was dried by heating at 100°C to give brownish red spots.

d) Ehrlich reaction

The agent was prepared by dissolving 1 g of p-dimethylaminobenzaldehyde in 90 ml of acetone and adding 10 ml of concentrated hydrochloric acid to the solution. When it was sprayed upon the chromatographed paper, blue purple spots were observed.

e) Sakaguchi’s reaction

The chromatographed paper was sprayed with an acetone solution containing 0.1% 8-hydroxyquinoline, and after drying, with 0.5 N NaOH solution containing 0.2% bromine. Red spots were detected.

f) Reaction by the iodoplatinic acid agent

The agent was prepared mixing 4 ml of 0.002 M chloroplatinic acid solution, 0.25 ml of 1 M KI solution, 0.4 ml of 2 N HCl and 76 ml of acetone immediately before use. The chromatographed paper was dipped in this agent, and when it was dried, white spots were observed on a reddish purple background.

RESULTS AND DISCUSSION

The comparison between Macaca mulatta monkey and human hemoglobins was shown in Fig. 2 by the results of electrophoresis on agar-gel and cellulose acetate membrane. In agar-gel electrophoresis at pH 7.2, as represented in this figure, a slight difference was recognized between monkey and human hemoglobins in the mobility of the main components, and a clear difference was observed in the mobility and the quantity of the minor component. In electrophoresis at pH 8.6, however, any perceivable difference could not be observed between them in both the cases of the main and minor components. In cellulose acetate membrane electrophoresis, the main components of monkey
and human hemoglobins presented no remarkable difference at pH 7.2 and pH 8.6 alike. The minor components, however, gave a clear difference in the mobility at pH 8.6. Judging from the fact that some difference could be observed between these two hemoglobins in the major and minor components by agar-gel electrophoresis at pH 7.2 and cellulose acetate membrane electrophoresis at pH 8.6, it can be considered that there is some difference in the structure of these hemoglobin components. Subsequently, Fig. 3 gives the comparison of results of column chromatography on CM-cellulose of Macaca mulatta monkey and human hemoglobins. Concerning column chromatography of human hemoglobin on CM-cellulose, HUISMAN and MEYERING have given a detailed report, but no report has been published yet on Macaca mulatta monkey hemoglobin.

At the present experiment, human hemoglobin gave a peak of the major component at about 52 ml and peaks of the preceding two minor components at about 23 ml and 33 ml, respectively. In contrast with
this, *Macaca mulatta* monkey hemoglobin, though it gave a peak of the major component at about 52 ml like human hemoglobin, was eluted with its two minor components at about 22 ml and about 27 ml, a little earlier than two minor components of human hemoglobin. From this result, some difference is thought to be existent between the structures of the two minor components of human hemoglobin and those of *Macaca mulatta* monkey hemoglobin. In Fig. 4, the comparisons between the hemoglobins from *Macaca mulatta* monkey and human adults, and between the hemoglobins from *Macaca mulatta* monkey fetuses and human cord blood were shown by the results of the alkali denaturation method. The alkali denaturation of adult and fetal hemoglobin from *Macaca*
mulatta monkey was reported by Beaven and Gratzer\textsuperscript{13}, and they recognized that both the adult and fetal types resembled human adult and fetal hemoglobins, respectively, with the fetal type having a strong resistance to alkali. At the present experiment also, any significant difference could not be observed between the adult types of monkey and human hemoglobins and between the fetal types of those hemoglobins. This close resemblance in alkali denaturation between Macaca mulatta monkey and human hemoglobins may be well considered to suggest a high similarity in the structures of these hemoglobins. Next, fingertints of the tryptic digests from Macaca mulatta monkey and human hemoglobins, reacting on the ninhydrin agent, are shown in Fig. 5 and Fig. 6, respectively. These fingerprints were also examined by several specific reactions besides the ninhydrin reaction, such as the Pauli reaction, $\alpha$-nitroso-$\beta$-napthol reaction, Ehrlich reaction, Sakaguchi’s reaction and reaction on the iodoplatinic acid agent, which proved the existence of the residues of histidine, tyrosine, tryptophan, arginine, methionine and some others. The results are given in Fig. 7 and Fig. 8. Fig. 7 is for
Fig. 5  Fingerprint of Tryptic Peptides from Macaca mulatta Monkey Hemoglobin. (Ninhydrin reaction)
Fig. 6  Fingerprint of Triptic Peptides from Human Hemoglobin (Ninhydrin reaction)
Macaca mulatta monkey hemoglobin and Fig. 8 for human hemoglobin. The numbers in Fig. 5 and Fig. 6 represent INGRAM's peptide numbers. What strikes us at a glance of these fingerprints is a great resemblance between these tryptic peptides obtained from Macaca mulatta monkey hemoglobin and from human hemoglobin. This fact may suggest that the protein structures of these two hemoglobins have a close similarity to each other like the cases of the experiments by means of electrophoresis, column chromatography, and the alkali denaturation test above mentioned. However, a close observation comparing these individual peptides indicates some differences between them. For example, some change is recognized between the fingerprints shown in Fig. 5 and 6 in the interrelationship of the so-called neutral peptides No. 3, 4, 5 and 9. It
is probably due to a movement of peptide No. 5 of *Macaca mulatta* monkey hemoglobin slightly toward the cathode compared with that of human hemoglobin. This peptide No. 5 is a tyrosine-containing peptide and clearly distinguishable from the other three, No. 3 and No. 4 containing histidine, and No. 9 containing histidine and methionine. Besides, the relationships of peptides No. 12 and 13, more distance in *Macaca mulatta* monkey hemoglobin than in human hemoglobin, also suggest the difference between these two hemoglobins. Peptide No. 14 could not be found with the ninhydrin reaction in both the cases of the hemoglobins, but clearly recognized with the specific reaction on arginine, tryptophan and tyrosine residues. In addition, peptide No. 17
though it appears in the same position in the two hemoglobins, presents itself more remarkably with the ninhydrin reaction in the case of *Macaca mulatta* monkey hemoglobin than in the case of human hemoglobin. Therefore, the spot of peptide No. 17 on the fingerprinted paper was cut out of the paper and extracted, and the amino acids in the extract were analyzed with an automatic amino acid analyzer (HITACHI KLA-2). As the results, human adult hemoglobin presented 1 mole each of threonine, aspartic acid, valine and lysine, and *Macaca mulatta* monkey adult hemoglobin 1 mole each of serine, aspartic acid, valine and lysine. As the amino acid sequence of peptide No. 17 obtained from human hemoglobin is known to be Thr-Asp-Val-Lys, *Macaca mulatta* monkey hemoglobin can be considered to have a sequence of Ser-Asp-Val-Lys. Another peptide No. 11, though it appears in the same position in the two hemoglobins like peptide No. 17, gives a light-colored spot in the case of *Macaca mulatta* monkey hemoglobin and a deep-colored spot in human hemoglobin contrary to peptide No. 17. No. 11 from human hemoglobin has been known to be a peptide containing two kinds of tryptic peptides produced with an incomplete hydrolysis by trypsin, and to have peptide No. 17 at the C-terminal. Therefore, the differences in the relative rates of peptide No. 11 and peptide No. 17 from human and *Macaca mulatta* monkey hemoglobin which are shown in Fig. 5 and Fig. 6 can be thought to be due to the change which the rate of tryptic hydrolysis suffered because of the interchange of threonine and serine at the N-terminal of peptide No. 17. As stated before, ZUCKERKANDL et al. recognized some differences between the fingerprints of the tryptic peptides from human adult and from *Macaca mulatta* monkey adult hemoglobin, while contrary to them MÄSIAR recognized no difference between the fingerprints of the water-soluble peptides of this tryptic digests from these two kinds of hemoglobin. The author followed their experiments of fingerprinting and obtained the results which rather supported ZUCKERKANDL et al. Moreover, it is noteworthy that the corresponding peptides in the same position on the fingerprints were found to have different types of amino acid composition. In this experiment, *Macaca mulatta* monkey adult hemoglobin and human adult hemoglobin have been compared by the methods of agar-gel electrophoresis, cellulose acetate membrane electrophoresis, column chromatography, alkali denaturation and fingerprinting of the tryptic digests of these hemoglobins, and confirmed, though bearing a close resemblance, to have still some difference between them. The detailed difference in their primary structures will be explained after the determination of the whole structure of *Macaca mulatta* monkey hemoglobin.
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